**Supplemental Data: A circadian egg timer gates ovulation**

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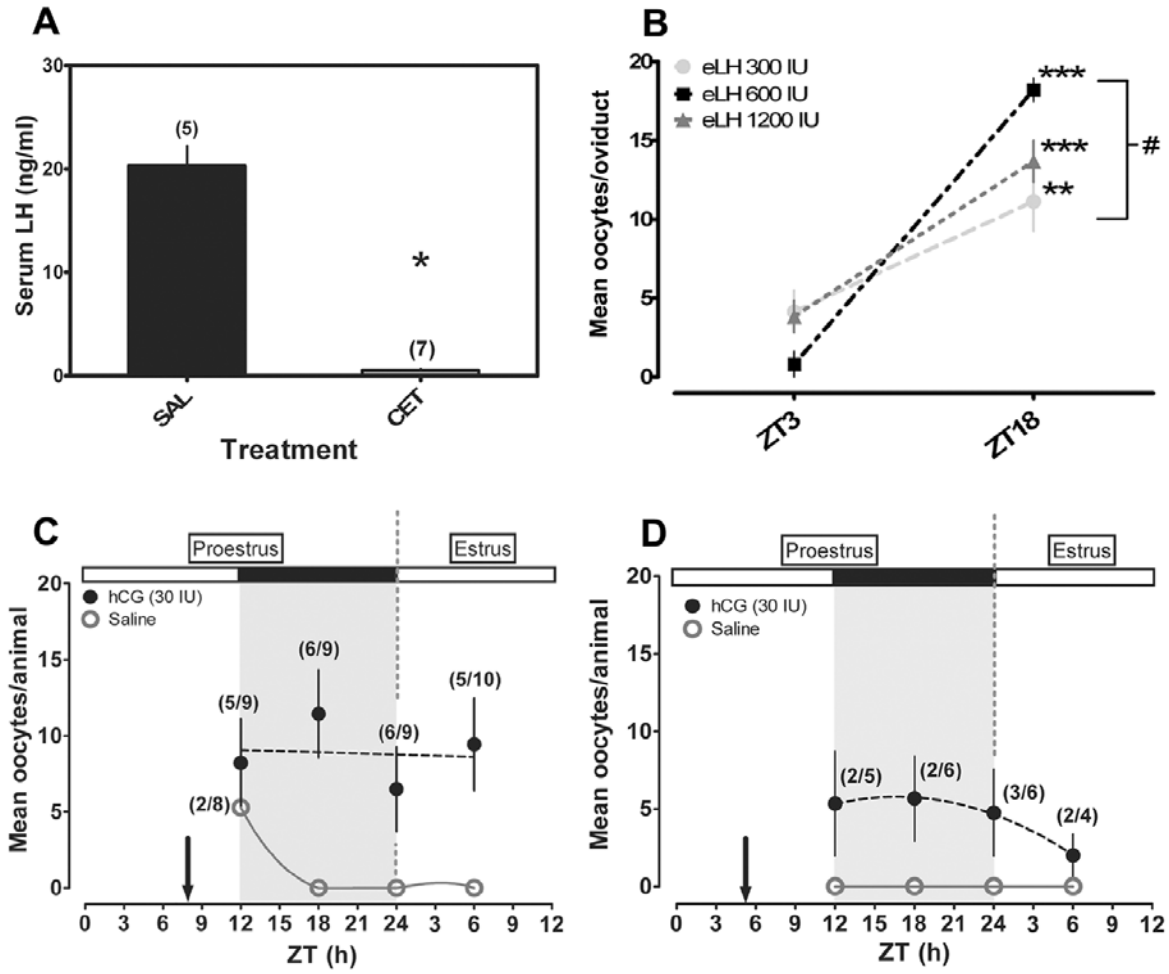


**Figure S1 (related to Fig. 1A and B). (A) Cetrorelix pamoate depot suppresses LH secretion in Period1-luciferase transgenic rats.** To determine the efficacy of CET to suppress the proestrous LH surge, we injected a group of cycling animals at ZT5 with either CET (1mg/0.1ml; i.m., n=7) or vehicle (0.1ml saline; n=5). These animals were euthanized 7h later (ZT12; the approximate onset of the LH surge) and trunk blood was analyzed with RIA for rat LH. As expected, CET suppressed the proestrous

surge of LH secretion in cycling rats. Animals injected with SAL at ZT5 displayed significantly higher serum LH levels (20.3±1.95 ng/ml) than animals treated with CET (0.53±0.17 ng/ml; $p<0.001$) at the same time of day. Data are presented as mean ng/ml LH ± SEM. The numbers above the bars indicate the number of animals per group. Asterisk indicates a significant difference between the CET and saline treated groups. **(B) Treatment with eLH after blockade of endogenous LH with cetrorelix displays both time- and dose- dependent effects on ovulation.** To more closely examine the time- and dose-dependent nature of the response to eLH we treated animals housed in a 12:12 L:D cycle with three concentrations of eLH (300, 600 or 1200 IU) at both the nadir (ZT3) and peak (ZT18) of the established ovulation rhythm. Animals injected at ZT18 ovulated more often and produced significantly more oocytes per ovulation than animals treated at ZT3 ($p<0.01$). Though we did not observe a significant main effect of dose ($p>0.05$, $F=0.92$), comparisons did reveal a dose-dependent effect at ZT18, with significantly more oocytes produced with a 600 IU eLH treatment compared with a 300 IU eLH treatment. This result indicates that the 600IU dose (previously determined to be above a threshold necessary for ovulation; data not shown) elicits a robust ovulatory response in our experiments that is dependent on the time of administration. Asterisks indicate significant differences in mean oocyte number between ZT3 and ZT18 (** $p<0.01$, ***$p<0.001$). The # indicates a significant difference between 300IU and 600IU eLH treatments at ZT18 ($p<0.01$). Data are presented as mean ± SEM.

**(C,D) Effects of human chorionic gonadotrophin (hCG) following Pentobarbital or CET -mediated suppression of LH secretion.** Timed treatment with hCG after pentobarbital (PB; 40mg/kg) or CET mediated blockade of LH secretion failed to reveal a diurnal rhythm of ovulation. **(C)** The LH surge was blocked using a single treatment with pentobarbital (PB; 40mg/kg) at ZT8 (ZT=zeitgeber time where ZT0=lights on or 05:00h; ZT8=13:00h) on the afternoon of proestrus as previously described [S1, S2]. Separate groups of animals injected with PB were then treated with human chorionic gonadotrophin (hCG, 30 IU) or sterile saline (SAL) every 6h for 24h (ZT0, 6, 12 and 18). Except for 2 of 8 rats given SAL at ZT12 (5.26±3.41 oocytes/rat), all animals treated with PB followed by saline treatment failed to

ovulate, regardless of the time of injection. Approximately 50-60% of rats injected with hCG ovulated, regardless of the time of hCG treatment. **(D)** A separate group of animals was treated with CET (1mg/0.1ml; i.m.) at ZT5 on proestrus and subsequently treated with hCG (30 IU) every 6h for 24h. Treatment with sterile saline failed to produce ovulation regardless of injection time in CET-treated rats. The numbers in parentheses indicate the number of animals that ovulated/ total animals injected with hCG. Approximately 60% of rats injected with hCG ovulated regardless of injection time. The open and solid bars at the top of the figure indicate the light and dark portions of the L:D cycle. The dashed black lines are non-linear regressions generated with second-order polynomials. The black arrow indicates the time of (A) PB or (B) CET-treatment. We believe the difference between these results and those reported in Fig. 1 is a consequence of the difference between the half-life in serum of eLH (<1h) and hCG (>6h). Data are presented as mean ± SEM

**Table S1 (related to Figure 1). The percentage of animals that ovulated in response to eLH injection after suppression of endogenous LH secretion with Cetrorelix as a function of time of day and lighting condition**

| *Experimental Group* | *Percent Ovulated* | *Ovulated /Total* | *Mean Eggs/Rat* |
|---|---|---|---|
| ***12:12 L:D cycle (Diestrus)*** | | | |
| ZT18 | 67 | (6/9) | 10 |
| ZT21 | 63 | (5/8) | 7.9 |
| ZT24 | 63 | (5/8) | 5.9 |
| ZT3 | 50 | (4/8) | 4.1 |
| ZT6 | 14 | (1/7) | 0.1 |
| ZT9 | 88 | (9/10) | 5.3 |
| ZT12 | 78 | (9/11) | 5.5 |
| ***12:12 L:D cycle (Proestrus)*** | | | |
| ZT12 | 88 | (7/8) | 13.4 |
| ZT15 | 83 | (5/6) | 13.3 |
| ZT18 | 100 | (5/5) | 18.2 |
| ZT21 | 80 | (4/5) | 12.6 |
| ZT24 | 50 | (3/6) | 2.8 |
| ZT3 | 20 | (1/5) | 0.8 |
| ZT6 | 60 | (3/5) | 2.2 |
| ZT9 | 16 | (1/6) | 0.3 |
| ***Dim constant light (dim LL; Proestrus)*** | | | |

| | | | |
|---|---|---|---|
| CT12 | 86 | (6/7) | 14 |
| CT18 | 100 | (5/5) | 14 |
| CT24 | 50 | (3/6) | 3.67 |
| CT6 | 43 | (6/7) | 1.86 |

**Supplemental Experimental Procedures**

**Animals:** Adult female (>60 day old) transgenic Period1-luciferase rats on a Wistar background were raised in our colony. Animals were housed in light and temperature controlled chambers with ad libitum access to food and water under a 12:12 L:D cycle with lights on from 04:00h-16:00h unless otherwise noted (>100 lux at cage level). For constant dim light experiments (dim LL), animals were transferred at lights off (16:00) to constant dim light [intensity at cage level was <1 lux ( <0.30 $\mu w/cm^2$)]. All experiments were conducted according to the guidelines of the University of Virginia Institutional Animal Care and Use Committee and NIH guidelines for the use of experimental animals.

**Vaginal cytology:** Daily vaginal smears were taken using cotton tipped applicators moistened with sterile water. Slides were stained with Methylene Blue and Eosin (Quick III stat-pak staining kit, Astral Diagnostics Inc., Deptford NJ) and imaged with an Olympus CKX-41 upright compound microscope. An individual blind to the experimental conditions determined the estrus stage according to the cytology described in [S3]. For experiments in dim LL, vaginal smears were collected in dim light with the aid of an infrared viewer. Only those animals that displayed a minimum of two consecutive 4 day estrous cycles were included in these experiments.

**Proestrous LH surge block and Equine LH treatment:** To suppress LH secretion, cetrorelix$^{tm}$ pamoate depot (CET; Aeterna Zentaris; Frankfurt) injections (1mg/0.1 ml; i.m.) were given under light isoflurane anesthesia. The depot formulation of CET allowed us to make a single intramuscular injection in the

medial biceps muscle of the right hind leg which provides a minimum of 48h of inhibition. An additional group of animals were treated at ZT6 (10:00h) with pentobarbital (40 mg/kg in sterile saline; i.p) without isoflurane anesthesia. At the appropriate times, rats were removed from their light-controlled chamber and injected i.p. with equine LH (300-1200 i.u./0.3ml sterile saline; Sigma-Aldrich) or human chorionic gonadotrophin (hCG; 30 IU/0.3ml sterile saline). In a preliminary experiment it was determined that concentrations of 30IU hCG or 600IU eLH were above the threshold necessary to induce ovulation (data not shown). Initially, we used hCG due to its established efficacy as an LH/CG receptor agonist and its well documented use for super-ovulation paradigms in rodents and ovulation trigger in human assisted reproduction technology (ART; [S4]). However, the serum half-life of hCG is significantly longer than the half-life of LH [S5-S7]. Given its' half-life in serum (>6h), injections of hCG are likely to obscure the window of sensitivity (see Supplementary Fig. 3). Therefore, we repeated our experiments using equine LH (eLH). Its half-life (<1h), strong molecular similarity to rat LH and its known affinity for the common LH/CG receptor [S8] made it a better choice. For experiments in dim LL, animals were injected in dim light with the aid of an infrared viewer.

**Locomotor Activity Recording:** General locomotor activity at cage level was recorded using Quorom 150 motion sensors. Locomotor activity was recorded in one minute bins and analyzed using ClockLab software (Actimetrics, Evanston, IL.). Chi-squared periodograms ($p<0.001$) were used to calculate the free-running period of activity (mean $\tau=24.64\pm0.04$h) in constant dim light.

**Tissue Dissection, Oocyte Recovery and Serum Sampling:** Animals were euthanized with carbon dioxide and decapitated by guillotine. Trunk blood was collected into 15 ml conical tubes and allowed to clot at room temperature. Samples were then centrifuged at 2000 rpm for 15 minutes at room temperature. Serum was recovered and stored at -80C until assayed for serum LH. To recover the oocytes, an abdominal incision was made and the ovary was isolated from the abdominal fat pad. Using fine forceps and scissors, the ovary and oviduct were separated from the uterine horn and fat pad and placed in chilled Hank's Balanced Salt Solution (HBSS) in a 50 mm plastic petri dish. With the aid of a

dissecting microscope, vascular scissors and fine forceps were used to separate the oviduct from the remaining abdominal fat and the ovary. The oviduct was then examined for a bulge in the lumen of the duct which contained the Cumulus Oocyte Complexes (COCs). Using fine forceps, the wall of the oviduct at the site of the bulge was gently ripped to create a small opening. Gentle pressure was then applied to the surrounding duct and the COCs were extracted from the lumen into the surrounding HBSS. The number of oocytes present within the COCs was then counted and logged. Data are presented as mean oocytes recovered ± SEM.

**Radioimmunoassay for Luteinizing Hormone:** Immunoassays for rat LH were conducted by the ligand Analysis Core at the Center for Reproduction Research at the University of Virginia. All assays were conducted by an independent technician that was blind to treatment group. Values for rat LH were normalized to the rat RP-3 standard and are reported as mean ng/ml $\pm$ SEM (Fig. S1).

**Data Analysis:** Within and between treatments comparisons were made with one-factor ANOVA followed by Student Neuman Keul's post-hoc statistical test, two-factor ANOVA followed by Bonferroni post-hoc tests and unpaired student's t-test. Non-linear "best-fit" curves in each graph are second to fourth-order polynomials generated to emphasize the rhythm. In addition to non-linear regressions, a linear harmonic regression fitting analysis that describes the data by adding harmonics to the principal wave function was used to determine the significance of the rhythms (CircWave, R.A. Hut, University of Groningen, Haren, The Netherlands, [S9]). For each data set, a single harmonic (sine-wave) was used to fit the data and F-testing was used for the primary fit with a significance level for rhythmicity of $p<0.05$. All graphs, non-linear regressions and data analyses were generated and/or performed with GraphPad Prism software (GraphPad Inc., La Jolla, CA). Differences were considered significant at $p<0.05$. All data are presented as mean ±SEM.

**Supplemental References**


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