Electronic supplementary material

Methods

Mice Mice were entrained to a 12h light–dark cycle for 2 weeks prior to all physiological experiments. Genotyping by PCR for wild-type, conditional *Bmal1* and deleted *Bmal1* was performed as described [1]. Primers for the *Cre* transgene were: forward primer 5′-gcg gtc tgg cag taa aaa cta tc, reverse primer 5′-gtg aaa cag cat tgc tgt cac tt.

Real-time recordings of circadian bioluminescence Explants of pancreas from *Bmal1-Luc* mice [2] were dissected into tissue culture medium (2 ml DMEM) containing protease inhibitors (Complete Mini EDTA-Free tabs; Roche, Penzberg, Germany), 10% (vol./vol.) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 μmol/l D-luciferin (PJK, Kleinblittersdorf, Germany). Explants were cultured and luminescence recorded with a luminometer as described [2]. Data analysed were between days 1 and 5 of the time series; trend elimination was performed by fitting a polynomial (order 3) to the raw data.

Glucose tolerance tests For glucose tolerance tests at ZT 4.5 h, mice were placed in clean cages (without food) 2 h prior to the experiment and injected with glucose (2 mg/g bodyweight; 20% [wt/vol.] glucose in 0.9% [wt/vol] NaCl). Blood glucose levels were measured with a glucometer (Contour; Bayer, Leverkusen, Germany) with blood drawn from the lateral tail vein just before the injection of glucose and at 15, 30, 60, 120 and 180 min afterwards.

Glucose tolerance tests across the circadian cycle were performed similarly, but were done in the animal's home cage, with food removed 1 h before glucose injection. Mice were housed in a 12 h light–dark cycle, but were transferred to constant darkness on the day of

glucose injection. Injections and all measurements were performed under dim red light (conditions we had previously documented to produce no detectable phase shift of behavioural circadian rhythms [L.A. Sadacca and C.J. Weitz, unpublished observations]). Each mouse received one glucose injection at one time-point on each experimental day. Mice were then returned to a light–dark cycle for at least 1 week before the next experimental day. Over the 4 week experiment, each mouse was tested at all four time-points, i.e. circadian times (CT) 4.5, 10.5, 16.5 and 22.5, with no less than 1 week between experimental days. The timing of the injections was arranged so as to randomise as much as possible the order of injection times, e.g. some mice received injections at CT 4.5 in one week followed by CT 10.5 the next, whereas other mice received injections at CT 16.5 in the first week and CT 22.5 the next week. AUC was calculated for each mouse using the formula $\Sigma(x_j - x_i)((y_i + y_j)/2)$, where ($x_i$, y_i) represents the blood glucose value (y) at one timepoint (x). Background subtraction was performed by subtracting the area from zero to the lowest glucose value for each mouse. Circadian rhythm of glucose tolerance was assessed by ANOVA on AUC values from paired genotypes to assess effect of genotype, time or genotype × time interaction.

Insulin tolerance tests Mice were placed in clean cages (without food) at ZT 6.5 h and were injected intraperitoneally 2 h later with 0.5 to 1.0 units per kg bodyweight of Novolin-R (Novo-Nordisk, Bagsvaerd, Denmark) in 0.9% NaCl. Blood glucose was measured just before injection of insulin and at 15, 30, 45, 60 and 90 min thereafter.

Insulin determination Mice were placed in clean cages (without food) at ZT 0. After 2 h mice were injected with i.p. glucose (2 mg/g bodyweight; 20% glucose in 0.9% NaCl) and at 3 and 10 min after the injection, blood was collected (~50 ml), placed on ice, allowed to clot for 20 min at room temperature and cleared by centrifugation for 5 min at 3,000 *g*. Serum insulin

was measured using an ELISA kit (UltraSensitive Rat Insulin; CrystalChem, Downers Grove, IL, USA).

Immunohistochemistry and in situ hybridisation Mice (*Panc-Bmal1*$^{-/-}$) and *Pdx1-Cre* littermates (~7 weeks old, female, n = 3 per genotype) were killed and the entire pancreas dissected into 4% (wt/vol.) formaldehyde in PBS. Pancreases were post-fixed (2 h at 4ºC), fixation solution was removed by washes in PBS and tissue was cryoprotected overnight (4ºC, 30% [wt/vol] sucrose in PBS). Pancreases were then embedded in Tissue Tek (Sakura, Torrance, CA, USA) and frozen, and 10 μm sections were cut on a cryostat. Sections were dried and stored at -80ºC.

Insulin immunohistochemistry was performed using guinea pig anti-insulin (1:700; Dako), followed by donkey anti guinea pig secondary antibody (1:200; Invitrogen, Carlsbad, CA, USA). For double labelling of insulin and *Per1* or *Bmal1*, fluorescence in situ hybridisation was performed as described [3], using digoxigenin-labelled *Per1* and *Bmal1* probes. After development of the in situ hybridisation signal, insulin immunohistochemistry was performed as described above.

Islet number was estimated by counting the number of islets (identified by insulin immunohistochemistry) per 10 μm section in four to seven sections per mouse (n=3 per genotype). Islet area and staining intensity were measured by circling islets in images from insulin immunohistochemistry and measuring area and mean grey value with ImageJ software [4]. A total of 12 to 14 islets were analysed per genotype.

Glucose-stimulated insulin secretion Pancreatic islets were isolated from mice of the indicated genotypes and incubated overnight for recovery in DMED containing 1 mg/ml glucose (Sigma, St Louis, MO, USA) and 10% (vol./vol.) FBS. For glucose-stimulated

insulin secretion, the isolated islets were incubated in KRBH (129 mmol/l NaCl, 4.8 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 5 mmol/l $NaHCO_3$, 10 mmol/l HEPES, 0.1% (vol./vol.) BSA) with 2.8 mmol/l D-glucose for 1 h for wash. Triplicates of ten islets were then incubated for 75 min in KRBH buffer containing either low (2.8 mmol/l) or high (16.7 mmol/l) D-glucose. The insulin levels in the supernatant fractions were measured using an ELISA kit (Mouse Insulin Ultrasensitive; Alpco Diagnostics). For total insulin content, groups of ten isolated islets were incubated overnight in acidic ethanol (1.5% [vol./vol.] HCl in 70% [vol./vol.] ethanol) at −80ºC, followed by centrifugation for 10 min at 20,000 *g* and measurement of insulin content in the supernatant fraction as above.

Western blots Nuclear extracts of the indicated tissues were made by standard methods and western blot analysis performed with rabbit anti-BMAL1 antiserum (1:1000; Covance, Princeton, NJ, USA).

Behavioural recordings and analysis Running-wheel activity and feeding activity using an infrared feeding monitor (Mini Mitter, Bend, OR, USA) were recorded (Clocklab; Actimetrics, Wilmette, IL, USA) for a period of 2 weeks with mice under a 12:12 h light–dark cycle and for 3 subsequent weeks with mice in constant darkness. Behavioural variables were analysed using Clocklab analysis software. Free access to food was provided throughout the experiment.

Cre recombinase indicator assays Mice (*Pdx1-Cre*) were crossed with *R26r Cre* indicator mice (Jackson Laboratories, Bar Harbor, ME, USA) [5]. Mice heterozygous for the *R26r* allele and with a single copy of the *Pdx1-Cre* transgene were analysed. Mice were killed and tissues dissected, embedded in Tissue Tek and frozen in liquid nitrogen. Sections (20 μm)

were cut on a cryostat and slides were processed with X-gal (Gold Biotechnology, St Louis, MO, USA) to identify cell lineages marked by *Cre* recombination.

Body composition analysis Mice (24 weeks of age) were killed by CO_2 inhalation (ZT7-ZT9), and the stomach and intestines removed. The carcasses were weighed, dried at 60ºC and saponified in a solution of two parts ethanol to one part 30% (wt/vol.) potassium hydroxide (KOH) at 60ºC , which converts all triacylglycerol to glycerol by de-esterification. The resulting carcasates were analysed for glycerol content using a reagent (Free Glycerol Reagent; Sigma) according to the manufacturer's protocol.

References

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