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, Rora-β-γ and Rev-Erbα-β (Takahashi et al. 2008). REV-ERBa 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-ERBa 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-ERBa (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 orexergic 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 (Chung 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 – 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 2 days. This concentration was determined to be highly preferred in C57BL/6J 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 ZT18-24). 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 [35S] 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 35S 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). Sections were then incubated in avidin-biotin complex (ABC; Vector Elite kit; ImmunoResearch, West Grove, 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 PF A. 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 H2O2. 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 H2O2 (refer to supplemental information for detailed Immunohistochemistry protocol).

**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 ORX antagonists on palatable feeding behaviour in mice, the ORX-1R antagonist SB-649868 (1-{2-methylbenzoxazol-6-yl)-3-[1.5]naphthyl-rind-4-yl urea hydrochloride; Tocris, Ellsville, Missouri) was tested in both WT and KO mice. Mice were exposed during 1 week to unlimited palatable and regular diets. Animals were injected intraperitoneally 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-hydroxypropyl)-β-cyclo-dextrin/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 coding region of the orexin gene, it was replaced by 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/6J 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* = 13.0, *p* < 0.001) and preference (Fig. 1C; *F* = 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* = 3.06, *p* = 0.04; Fig. 1C; *F* = 3.23; *p* = 0.03). No differences were found in rhythms of regular feeding behaviour between genotypes (Fig. 1A; *F* = 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* = 5.05, *p* = 0.008) and palatable food (Fig. 1E; *F* = 12.33, *p* < 0.001) with maximal intake at night. Similarly to sucrose consumption, KO mice showed a higher intake 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).
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 (\( F_{3, 28} = 3.54, p = 0.01 \)). Furthermore, adipose tissue and plasma leptin concentrations were higher in KO mice eating regular food + 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: \( F_{1, 27} = 33.12, p < 0.001 \)). 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, \( F_{1, 27} = 3.53, p = 0.02 \)).

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-erba 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 (\( F_{5, 12} = 3.38, p = 0.01 \)) in Orx mRNA concentration (Fig. 2A and 2C), with a main difference between ZF-4 (lower) and ZF-12 (higher) in WT (Post-hoc LSD test, \( p = 0.004 \)) and KO mice (Post-hoc LSD test, \( p = 0.002 \)). However, the factor genotype (\( F_{1, 12} = 0.18, p = 0.66 \)) or the interaction time X genotype (\( F_{5, 12} = 0.63, p = 0.67 \)) were not significant.

At the protein level, whereas no significant differences were found neither for the genotype factor (\( F_{1, 12} = 3.31, p = 0.07 \)) nor for the factor time (\( F_{5, 12} = 2.39, p = 0.05 \)), we found a significant effect of the interaction genotype X time for the intensity of ORX-positive cells (Fig. 2B and 2D; \( F_{5, 12} = 3.79, p = 0.008 \)). In the LH of KO animals, intensity of staining was significantly higher than in WT mice at ZF-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 z (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-erba 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::pGL3.2 vector with Rev-erba in NG108-15 neuroblastoma cells resulted in a decreased activation at any dose used (0.02, 0.05 and 0.1 μg; Fig. 4B; \( F_{3, 20} = 36.34, p < 0.001 \)). Co-transfection of the reporter construct with Rev-erba dampened the activation of Orx in a concentration-dependent manner (Fig. 4B; \( F_{1, 28} = 8.19, p = 0.004 \)). Mutation of the RRE sequence in the Orx promoter (blue in Fig. 4A) reversed repression of luciferase activity by Rev-erba, leading to increased luminescence at the lower Rev-erba concentration (0.02 μg; Fig. 4B: \( F_{1,19} = 4.24, p = 0.01 \)).

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.
in both structures with significantly higher values at ZT-17 (Fig. 4C; \( F_1, 8 = 111.89, p < 0.001 \)). ChIP for REV-ERB\(\alpha\) 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-ERB\(\alpha\) 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-Erba* 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-Erba* 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 extent drug-seeking persists regardless of negative consequences (Sanchis-Segura & Spanagel, 2006). Compulsive-feeding
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). Thus, palatable food seeking behaviours observed in animals may model eating behaviours observed in humans with compulsive overeating disorders (Avena et al. 2012).

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 with 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]
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-Erb 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-ERBa/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 Orexin be modulated by the circadian clock? ROR response elements (RREs) are binding sites for REV-ERBa and ROR (Takahashi et al. 2008). The Orexin 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 Orexin reporter construct with Rev-Erbα dampened the activation of Orexin, indicating the Orexin 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-ERBa protein binds the Orexin promoter in the LH at ZT-17 and subsequently reduces Orexin transcription. One can assume that Orexin-induced behaviours (including palatable feeding) will go down thereafter (i.e. at the end of the night). Through this timed control, Orexin 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 Orexin activation (present study) via RREs in the promoters of Th and Orex genes, respectively. Therefore, Rev-Erbα links the molecular clock and brain pathways controlling reward-related behaviour. 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-ERBa to modulate reward-feeding behaviour by affecting the central ORX pathway, with the Orex promoter being a primary functional target.

Our study, together with recent work on the role of REV-ERBa in the regulation of metabolism and behaviour (Choo 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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We would like to thank Prof. Ueli Schibler (University of Geneva, Switzerland) for the original breeding of our Rev-Erbα mice colony. We thank Prof. Michihiro Mieda (Kanazawa University) for the Orexin plasmid. We thank Drs. Marie Paule Felder and Etienne Challet for comments on the manuscript and Daniel Clesse for technical assistance. Funding sources of the present study were provided by the Agence National de la Recherche (ANR-14-CE13-0002-01 ADDiCLOCK JCJC to JM and NLS PhD fellow), the Centre National de la Recherche Scientifique (JM) and the Swiss National Fund (UA). ABV is a PhD NeuroTime programme fellow. Authors declare no competing financial interests.

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 were 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).