Chronic Stress Induces Brain Region Specific Alterations of Molecular Rhythms in Mice that Correlate with Depression-Like Behavior

Supplemental Information

Supplemental Methods & Materials

Animals

Mice were maintained under standard conditions unless otherwise stated (group housed, 12:12-hour light/dark cycle, 22 ± 1°C, food and water *ad libitum*). *Period2::*luciferase mice were obtained from Joseph Takahashi (U. Texas Southwestern Medical Center) and were bred in homozygous pairs. The following experimental cohorts of male mice were used: telemetry recording of activity and body temperature (*n* = 23 male C57BL/6J mice; 3 months old); lumicycle recording of brain region specific *Period2* reporter activity (*n* = 15-22 male *Per2::luc* mice per group; 3 months old); and gene expression (*n* = 6 male B6 mice per time-point per group; 3 months old).

Unpredictable Chronic Mild Stress

UCMS consisted of a randomized schedule of 1-2 mild stressors per day, seven days a week. Stressors were administered at random times through the day to avoid circadian entrainment of acute stress. Stressors included forced bath (~4 cm of water in a rat-sized cage for 15 min), wet bedding, aversive smell (1 h exposure to fox urine), dirty bedding (rotate mice into previously occupied “dirty” cages), tilt cages (45° tilt), restraint (50 ml tube for 15 min), reduced cage space, no bedding, and bedding change (replaced soiled bedding with clean bedding). Two or three stressors were intermittently used simultaneously to contribute to the random nature of the paradigm. No light manipulations were used. Fur rating and body weights

were measured weekly to track the progression of the “UCMS syndrome”. Body weight and fur rating were assessed weekly during and following UCMS.

Behavioral Assays

Elevated Plus Maze: The EPM test consisted of four elevated runways (81 cm height): two open arms (without walls) and two closed arms (with walls) (36 cm x 6 cm arms). Mice were placed in the EPM for 10 minutes and anxiety-like behaviors were assessed by measuring the amount of time spent on the open arms (relative to the 10 minute total time in the maze) and the ratio of crosses into the open arms. Total number of crosses into either open or closed arms was used as a measure for overall locomotor activity.

Open Field: The OF test consisted of a square plexiglass arena with a clear floor and solid black walls (61 cm x 61 cm). Mice were placed in the OF for 10 minutes and anxiety-like behavior was measured by time spent in the center and the ratio of distance traveled in the center. Locomotor activity was measured by total distance traveled.

Forced Swim Test: Mice were placed in a one liter beaker filled with room temperature water (~22°C) for 6 minutes. The time-spent swimming during the last four minutes of testing was used as a measure of antidepressant-like behavior.

Novelty Suppressed Feeding: The NSF test consisted of food-depriving mice overnight (~16 hours), then providing them with a single food pellet placed in the middle of a novel, aversive environment (a brightly lit 61 cm x 61 cm enclosure). The latency to start feeding (during 12 min assay) was used as a measure for depressive-like behavior. Food consumption (8 minutes post-test) and weight loss were measured as controls for potential feeding differences.

Dark/Light Box: The dark/light box (Med Associates, Inc., St. Albans, VT) consisted of a clear plexiglass rectangular box with a black plexiglass insert that divided the chamber into a light and dark side. Mice were initially placed in the dark chamber and the door to the light side

was removed at the beginning of the test. Mice were placed in the apparatus for 10 minutes and the time in light and latency to enter the light chamber were recorded as measures of anxiety-like behavior.

Quantitative Real-Time RT-PCR

Mice were sacrificed at six time-points over a 24 h period by cervical dislocation followed by decapitation and brains were flash frozen on dry ice. Brains were flash frozen and sectioned (200 μM) on a cryostat to punch (1 mm diameter corer) specific regions (see main text). Select brain regions were micro-punched on a cryostat and total RNA was extracted using an RNeasy Micro Kit (Qiagen, Germantown, MD). Total RNA was converted into cDNA using the Superscript III first strand synthesis kit (Life Technologies, Grand Island, NY). Real-time RT-qPCR reactions were assessed by SYBR green fluorescence signal (Power SYBR Green PCR Master Mix, Life Technologies) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY). Samples were run in duplicates and ΔCt values were determined by comparison with the housekeeping gene *Gapdh*. Primer sequences for *Per2*: F—5′-GAGTGTGTGCAGCGGCTTAG-3′, Reverse—5′-GTAGGGTGTCATGCGGAAGG-3′; and *Gapdh*: F—5′-AACGACCCCTTCATTGAC-3′, Reverse—5′-TCCACGACATACTCAGCAC-3′.

Lumicycle Recording and Data Analysis

Brain sections (1 mm; except SCN 250 μM) were obtained by micro-punching (0.5-1 mm diameter). *Per2* rhythms in brain region explants were first de-trended using a polynomial baseline correction function (Lumicycle Analysis, Actimetrics). Following de-trending, rhythms were assessed for period, amplitude, and phase over a period of four days using the Lomb-scargle periodogram (LSP) (Circadian Rhythm Laboratory Software, Circadian Physiology by Roberto Refinetti). To eliminate variability as the samples acclimate to culture, the first 0.8 days of the each sample were excluded. Samples were eliminated that did not meet rhythmicity

criteria (e.g., period was not significant in LSP analysis or goodness of fit did not reach significance in acrophase/amplitude analysis).

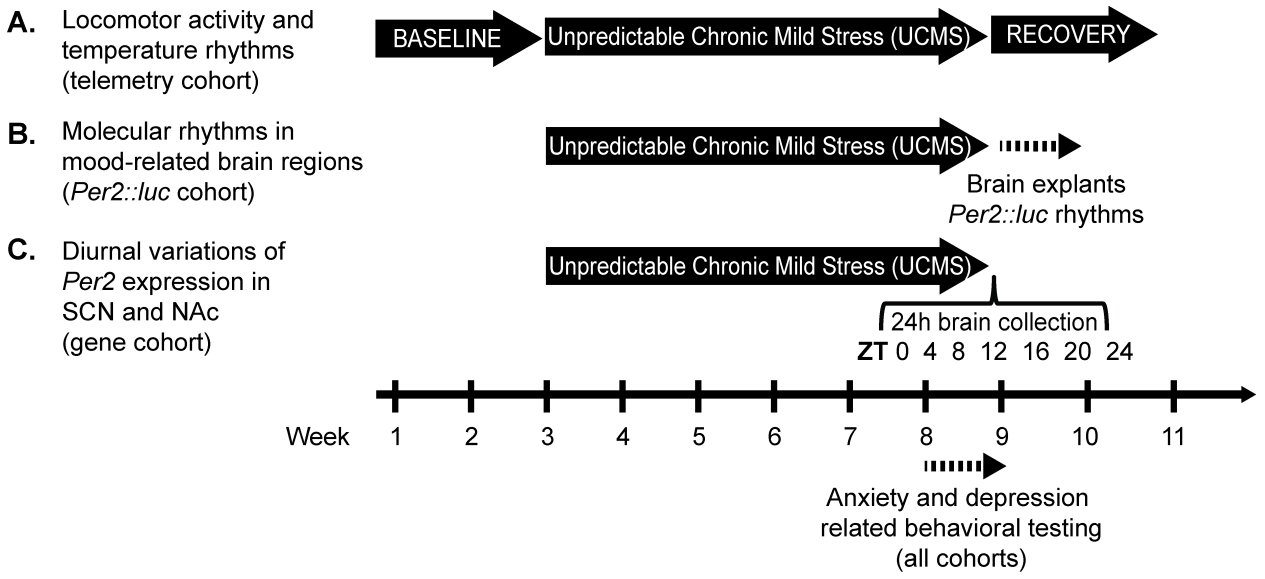

Figure S1. Timeline of UCMS experiments. Telemetry devices were implanted in the abdomen of male B6 mice and locomotor activity and body temperature rhythms were recorded continuously prior to UCMS (baseline), during and following (recovery) UCMS **(A)**. *Per2::luc* mice were exposed to UCMS and brain region specific molecular rhythms were measured continuously for 1 week (week 7) **(B)**. A separate cohort of male B6 mice were exposed to UCMS and brain regions were micro-punched every 4 h across the day then processed for gene expression assays **(C)**. Each cohort of mice underwent behavioral testing during the last week of UCMS **(A-C)**.

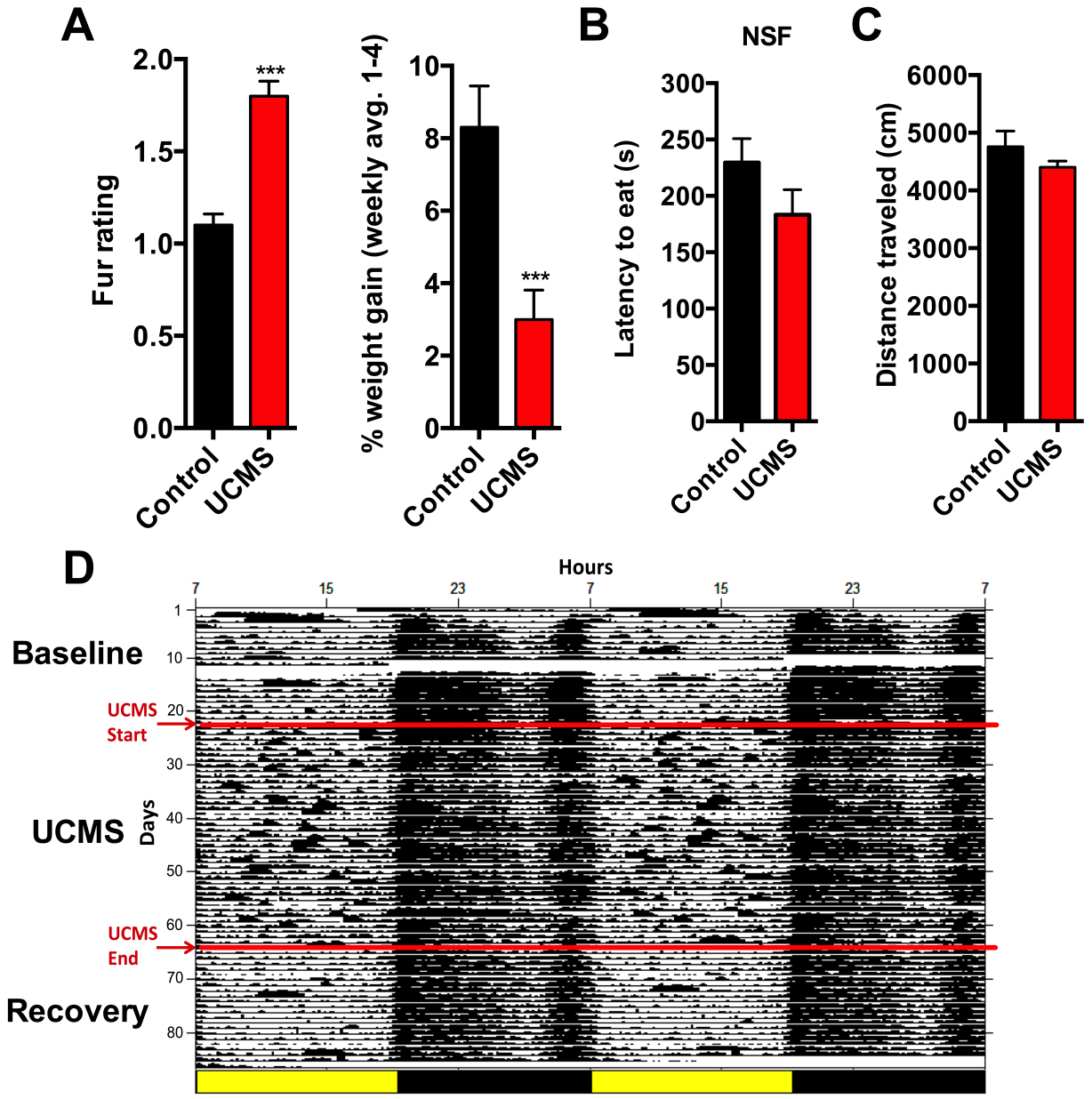

Figure S2. Effects of UCMS on fur rating, weight gain, and novelty suppressed feeding (NSF). UCMS increased fur rating and reduced weight gain in the *Per2::luc* mouse cohort similar to other cohorts **(A)**. Latency to eat during the NSF behavioral assay was similar across cohorts between control and UCMS exposed mice **(B)**. Representative double plotted actogram with experimental phases marked by red lines. Note: missing data during the baseline period of experiment marked by white bars beginning around day 10 was due to a software malfunction and this data was removed from the analyses. *** $p < 0.001$; independent samples *t* tests. Significance was set at $\alpha = 0.05$. Data is represented as the mean $\pm$ SEM.

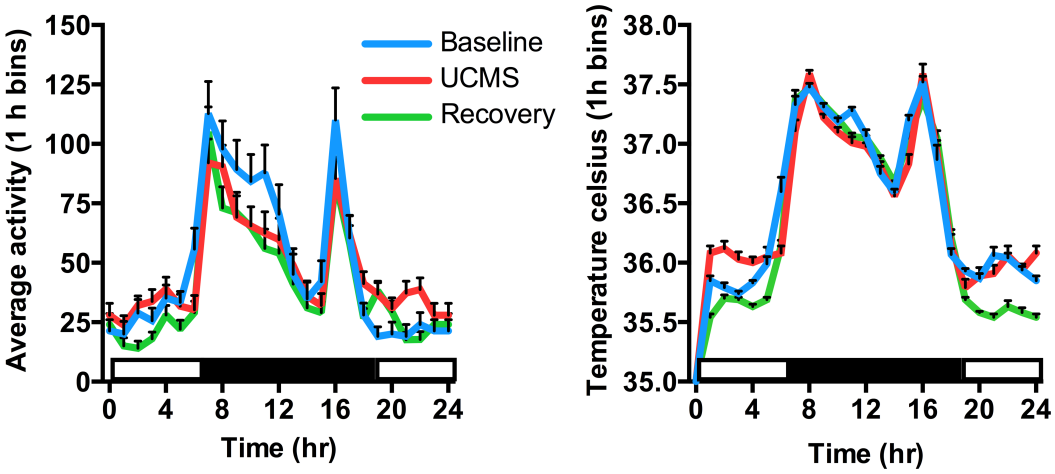

Figure S3. Circadian locomotor activity and body temperature averaged across all weeks of each period of the experiment. Circadian activity was averaged over the entire duration of each experimental phase (i.e., baseline, 2 weeks; UCMS, 6 weeks; recovery, 2 weeks) and circadian waveforms show reduced locomotor activity during the dark phase of light-dark cycle during and following UCMS, while body temperature was increased during the day phase of the light-dark cycle during UCMS. Figure 4 depicts circadian waveforms during only last week of baseline, UCMS, and recovery.

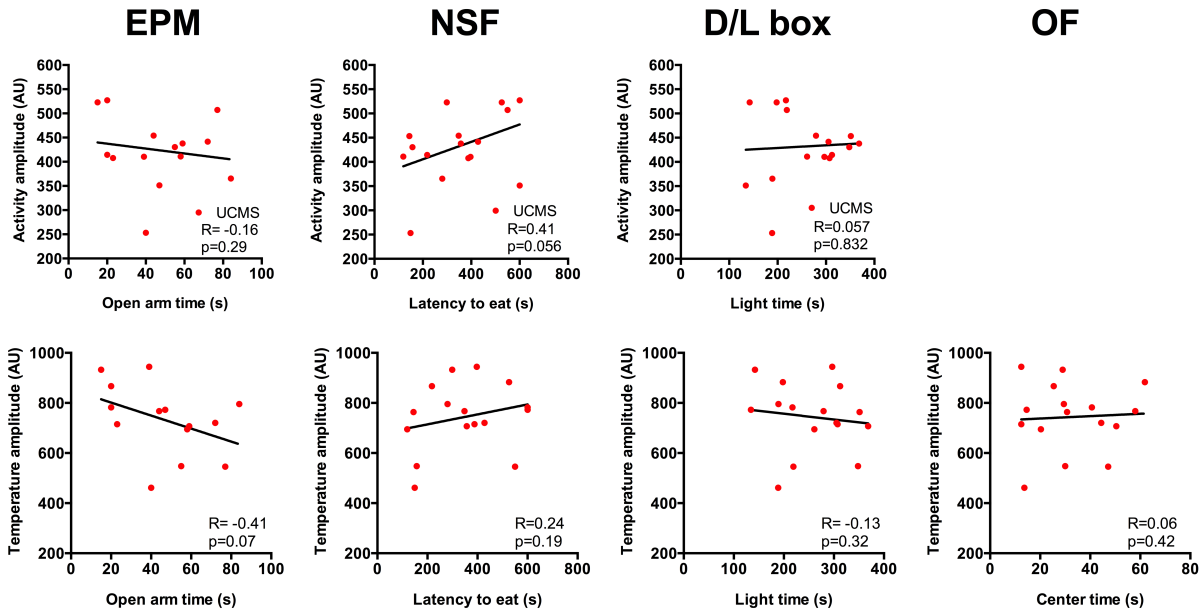

Figure S4. Correlations between circadian amplitudes of locomotor activity and body temperature with mood-related behaviors. Circadian amplitude of activity and body temperature were not significantly correlated with other behavioral measures in the elevated plus maze (EPM), novelty suppressed feeding (NSF), dark-light box (D/L box), and open-field (OF). One-tailed Pearson analyses (R correlation coefficient) for amplitude and behavioral measures. Significance was set at $\alpha = 0.05$.

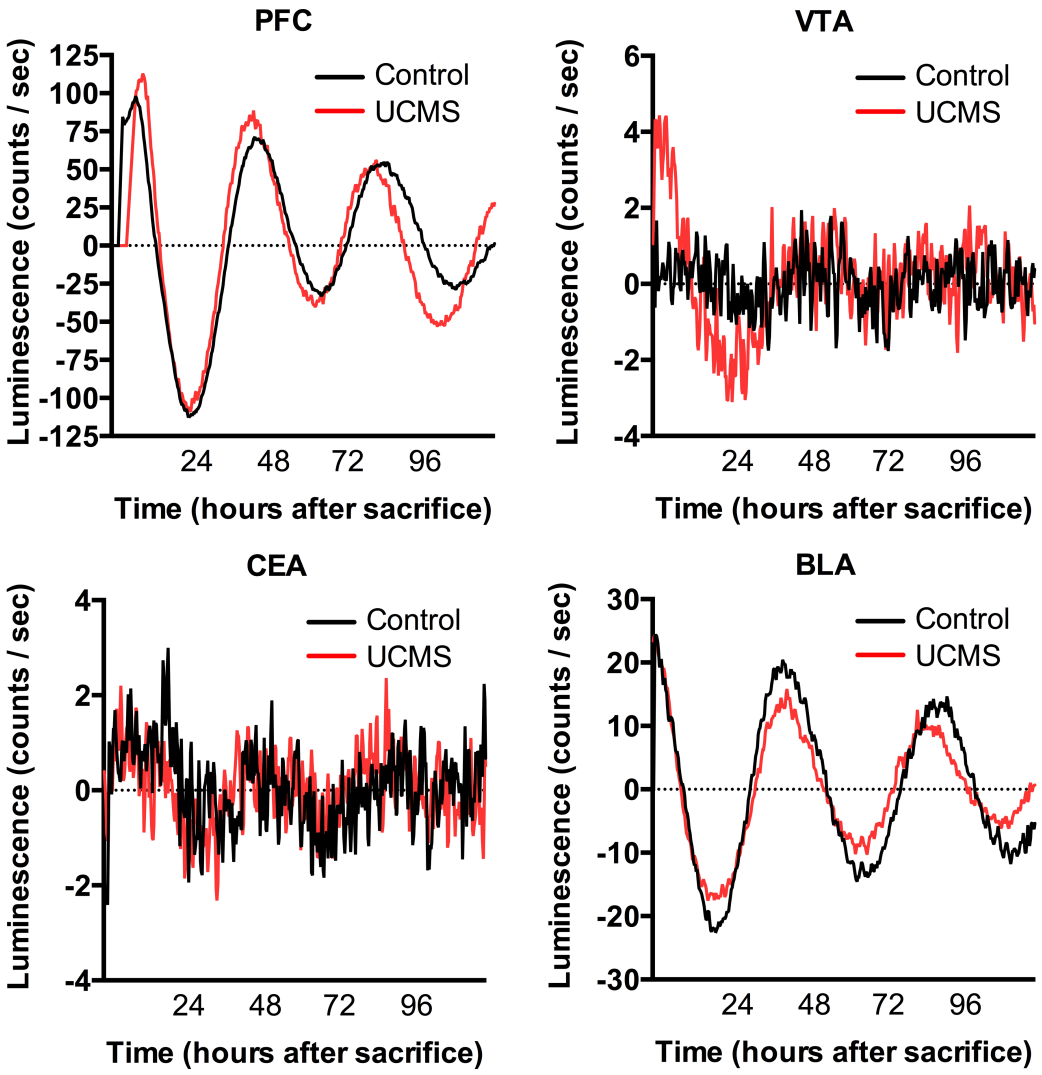

Figure S5. Representative traces of *Per2::luc* molecular rhythms from mood-related brain regions in control and UCMS exposed mice. Multiple brain regions, including the prefrontal cortex (PFC), ventral tegmental area (VTA), central nucleus of the amygdala (CEA), and the basolateral nucleus of the amygdala (BLA), were micro-punched from live brain tissue in control mice and mice exposed to UCMS. *Per2::luc* reporter activity was continuously recorded for up to one week following sacrifice. No differences in molecular rhythms for amplitude, phase, and period were found for these mood-related brain regions following UCMS. Note: PFC and BLA retain molecular rhythmicity after separation from SCN inputs, whereas the VTA and CEA quickly lose rhythmicity.

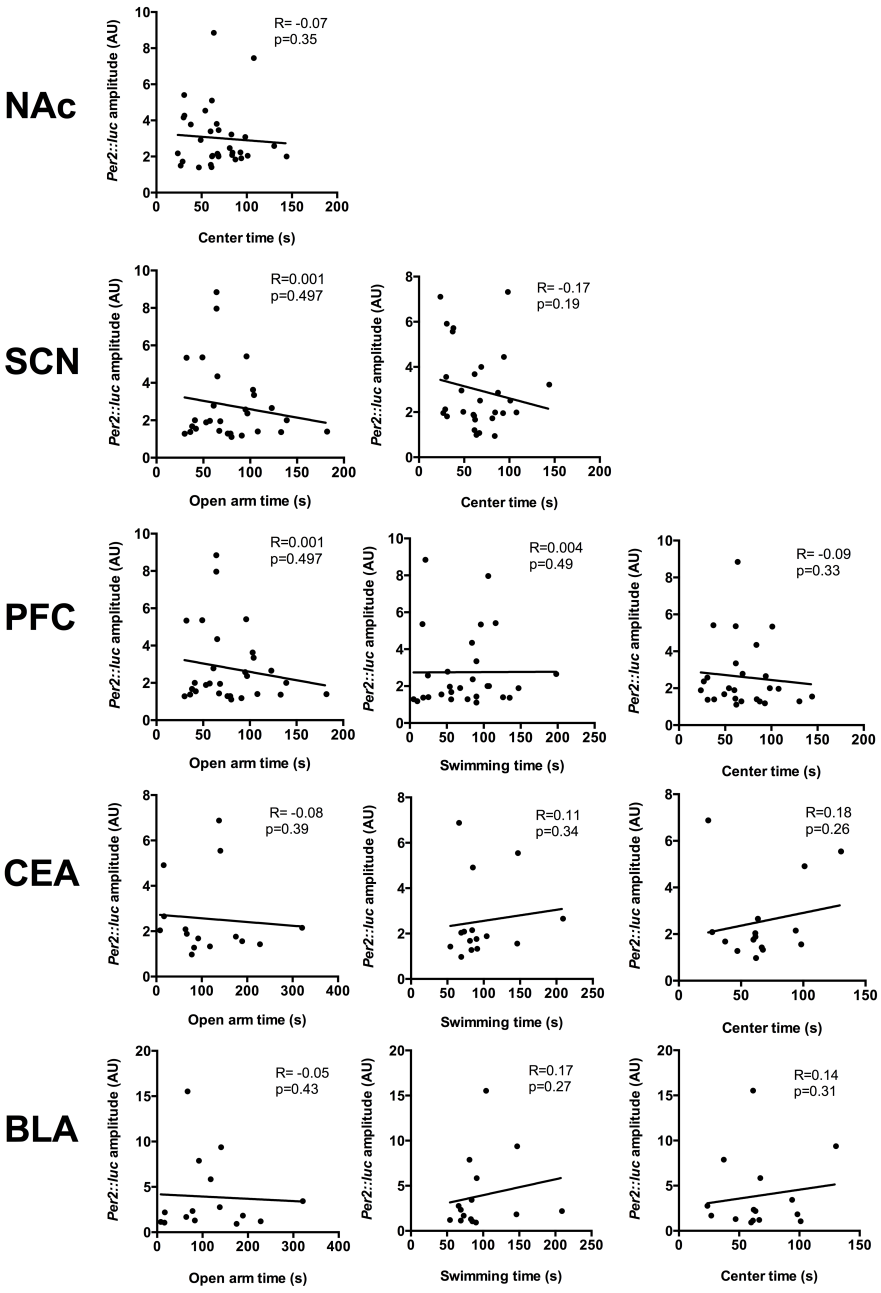

Figure S6. Correlations between circadian amplitude of *Per2::luc* reporter activity in specific mood-related brain regions and behavioral measures. Molecular rhythm amplitude in the nucleus accumbens (NAc), suprachiasmatic nucleus (SCN), prefrontal cortex (PFC), central nucleus of the amygdala (CEA), and basolateral nucleus of the amygdala (BLA) were not significantly correlated with time spent on the open arms in the elevated plus maze, time spent swimming in the forced swim test, and time spent in the center of the open-field. One-tailed Pearson analyses (R correlation coefficient) for amplitude and behavioral measures. Significance was set at $\alpha = 0.05$.

Table S1. Gene expression analyses in the SCN and NAc for core circadian genes. Two-way ANOVA analyses revealed significant main effects for group and/or time, and also their interactions for certain genes in the SCN or NAc. Bonferroni post-hoc analyses were used to investigate interactions, which revealed significant reductions of expression at specific time-points due to chronic stress.

	Per1	***Per2***	***Clock***	***Bmal1***
SCN				
Group (Cont vs. UCMS)	n.s.	$F_{1,59} = 4.902$, $p = 0.03$	$F_{1,59} = 13.61$, $p < 0.001$	n.s
Time (ZT)	n.s	$F_{6,59} = 6.803$, $p < 0.0001$	$F_{6,59} = 3.6$, $p < 0.01$	n.s
Interaction	$F_{6,59} = 4.752$, $p < 0.001$	n.s	$F_{6,59} = 5.68$, $p < 0.001$	$F_{6,59} = 3.761$, $p < 0.01$
Post-hoc	ZT24, $p < 0.05$	-	ZT24, $p < 0.001$	ZT12, $p < 0.05$
NAc				
Group (Cont vs. UCMS)	$F_{1,59} = 21.62$, $p < 0.0001$	n.s	n.s	$F_{1,59} = 36.34$, $p < 0.0001$
Time (ZT)	$F_{6,59} = 4.76$, $p < 0.001$	n.s	$F_{6,59} = 3.02$, $p < 0.05$	$F_{6,59} = 12.95$, $p < 0.0001$
Interaction	n.s	$F_{6,59} = 3.13$, $p < 0.01$	n.s	$F_{6,59} = 4.61$, p < 0.001
Post-hoc	-	n.s	-	ZT0 & 4, $p < 0.05$