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Rev-erbα modulates the hypothalamic orexinergic system to influence pleasurable feeding behaviour in mice

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ABSTRACT

The drive to eat is regulated by two compensatory brain pathways termed as homeostatic and hedonic. Hypothalamic orexinergic (ORX) neurons regulate metabolism, feeding and reward, thus controlling physiological and hedonic appetite. Circadian regulation of feeding, metabolism and rhythmic activity of ORX cells are driven by the brain suprachiasmatic clock. How the circadian clock impacts on ORX signalling and feeding-reward rhythms is, however, unknown. Here we used mice lacking the nuclear receptor REV-ERBα, a transcription repressor and a key component of the molecular clockwork, to study food-reward behaviour. *Rev-Erbα* mutant mice showed highly motivated behaviours to obtain palatable food, an increase in the intake and preference for tasty diets, and in the expression of the ORX protein in the hypothalamus. Palatable food intake was inhibited in animals treated with the ORX1R antagonist. Analyzing the *Orx* promoter, we found Retinoic acid-related Orphan receptor Response Element binding sites for *Rev-Erbα*. Furthermore, *Rev-Erbα* dampened the activation of *Orx in vitro* and *in vivo*. Our data provide evidence for a possible repressive role of *Rev-Erbα* in the regulation of ORX signalling, highlighting an implication of the circadian clockwork in modulating food-reward behaviours with an important impact for the central regulation of overeating.

Keywords circadian, compulsive, food-reward, orexin, palatable, *Rev-Erbα*.

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INTRODUCTION

In industrialized countries, living in a food-rich world, the incidence of obesity has increased alarmingly, caused in part by calorie overconsumption. Beyond metabolic need, hedonic properties of food are a major determinant for overeating (Berridge *et al.* 2010). In the brain, food intake is regulated by complementary homeostatic and hedonic mechanisms (Saper *et al.* 2002). While hypothalamic nuclei are mainly regulating the homeostatic drive of feeding, cortico-limbic structures control rewarded feeding behaviours (Berridge *et al.* 2010). The circadian clock in the hypothalamic suprachiasmatic nucleus (SCN) regulates daily rhythms of feeding behaviour (Bass & Takahashi, 2010). Circadian cycles of SCN activity rely on a molecular machinery, which consists of interlocked feedback loops encoded by clock genes such as *Clock*, *Npas2*, *Bmal1*, *Per1-2*, *Cry1-2*, *Rorα-β-γ* and *Rev-Erbα-β* (Takahashi *et al.* 2008). REV-ERBα is a nuclear receptor protein expressed rhythmically in the SCN and peripheral organs (Preitner *et al.* 2002). The *Rev-Erbα* promoter itself contains a response element for CLOCK::BMAL1. Within the promoter of *Bmal1*, REV-ERBα proteins compete with ROR proteins for Retinoic acid-related Orphan receptor Response Element (RORE) binding sites. While ROR induces *Bmal1* transcription, it is inhibited by REV-ERBα (Preitner *et al.* 2002).

Rev-Erbα is an important molecular link between circadian rhythms, metabolism and behaviour (Preitner *et al.* 2002; Everett & Lazar, 2014). Interestingly, recent data showed a hyper-dopaminergic activity accompanied by cognitive dysfunction and mood-related behaviours in *Rev-Erbα* mutant mice (Jager *et al.* 2014; Chung *et al.* 2014). Dopamine (DA) is a critical neurotransmitter

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implicated in motor behaviours directed to obtaining desired food (Berridge & Robinson, 1998). Thus, *Rev-Erbα* plays an important role in circadian and non-circadian functions. Earlier results suggest that some clock genes participate in the development of drug consumption (Abarca *et al.* 2002; Spanagel *et al.* 2005) and overeating (Turek *et al.* 2005). However, the role of *Rev-Erbα* in the regulation of compulsive or addictive feeding remains unknown.

In the lateral hypothalamus (LH) the orexinergic system (ORX) has been implicated in the regulation of sleep and feeding (Harris *et al.* 2005; Cason *et al.* 2010; Mahler *et al.* 2014; Sakurai, 2014), and in modulation of the DAergic mesolimbic reward circuit (Choi *et al.* 2010; Mahler *et al.* 2014). Moreover, the SCN clock controls the daily rhythms of ORX activity (Deboer *et al.* 2004; Zhang *et al.* 2004).

Therefore, we hypothesize that *Rev-Erbα*, as for the DAergic system (Chung *et al.* 2014), has a repressive action on the activity of the ORX system, which in turn is involved in the regulation of hedonic feeding behaviour.

MATERIALS AND METHODS

Animals and housing

Four- to six-week-old C57BL/6 J wild-type (WT) and *Rev-Erbα* mutant (KO) male mice were housed in individual cages with a 12-h light/12-h dark cycle (LD - zeitgeber time (ZT) 0 represents lights on at 8:00 AM) with controlled temperature (24 ± 1°C) and humidity (55 ± 5 percent). Mice were allowed free access to pellet chow (UAR, Epinay sur Orge, France) and water. All animal protocols were in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication 86–23, revised 1985), a local ethics committee (licence no. 67–378 to J.M.), and the French and Swiss laws of animal experimentation.

Two-bottle choice test for sucrose consumption

First, a two-bottle choice paradigm between water and 10 percent sucrose was conducted in WT ($n = 6$) and KO mice ($n = 5$). Mice were given unlimited access to two bottles of water for 1 week to evaluate whether they present place preference. It was followed by a choice between either water or 10 percent sucrose (Sigma-Aldrich) for 1 week. This concentration was determined to be highly preferred in C57BL/6 J mice according to previous data (Lewis *et al.* 2005). Sucrose intake (ml), preference (percent) and food intake were evaluated every 6 h (ZT0-6, ZT6-12, ZT12-18 and ZT18-24) for 24 h to obtain daily rhythms. Note that in all the two-bottle choice procedures, the position of the two bottles was changed daily to avoid location preference.

Sucrose deprivation test

In a second experiment, (sucrose-relapse test) mice (WT, $n = 8$; KO, $n = 6$) were given unlimited access to two bottles of water for 2 days, followed by water and 10 percent sucrose for 7 days. Sucrose deprivation (two bottles of water only for 3 days) followed, and then a re-exposure to 10 percent sucrose for 5 days (from ZT-12). Sucrose intake was measured before and after the 3 days of deprivation.

Saccharin intake and preference

To evaluate saccharin intake and preference both WT ($n = 6$) and KO ($n = 5$) mice were exposed to a two-bottle choice paradigm. Animals were first exposed to two bottles of water for 2 days. Then, one bottle was filled with saccharin at 1 percent (Sucralose Auchan, France). To avoid neophobia to saccharin, we evaluated intake and preference 2 days after the exposure.

Two-feeding choice test for food consumption

Both WT ($n = 4$) and KO ($n = 5$) mice maintained on laboratory chow food were permanently exposed to highly palatable snacks (commercial chocolate, KINDER® bar, 550 kcal/100 g) for 3 weeks. Regular food and chocolate position changed (right versus left) daily to avoid location preference. Food intake (Kcal) and preference (percent) of both regular and palatable food were measured every 6 h (ZT0-6, ZT6-12, ZT12-18 and ZT-18-24). Body weight was recorded weekly.

For c-Fos/ORX experiments, a control group (regular food only; WT and KO, $n = 5$-6) and a group receiving both food and chocolate for three weeks were used (WT and KO, $n = 6$). Animals were killed at ZT-14 (when both WT and KO ingest the highest amount of palatable food).

Food-motivation test

We used a test of motivation for food, in which 2 g of chocolate is suspended, at a height difficult to reach for mice, according to a previous study (Gondard *et al.* 2013). Both WT ($n = 6$) and KO ($n = 5$) mice were first habituated to the setup. Regular food (day 2) or chocolate (days 3–5) were then suspended in the cage. Mice behaviour was recorded every day for 5 min before lights off (between ZT-11.5 and ZT-12: higher intake and preference for chocolate) using a video camera. We quantified (with a double blind paradigm) the number of events selected as behaviours directed to the stimulus (biting, touching, jumping and carrying; Gondard *et al.* 2013) every 5 s on day 2 (chow food), and on the 3 days of chocolate exposure. We also quantified the latency to initiate directed behaviours on days 3–5 (Gondard *et al.* 2013).

In situ hybridization

Mice were killed by cervical dislocation at ZT-0, ZT-4, ZT-8, ZT-12, ZT-16 and ZT-20 ($n = 3–7$ per genotype and time point). Brains were rapidly removed and frozen. Eighteen-micrometre-thick coronal sections through the mediobasal hypothalamus (interaural 2.58 to 2.10 mm; Paxinos & Franklin 2001), were cut and mounted. *In situ* hybridization was performed as described previously in the same species (Mendoza *et al.* 2010). The antisense [^{35}S] UTP-labelled RNA probe for *mOrx* (a generous gift from Prof. Mieda, Kanazawa University) was synthesized using the MAXI-script T3/T7/SP6 kit (Ambion, Austin, TX, USA). Sections were exposed to a Kodak Biomax MR film (Sigma, Lyon, France). The intensity of hybridization signal was quantified in three consecutive sections and normalized to ^{35}S calibration microscales (Amersham Biosciences, France). The average value for each mouse was used to calculate the group means. Data were expressed as relative optical density (OD) values.

Immunohistochemistry

Slides were fixed in phosphate-buffered 4 percent paraformaldehyde (Sigma, 4 percent PFA), blocked with 10 percent normal donkey serum (NDS) and incubated with a goat anti-orexin A antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), and then in biotinylated donkey anti-sheep secondary antibody (1:1000; Jackson ImmunoResearch, West Grove, PA). Slides were then incubated in avidin-biotin complex (ABC; Vector Elite kit; Vector Laboratories, Burlingame, CA; 1:500 in PBS) and finally in 0.05 percent 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma), nickel sulfate 1 percent (Fisher Scientific, Pittsburgh, PA) and 0.015 percent hydrogen peroxide.

For double c-Fos/ORX labelling mice were deeply anesthetized with isoflurane and perfused with saline (Sigma, 0.9 percent), followed by 4 percent PFA. Brains were postfixed and frozen. Sections (30 µm) were cut across the hypothalamus. Following a blocking step with 10 percent NDS, sections were incubated in a rabbit anti-c-Fos primary antibody (Santa Cruz, 1:10,000), and then in biotinylated donkey anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch). Next, sections were incubated in ABC, followed by 0.05 percent DAB, 1 percent nickel ammonium sulfate and 0.015 percent H_2O_2. c-Fos staining was followed by labelling for ORX-A (1:5000). The secondary antibody, biotinylated donkey anti-sheep IgG (1:1000), was then applied. Sections were incubated in ABC followed by DAB containing 0.03 percent H_2O_2 (refer to supplemental information for detailed Immunohistochemistry protocol).

Cell counting procedures

Sections were visualized using a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France). For counting the intensity of ORX-positive cells, three bilateral images were collected in the LH (Paxinos & Franklin, 2001) using the NIH ImageJ software (Rasband, W.S., US National Institutes of Health, Bethesda MD, USA). The region of interest was determined using a circle surrounding the labelled neurons, and the mean integrated density of labelled neurons was measured and subtracted from a background value taken from neighbour tissue with no staining. The labelling intensity was calculated as the mean ± SEM.

For quantitative assessment of c-Fos expression in ORX cells, the total number of c-Fos/ORX double- labelled cells over total number of ORX-positive cells was estimated (percent) (complete procedure described in the Supporting Information).

Elisa ORX determination

For ORX determination in cerebrospinal fluid (CSF), samples were collected in WT and KO mice from the cistern magna at ZT-14 (Liu & Duff, 2008). We used two groups of mice receiving either only regular food or both food and chocolate ($n = 7$-8 per genotype and feeding condition) for 3 weeks. Seven to ten microlitres of CSF can be obtained per mouse; thus, CSF samples were pooled for each group to obtain the 50 µl required for the assay. Moreover, frozen brain regions containing the ventral tegmental area (VTA—about 25 mg) were microdissected from 600 µm sections. Animals were killed at ZT-14 ($n = 3–4$ per genotype and feeding condition), after 3 weeks of either food regimen. Brain tissue was homogenized, centrifuged and ORX concentration was determined in 50 µl supernatant using an ORX-A ELISA Kit (FEK-003-30; Phoenix Pharmaceuticals) according to manufacturer's protocol.

Effect of SB-649868 on palatable feeding behaviour

To evaluate the role of OXR antagonists on palatable feeding behaviour in mice, the OXR-1R antagonist SB-649868 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl urea hydrochloride; Tocris, Ellisville, Missouri) was tested in both WT and KO mice. Mice were exposed during 1 week to unlimited palatable and regular diets. Animals were injected intraperitonealy at ZT-12 (when the highest amount of palatable food is ingested) with either vehicle or SB-334867 at 20 mg/kg ($n = 5–7$). Feeding behaviour was measured 24 h later. SB-334867 was dissolved in 10 percent (w/v) (2-hyroxypropyl)-β-cyclodextrin/10 percent dimethyl sulfoxide in sterile water.

The 20 mg/kg dose of SB-334867 used was selected according to previous studies (Sharf *et al.* 2010).

Locomotor activity recordings

To evaluate the effects of SB-334788 on locomotion, animals were housed in individual cages with food and water *ad libitum* under a LD cycle 12–12 h. General locomotion was recorded in 5 min bins using infrared sensors (Circadian Activity Monitoring System, Lyon, France) and plotted as actograms. Clocklab software (Actimetrics, Wilmette, IL) was used to determine total activity of each animal under different experimental conditions (vehicle versus antagonist).

Effects of SB-334788 on locomotion and feeding

After at least 1 week of habituation animals were treated with vehicle or the ORX-1R antagonist (20 mg/kg) at ZT12 (lights off) in counterbalance design. WT ($n = 5$) and KO mice ($n = 4$) were treated at least twice with vehicle and SB-334788. We evaluated activity changes 3 h after the treatment in 30 min blocks. Moreover, we compared the percent of activity change of nighttime activity on the day of injections with the preceding day (baseline).

Molecular analysis of the ORX promoter: plasmids for luciferase assay

Full-length mouse cDNAs encoding REV-ERBα and LAC-Z were cloned into a pSCT1 expression vector. *Bmal1* promoter was cloned into a pGL2 basic vector (Promega). This construct expresses the firefly luciferase under control of the *Bmal1* promoter (refer to Supporting Information).

Orexin promoter (ORX) cloning and mutagenesis

The 1.6 kb region upstream the coding sequence of the orexin gene was subcloned by PCR using a Phusion™ kit (Finnzymes) according to manufacturer's manual and was ultimately cloned into a pGL2 basic vector. To mutate the RORE site (TGACCT) situated 1.6 kb upstream of the coding region of the orexin gene, it was replaced by an EcoRV site (GATATC) to allow both mutation of the RORE sequence and selection of positive clones (refer to Supporting Information).

Luciferase assay

Proliferating NG108-15 cells were transfected with 0.5 µg of either a *Bmal1*::pGL2 vector (positive control), 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. Cells were harvested 24 h after transfection and cell lysates were processed to perform a luciferase assay. Luciferase values were normalized to a 4-methylumbelliferyl β-D-galactopyranoside assay that was performed on the same cell lysates (refer to Supporting Information for detailed procedure).

Chromatin immunoprecipitation (ChIP)

WT C57BL/6 J mice were sacrificed at ZT-5 and ZT-17. Brain tissue (LH or cerebellum, $n = 3$ per structure and time point) was homogenized in 1 percent formaldehyde, cross-linked and layered in 2.05 M sucrose. After sonication, coimmunoprecipitated DNA fragments were isolated and used in subsequent real-time PCR reactions for quantification (refer to Supporting Information for detailed procedure).

Statistical analysis

All results are indicated as mean ± SEM. We used one-way or two-way ANOVA's and LSD (least significant difference) as *post hoc* test. In some cases repeated measured two-way ANOVA was used. Significance level was set at $p < 0.05$. Statistical analysis was performed with the statistical package Statistica (version 8.0; StatSoft Inc., 2007, FRANCE).

RESULTS

Feeding-reward phenotype in Rev-erbα mutant mice

We investigated the feeding behaviour for both regular and palatable foods in WT and KO mice. First, we used a two-bottle choice paradigm with a 10 percent sucrose solution. Both WT and KO mice did show a daily rhythm of sucrose intake (Fig. 1B; $F_{3,\ 27} = 13.0$, $p < 0.001$) and preference (Fig. 1C; $F_{3,\ 27} = 4.97$, $p = 0.007$), and drank more sucrose than water mainly at night. KO mice had increased intake and preference for sucrose compared with WT animals at night (Fig. 1B; $F_{3,\ 27} = 3.06$, $p = 0.04$; Fig. 1C; $F_{3,\ 27} = 3.23$; $p = 0.03$). No differences were found in rhythms of regular feeding behaviour between genotypes (Fig. 1A; $F_{3,\ 27} = 0.79$; $p = 0.5$). Moreover, we did not observe any difference in the intake and preference for a non-caloric sweet solution between KO and WT mice (Fig. S1).

To establish if this behaviour applied to other types of reward, we evaluated whether the intake of a highly palatable food (chocolate) was also increased in KO mice. Both WT and KO animals showed a significant daily rhythm of intake for chow (Fig. 1D; $F_{3,\ 21} = 5.05$, $p = 0.008$) and palatable food (Fig. 1E; $F_{3,\ 21} = 12.33$, $p < 0.001$) with maximal intake at night. Similarly to sucrose consumption, KO mice showed a higher intake

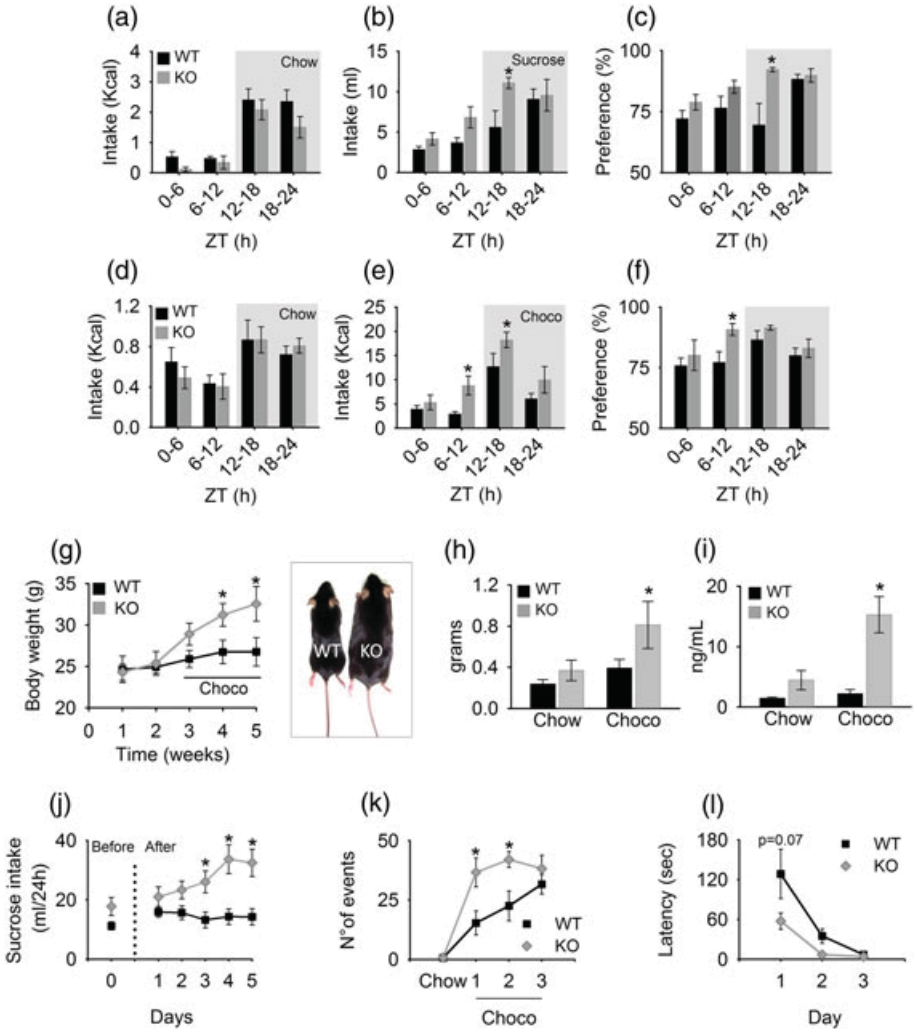

Figure 1 Feeding-reward phenotype in *Rev-Erbα* mutant mice. (a) Daily intake of regular chow food, (b) 10 percent sucrose and (c) preference for sucrose versus water in WT and KO mice exposed to the sucrose preference test. KO mice showed a significant intake and preference for sucrose at the early night (from ZT-12 to ZT-18; *$p < 0.05$ LSD post-hoc). (d) Daily intake of regular food, (e) chocolate and (f) preference for chocolate versus chow food in WT and KO mice during the chocolate preference test. KO mice showed a significant chocolate intake during the night period (*$p < 0.05$ LSD post-hoc). Preference for chocolate was significantly higher in KO mice only at the late day (from ZT-6 to ZT-12; *$p < 0.05$ LSD post-hoc). Shaded gray areas on graphs represent the night period. (g) Body weight changes in both WT and KO mice exposed to palatable food (chocolate). KO mice gained significantly more weight during the second and third week of chocolate exposure (*$p < 0.05$ LSD post-hoc between genotypes). (h) Mass of adipose tissue and plasma leptin (i) concentrations are higher in KO mice eating regular food + chocolate. Data are plotted as mean ± SEM ($n = 4$-5 per genotype and feeding condition; *$p < 0.05$ LSD post-hoc between genotypes). Refer to Supporting Information for detailed protocols. (j) Sucrose deprivation test. Total daily intake of 10 percent sucrose in WT and KO animals before (day 0) and after sucrose deprivation (day 1–5). Sucrose intake was significantly increased by re-introduction of sucrose solution following the period of deprivation as compared with the baseline drinking intake in KO mice (*$p < 0.05$ LSD post-hoc between genotypes). (k) Total number of behaviours (biting, touching, jumping and carrying; Gondard *et al.* 2013) in WT and KO mice directed to chow or chocolate suspended (days 1–3). From days 1 and 2, KO animals showed significantly more direct behaviours to the chocolate than WT mice (*$p < 0.05$ LSD post-hoc between genotypes). (l) Latency (s) to initiate behaviours directed to manipulate and eat the suspended chocolate during the 5 minutes of recording. KO animals showed shorter latency on days 1 and 2, although it was only close to statistical significance (ANOVA $p = 0.07$ between genotypes). All results are indicated as mean ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

(Fig. 1E; $F_{1,\ 21} = 13.7$, $p = 0.007$) and preference (Fig. 1F; $F_{1,\ 21} = 18.4$, $p = 0.003$) for chocolate compared with WT mice.

KO mice eating chocolate showed progressive body weight gain (Fig. 1G; $F_{4,\ 28} = 10.94$, $p < 0.001$) which was significantly higher than WT animals ($F_{4,\ 28} = 3.54$, $p = 0.01$). Furthermore, adipose tissue and plasma leptin concentrations were higher in KO mice fed with chocolate (Fig. 1H; $F_{1,18} = 6.14$, $p = 0.02$, Fig. 1I; $F_{1,14} = 11.02$, $p = 0.005$).

To evaluate the motivational state of animals for palatable foods, in a second experiment, mice were

submitted to a sucrose deprivation test. In the last part of the experiment, KO animals exhibited a significant increase in sucrose intake compared with WT mice after deprivation (relapse-like behaviour; Fig. 1J; $F_{1, 60} = 10.03$, $p = 0.008$), which was progressively increasing during the last 3 days of sucrose re-exposure (Fig. 1J; ANOVA with repeated measures: time, $F_{5, 60} = 23.9$, $p < 0.001$; interaction genotype X time, $F_{5, 60} = 18.68$, $p < 0.001$).

Additionally, we quantified the behavioural attempts to obtain palatable food. Whereas both genotypes did not show interest in obtaining suspended regular food (chow diet), on days 1–3 both genotypes showed direct behaviours to obtain and eat chocolate (Fig. 1K; $F_{3, 27} = 33.12$, $p < 0.001$). The number of events was significantly higher in KO mice than in WT animals (Fig. 1K; ANOVA with repeated measures: genotype, $F_{1, 27} = 6.91$, $p = 0.02$; ANOVA with repeated measures: interaction genotype X time, $F_{3, 27} = 3.53$, $p = 0.02$).

Moreover, on day 1 of chocolate presentation, KO mice tended to take less time than WT animals to initiate the attempts to obtain palatable diet, although this difference failed to reach significance (Fig. 1L; $F_{1,16} = 4.18$, $p = 0.07$).

ORX expression is altered in Rev-erbα mutant mice

Among the hypothalamic systems regulating feeding behaviour, ORX cells in the LH play an important role. We found significant differences for the factor time ($F_{5, 32} = 3.38$, $p = 0.01$) in *Orx* mRNA concentration (Fig. 2A and 2C), with a main difference between ZT-4 (lower) and ZT-12 (higher) in WT (Post-hoc LSD test, $p = 0.04$) and KO mice (Post-hoc LSD test, $p = 0.002$). However, the factor genotype ($F_{1, 32} = 0.18$, $p = 0.66$) or the interaction time X genotype ($F_{5, 32} = 0.63$, $p = 0.67$) were not significant.

At the protein level, whereas no significant differences were found neither for the genotype factor ($F_{1, 32} = 3.31$, $p = 0.07$) nor for the factor time ($F_{5, 32} = 2.39$, $p = 0.05$), we found a significant effect of the interaction genotype X time for the intensity of ORX-positive cells (Fig. 2B and 2D; $F_{5, 32} = 3.79$, $p = 0.008$). In the LH of KO animals, intensity of staining was significantly higher than in WT mice at ZT-12, a time when animals initiate activity and feeding behaviour (Post-hoc LSD test, $p < 0.001$).

Then, we measured the percentage of active ORX cells (c-Fos expression) and ORX concentration in the CSF of WT and KO mice (Fig. 3A and 3B). For double c-Fos/ORX cells the ANOVA indicates a significant difference for both the genotype ($F_{1, 19} = 4.85$, $p = 0.04$) and the feeding condition ($F_{1, 19} = 23.14$, $p < 0.001$), with a marked effect of chocolate ingestion in the KO group (Post-hoc LSD test, $p = 0.04$) which was not observed for the regular feeding condition (chow diet; post-hoc LSD test, $p = 0.33$; Fig. 3A). CSF concentrations were not different between genotypes under regular feeding condition, but an increase in KO mice compared with WT animals was observed when they were under the chocolate feeding condition (Fig. 3B).

In the VTA, a main target of ORX neurons, ORX concentration showed a tendency to be higher in KO mice compared with WT animals under normal chow food conditions (Fig. 3C). However, in animals challenged with palatable chocolate, we found that ORX concentration in the VTA was higher in KO mice than in WT animals (Fig. 3C). The ANOVA indicates a significant difference for the factor genotype ($F_{1, 10} = 8.05$, $p = 0.01$) for chocolate intake (Post-hoc LSD test, $p = 0.02$).

In a last experiment, after 1 week of a food-choice paradigm, the effect of the ORX-1R antagonist SB-334867 ꟷ (20 mg/kg) on feeding behaviour was investigated at ZT-12, in both WT and KO mice. This time point correlates with both the main ingestion of chocolate and the increase in the number of active ORX cells. I.P. injections of SB-334867 at 20 mg/kg significantly decreased chocolate intake in both genotypes when compared with vehicle injection (Fig. 3D; WT, $F_{1, 8} = 11.54$, $p = 0.009$; KO, $F_{1, 12} = 10.38$, $p = 0.007$). However, for both genotypes, no significant effects of SB-334867 on chow food intake and locomotor activity were found (Figs S1 and S2).

The orexin gene is regulated by Rev-erbα *in vitro* and *in vivo*

ROR response elements (RREs) are binding sites for REV-ERBα. Mapping of the Orexin (*Orx*) promoter revealed 6 E-box like (CANNTG), 1 RRE (AGGTCA) and 1 RRE-like (one mismatch; Fig. 4A) sequences. To determine whether *Orx* is regulated by clock components, we cloned the 1.6 kb region upstream the coding sequence of the mouse *Orx* gene into a luciferase reporter vector. As expected, co-transfection of a *Bmal1*::pGL2 vector with *Rev-Erbα* in NG108-15 neuroblastoma cells resulted in a decreased activation at any dose used (0.02, 0.05 and 0.1 µg; Fig. 4B; $F_{3, 20} = 363.4$, $p < 0.001$). Co-transfection of the reporter construct with *Rev-Erbα* dampened the activation of *Orx* in a concentration-dependent manner (Fig. 4B; $F_{3, 28} = 8.19$, $p = 0.0004$). Mutation of the RRE sequence in the *Orx* promoter (blue in Fig. 4A) reversed repression of luciferase activity by *Rev-Erbα*, leading to increased luminescence at the lower *Rev-Erbα* concentration (0.02 µg; Fig. 4B; $F_{3,19} = 4.24$, $p = 0.01$).

To evaluate whether REV-ERBα protein is regulating *Orx in vivo* we performed chromatin immunoprecipitation (ChIP) at two different time points (ZT-5 and ZT-17) in the LH and cerebellum of mice. As expected, we found a rhythmic fixation of REV-ERBα on the *Bmal1* promoter

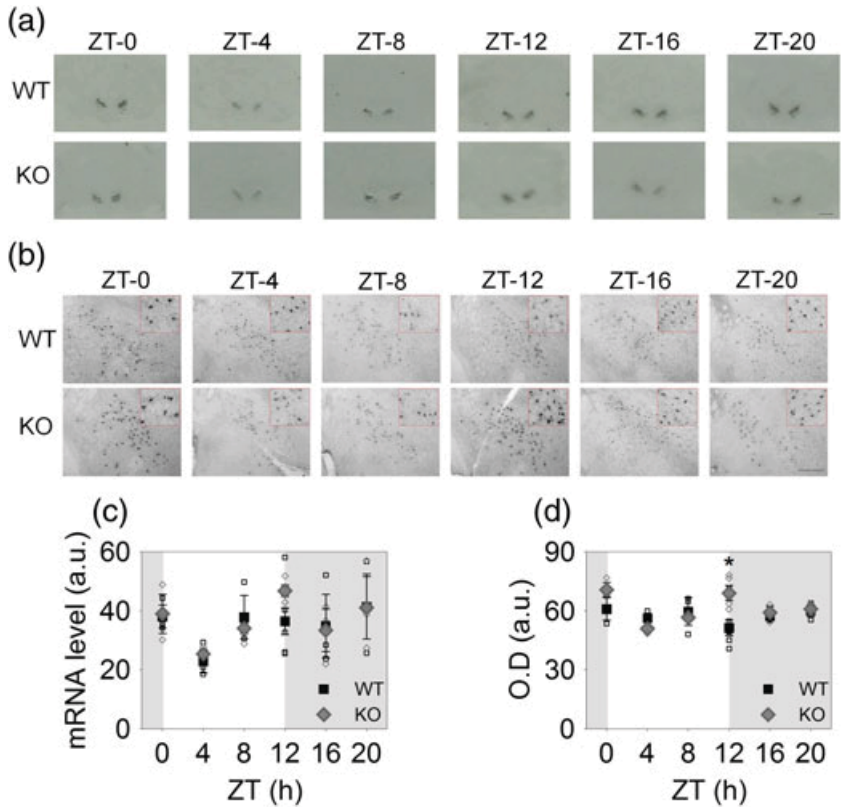

Figure 2 Daily profiles of *mOrx* and ORX expression in the LH of WT and KO mice. (a) Representative pictures showing *Orx* mRNA (*in situ* hybridization) and (b) protein (immunoreactivity) expression in the LH of WT and KO mice (scale bar, 1 mm and 100 μm, respectively). In B inset shows a close up of the LH region, in which ORX neurons intensity was quantified at the lateral part of the nucleus (from interaural 2.58 mm to 2.10 mm; Paxinos & Franklin 2001). (c) Daily profiles of the *mOrx* gene expression in WT and KO mice under a LD cycle and regular feeding conditions (ZT-0 beings lights on). A significant time effect was observed in both genotypes (ANOVA $p = 0.01$) with a higher expression at ZT-12 (lights off). This rise in the expression of *mOrx* was more important in KO mice; however, the difference did not reach the threshold of significance (ANOVA, genotype factor $p = 0.66$). (d) Daily profiles of the ORX protein expression in WT and KO mice under a LD cycle and regular feeding conditions. Relative intensity (mean optical density) of ORX immunoreactivity in the LH was significantly higher in KO mice at ZT-12 (*$p < 0.001$, LSD post-hoc between genotypes). Shaded gray areas on graphs represent the night period. Open symbols in C and D show the individual values for each animal and the closed symbols the mean value (±SEM) for each time point and genotype. [Colour figure can be viewed at wileyonlinelibrary.com]

in both structures with significantly higher values at ZT-17 (Fig. 4C; $F_{1,\ 8} = 111.89$, $p < 0.001$). ChIP for REV-ERBα on the albumin D site-binding protein gene (*Dbp*) promoter showed no rhythmic binding (negative control; Fig. 4C; $F_{1,\ 8} = 0.042$, $p = 0.84$). Interestingly, ChIP for REV-ERBα on the *Orx* promoter in the LH, but not in the cerebellum, revealed a day-night rhythm of fixation with a significant increase at ZT-17 (Fig. 4C; $F_{1,\ 8} = 35.44$, $p = 0.0003$).

DISCUSSION

Perturbations of the circadian clock influence food intake. *Clock* and *Rev-Erbα* mutant mice develop obesity (Turek *et al.* 2005; Delezie *et al.* 2012), indicating that the molecular circadian clock regulates metabolism and feeding behaviour. The present study shows that the nuclear receptor *Rev-Erbα* is important for modulating the ORX activity in the LH of mice in response to food-reward signals.

KO mice express an increase in the intake of palatable diets (sucrose, chocolate) and also in behaviours directed to food-rewards. However, this is not the case for non-caloric sweet solutions (saccharine). Thus, this phenotype may be because of both a metabolic alteration and a disruption in the central system regulating motivational behaviours. In fact ORX neurons from the perifornical area have been implicated in the endogenous production of glucose by the liver *via* the autonomus nervous system (Yi *et al.* 2009). So ORX may regulate feeding and motivational behaviour via its major brain targets but can also modulate the metabolic state of the organism through its functional links with peripheral organs.

The relapse-like behaviour observed in mutant animals after a sucrose deprivation test suggests that these mice show compulsive-like behaviours for palatable diets.

Compulsive-seeking behaviour has mainly been studied for drug addiction to evaluate to what extend drug-seeking persists regardless of negative consequences (Sanchis-Segura & Spanagel, 2006). Compulsive-feeding

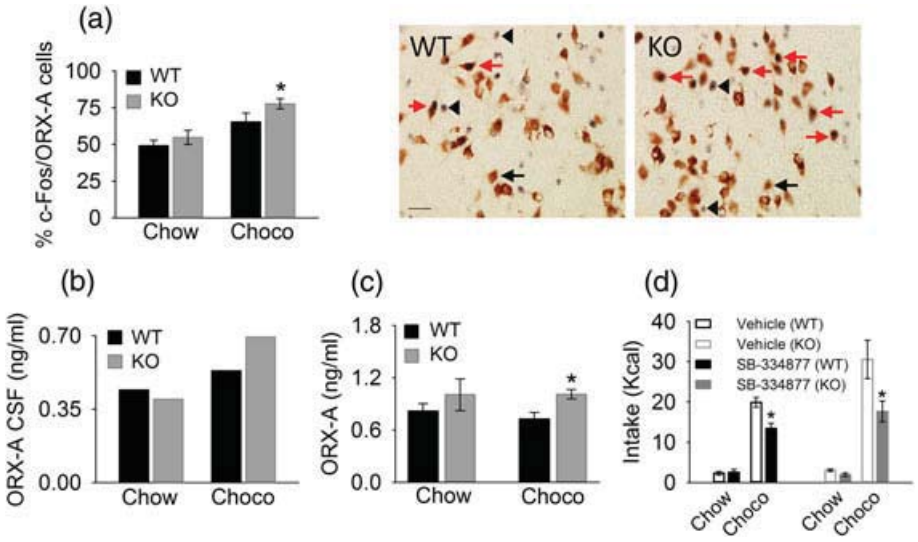

Figure 3 ORX activity in WT and KO mice challenged with chocolate. (a) *Left:* c-Fos expression in ORX cells in WT and KO mice. Mean values (±SEM) of the percent of double IHC of c-Fos/ORX neurons in WT and KO mice (*$p < 0.05$, LSD post-hoc test between genotypes). *Right:* representative images of LH sections from WT and KO mice. Red arrows point to dual-labelled cells, arrowheads c-Fos staining and black arrows ORX cells (scale bar 20 μm). Animals were killed at ZT-14 under regular *ad libitum* food conditions (Chow) or food + chocolate (Choco) access. (b) ORX concentrations in the cerebrospinal fluid (CSF) of KO and WT mice (at ZT-14) under the two feeding conditions (Chow versus Choco). (c) ORX concentration obtained in brain punches from the VTA of WT and KO mice at ZT-14 (*$p < 0.05$, LSD post-hoc test). (d) ORX antagonist reverses the food-reward phenotype in mice. Chow and choco intake in WT and KO mice treated with vehicle (control) or 20 mg/kg of the ORX-1R antagonist SB-334867 at ZT-12. Both WT and KO mice showed a significantly reduced palatable food intake following the drug injection (*$p < 0.05$, LSD post-hoc, vehicle versus antagonist). All results are indicated as mean ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

or addiction for natural rewards such as sucrose is a controversial issue, although we can propose that in the sucrose deprivation test *Rev-Erbα* KO mice showed an increase in the motivational drive to ingest sucrose. Interestingly in rats, highly sweet solutions can even override the rewarding effects of cocaine leading to an addiction-like state (Lenoir *et al.* 2007). Thus, palatable food seeking behaviours observed in animals may model eating behaviours observed in humans with compulsive overeating disorders (Avena *et al.* 2012).

Knowing that ORX neurons regulate energy balance, feeding and reward (Harris *et al.* 2005; Cason *et al.* 2010; Sakurai & Mieda, 2011; Mahler *et al.* 2014; Sakurai, 2014) and that *Rev-Erbα* impacts on the ORX system at a molecular level, one may infer that the observed phenotype in *Rev-Erbα* KO mice is at least partly because of deregulation of this system. Interestingly, *Rev-Erbα* KO mice also show dysfunction in learning tasks linked to higher levels of DA in the hippocampus (Jager *et al.* 2014). It was recently demonstrated that *Rev-Erbα* exerts a circadian control on DA, and mood-related behaviours, in the midbrain of mice (Chung *et al.* 2014). DA is a critical neurotransmitter mediating responses to natural rewards like food (Berridge & Robinson, 1998). DAergic neurons in the VTA receive projections from ORX cells in the LH and vice versa (Nieh *et al.* 2015), and activation of the ORX receptors in the VTA modulates behaviours related to motivation and addiction (Fadel & Deutch, 2002; Borgland *et al.* 2006). Therefore, it is possible that the increased preference for palatable food in *Rev-Erbα* KO mice is also modulated by DA via the ORX pathway.

A recent study has reported that while activation of the LH-VTA pathway increases seeking behaviour for sucrose, inhibition of this pathway reduces compulsive sucrose seeking (Nieh *et al.* 2015). Thus, in *Rev-Erbα* KO mice the observed rise in *Orx* activity could be a feedback signal to the DAergic system in the VTA leading to an increase in locomotion and reward-feeding behaviour.

Similarly to *Rev-Erbα* KO animals, *Clock* mutant mice are obese, hyperdopaminergic and show higher sensitivity to cocaine (McClung *et al.* 2005; Turek *et al.* 2005). Whether obesity and addiction share common mechanisms at a central level is not fully known, but similar central changes can occur following food or drug consumption, mainly in the DAergic and ORXergic systems (Cason *et al.* 2010; Kelley *et al.* 2005). Altogether, these findings lead to the hypothesis that clock genes participate in brain mechanisms regulating addictive behaviours for both food and drugs (Logan *et al.* 2014).

In rodents, a daily fluctuation of extracellular ORX content in the LH has been found, which is dependent on the SCN clock (Yoshida *et al.* 2001; Zeitzer *et al.* 2003; Deboer *et al.* 2004; Zhang *et al.* 2004). ORX expression rises at the activity onset and is higher at night in nocturnal rodents (Yoshida *et al.* 2001; Zeitzer *et al.* 2003). Similarly, in both WT and KO mice entrained to

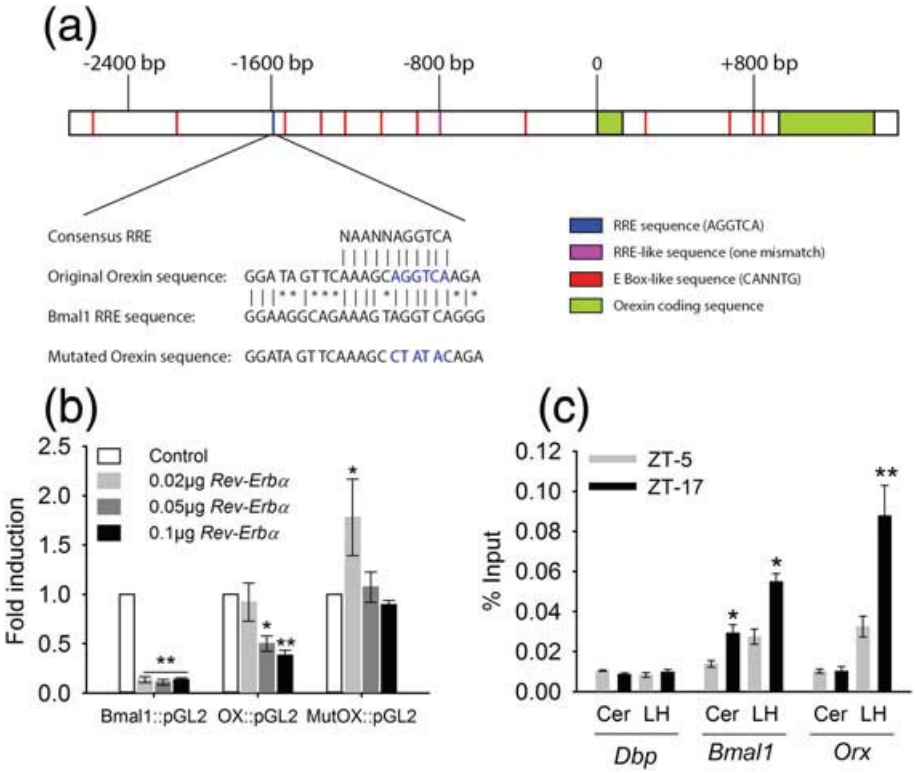

Figure 4 Regulation of the *Orx* promoter by *Rev-Erbα*. (a) Schematic representation of the mouse *Orx* promoter. Consensus RRE and *Bmal1* RRE have been aligned with the *Orx* promoter around its RRE sequence. Mutated sequence is also represented. (b) Transcriptional regulation of the *Orx* gene by *Rev-Erbα* in NG108-15 cells. Luciferase reporter plasmid containing the 1.6 kb region upstream the coding sequence of the orexin gene (OX::pGL2), *Bmal1* (positive control; *Bmal1*::PGL2) and MutOX (mutated RRE; MutOX::pGL2) were used for the transcriptional assays. Co-transfection of a *Bmal1*::pGL2 vector with *Rev-Erbα* in NG108-15 neuroblastoma cells resulted in a decreased activation at any dose used (0.02, 0.05 and 0.1 µg). Co-transfection of the reporter construct with *Rev-Erbα* dampened the activation of *Orx* in a concentration-dependent manner. Mutation of the RRE sequence in the *Orx* promoter (blue in Figure 4A) reversed repression of luciferase activity by *Rev-Erbα*, leading to increased luminescence at the lower *Rev-Erbα* concentration (0.02 µg). Each value represents the mean ± SEM (*$p < 0.05$, **$p < 0.01$ LSD post-hoc test, different from control). (c) Binding of REV-ERBα to the *Orx*, *Dbp* (negative control) and *Bmal1* (positive control) promoters as revealed by ChIP. Mouse brain tissue containing the LH or the cerebellum were collected at ZT-5 and ZT-17 ($n = 3$ per time point and brain structure, *$p < 0.05$, **$p < 0.01$ LSD post-hoc test, between time points). Each value represents the mean ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

a 12–12 h LD cycle, we observed higher expression of both the mRNA and protein expression of ORX at night. Interestingly, at the activity onset ORX protein expression was higher in KO mice compared with WT animals. However, when we determined ORX cell activity (c-Fos expression) or CSF concentrations in regular chow-fed animals, no differences were found between genotypes. This suggests that the peptide concentration is higher in mutant mice but needs a stimulus to trigger its release. The ORX system regulates the homeostatic drive to eat, and integrates motivational factors to control the hedonic drive of feeding (Cason *et al.* 2010; Choi *et al.* 2010; Mahler *et al.* 2014; Sakurai, 2014). In rats facing conditions of hedonic challenge (high saccharine intake) the number of ORX positive neurons is higher than in control animals (Holtz *et al.* 2012). Also, ICV administration of ORX stimulates food intake (Clegg *et al.* 2002). Conversely, ORX mutations decrease seeking behaviours for food intake and preference for sucrose solutions (Akiyama *et al.* 2004; Mieda *et al.* 2004; Baird *et al.* 2009; Matsuo *et al.* 2011). Therefore, *Rev-Erbα* KO mice could display alterations in ORX expression in animals challenged with highly palatable food. Seeking to test this hypothesis, we found that the number of ORX cells expressing c-Fos, ORX concentration in the CSF and in the VTA were higher in KO mice fed with palatable chocolate, confirming our hypothesis and suggesting that behaviours regulated by ORX are altered in *Rev-Erbα* KO mice.

The ORX receptors, orexin 1 (ORX-1R) and orexin 2 (ORX-2R), are G-protein-coupled receptors located in numerous brain regions including DA cells in the VTA, a key site to regulate drug addiction and reward (Sakurai & Mieda, 2011). I.P. injections of the ORX-1R antagonist SB-334867 significantly decreased chocolate intake, but not regular food, in both genotypes when compared with vehicle injection, suggesting that ORX signaling is much more related to motivational feeding. Similarly,

the ORX-1 receptor antagonist SB-334867 reduced high-fat pellet self-administration in rats (Nair *et al.* 2008).

Because the ORX system is also implicated in the regulation of arousal, we evaluated the effects of the ORX-1R receptor in locomotion. However, we do not observe significant changes in locomotor activity in both WT and KO. Consistent with our finding, a previous study reported that I.P. injections of SB-334867 decreased binge-like consumption of sucrose and saccharin without any effect on locomotor activity (Alcaraz-Iborra *et al.* 2014).

REV-ERBα/β agonists *in vivo* affect circadian behaviour and the expression of clock genes in the brain and peripheral oscillators (Solt *et al.* 2012). Moreover, treating diet-induced obese mice with these agonists reduced fat mass stores and altered regulation of lipid and glucose metabolism leading to a reduction of obesity (Solt *et al.* 2012). Therefore, targeting *Rev-Erbα* may have clinical applications for the treatment of metabolic disorders, including obesity and food addiction.

At the molecular level, how could *Orx* be modulated by the circadian clock? ROR response elements (RREs) are binding sites for REV-ERBα and ROR (Takahashi *et al.* 2008). The *Orx* promoter contains a 1 RRE (AGGTCA) and 1 RRE-like sequence. They are conserved among mouse and human, suggesting comparable regulation in these species. Co-transfection of the *Orx* reporter construct with *Rev-Erbα* dampened the activation of *Orx*, indicating the *Orx* promoter is prone to specific regulation by a circadian clock component *Rev-Erbα*, *via* RRE elements *in vitro*.

Moreover, our ChIP data indicate that the REV-ERBα protein binds the *Orx* promoter in the LH at ZT-17 and subsequently reduces *Orx* transcription. One can assume that *Orx*-induced behaviours (including palatable feeding) will go down thereafter (i.e. at the end of the night). Through this timed control, ORX signalling will be re-established in coordination with food intake.

Rev-Erbα expression in the midbrain, and more precisely in DAergic neurons from the VTA, exerts a circadian control on TH expression (Chung *et al.* 2014). In the circadian molecular machinery, *Rev-Erbα* competes with ROR for RREs (Preitner *et al.* 2002). *Rev-Erbα* also competes with the nuclear receptor-related 1 protein in DAergic neurons to regulate the circadian activity of TH (Chung *et al.* 2014).

Thus, *Rev-Erbα* is a circadian clock gene which regulates circadian DAergic activity (Chung *et al.* 2014; Jager *et al.* 2014), and the food- induced ORX activation (present study) *via* RREs in the promoters of *Th* and *Orx* genes, respectively. Therefore, *Rev-Erbα* links the molecular clock and brain pathways controlling rewarded behaviours. This result reinforces the importance of circadian genes and rhythmicity in reward systems (McClung *et al.* 2005; Hampp *et al.* 2008; Chung *et al.* 2014), revealing a critical role for the nuclear receptor REV-ERBα to modulate reward-feeding behaviour by affecting the central ORX pathway, with the *Orx* promoter being a primary functional target.

Our study, together with recent work on the role of REV-ERBα in the regulation of metabolism and behaviour (Cho *et al.* 2012; Solt *et al.* 2012), indicates how the circadian clock can modulate not only the timing of feeding but food intake as well. Hence, the present work may define potential targets for treatment of obesity, compulsive feeding behaviours and addiction.

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Authors Contribution

JM, CAF and UA conceived and designed the experiments. JM, CAF, CB, MM, ABV, NLS and JAR carried out the experiments and analysed the data. JM, CAF and UA contributed to interpretation of findings and drafted the manuscript. All authors critically reviewed the first draft, edited, gave comments and suggestions and approved final version for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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 ($F3, 27 = 11.75$; $p < 0.01$). However, no differences were found between genotypes ($F1, 9 = 0.66$; $p = 0.43$). (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, $F1, 9 = 1.49$; $p < 0.25$, preference, $F1, 9 = 3.21$; $p = 0.1$), although there was a significant daily rhythm of both intake and preference (Intake, $F3, 27 = 33.17$; $p < 0.01$, Preference, $F3, 27 = 4.36$; $p = 0.01$).

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, $F1, 8 = 1.16$; $p = 0.31$; KO, $F1, 6 = 0.007$; $p = 0.93$). 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 ($F1, 14 = 0.005$; $p = 0.94$). 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, $F1, 8 = 0.29$; $p = 0.6$; KO, $F1, 6 = 0.02$; $p = 0.87$). Open symbols show the individual values for each animal and the closed symbols the mean value (±SEM) for each condition (vehicle vs. SB–334877).