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Circadian *Clock* Mutation in Dams Disrupts Nursing Behavior and Growth of Pups

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To understand the role of the circadian molecular clock in mouse reproduction, we investigated the daily rhythms associated with nursing and pup growth in *Clock*-mutant mice maintained under light-dark housing conditions. The daily rhythm associated with the maternal behavior of crouching had a strong diurnal peak and two weak nocturnal peaks in wild-type dams, whereas homozygotes (*Clock*/*Clock*) exhibited no significant peaks in activity. Wild-type, but not *Clock*-mutant, dams showed high, rhythmic levels of prolactin content in serum that corresponded with crouching. Pup body weight increased at a significantly slower rate in *Clock*-mutant dams compared with wild-type dams under all experimental conditions when the pups ranged from 10–15 in number. Heterozygote dams equally bred wild-type, heterozygote, or homozygote pups. The amount of milk secreted from dams, as calculated by the increase in pup body weight through suckling, was lower in *Clock*-mutant mothers *vs.* wild-type mice. When *Clock*-mutant dams gave birth to more than 10 pups, survival was poor for offspring until the time of weaning. The present results demonstrate that *Clock* mutation disrupts daily maternal behavior and the growth and survival rate of pups, especially with the breeding of more than 10 pups. (*Endocrinology* 147: 1916–1923, 2006)

IN MAMMALS, THE MASTER circadian pacemaker (or circadian clock) is located in the suprachiasmatic nuclei (SCN) of the hypothalamus and coordinates circadian physiological and behavioral rhythms. Circadian output from the SCN plays a major role in the regulation of reproduction. SCN ablation or the severing of neuronal pathways between the SCN and the preoptic hypothalamic area results in estrous acyclicity and infertility (1, 2). Molecular clock components such as *Clock* (3), *Bmal1* (4), *Per1* and *Per2* (5, 6), and *Cry1* and *Cry2* (7, 8) participate in circadian oscillation. Recent reports suggest that circadian *Clock* mutation disrupts estrous cyclicity, interferes with pregnancy (9), and results in perinatal and postpartum problems and poor pup survival until weaning (10). *Clock*-mutant female mice also show an unexpected decline in progesterone levels at midpregnancy and a shortened duration of pseudopregnancy, suggesting that maternal prolactin release may be abnormal (9). We conducted the present experiments to elucidate the role of *Clock* mutation in association with maternal behavior during lactation.

Prolactin plays multiple roles in reproduction, including lactation, luteal function, and reproductive behavior (reviewed in Ref. 11). The varied effects of prolactin on the mammary gland include the growth and development of the mammary gland, milk synthesis, and the maintenance of milk secretion (11). Probably the best-characterized prolactin-driven reproductive behaviors are seen in the parents. Maternal behaviors such as nest building, gathering, grouping, cleaning, and crouching behavior have been most extensively studied in rats (12, 13) and less extensively in mice (14). Mice lacking a functional prolactin receptor exhibit profound deficits in the expression of maternal cues (14), clearly demonstrating that prolactin functions in mediating this behavior. Consistent with what is known about the neuronal circuitry regulating maternal behavior, it appears that the action of prolactin to stimulate maternal behavior (15) is exerted in the medial preoptic nucleus of the hypothalamus in rats (16).

There is ample chronobiological evidence that the temporal organization of prolactin secretion in rats is controlled by circadian input (17, 18). For example, the rhythm of prolactin release is maintained in constant conditions and abolished by SCN lesion in rats (17, 19). The best-known physiological stimulus affecting prolactin secretion is suckling of the nipple by neonates (20), which involves a suckling-induced decrease in dopamine neuronal activity in the arcuate nucleus (21). The prolactin secretory response to nursing is reported to be superimposed by the daily rhythm of prolactin secretion (22, 23).

In taking all previous findings into consideration, mutation of the circadian clock may affect reproductive behavior not only during pregnancy, but also during lactation. In this study we examined daily rhythms associated with the maternal behavior of crouching as well as the prolactin level and the growth and survival rates of pups from *Clock*-mutant dams.

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Abbreviations: SCN, Suprachiasmatic nuclei; TR-FIA, time-resolved fluoroimmunoassay; ZT0, lights-on time; ZT12, lights-off time.

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Materials and Methods

Animals and housing

Clock-mutant mice were purchased from The Jackson Laboratory (stock no. 002923; Bar Harbor, ME) and backcrossed to ICR strains more

than eight times. Animals were maintained on a 12-h light, 12-h dark cycle with lights on at 0800 h (room temperature, 23 ± 2 C) and allowed access to commercial chow (Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*. During the light period, light intensity was 100–150 lux at cage level. ZT0 was defined as the lights-on time, and ZT12 as the lights-off time. Female wild-type and *Clock*-mutant mice were mostly mated with male wild-type and *Clock*-mutant mice, respectively, but in some experiments, they were mated with opposite genotype male mice (wild-type female × *Clock*-mutant male; *Clock*-mutant female × wild-type male). To examine the effect of *Clock* mutation on pup growth minus the effects of the *Clock* mutation in dams, heterozygote females and males were mated. Wild, heterozygote, and homozygote pups were bred by heterozygote dams. In this experiment, pups ears were punched for individual identification until postpartum d 7, and the genotypes of the pups were determined after weaning.

Genotypes were determined using a PCR mutagenesis method that introduces a restriction site that allows for direct detection of the *Clock* mutation (24). The mutant allele has an A to T transversion in the third nucleotide of the intron splice donor site (from CAGgtaac to CAGgttac, where lower case represents the intronic sequence), resulting in the skipping of exon 19. The 5′ primer (63 nucleotides; 5′-GCAAGAAGA-ACTAAGGAAAATTCAAGAGCAACTTCAGATGGTCCATGGTCA-AGGGCTACAGTT-3′) is based on the sequence of exon 18 and includes the first two nucleotides of the intron, but terminates with CAGtt (rather than CAGgt). The 3′ primer (5′-TAGTGCCCTAGATGGCCCTGTTGG-3′) is the reverse complement of the intronic sequence terminating 398 bp 3′ of the mutation. PCR amplification results in the introduction of a *Hin*cII site (Gttaac) in the wild-type allele, whereas the corresponding section of the mutant allele (Gtttac) is not cut by *Hin*cII. Mouse genomic DNA was extracted from tail biopsies by proteinase K digestion and isopropanol precipitation. Genomic DNA was subjected to PCR amplification using a hot start protocol and *Taq* DNA polymerase. Cycling parameters were 94 C for 3 min; 30 cycles of 94 C for 1 min, 60 C for 2 min, and 72 C for 3 min; followed by a final extension at 72 C for 10 min. Amplification products were digested with *Hin*cII (TakaraBio, Osaka, Japan) without extraction and were separated by 1.5% agarose gel electrophoresis. The *Clock* allele produces an amplified product of 460 bp that is unaffected by *Hin*cII. The wild-type allele is sensitive to *Hin*cII; cleavage of the 5′ primer results in a 398-bp band.

To reduce the gender effects in experiments on growth and survival rates, crouching behavior, milk secretion, and prolactin content, the male and female ratio between wild and mutant pups was adjusted. Some dams were killed to measure prolactin in serum. Experimental animal care was conducted under the permission of the experimental animal welfare committee of the School of Science and Engineering at Waseda University).

Video observation of crouching behavior

Crouching, which has previously been reported in rats (25) and mice (26), is a maternal behavior associated with nursing. In this experiment, crouching behavior, including arched back nursing, was observed in 12 pups from both wild-type and *Clock*-mutant mice and was recorded by video for evaluation later. Crouching for over 5 min was counted as one event, and the duration was marked on an event sheet. Behavioral rhythmicity was assessed for approximately 4 wk after pups were born using a χ^2 periodogram (27) in the range of 20–28 h. Crouching behavior was analyzed from delivery day to weaning day by an experimenter who was blind to the animals' genotypes.

Measurement of milk secretion

On postpartum d 3 or 7, the dams were separated from their pups for 8 h from ZT2 to ZT10. After an 8-h separation, the pups were returned to the dams. We then measured the increase in pup body weight 1 h after reunion. Because the amount of prolactin released is related to stimulus intensity and is somewhat commensurate with the number of pups nursing (28), the pup number was set at 12.

Nest-building behavior

To evaluate nest-building behavior, we measured the height from the base to the top of the nest. If the height was more than 4.5 cm and included a ridge formation, we designated this nest completed. The nest was defined as incomplete if it did not meet these conditions. A researcher who was blind to the animals' genotypes measured each nest, and the percentage of complete or incomplete nests was calculated.

Exchange of pups among wild-type and Clock-mutant dams

Half the litters were cross-fostered on postpartum d 3 or 4. In this experiment, we only used litters with more than 12 pups. Total pup number was adjusted to 12, and the gender and genotype of pups were also adjusted to equal numbers. Some dams were killed to measure prolactin in the serum.

Sample preparation

Mice were deeply anesthetized with ether, and blood was intracardially collected. Serum was separated and stored at −80 C until it determination of the prolactin level.

Measurement of prolactin levels in serum

In this experiment, we measured prolactin secretion during crouching. To ensure controlled conditions, blood samples were collected during times of stimulation by pup suckling. Dams were killed when they demonstrated an arched back or blanket nursing behavior toward pups for 20–30 min during video surveillance. A previous study (29) showed that in rats, blood prolactin concentrations begin to rise within 3 min of nursing initiation, peak within 10 min, and reach a constant level with continuous nursing. Although we do not know whether the same situation occurs in mice, with these experimental conditions we could measure prolactin secretion under suckling-stimulation conditions. Because a significant delay in pup growth was observed when pup number ranged from 10–15, we consistently examined wild-type and *Clock*-mutant dams who were nursing 10–15 pups. We also measured the prolactin secretion of both wild-type and *Clock*-mutant dams under basal conditions in the absence of nursing.

Prolactin levels in serum were determined using a newly developed time-resolved fluoroimmunoassay (TR-FIA) for mouse prolactin. The mouse prolactin RIA kit (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases) was provided by Dr. Masanobu Yamada (Gunma University, Maebashi, Japan). Highly purified mouse prolactin for iodination (AFP-10777D; 2 μg) was labeled with europium using the DELFIA Eu-N1 ITC labeling kit (PerkinElmer Japan, Tokyo, Japan) at 22 C for 48 h according to the manufacturer's instructions. Free europium and europium-labeled prolactin were then separated by PD-10 (Amersham Biosciences, Tokyo, Japan).

A second antibody (antirabbit IgG; 20 μg/ml in 50 mM K_2HPO_4 containing 0.9% NaCl and 0.5% NaN_3, 200 μl; Shibayagi, Shibukawa, Japan) was immobilized to the surface of the microtiter plate (no. 437958; Nunc, Rosklide, Denmark) at 4 C for 18 h. After three washes with the washing solution (150 mM NaCl containing 0.02% Tween 20 and 0.05% NaN_3), the plate was blocked with 0.1% BSA (Sigma-Aldrich Corp., St. Louis, MO) in 50 mM Na_2HPO_4 and 0.05% NaN_3 (300 μl) at room temperature for 1 h. The plate was washed three times, and 100 μl antimouse prolactin (AFP 131078, ×400,000 in DELFIA Assay Buffer, PerkinElmer Japan) was dispensed into the wells. After incubation at 4 C for 18 h, the plate was washed three times, and 50 μl mouse prolactin reference preparation for RIA (AFP-6476C; 0.078–40 ng/ml) or mouse serum samples diluted with the assay buffer were dispensed into the wells. Usually, 5-μl serum samples were used. After incubation at room temperature for 1 h, europium-labeled mouse prolactin (50 μl, ~10,000 counts per second) was dispensed into the wells and further incubated at 4 C for 18 h. After three washes, DELFIA Enhancement Solution (100 μl; PerkinElmer Japan) was added to dissociate europium from the antibody-antigen complex on the surface of the plate, and the plates were shaken for 5 min at room temperature. Time-resolve fluorescence was measured using an ARVO-Sx (PerkinElmer Japan). Prolactin levels in serum were calculated using PRISM (version 2.0; GraphPad, Inc., San Diego, CA).

To validate TR-FIA, the parallelism of the inhibition curves for a serial 2-fold dilution of the mouse serum and the mouse prolactin standard was tested using a parallel line assay (2 × 3 points). The inhibition curve

for the serial 2-fold dilution of the serum was parallel to the curve for the standard (data not shown). Intra- and interassay coefficients of variation of the TR-FIA were 7.4% (n = 3) and 9.1% (n = 4) at the 0.06 ng/tube level, respectively. The minimum detectable level, defined as 2 SD from the buffer controls, was approximately 5 pg/tube.

Statistical analysis

The data are expressed as the mean ± SEM. For statistical analysis, one- or two-way ANOVA was applied, followed by the Bonferroni-Dunn or Student's *t* test. In some experiments, we used the nonparametric Mann-Whitney *U* test for statistical analysis. The Fisher exact probability test was applied for incidence analysis. StatView for Windows (version 5.0; SAS Institute, Cary, NC) statistical analysis software was used.

Results

Body weight increase, survival rate, and number of pups in wild-type and Clock*-mutant mice*

We obtained a total of 746 pups from 65 wild-type pregnancies and 741 pups from 72 *Clock*-mutant pregnancies. There were significant differences in the number of pups born to wild-type (11.5 ± 0.28) and *Clock*-mutant mothers (10.3 ± 0.32; $P < 0.01$, by Student's *t* test; Fig. 1A). Although the body weight of pups born to *Clock*-mutant mice tended to be slightly lower, there were no significant differences between the two genotypes ($P > 0.05$, by Student's *t* test; Fig. 1B). When we observed the developmental pattern of pups obtained from 55 wild-type and 64 *Clock*-mutant dams, interrupted nursing behavior occurred in 13 of 64 *Clock*-mutant dams ($P < 0.05$ *vs.* wild-type, by Fisher probability test) and in three of 55 wild-type dams (Fig. 1C), and all pups died within 1 wk of birth. A reduction in the number of pups was observed in 13 of 64 *Clock*-mutant dams ($P < 0.05$ *vs.* wild-type, by Fisher probability test) and five of 55 wild-type dams up until the day of weaning (postpartum d 28; Fig. 1C).

Nursing dams were divided into two groups depending on pup number (less than nine or more than 10), because mothers with less than nine pups did not show a reduced pup number until weaning. Data from dams with interrupted nursing were excluded from the following results. Under these criteria, dams with more than nine pups had no significant differences in pup survival between wild-type (12.2 ± 0.24; n = 52) and *Clock*-mutant (11.8 ± 0.23; n = 47; $P > 0.05$, by Student's *t* test) dams. *Clock*-mutant dams exhibited a significant decrease in pup survival rate compared with wild-type dams ($P < 0.05$, by Student's *t* test) only when they bred 10–15 pups (Fig. 1D). Two-way ANOVA revealed that weight gain corresponding to postnatal week was significantly slower in *Clock*-mutant pups *vs.* wild-type (df = 4,60; F = 3.4; $P < 0.05$, genotype × week), when pups numbered between 10 and 15 at birth (Fig. 1E). If pups numbered from six to nine, both wild-type and *Clock*-mutant pups grew faster, and there were no significant differences between the two groups (Fig. 1E).

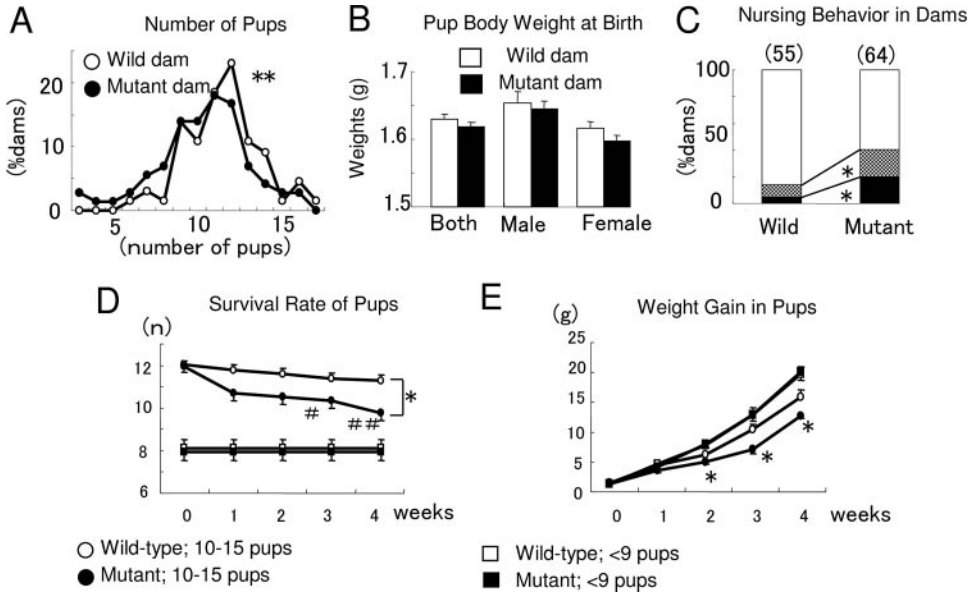

FIG. 1. Number of pups birthed by wild-type and *Clock*-mutant dams, their body weights at birth, and weight development. A, Distribution of dams (percentage of total dams) compared with the number of pups from each mother. Data are based on 65 (total number of pups, 746) wild-type and 72 (total number of pups, 741) *Clock*-mutant mothers. **, $P < 0.01$ between mean number of pups (11.5 ± 0.28 for wild-type and 10.3 ± 0.32 for *Clock*-mutant mice; by Student's *t* test). ○, Wild-type mice; ●, *Clock*-mutant mice. B, Birth weights of pups born to wild-type (n = 65) and *Clock*-mutant (n = 72) dams. □, Wild-type mice; ■, *Clock*-mutant mice. C, Percentage of dams showing abnormal behavior during nursing. □, Normal dams; ▨, dams that had a reduction in the number of nursing pups; ■, dams that completely interrupted nursing within 1 wk after delivery. *Numbers in parentheses* refer to the number of dams. *, $P < 0.05$ *vs.* wild-type mice (by Fisher exact probability test). D, Survival rate of pups. ● and ○, Survival rate of pups numbering between 10–15 (n = 19 for wild-type; n = 22 for *Clock*-mutant mothers); ■ and □, dams with fewer than nine pups (n = 4 for wild-type; n = 6 for mutant mothers); ○ and □, wild-type mice; ■ and ●, *Clock*-mutant mice. #, $P < 0.05$; ##, $P < 0.01$ (*vs.* postpartum d 0, by Bonferroni/Dunn test). *, $P < 0.05$ (*vs.* wild-type mice, by Student's *t* test). E, Weight gain of pups numbering 10–15 (● and ○) or less than nine (■ and □) from wild-type (○ and □) or Clock-mutant (● and ■) dams. Data came from seven wild-type and *Clock*-mutant mothers that bred 10–15 pups and five wild-type and mutant mothers that bred fewer than nine pups. *, $P < 0.05$ (*vs.* wild-type mice, by Student's *t* test).

Daily rhythm of crouching behavior in wild-type and Clock-mutant dams

The daily bouts of crouching were recorded and then evaluated at a later time. If dams showed arched back nursing or blanket nursing for at least 5 min, we measured the event duration time. Daily crouching patterns of the representative mice were plotted as an actogram (Fig. 2A), and the averaged daily pattern of crouching events during 30-min epochs from birth until weaning was plotted (Fig. 2, A and B). Wild-type dams exhibited a daily pattern of events with a major peak between 0900 and 1500 h and two minor peaks between 2100 and 0000 h and between 0200 and 0500 h. *Clock*-mutant dams did not display clear rhythmicity. Periodogram analysis revealed significant 24-h and weak 12-h rhythmicity in wild-type mothers, but not in *Clock*-mutant mothers (Fig. 2D). The average of daily crouching episodes was calculated for three wild-type and three *Clock*-mutant mice (Fig. 2C), and two-way ANOVA demonstrated a significant difference in the daily pattern of crouching events between the two groups (df = 47, 192; F = 2.2; $P < 0.01$, genotype × clock time; Fig. 2C). The duration of crouching per event (Fig. 2E, *left*) and the total length per day (Fig. 2E, *middle*) were significantly longer ($P < 0.05$, by Student's *t* test) in wild-type mice *vs.* *Clock* mutants, and event number per day was slightly lower in wild-type mice (Fig. 2E, *right*).

Daily rhythms of prolactin content and milk secretion in wild-type and Clock-mutant dams

Prolactin content was higher in nursing mothers than in virgin female mice (Fig 3A). As we did not control the suckling duration for each dam, prolactin content fell over a wide range. Therefore, we statistically analyzed prolactin content in serum using the nonparametrical Mann-Whitney *U* test and found that wild-type mice exhibited a clear rhythmic daily change ($P < 0.05$, ZT6 and ZT18 *vs.* ZT12), whereas *Clock*-mutant mice did not ($P > 0.05$; Fig. 3A). The mean daily prolactin content was slightly higher in wild-type mice *vs.* *Clock* mutants (227 ng/ml for wild-type mice; 198 ng/ml for *Clock*-mutant mice), but this was not a significant difference ($P > 0.05$, by Student's *t* test). Interestingly the prolactin content in wild-type and *Clock*-mutant dams without pups suckling for 2 d was almost the same (Fig. 3A).

Milk secretion was estimated by the weight gain of 12 suckling pups. The increase in body weight after suckling was significantly lower ($P < 0.01$, by Student's *t* test) in *Clock*-mutant pups *vs.* wild-type pups (Fig. 3B). Wild-type

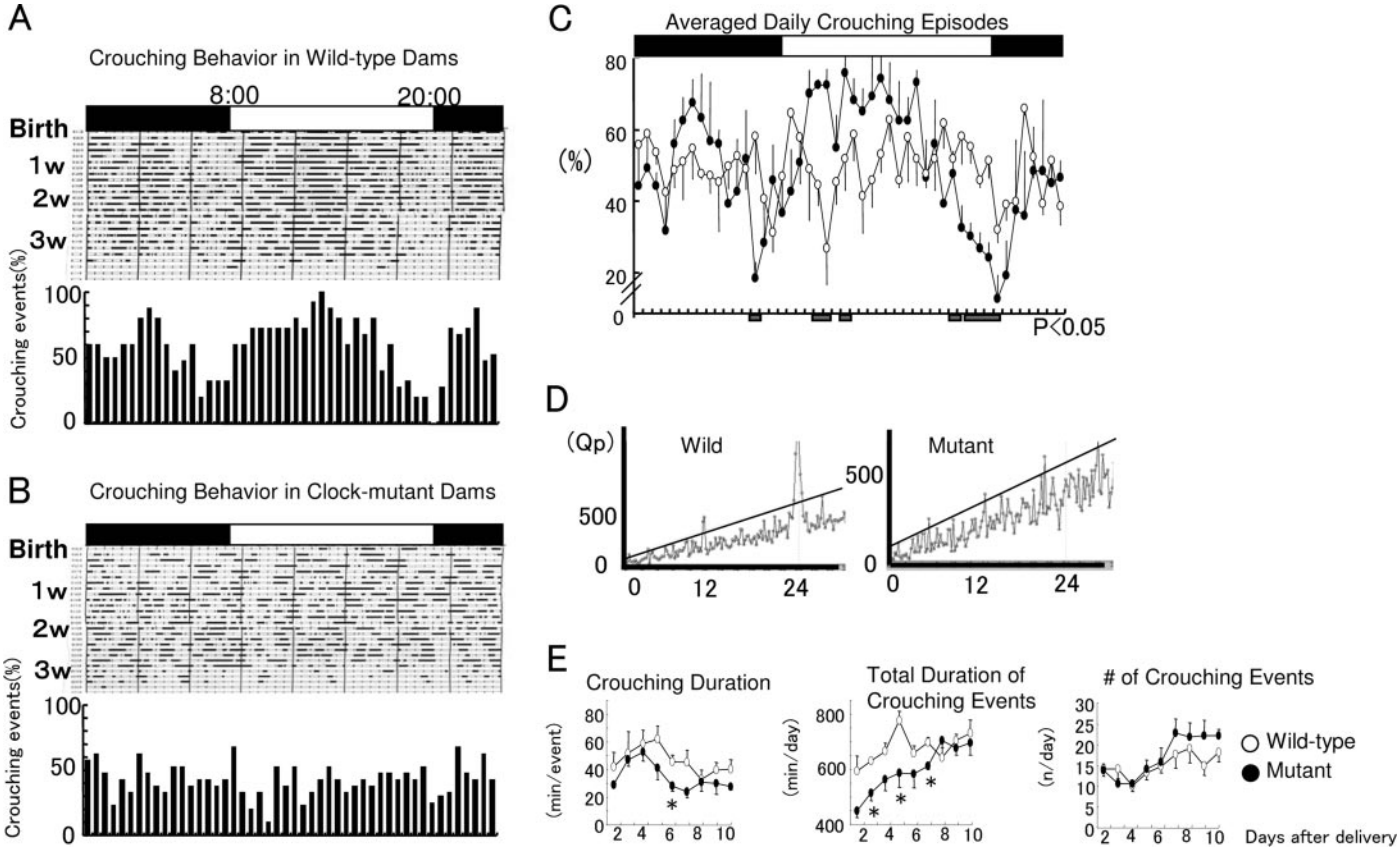

FIG. 2. Daily rhythm of crouching behavior in wild-type and *Clock*-mutant mice. A and B, *Upper panel*, Representative actogram of crouching activity in wild-type (A) and *Clock*-mutant dams (B) maintained under a light-dark cycle. *Lower panel*, Crouching as a percentage of events from 30-min intervals taken from delivery to weaning. C, Percentage of crouching episodes in three wild-type and three *Clock*-mutant mice. *Shaded areas* under the horizontal clock timeline show a significant change between wild-type and mutant mice ($P < 0.05$, by Student's *t* test). D, *Left* and *right* periodograms were obtained from wild-type (A) and *Clock*-mutant (B) dams, respectively. E, *Left*, Duration of crouching per event until d 10 after delivery; *middle*, number of crouching events per day; *right*, total duration of crouching events per day. ○, Wild-type mice; ●, *Clock*-mutant mice. *, $P < 0.05$ (*vs.* wild-type mice, by Student's *t* test).

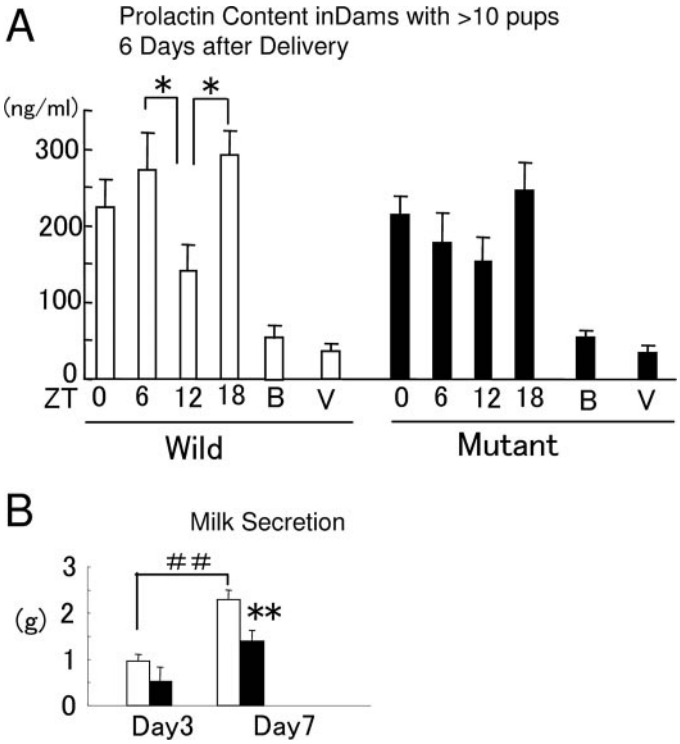

FIG. 3. Daily profile of prolactin content in serum and milk secretion of wild-type and *Clock*-mutant dams. A, Prolactin content in serum of wild-type and *Clock*-mutant dams nursing 10–15 pups on d 6 after delivery. Each *point* consists of three to eight wild-type and three to eight *Clock*-mutant dams. *, $P < 0.05$ (by Mann-Whitney *U* test).V, Virgin female killed at ZT8. B, Basal level of prolactin content in dams not nursing. B, Estimated milk secretion according to weight gain. After starvation from ZT2 to ZT10, average body weight increase in 12 pups was measured for the next 1 h of suckling. **, $P < 0.01$ (*vs.* wild-type mice, by Student's *t* test); ##, $P < 0.01$ (*vs.* d 3, by Student's *t* test).

($P < 0.01$, by Student's *t* test), but not *Clock*-mutant ($P = 0.06$, by Student's *t* test), pups showed significantly increased body weight from d 3 to 7. When there were fewer than nine pups, no difference in milk secretion was observed (data not shown).

Nest building in wild-type and Clock-mutant mice

Because the *Clock*-mutant mouse exhibited a deficit in crouching behavior and prolactin release in the serum after pup delivery, we investigated whether maternal behavior was also disturbed before delivery. Up until the time of delivery, many of the *Clock*-mutant mice made incomplete nests compared with wild-type mice, with significant differences between the two groups ($P < 0.01$ and $P < 0.05$, by Fisher's probability test; Fig. 4A). However, there were no significant differences in weight gain during pregnancy between wild-type and *Clock*-mutant females (Fig. 4B).

Slow increase in body weight related to Clock-mutant dams, but not to pups

Mating of heterozygote males and females produced wild-type, heterozygote, and homozygote pups. Heterozygote mothers bred 10–15 pups of all genotypes equally, and there

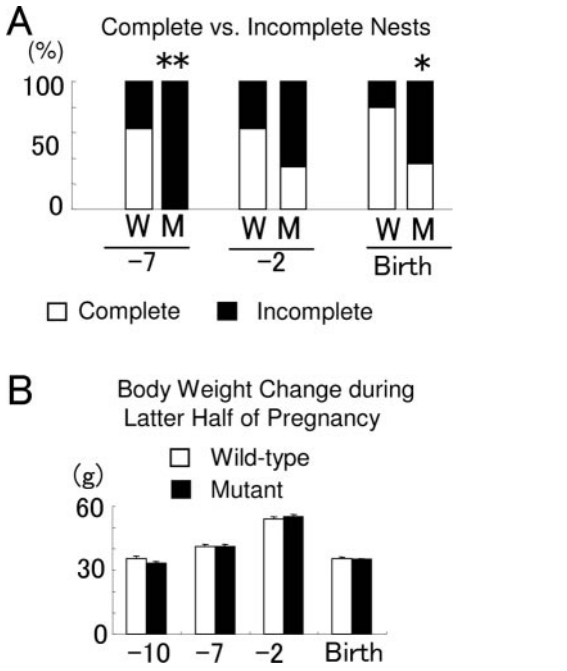

FIG. 4. Nest-building behavior and body weight change in pregnant wild-type and *Clock*-mutant mice. A, Percentage of complete (□) or incomplete (■) nests built by pregnant wild-type (n = 15) and *Clock*-mutant (n = 22) dams. *, $P < 0.05$; **, $P < 0.01$ (*vs.* wild-type mice, by Fisher's exact probability test). B, Change in body weight of pregnant mice in the latter half of pregnancy. □, Wild-type mice; ■, *Clock*-mutant mouse.

were no significant differences in the body weight increase among the pups (Fig. 5A). In a second experiment, wild-type and *Clock*-mutant females were mated with *Clock*-mutant and wild-type males, respectively. Wild-type and *Clock*-mutant dams bred heterozygous pups only. The body weight gain in pups bred by wild-type dams was faster than that in pups bred by *Clock*-mutant dams when pups numbered between 10 and 15 (Fig. 5B). When there were less than nine pups, there was no difference in body weight gain (data not shown).

Clock-mutant foster mother and nursing behavior

To confirm that *Clock*-mutant dams have a deficit in nursing behavior, we conducted cross-fostering experiments. In this case, half the litters were cross-fostered on postpartum d 3 or 4. The body weight increase in pups bred by *Clock*-mutant dams was slow compared with that in pups of wild-type dams, even when pups were wild type or mutant (Fig. 6A). Although the number of pups was adjusted to 12 on the day of exchange, the pup survival rate significantly decreased in *Clock*-mutant dams *vs.* wild-type dams (Fig. 6B). A decreased prolactin content in the serum was more evident in the *Clock*-mutant foster dams than in wild-type foster dams (Fig. 6C), but this difference was not significant ($P > 0.05$, by Student's *t* test).

Discussion

In the present study we examined whether *Clock* mutation affected the maternal behavior of crouching during nursing,

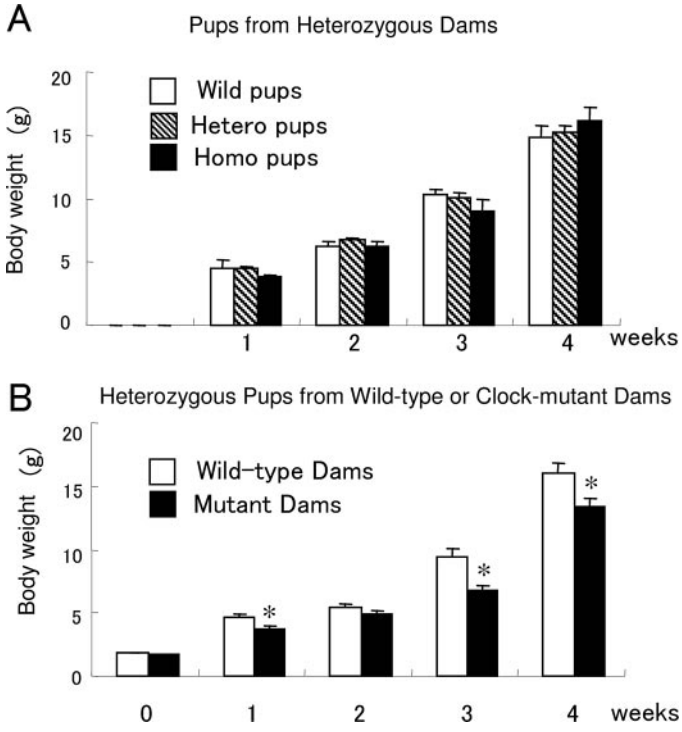

FIG. 5. Slow increase in body weight related to clock mutation of dams and their pups. A, Four heterozygous dams bred 10–15 pups that were homozygous (■), heterozygous (▨), or wild type (□). B, Wild-type (n = 5) and *Clock*-mutant homozygous (n = 5) mice bred heterozygous pups only. When the number of pups ranged from 10–14, a slow increase in body weight was seen in pups bred by *Clock*-mutant dams. *, $P < 0.05$ (*vs.* wild-type mice, by Student's *t* test).

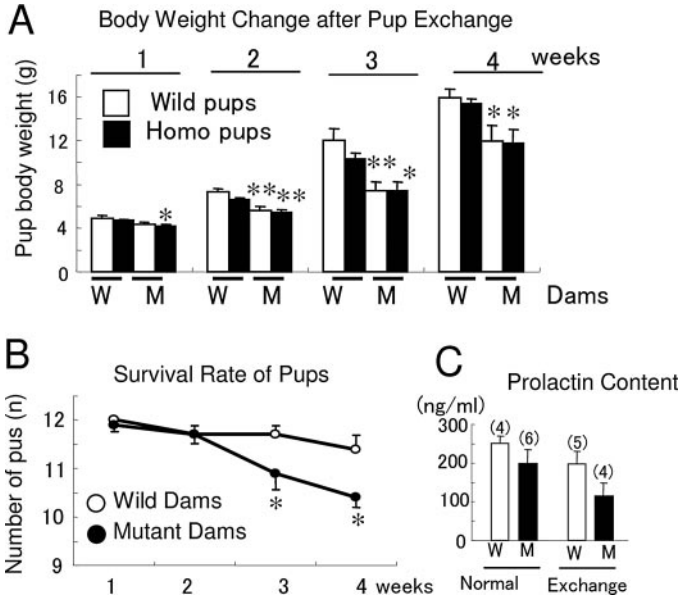

FIG. 6. Effect of cross-fostering pups between wild-type (W) and *Clock*-mutant (M) mothers on nursing behavior. A, Wild-type (n = 7) and *Clock*-mutant (n = 7) dams bred their 12 pups (six were from a true or foster mother). *, $P < 0.05$; **, $P < 0.01$ (*vs.* wild-type mice, by Student's *t* test). B, Survival rate of pups. ○, Wild-type mice (n = 7); ●, *Clock*-mutant mice (n = 7). *, $P < 0.05$ (*vs.* wild-type mice, by Student's *t* test). C, Half the litters were cross-fostered, and the prolactin content of dams with their own and foster pups was measured. *Numbers in parentheses* represent the number of mothers.

prolactin release, and weight gain in pups. A clear daily rhythm in the occurrence of crouching was present in wild-type dams, whereas *Clock*-mutant dams exhibited a loss of daily rhythm. *Clock*-mutant dams also demonstrated shorter durations of crouching per event and a low total time spent crouching per day. In rodents, the prolactin secretory response to nursing is superimposed by the daily rhythm of prolactin secretion; that is, the same intensity of suckling stimulus can elevate prolactin levels more effectively at certain times of the day when the daily input enhances the suckling stimulus-evoked secretory response (22, 23). In the present experiment, *Clock* mutation did not seem to enhance the suckling stimulus-evoked prolactin secretion on a daily basis. Based on all findings, we suggest that longer durations of crouching, which occur most often during the day in wild-type mothers, may be vital to normal pup growth. Alternately, disruption of the daily feeding cycle in *Clock*-mutant mice (30) could then be associated with altered behavior toward the pups.

Turek *et al.* (30) recently reported that *Clock*-mutant mouse pups showed an initial body weight gain similar to wild-type mice, but then became obese after weaning. In the present experiment, there were no significant differences in the weight gain and survival rate of pups born to wild-type and *Clock*-mutant mothers when the number of pups was less than nine. Thus, *Clock* mutation of dams did not affect pup growth when the number of pups was small; however, it severely disrupted pup growth when the number of pups ranged from 10–15. Thus, we believe that lower milk production can sustain only a certain number of pups, beyond which it becomes more difficult regardless of whether *Clock*-mutant dams carry the C57black or ICR strains.

Because a larger number of pups was born to dams carrying the ICR strain than to those carrying the C57black strain, Turek *et al.* (30) may not have found a difference in weight gain due to the small number of pups born to *Clock*-mutant mice with a C57black background gene. Our *Clock*-mutant pups gained weight after weaning and caught up with wild-type mice within 5–6 wk after birth (data not shown). The ICR mouse strain generally grows larger in size than the C57black strain; therefore, we believe there were no significant differences in body weight between wild-type and *Clock*-mutant ICR mice in the adult stage (data not shown).

Miller *et al.* (9) reported that circadian *Clock* mutation both disrupts estrous cyclicity and interferes with the ability of mice to carry a pregnancy to full-term. *Clock*-mutant C57black strain females exhibit increased fetal reabsorption during pregnancy and have a high rate of full-term pregnancy failure. Interestingly, a recent report demonstrated that *Clock*-mutant mice carrying the C57black strain also show more perinatal and postpartum problems and very poor offspring survival until weaning (10). Findings from our present experiment indicated that *Clock* mutation in ICR mice had a significant, but weak, effect on reproductive performance. Recently, Kennaway *et al.* (31) reported that *Clock*-mutant C57black strain mice backcrossed to CBA strain mice showed a redundancy of genes in the part of the circadian system that allows for reproductive cyclicity. Considering all

findings, we suggest that the C57black strain carrying the *Clock* mutation exhibits a more severe, abnormal pattern of reproduction performance than the CBA and ICR strains also carrying the *Clock* mutation.

The live birth of pups has never resulted from homozygous pairings of *Bmal1*-null mutants (32). Nevertheless, *Bmal1* females ovulate, mate, and fertilize ova after mating with wild-type males, but either delayed embryo development or early embryo loss prevents full-term pregnancies. Thus, normal clock function seems necessary to maintain normal reproduction.

In this study, *Clock*-mutant dams exhibited a disrupted daily rhythm of prolactin release that corresponded with the disrupted daily rhythm of crouching. Disrupted crouching rhythms may affect suckling behavior, resulting in the disrupted rhythm of prolactin release. Abnormal prolactin release may, in turn, affect not only milk production and secretion, which would then affect pup growth, but also other various maternal behaviors. Prolactin has actually been found to modulate maternal behaviors through the prolactin receptor located in the medial preoptic nucleus (20). We saw in this investigation that a smaller amount of milk was secreted in *Clock*-mutant dams *vs.* wild-type dams. However, the present results simply show a correspondence between two phenotypes, such as prolactin secretion and milk production. The direction of causality remains unclear.

There are no clear answers to the questions of how and where the *Clock* mutation alters maternal behavior and breeding success. It is possible that the absence of the *Clock* gene causes errors in prolactin production/secretion by virtue of disrupted circadian rhythm regulation of prolactin synthesis/release. There is important literature on the necessity of circadian clock for maintenance of luteal function during pregnancy (9). Taken together, these findings suggest that at least some of the *Clock*-mutant pregnancy abnormalities and nursing abnormalities are probably due to abnormal daily 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 (33). However, we still do not know whether the present findings are the result of a defective circadian clock in the SCN or the absence of normal clock gene function at one or more loci outside the SCN. Regarding to this hypothesis, a previous paper suggested that *Clock*-mutant females display irregular estrous cycles and failed 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 (9). The daily prolactin surge induced by continuous exposure of ovariectomized rats to high estradiol levels is eliminated by SCN lesions (34, 35). Vasopressin (33) and vasoactive intestinal polypeptide (36) outputs from the rat SCN have been reported to be neurotransmitters for the regulation of this daily prolactin surge. Although a direct effect of the *Clock* mutation on prolactin synthesis/release of lactotrophs *in vivo* remains to be examined, we hypothesize that *Clock*-mutant dams failed to have daily prolactin release because the SCN in mutant animals does not provide a coordinated time of day signal to prolactin surges.

In contrast, the *Clock* genes may directly regulate prolactin synthesis through the control of prolactin promoter activity in MMQ prolactin cells (37). Imaging studies of isolated brain regions from transgenic rats using a luciferase reporter driven by the *mPer1* promoter established that average *Per1*-luciferase expression is rhythmic in the arcuate nucleus (38). Furthermore, a circadian rhythm associated with the *Per1*: green fluorescence protein reporter signal was observed in tyrosine hydroxylase-positive neurons in the arcuate nucleus (39). Thus, we could not rule out the possibility that maternal behavior is disrupted by the deficits of *Clock* gene function at one or more loci outside the SCN. Additional experiments are necessary to elucidate the detailed mechanism of deficits of maternal behavior observed in *Clock*-mutant mice.

Weight gain was similar in wild-type, heterozygous, and homozygous pups bred by heterozygous dams, whereas heterozygous pups bred by *Clock*-mutant mothers exhibited a slow increase in body weight compared with heterozygous pups bred by wild-type dams. In addition, the cross-fostering experiment showed disrupted weight gain in pups bred by *Clock*-mutant mothers. Thus, we suggest that the slow increase in pup body weight relates not to a *Clock* mutation in pups, but to that in dams.

It is known that *Clock* mutation disrupts the reproductive process of estrous cyclicity during ovulation and the ability to carry a pregnancy to full term (9). We found in the present study that *Clock* mutation disrupted maternal behavior and interfered with weight gain in pups, thereby reducing the survival rate of offspring until the time of weaning.

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K.H., Y.W., M.I., and S.S. have nothing to declare.

References

1. **Brown-Grant K, Raisman G** 1977 Abnormalities in reproductive function associated with the destruction of the suprachiasmatic nuclei in female rats. Proc R Soc Lond B Biol Sci 198:279–296
2. **Wiegand SJ, Terasawa E, Bridson WE, Goy RW** 1980 Effects of discrete lesions of preoptic and suprachiasmatic structures in the female rat. Alterations in the feedback regulation of gonadotropin secretion. Neuroendocrinology 31:147–157
3. **Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS** 1994 Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science 264:719–725
4. **Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA** 2000 Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103:1009–1017
5. **Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC** 2001 Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105:683–694
6. **Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR** 2001

Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30:525–536
7. **Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher RJ, Ishikawa T, Miyazaki J, Takahashi JS, Sancar A** 1999 Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. Proc Natl Acad Sci USA 96:12114–12119
8. **van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A** 1999 Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627–630
9. **Miller BH, Olson SL, Turek FW, Levine JE, Horton TH, Takahashi JS** 2004 Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. Curr Biol 14:1367–1373
10. **Dolatshad H, Campbell EA, O'hara L, Maywood ES, Hastings MH, Johnson MH** 2005 Developmental and reproductive performance in circadian mutant mice. Hum Reprod 21:68–79
11. **Freeman ME, Kanyicska B, Lerant A, Nagy G** 2000 Prolactin: structure, function, and regulation of secretion. Physiol Rev 80:1523–1631
12. **Bridges RS, DiBiase R, Loundes DD, Doherty PC** 1985 Prolactin stimulation of maternal behavior in female rats. Science 227:782–784
13. **Bridges RS, Robertson MC, Shiu RP, Friesen HG, Stuer AM, Mann PE** 1996 Endocrine communication between conceptus and mother: placental lactogen stimulation of maternal behavior. Neuroendocrinology 64:57–64
14. **Lucas BK, Ormandy CJ, Binart N, Bridges RS, Kelly PA** 1998 Null mutation of the prolactin receptor gene produces a defect in maternal behavior. Endocrinology 139:4102–4107
15. **Bridges RS, Numan M, Ronsheim PM, Mann PE, Lupini CE** 1990 Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. Proc Natl Acad Sci USA 87:8003–8007
16. **Bridges RS, Robertson MC, Shiu RP, Sturgis JD, Henriquez BM, Mann PE** 1997 Central lactogenic regulation of maternal behavior in rats: steroid dependence, hormone specificity, and behavioral potencies of rat prolactin and rat placental lactogen I. Endocrinology 138:756–763
17. **Bethea CL, Neill JD** 1979 Prolactin secretion after cervical stimulation of rats maintained in constant dark or constant light. Endocrinology 104:870–876
18. **Kizer JS, Zivin JA, Jacobowitz DM, Kopin IJ** 1975 The nyctohemeral rhythm of plasma prolactin: effects of ganglionectomy, pinealectomy, constant light, constant darkness or 6-OH-dopamine administration. Endocrinology 96:1230–1240
19. **Bethea CL, Neill JD** 1980 Lesions of the suprachiasmatic nuclei abolish the cervically stimulated prolactin surges in the rat. Endocrinology 107:1–5
20. **Grattan DR** 2002 Behavioural significance of prolactin signalling in the central nervous system during pregnancy and lactation. Reproduction 123:497–506
21. **Selmanoff M, Gregerson KA** 1985 Suckling decreases dopamine turnover in both medial and lateral aspects of the median eminence in the rat. Neurosci Lett 57:25–30
22. **Arey BJ, Kanyicska B, Freeman ME** 1991 The endogenous stimulatory rhythm regulating prolactin secretion is present in the lactating rat. Neuroendocrinology 53:35–40
23. **Kacsoh B, Nagy G** 1983 Circadian rhythms in plasma prolactin, luteinizing hormone and hypophyseal prolactin levels in lactating rats. Endocrinol Exp 17:301–310
24. **Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ, Reppert SM** 1999 A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell 96:57–68
25. **Myers MM, Brunelli SA, Squire JM, Shindeldecker RD, Hofer MA** 1989 Maternal behavior of SHR rats and its relationship to offspring blood pressures. Dev Psychobiol 22:29–53
26. **Kikusui T, Isaka Y, Mori Y** 2005 Early weaning deprives mouse pups of maternal care and decreases their maternal behavior in adulthood. Behav Brain Res 162:200–206
27. **Sokolove PG, Bushell WN** 1978 The χ square periodogram: its utility for analysis of circadian rhythms. J Theor Biol 72:131–160
28. **Mena F, Grosvenor CE** 1968 Effect of number of pups upon suckling-induced fall in pituitary prolactin concentration and milk ejection in the rat. Endocrinology 82:623–626
29. **Grosvenor CE, Whitworth N** 1974 Evidence for a steady rate of secretion of prolactin following suckling in the rat. J Dairy Sci 57:900–904
30. **Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J** 2005 Obesity and metabolic syndrome in circadian Clock mutant mice. Science 308:1043–1045
31. **Kennaway DJ, Boden MJ, Voultsios A** 2005 Reproductive performance in female Clock(Delta19) mutant mice. Reprod Fertil Dev 16:801–810
32. **Boden MJ, Kennaway DJ** 2005 Reproduction in the arrhythmic Bmal1 knockout mouse. Reprod Fertil Dev 17:126 (Abstract 297)
33. **Palm IF, van der Beek EM, Swarts HJ, van der Vliet J, Wiegant VM, Buijs RM, Kalsbeek A** 2001 Control of the estradiol-induced prolactin surge by the suprachiasmatic nucleus. Endocrinology 142:2296–2302
34. **Kawakami M, Arita J, Yoshioka E** 1980 Loss of estrogen-induced daily surges of prolactin and gonadotropins by suprachiasmatic nucleus lesions in ovariectomized rats. Endocrinology 106:1087–1092
35. **Neill JD** 1972 Sexual differences in the hypothalamic regulation of prolactin secretion. Endocrinology 90:1154–1159
36. **Egli M, Bertram R, Sellix MT, Freeman ME** 2004 Rhythmic secretion of prolactin in rats: action of oxytocin coordinated by vasoactive intestinal polypeptide of suprachiasmatic nucleus origin. Endocrinology 145:3386–3394
37. **Leclerc GM, Boockfor FR** 2005 Pulses of prolactin promoter activity depend on a noncanonical E-box that can bind the circadian proteins CLOCK and BMAL1. Endocrinology 146:2782–2790
38. **Abe M, Herzog ED, Yamazaki S, Straume M, Tei H, Sakaki Y, Menaker M, Block GD** 2002 Circadian rhythms in isolated brain regions. J Neurosci 22:350–356
39. **Kriegsfeld LJ, Korets R, Silver R** 2003 Expression of the circadian clock gene Period 1 in neuroendocrine cells: an investigation using mice with a Per1::GFP transgene. Eur J Neurosci 17:212–220

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