Supplemental information

MATERIALS AND METHODS

Immunohistochemistry

To determine the daily rhythm of ORX expression we used adjacent series of sections from the same animals used for *in situ* hybridization. Sections were fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4 (Sigma, 4% PFA) for 15 min. Following Phosphate buffered Saline (PBS) and 0.05% Tween-20 washing steps, sections were blocked with 10% normal donkey serum for 1h diluted in PBS and 0.3% Tween-20 (PBS-T), and then incubated overnight at 4°C with a goat anti-orexin A antibody diluted in PBS-T and 5% serum (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). For secondary labeling, sections were incubated in biotinylated donkey anti-sheep secondary antibody for 2h at room temperature (1:1000; Jackson ImmunoResearch, West Grove, PA) diluted in PBS-T. Sections were then washed in PBS and incubated in avidin-biotin complex (ABC; Vector Elite kit; Vector Laboratories, Burlingame, CA; 1:500 in PBS) for 1h at room temperature. After PBS-T washes, sections were incubated in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma), nickel sulfate 1% (Fisher Scientific, Pittsburgh, PA) and 0.015% hydrogen peroxide dissolved in distilled water.

For double c-Fos/ORX labeling, we used the ABC/nickel-enhanced 3,3′-diaminobenzidine tetrahydrochloride (NiDAB) method. Mice were deeply anesthetized with isoflurane and transcardialy perfused with 50 ml of saline solution (Sigma, 0.9%), followed by 50 ml of 4% PFA. Brains were extracted and postfixed in the same fixative overnight. Frozen sections (30 µm) were cut across the entire rostro-caudal hypothalamus (interaural 2.58mm to 2.10mm; Paxinos & Franklin 2001) and stored at 4°C. Following a blocking step with 10% normal

donkey serum for 1h diluted in PBS-T, sections were incubated in the rabbit anti-c-Fos primary antibody (Santa Cruz, Santa Cruz, CA, USA; 1:10,000) overnight at 4°C, and then in biotinylated donkey anti-rabbit secondary antibody for 2h at room temperature (1:1000; Jackson ImmunoResearch, West Grove, PA). Next, sections were incubated in avidin-biotin complex (ABC; Vector Elite kit; Vector Laboratories, Burlingame, CA; 1:500 in PBS) for 1h at room temperature. After washing in PBS-T, sections were incubated in 0.05% DAB, 1% nickel ammonium sulfate and 0.015% hydrogen peroxide dissolved in distilled water. c-Fos staining was followed by labeling for ORX-A. Sections were re-incubated with the blocking solution before incubation in the second primary antiserum, goat anti-ORX (1:5000) overnight at 4°C. The secondary antibody, biotinylated donkey anti-sheep IgG (1:1000), was applied for 2h at room temperature. After, sections were incubated in avidin-biotin complex for 1h. Sections were then washed again in PBS and incubated in a 0.05% solution of DAB containing 0.03% H_2O_2 without nickel ammonium sulfate to obtain a brown staining.

Cell counting procedures

Cells were visualized using a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with an Olympus DP50 digital camera (Olympus France, Rungis, France). Image caption was standardized using fixed lighting parameters on the microscope and camera software (Viewfinder Lite, Olympus). Since ORX are present in a large part of the LH (medial, lateral perifornical area), for quantification we selected neurons in the lateral part of the nucleus (Paxinos & Franklin 2001). For counting the intensity of ORX-positive cells, three bilateral images were collected in the region of the whole lateral hypothalamus with the highest concentration of positive cells (interaural 2.58-2.10mm; Paxinos & Franklin, 2001) using the NIH ImageJ software (Rasband, W.S., US National Institutes of Health, Bethesda MD, USA). For each animal, 1 rostral, 1 mid and 1 caudal LH sections were photographed and converted to 8-bit grayscale. The region of interest was

determined using a circle surrounding the labelled neurons and the integrated density of individual neurons was measured and subtracted from a background value taken from neighbour tissue with no staining. An average of 10 neurons per LH section was analysed and the mean integrated density was determined for each animal. For each time point the labeling intensity was calculated as the mean ± SEM.

For quantitative assessment of c-Fos expression in ORX cells, three sections where ORX staining was the highest were selected. Neurons were classified as double labeled according to the presence above background of black NiDAB (c-Fos) reaction product in the cell nucleus and brown (DAB) ORX in the cytoplasm. For c-Fos colocalization, ORX cells were counted for each animal. Total number of c-Fos/ORX double-labeled cells was estimated as the percentage of c-Fos/ORX co-labeled cells on the total number of ORX-positive cells.

Adipose tissue and hormonal determinations

For adipose tissue determination, total epididymal fat (mass of white adipose tissue) was obtained in both WT and KO mice. After decapitation, trunk blood was collected into 2 ml Eppendorf heparinised tubes containing 10µl of 4% EDTA. Blood samples were centrifuged at 5000 rpm for 10 min, and plasma was stored at -80°C before determination of plasma leptin concentration by radioimmunoassay. We used a mouse Leptin ELISA Kit (EZML-82K, Millipore, USA). The limit of sensitivity of the leptin assay was 0.05 ng ml−1.

Molecular analysis of the ORX promoter

Plasmids for luciferase assay

Full-length mouse cDNAs encoding, LAC-Z and REV-ERBα were cloned into a pSCT1 expression vector. REV-ERBα was fused in-frame to 2 N-terminal HA tags. *Bmal1* promoter was cloned into a pGL2 basic vector (Promega). This construct express the firefly luciferase

under control of the *Bmal1* promoter. It was used as positive controls for BMAL1/NPAS2 and REV-ERBα assays.

Orexin promoter (ORX) cloning and mutagenesis

The 1.6 kb region upstream the coding sequence of the orexin gene was subcloned by PCR into a pJet vector using a Phusion™ kit (Finnzymes) according to manufacturer's manual. PCR product was ultimately cloned into a pGL2 basic vector (Promega) to allow expression of the firefly luciferase under control of the *Orx* promoter.

To mutate the RORE site situated 1.6 kb upstream of the coding region of the orexin gene, a PCR using the Pfu polymerase was performed directly on the original Ox-pGL2 template with 48 bases long forward and reverse primers overlapping the RORE sequence. The RORE sequence (TGACCT) was replaced by an EcoRV site (GATATC) to allow both mutation of the RORE sequence and selection of positive clones. After the PCR, original templates were digested using Dpn1. E.coli bacteria were then transformed with the PCR product. All positive clones were isolated and digested with EcoRV to check for successful insertion of the restriction site. Plasmids were ultimately sequenced to confirm the mutation.

Luciferase assay

Proliferating NG108-15 cells cultured in DMEM High Glucose (Sigma-Aldrich) supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin (Amimed) were transfected with 0.5 µg of either a *Bmal1*::pGL2 vector (positive controls), a basic pGL2 (negative control), an *Ox*::pGL2 or a *mutated Ox*::pGL2. These plasmids were co-transfected with 0.1 µg of a LAC-Z expression vector to control for transfection efficiency, together with the indicated amounts of expression vectors using LINPEI Max (Polyplus Transfection) in 6-wells plates. Cells were harvested 24h after transfection. Cell lysates were then processed to perform a luciferase assay. Briefely 100 µl of Luciferase reaction buffer (25 mM Tris-HCl, 1

mM ATP, 10 mM MgAc, 0.1 mg/ml BSA) was added to 10 µl of the cell lysates in a 96-wells plate. 100 µl of Luciferin injection buffer (20 mg/l Coenzyme A, 30 mg/l Luciferin (Chimie Brunschwig), 12.5 mM PIPES) was added directly by a pump inside the luminometer and luminescence was measured for 10 sec. In parallel to the luciferase assay, 5 µl of the cell lysate was used to perform a 4-methylumbelliferyl β-D-galactopyranoside (MUG) assay. Briefely, 140 µl of a MUG solution (1mg/ml MUG in 100 mM Na3PO4 / 2 mM MgCl2 / pH=8.0) was added to cell lysates in a 96-wells plate and incubated 5 min at 37°C. The reaction was stopped by addition of 100 µl of 300 mM glycine / 15 mM EDTA / pH= 11.5. MUG is a fluorogenic substrate of ß-galactosidase and its hydrolysis product has an absorption/emission maxima between 360/449 nm that was measured directly in a plate reader. Luciferase values were normalized to MUG assay values. Luciferase activity was normalized using basic pGL2 as a control.

Chromatin immunoprecipitation

C57BL/6J WT mice, between 4 and 7 months of age, were kept in 12 h light-12 h dark conditions (LD 12:12; lights on at 8:00 AM) at an ambient temperature of 24.5–25.5°C. They were housed 2 to 5 per cage with food and water *ad libitum*. Mice were sacrificed at ZT-5 and ZT-17. Brain tissue (LH or cerebellum, n=3 per structure and time point) was immediately homogenized in PBS with 1% formaldehyde. Cross linking was continued for 5 min at room temperature. Cross-linking reactions were stopped by the addition of 25 ml of ice-cold 2.2 M sucrose in 150 mM glycine, 10 mM HEPES pH 7.6, 15 mM KCl, 2 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT and 0.5 mM PMSF. The homogenate was layered on top of a cushion of 2.05 M sucrose (containing the same ingredients and including 10% glycerol and 125 mM glycine) and centrifuged for 30 min at 24,000 rpm (100,000g). Nuclei were resuspended in 20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTAand sedimented at 1,500g. They were then resuspended in the same buffer supplemented with SDS (final

concentration, 1%), and sonicated 10 times for 15 s. The fragmented cross-linked chromatin was then diluted with the same buffer containing 1% Triton X-100. About 2x106 genomic copies were used as starting material for DNA quantification experiments. Coimmunoprecipitated DNA fragments were captured by protein A-agarose beads. Beads were collected by centrifugation, washed with lysis buffer supplemented with 1% Triton X-100 and 150 mM NaCl and cross-links were reversed overnight at 65 °C in 100 µl of 20 mM Tris pH 7.5, 0.1% NP-40, 10 µg/ml BSA, 0.1 mM EDTA, 0.02 µg/ µl salmon sperm DNA. Four microliters of each reaction were directly used in 20-µl real-time PCR reactions. The amount of DNA was quantified against a 2^{-CT} calibration curve prepared from mouse genomic DNA. Experiments were performed with 3 independent preparations of mouse brain chromatin. Antibodies against REV-ERBα are from a non-commercial batch made for U.A. lab. The specificity of the antibody was verified by competition experiments with peptides obtained from the same source.

Supplemental figure legends

Figure S1. (A) Daily rhythm of water drinking behavior in both WT and KO mice. Animals drank more water at night than at day and this difference is statistically significant ($F_{3, 27}$ = 11.75; $p < 0.01$). However, no differences were found between genotypes ($F_{1, 9}$ = 0.66; p = 0.43). **(B)** Daily intake (ml) and **(C)** preference (%) of saccharin at 1% in WT and KO mice. Intake and preference were not differences between genotypes (Intake, $F_{1, 9}$ = 1.49; $p < 0.25$, preference, $F_{1, 9}$ = 3.21; p = 0.1), although there was a significant daily rhythm of both intake and preference (Intake, $F_{3, 27}$ = 33.17; $p < 0.01$, Preference, $F_{3, 27}$ = 4.36; p = 0.01).

Figure S2. Representative double plot actograms of locomotor activity of WT **(A)** and KO **(B)** animals. On day 6 both animals received an i.p. injection the antagonist SB-334877 (20mg/kg), and 4 days later a vehicle injection. Vertical arrows indicate the time of injections (ZT12, lights off). Mean activity counts of WT **(C)** and KO **(D)** animals of the first three hours of activity onset (from ZT12-ZT15) in bins of 30 min. No differences were found between vehicle and the antagonist treated animals in both genotypes (WT, $F_{1, 8} = 1.16$; $p = 0.31$; KO, $F_{1, 6} = 0.007$; $p = 0.93$). For the percent of activity change **(E)**, in relation to night activity, after each treatment no differences where found between vehicle and the antagonist injection in both genotypes ($F_{1, 14} = 0.005$; $p = 0.94$). In the same animals we measured chow food intake, and no differences were found before vs. after treatments (vehicle vs. SB-334877) in both WT **(F)** and KO **(G)** mice (WT, $F_{1, 8} = 0.29$; $p = 0.6$; KO, $F_{1, 6} = 0.02$; $p = 0.87$). Open symbols show the individual values for each animal and the closed symbols the mean value (±SEM) for each condition (vehicle vs. SB-334877).

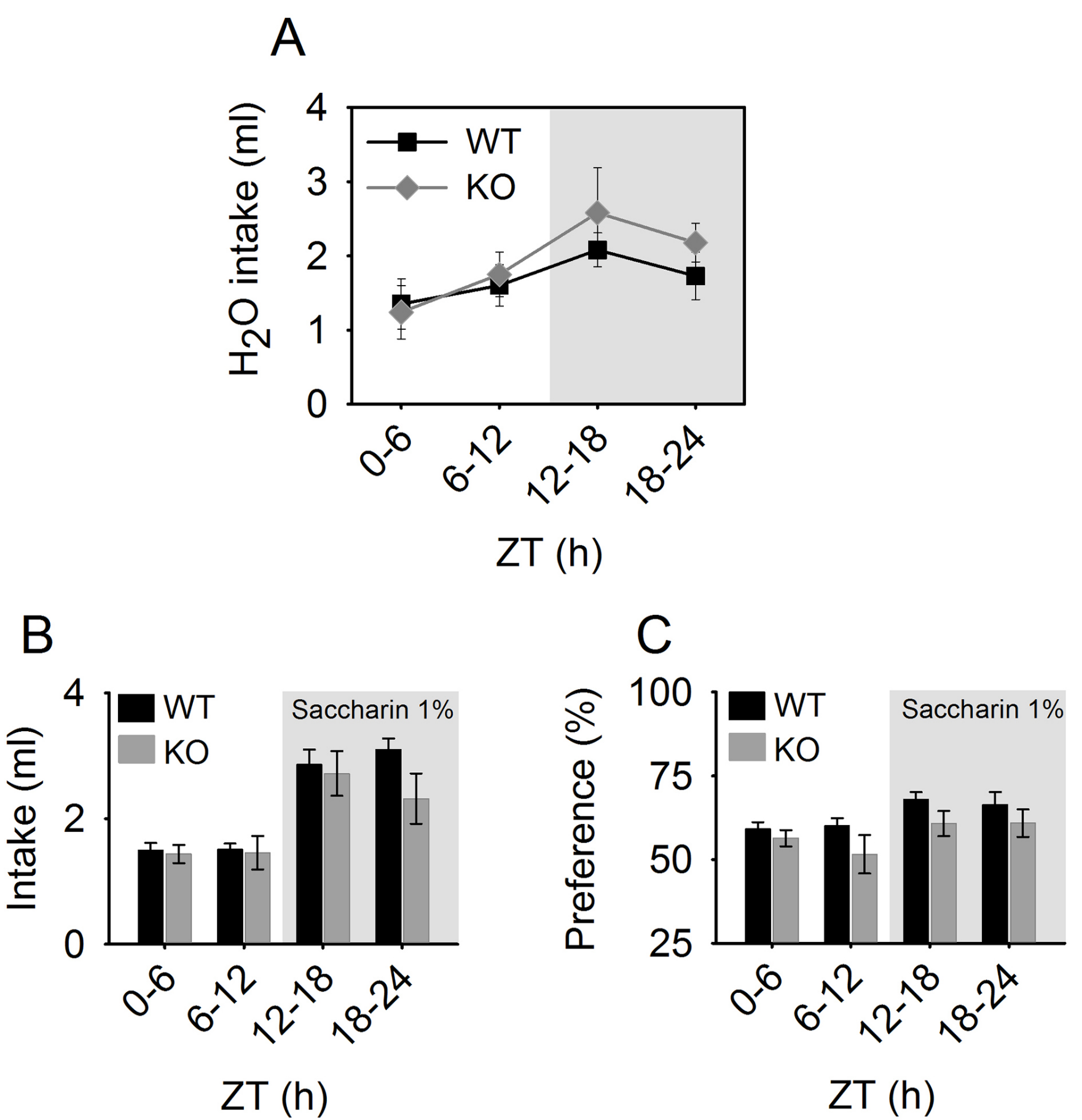
A
H2O intake (ml)
4
3
2
1
0
WT
KO
0-6
6-12
12-18
18-24
ZT (h)
B
Intake (ml)
4
2
0
WT
KO
Saccharin 1%
0-6
6-12
12-18
18-24
ZT (h)
C
Preference (%)
100
75
50
25
WT
KO
Saccharin 1%
0-6
6-12
12-18
18-24
ZT (h)

Figure S1

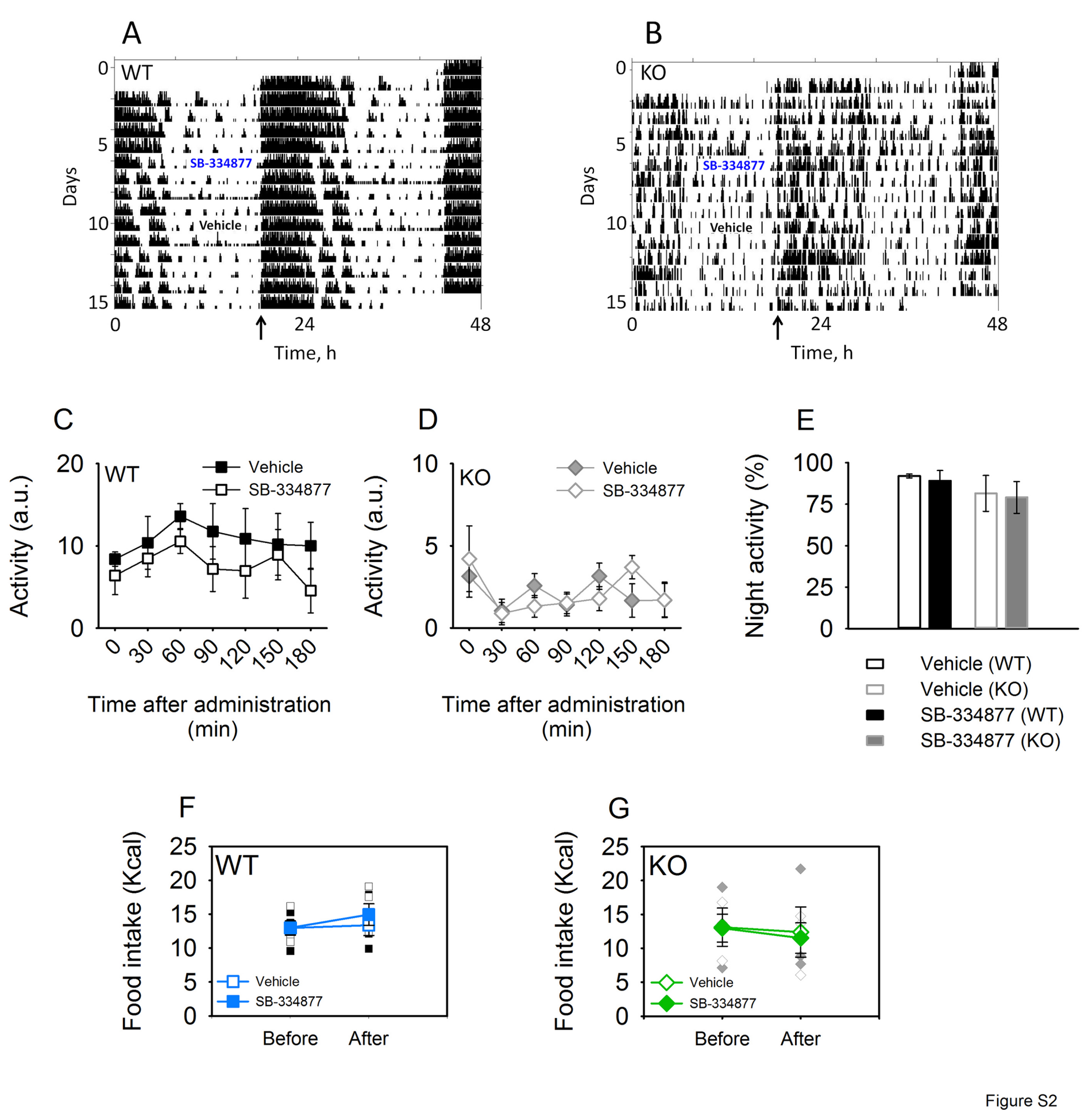
A
WT
SB-334877
Vehicle
Days
0
5
10
15
0
24
48
Time, h
B
KO
SB-334877
Vehicle
Days
0
5
10
15
0
24
48
Time, h
C
WT
Vehicle
SB-334877
Activity (a.u.)
20
10
0
0
30
60
90
120
150
180
Time after administration (min)
D
KO
Vehicle
SB-334877
Activity (a.u.)
10
5
0
0
30
60
90
120
150
180
Time after administration (min)
E
Night activity (%)
100
75
50
25
0
Vehicle (WT)
Vehicle (KO)
SB-334877 (WT)
SB-334877 (KO)
F
WT
Food intake (Kcal)
25
20
15
10
5
0
Vehicle
SB-334877
Before
After
G
KO
Food intake (Kcal)
25
20
15
10
5
0
Vehicle
SB-334877
Before
After

Figure S2