Genetic Disruption of Circadian Rhythms in the Suprachiasmatic Nucleus Causes Helplessness, Behavioral Despair, and Anxiety-like Behavior in Mice

Supplemental Information

Supplemental Methods and Materials

Learned Helplessness

The learned helplessness protocol consists of two training days and one testing day. On both training days, at *Zeitgeber time* 9 (ZT9, 9 hours after light on), mice were restrained and received 120 electric tail shocks, each lasting 5 sec, randomly timed within a 60 min session. Shock intensity was gradually increased from 0.25 mA to 0.60 mA: every 15 shocks, the current was increased by 0.05 mA. On the testing day, at ZT6, mice were transferred to shuttle boxes (San Diego Instruments, San Diego, CA, USA). Mice received 30 electric shocks to their feet through the grid floor of the shuttle box. During each test shock (0.10 mA, maximum duration 30 sec), the gate remained open, and mice had a chance to escape the shock by crossing a gate to an adjacent compartment. The schedule in trials #1-5 was fixed ratio (FR) 1 (crossing the gate once in order to escape the shock). In the remaining trials #6-30, the schedule was changed to FR-2 (crossing the gate twice in order to escape the shock).

Sucrose Preference

Mice received two bottles (A & B) for 24 hours. Bottle A was filled with 1% sucrose and bottle B with tap water. To avoid a position bias, bottles A and B were switched each time a new

measurement was done. Sucrose preference for each mouse was calculated as $100' \frac{VolA}{(VolA+VolB)}$.

Tail Suspension Test

Lack of active struggling behavior was measured in the tail suspension test at ZT9 as described previously (1). Briefly, adhesive tape was used to suspend mice from their tails on a metal bar located 30 cm above a flat surface for 6 min. Plastic tubes were put over the tail to prevent grabbing and climbing up the tail. Immobility was quantified by measuring the amount of time when no whole body movement was observed. Whole body movement was defined as movement of all 4 limbs. Flailing with the front limbs was not counted as movement.

Open Field Test

Exploratory locomotor activity in a 5 minute test period was measured in an open field (45 X 45 cm) at ZT9 by an AccuScan apparatus (AccuScan Instruments; Columbus, OH). Immobility time and time spent in the center were recorded automatically by infrared detectors, and data were transferred to a connected PC.

Light/Dark Box Test

At ZT9, mice were transferred to shuttle boxes (San Diego Instruments, San Diego, CA, USA) for 10 min. One compartment was illuminated with an integrated lamp (~200 lux) whereas the

other compartment stayed dark (<10 lux). Anxiety was quantified by measuring the amount of time mice spent in the illuminated compartment.

Novelty-Suppressed Feeding

Mice were starved for 15 hours from ZT12 to ZT3 the next day. The latency to assess a chow pellet and the amount of food intake within 10 min was measured in an open field (45 X 45 cm), an environment that was novel for the animals. The total daily food uptake was measured in their home cages over the course of two consecutive days and values of both days were averaged.

Pain Sensitivity

In the same shuttle boxes that were used for learned helplessness, SCN-*Bmal1*-KD and control mice were exposed to a gradually increasing, continuous foot shock. Electric shock intensities making the mice flinch and jump were measured. After mice jumped, shocks were immediately terminated.

Immunohistochemistry and BMAL1 Quantification

For each brain, two slices encompassing the SCN were used for immunohistochemistry. Free-floating slices were first blocked for 1 h in 0.1M phosphate buffer (PB) containing 3% normal goat serum (NGS), 0.2% Triton X-100 and 0.3% bovine serum albumin (BSA). After blocking, slices were incubated with anti-BMAL1 antibody (Santa Cruz Biotechnology) in PB/3% NGS/0.2% Triton X-100/0.3% BSA solution for 24 h at 4°C with constant shaking. After three washes in PB

buffer, slices were incubated with secondary antibody Alexa Fluor 568 (ThermoFisher Scientific) in PB/0.2% Triton X-100/0.3% BSA containing 1ug/ml Hoechst for 4 h at RT. Slices were washed and mounted using Vectashield mounting medium (Vector Laboratories). Images were taken with an FV1000 confocal microscope (Olympus). To quantify BMAL1 expression, two sections encompassing the SCN from each of three control sh*Scr*-injected mice and three sh*Bmal1*-injected mice were analyzed. Mean fluorescence was measured using ImageJ and background was subtracted. For each section, red fluorescence (BMAL1) in the SCN was normalized to the nuclei staining Hoechst fluorescence from the same area. Normalized BMAL1 fluorescence was averaged for each mouse.

Preparation of Blood Samples

To obtain blood, mouse tails were nicked using a sterile blade ~1-2 mm from the tip, and blood was gently milked and collected into heparinized capillary tubes (Fisher Scientific, Hampton, NH, USA). Blood was immediately transferred to dipotassium EDTA covered blood collection tubes (BD, Franklin Lakes, NJ, USA), centrifuged (20 min, 3000 rpm, 4°C), and plasma was transferred to a fresh tube and stored at -80°C. For repeated sampling, the tail was only nicked once and thereafter the scab was gently removed using sterile gauze. All samples were taken within 2 min in order to avoid stress-related hormone induction.

Supplemental Data

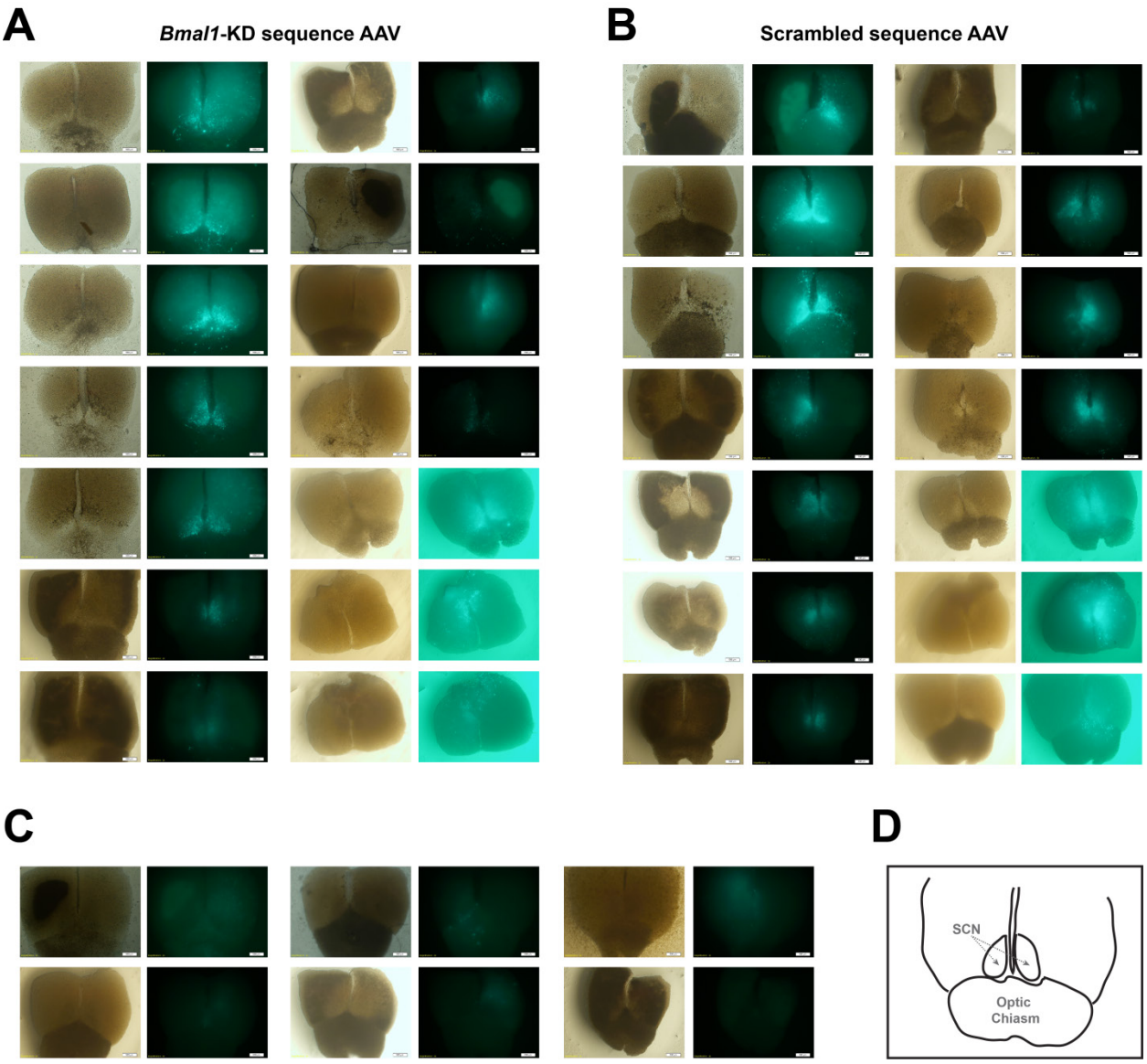

Figure S1. Additional representative images of coronal organotypic SCN explants of SCN-*Bmal1*-KD (**A**) and control mice (**B**) to confirm correct location of injections based on GFP expression patterns. (**C**) Animals that did not show GFP expression in the SCN were excluded from the study. (**D**) Schematic figure of cultured SCN explant showing approximate locations of SCN and optic chiasm.

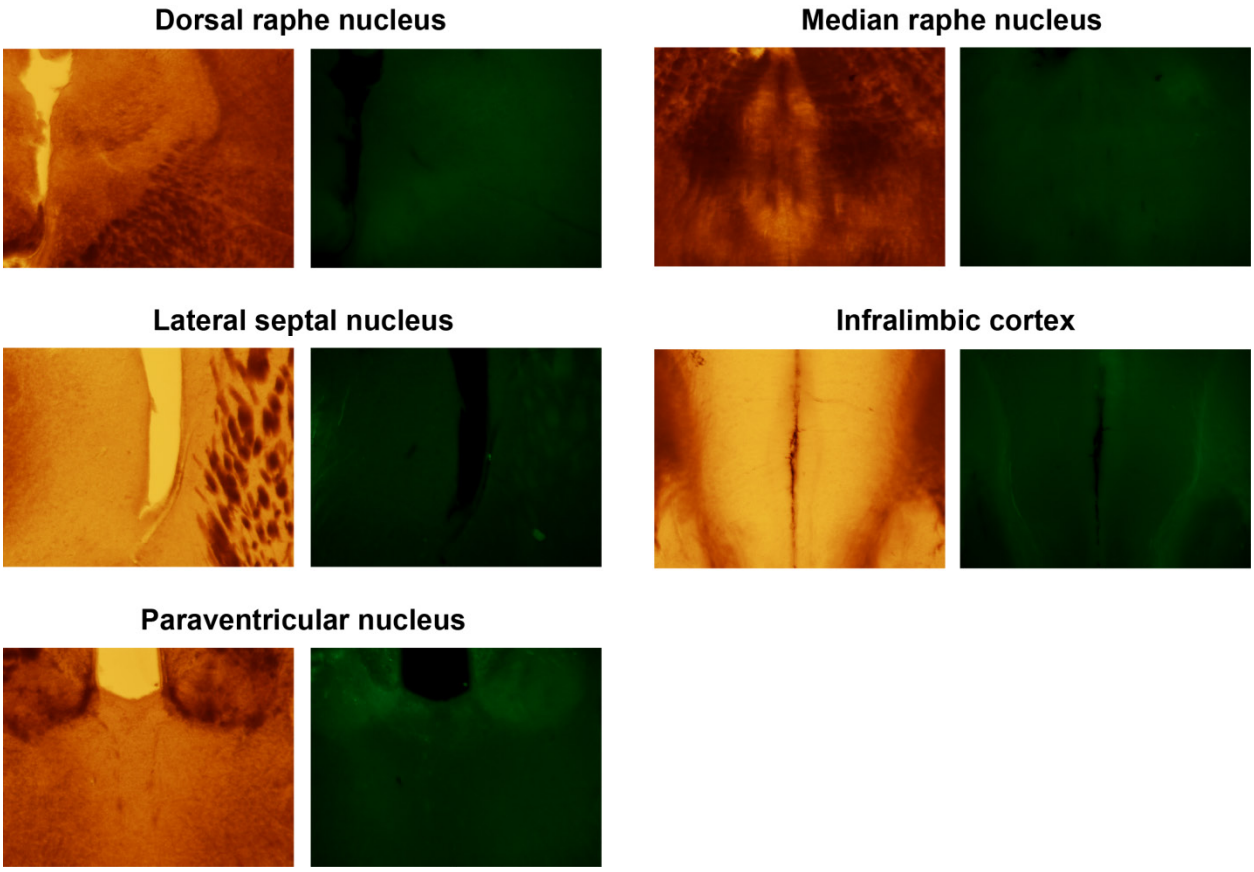

Figure S2. Based on GFP expression, no significant retrograde infection of *Bmal1*-KD AAVs injected into the SCN was detected in brain areas that project to the SCN.

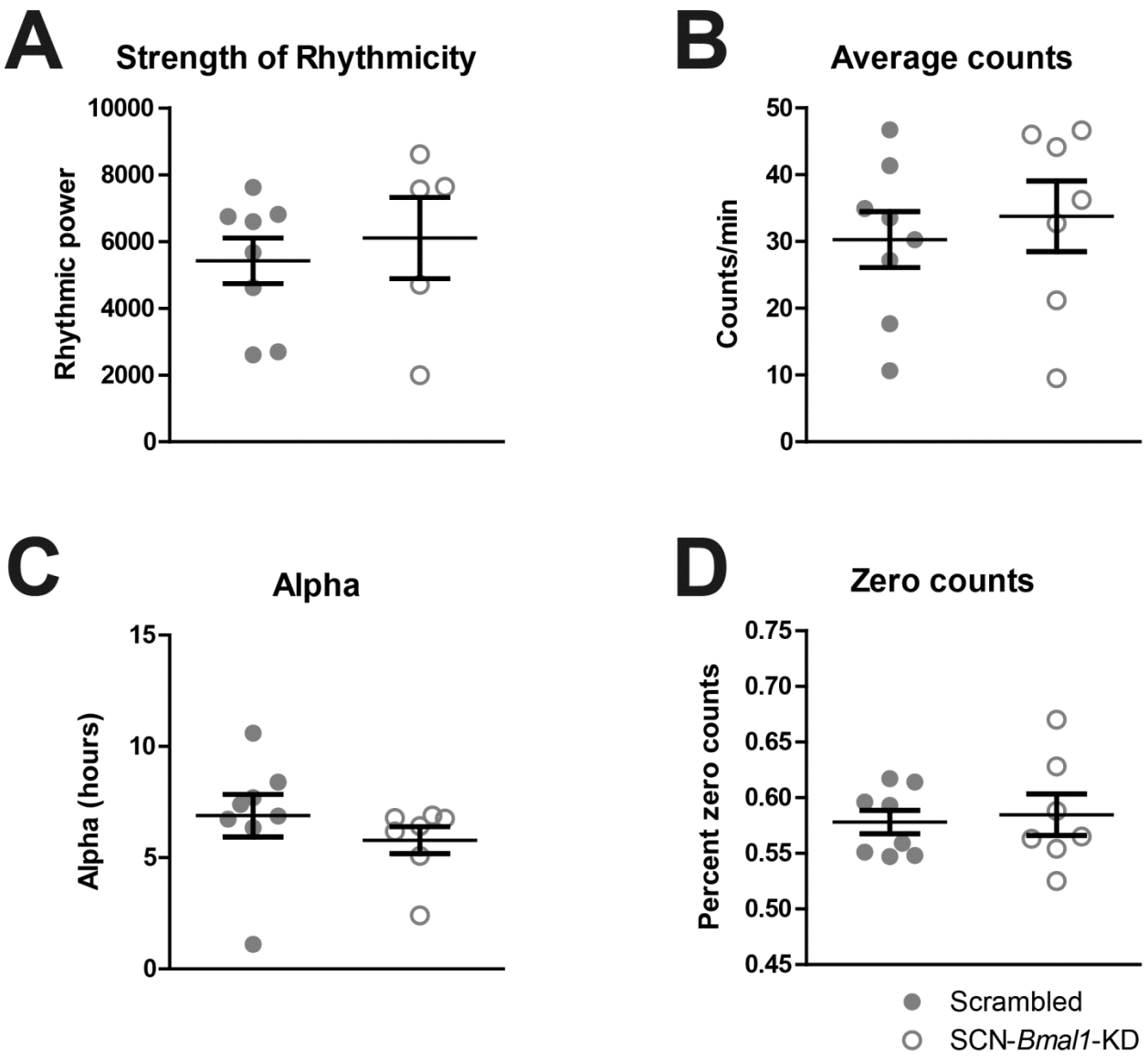

Figure S3. Circadian locomotor activity characteristics of SCN-*Bmal1*-KD and control mice. (**A**) Strength of rhythmicity (relative spectral power in the circadian range exceeding the level of statistical significance, in arbitrary units, chi-squared periodogram, ClockLab) [$t_{11} = 0.5311$]. (**B**) Average number of wheel-running activity counts per minute [$t_{11} = 0.2120$]. (**C**) Alpha (length of activity phase) [$t_{11} = 0.1034$]. (**D**) Percentage of 1 min intervals with zero wheel-running activity counts [$t_{11} = 0.1046$]. Data are shown as mean ± SEM; not significant; *t*-values and degree of freedom are given in square brackets (Student's *t*-test); $n = 8$, SCN-*Bmal1*-KD: $n = 5$.

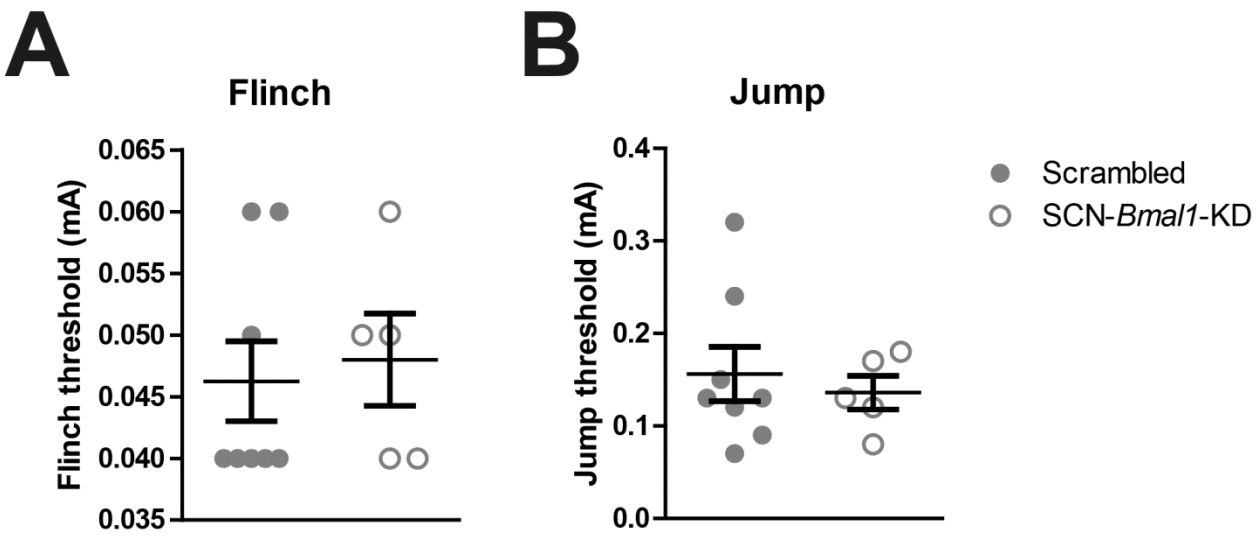

Figure S4. SCN-*Bmal1*-KD and control mice display similar pain sensitivity. Minimum electric shock intensities causing observable flinch (**A**) [t_{11} = 0.3457] or jump (**B**) [t_{11} = 0.5028] behaviors were measured. Data are shown as mean ± SEM; not significant; *t*-values and degree of freedom are given in square brackets (Student's *t*-test); *n* = 8, SCN-*Bmal1*-KD: *n* = 5.

Supplemental Reference

1. Landgraf D, Long J, Der-Avakian A, Streets M, Welsh DK (2015): Dissociation of learned helplessness and fear conditioning in mice: a mouse model of depression. PloS One 10:e0125892.