EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 39, pp. 207–217, 2014 doi:10.1111/ejn.12390

BEHAVIORAL NEUROSCIENCE

Ghrelin receptor-knockout mice display alterations in circadian rhythms of activity and feeding under constant lighting conditions

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Keywords: arcuate nucleus of the hypothalamus, dorsomedial nucleus of the hypothalamus, growth hormone secretagogue receptor, lateral hypothalamus, para-ventricular nucleus of the hypothalamus, suprachiasmatic nucleus

Abstract

Ghrelin is an orexigenic hormone produced by the stomach. Ghrelin, however, may also be a modulator of the circadian system given that ghrelin receptors are expressed in the master clock, the suprachiasmatic nucleus (SCN) and several outputs of this region. To investigate this, we performed analyses of running wheel activity and neuronal activation in wild type (WT) and growth hormone secretagogue receptor-knockout (GHSR-KO) mice under various lighting conditions. GHSR-KO and WT mice were maintained under constant dark (DD) or constant light (LL) with *ad libitum* access to food before being placed on a schedule of temporally restricted access to food (4 h/day) for 2 weeks. There were no differences between KO and WT mice in free-running period under DD, but GHSR-KO mice required more days to develop a high level of food anticipatory activity, and this was lower than that observed in WT mice. Under LL, GHSR-KO mice showed greater activity overall, lengthening of their circadian period, and more resistance to the disorganisational effects of LL. Furthermore, GHSR-KO mice showed greater activity overall, and greater activity in anticipation of a scheduled meal under LL. These behavioral effects were not correlated with changes in the circadian expression of the Fos, *Per1* or *Per2* proteins under any lighting conditions. These results suggest that the ghrelin receptor plays a role in modulating the activity of the circadian system under normal conditions and under restricted feeding schedules, but does so through mechanisms that remain to be determined.

Introduction

The circadian system controls daily rhythms of rest and activity, hormones, and motivated behaviors like feeding. Light is the primary synchroniser of the master circadian clock, the suprachiasmatic nucleus (SCN) (Reppert & Weaver, 2002). However, feeding also synchronises circadian rhythms (Stephan, 2002). Nocturnal rodents given food for only a few hours during daylight show increased activity prior to food availability (Stephan, 2002). This type of temporally restricted feeding (RF) schedule synchronises circadian oscillators in the limbic forebrain (Amir *et al.*, 2004; Lamont *et al.*, 2005; Waddington Lamont *et al.*, 2007) and can induce a diurnal rhythm of clock gene protein expression in the dorsomedial nucleus of the hypothalamus (DMH; (Verwey *et al.*, 2007).

Ghrelin is a stomach peptide that acts in the brain to regulate energy balance (Kojima *et al.*, 1999; Tschop *et al.*, 2000; Toshinai *et al.*, 2001). Ghrelin is secreted in response to fasting and hypoglycemia, and causes feeding when administered either peripherally or centrally (Tschop *et al.*, 2000; Toshinai *et al.*, 2001). Importantly, plasma ghrelin levels increase before, and are rapidly reduced following, a meal, suggesting a role in meal initiation (Cummings *et al.*, 2001; Toshinai *et al.*, 2001; Sanchez *et al.*, 2004; Drazen *et al.*, 2006).

The effects of ghrelin are mediated through the growth hormone secretagogue receptor (GHSR), found in brain regions associated with feeding and the regulation of circadian rhythms. For example, the message for GHSR is found in the SCN of rats, primates and, to a lesser extent, mice (Guan *et al.*, 1997; Mitchell *et al.*, 2001; Zigman *et al.*, 2006). Ghrelin receptors are also found in brain regions stimulated in anticipation of scheduled meals (Angeles-Castellanos *et al.*, 2004). These data suggest that ghrelin may play a role in circadian timing mechanisms, particularly entrainment to food availability. The latter hypothesis has been supported by studies showing that GHSR-knockout (KO) mice show attenuated anticipatory locomotor activity on an RF schedule (Blum *et al.*, 2009; LeSauter *et al.*, 2009), and cFOS expression is reduced in many brain areas in response to RF (Blum *et al.*, 2009; Lamont *et al.*, 2012). Moreover, and in spite of evidence for the presence of the ghrelin receptor in the circadian system, the role of ghrelin on circadian rhythms remains to be studied in detail. Here we looked for the presence of GHSR in the circadian system of mice using GHSR-KO mice with a LacZ reporter inserted into the promoter of the GHSR gene. To further investigate the circadian

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Received 1 April 2011, accepted 16 September 2013

phenotype of animals lacking the ghrelin receptor, analyses of running wheel activity and neuronal activation were performed under various lighting conditions. KO and WT mice were placed under a 12 : 12 h light : dark schedule (LD), constant darkness (DD) or constant light (LL); they were killed at different intervals to observe circadian rhythms of cFos expression. We also examined circadian rhythms of GHSR-KO and WT mice under conditions of DD and LL, and the ability of these animals to entrain to scheduled meals under these lighting conditions.

Materials and methods

Animals

Mice with targeted mutations to the ghrelin receptor gene (GHSR-KO) and their WT littermates were bred at the Carleton University Department of Neuroscience animal facilities. Mice originated from heterozygous breeding pairs obtained from Regeneron Pharmaceuticals (Tarrytown, New York, NY, USA). These mice were generated using a mixed C57BL/6J and DBA strain as background and the coding region of the *ghsr* locus was precisely deleted and replaced with an in-frame *lacZ* reporter gene (Abizaid *et al.*, 2006; Diano *et al.*, 2006). All animals had free access to tap water at all times and to food unless otherwise specified. Prior to the beginning of the experiments, animals were group-housed under an LD cycle with the onset of light set at 08:00 h [zeitgeber time (ZT) 0], with light intensity ranging between 120 and 180 lux at cage level. Research was conducted according to the guidelines of the Canadian Council on Animal Care and approved by Carleton University's Animal Care Committee.

Experiment 1: anatomical analysis of circadian brain oscillators under different lighting conditions

Location of GHRS receptors: beta-galactocidase staining

GHSR-KO mice ($n = 2$) living on an LD schedule were taken from their home cage at ≈ ZT 4–6, overdosed with sodium pentobarbital and perfused using a 2% paraformaldehyde solution. The brains were postfixed overnight in 2% paraformaldehyde, sliced into 50-μm sections on a Vibratome, and stained using the beta-galactocidase staining method described previously (Diano *et al.*, 2006). Briefly, sections were thoroughly rinsed with 10 mM phosphate-buffered saline (PBS; in mM: NaCl, 137; KCl, 2.7; Na_2HPO_4, 8; KH_2PO_4, 2.6), rinsed once quickly in cold PBS plus 2 mM $MgCl_2$ (PBS-$MgCl_2$), then incubated in PBS-$MgCl_2$ for 10 min at 4 °C. Permeability was then increased by incubating in cold PBS with detergent (0.01% sodium desoxycholate and 0.02% NP40) for 10 min at 4 °C, and placed in staining solution for 4 h at 37 °C in the staining solution containing (in mM) $K_3Fe(CN)_6$, 25; $K_4Fe(CN)_6$, 25; $MgCl_2$, 2 in PBS with 1 mg/mL of X-Gal.

Activation of circadian oscillators under LD: immunocytochemistry

For the LD condition, WT and GHSR-KO mice ($n = 4$ per group per time point) were taken from their home cage at ZT 0, ZT 6, ZT 12 or ZT 18. Pairs of animals consisting of one WT and one KO were injected with an overdose of sodium pentobarbital and perfused with 100 mL of saline (0.9%) followed by 100 mL of 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight and transferred to a 1% sodium azide solution until being sectioned at a thickness of 60 μm using a Vibratome. Sections were then cryoprotected in Watson's solution and frozen. One out of four 60-μm sections containing the hypothalamus were processed for cFos immunocytochemistry as described previously (Abizaid *et al.*, 2005). Separate sections through the SCN were processed for PERIOD1 (PER1) or PERIOD2 (PER2) as described previously (Amir *et al.*, 2004). Images from different hypothalamic nuclei were captured with a digital camera connected to an Olympus microscope (Olympus Canada, Markham, ON, Canada), and analysed using Image XSM software (v. 1.91, 2010,http://www.liv.ac.uk/~sdb/ImageSXM/). A total of seven or eight sections through each hypothalamic nucleus were used for cell quantification, and the mean number of cFos-immunoreactive cells for each hypothalamic nucleus of all animals, and of PER1- or PER2-immunoreactive cells for each SCN, were used to obtain group means and SEMs. An experimenter blind to the treatment groups performed all cell counts. Differences in these cell counts between groups and over circadian time were analysed using independent group two-way ANOVAs, with ZT and genotype as the grouping variables, using Prism 5 for Mac OSX (v. 5.0c, 2009, GraphPad Software, Inc., La Jolla, CA, USA).

Activation of circadian oscillators under DD and LL: determination of circadian time (CT) and immunocytochemistry

A total of 62 WT and GHSR-KO mice were transferred from the colony room to individual cages equipped with an activity wheel (Lafayette Instruments, Lafayette, IN, USA), and connected to a computer running Activity Wheel Monitor Software Running (Lafayette Instruments). Wheel activity was measured in 6-min bins throughout the experiment. Mice were housed in DD or LL for a minimum of 10 days, before being killed at one of four CT points ($n = 3$ or 4 animals per light cycle, genotype and time point) equally distributed over the rest–activity cycle. Circadian times were calculated using the last 10 days (2400 bins) of activity and producing an actogram, using Plot (R. Refinetti; http://www.circadian.org/softwar.html). Period length and acrophase were calculated using the Tau (v. 6.5, Mar. 2006) and Acro (v. 3.5, Jan. 2004) programs (R. Refinetti; http://www.circadian.org/softwar.html), using a χ^2 periodogram procedure and a fitted cosign wave function, respectively. These variables were used to produce an eye-fitted line projecting the time of activity offset (defined as CT0), the midpoint of the rest period (CT6), activity onset (CT12) or the mid-point of the active period (CT18). Whenever possible, pairs of animals consisting of one WT and one KO were killed at the same time by injection with an overdose of sodium pentobarbital and processed for immunocytochemistry as described above.

Circadian behaviour under DD and LL: activity analysis

All animals in this experiment were kept under DD or LL for at least 10 days, but some animals were kept for > 10 days due to the varying amounts of time required to assign animals to the appropriate CT time. Therefore, in order to standardise the behavioural analysis, calculations for activity levels (number of wheel revolutions), tau (Tau v. 6.5; Refinetti, 2006) and acrophase (Acro v. 3.5; Refinetti, 2004) were made on the first 2400 bins (10 days) of activity.

Experiment 2: analysis of wheel-running and feeding behaviour under different lighting conditions

Circadian behaviour under DD and LL: activity analysis

A total of 22 GHSR WT and KO mice were individually housed in running wheel-equipped cages (Lafayette Instruments). All animals

were allowed to acclimate to the equipment and lighting schedule under *ad libitum* feeding conditions for several days before beginning scheduled feeding (see below). A total of 10 animals (five WTs and five KOs) were exposed to an LD schedule (lights on at 02:00, lights off at 14:00 h) for 14 days followed by a 6-h delay of the LD (on at 08:00, off at 20:00 h), a few days of a 25-h day, and finally 24-h exposure to LL for ≈ 45 days (30 days *ad libitum* food access, followed by 16 days restricted feeding). Additional GHSR KO and WT mice (n = 6/group) were placed in DD for 28 days (14 days *ad libitum*, 14 days restricted feeding).

Entrainment to feeding schedules

For the LL condition, KO and WT mice were given temporally restricted access to food for a 4-h period at the same time each day for the last 16 days of LL. Body weights were recorded every 2–3 days during lighting manipulations and daily during scheduled feeding. After ≈ 1 month on an LL schedule, food was removed and returned the following day between 11:00 and 15:00 h. For the DD condition, WT and KO mice were exposed to 14 days of DD before undergoing a temporally restricted feeding schedule for 14 days in DD. During the first day of limited access, food was available for 8 h, starting during the inactive period, and on subsequent days food was removed 2 h earlier than on the previous day until the target duration of 4 h access per day was reached. Food was weighed daily during this period.

The amount of daily food anticipatory activity for animals housed in LL or DD was calculated by summing the total number of wheel revolutions in the 4 h immediately prior to food access and averaging across days. Past research suggests that entrainment to feeding occurs within ≈ 1 week (Blum *et al.*, 2009), so only the first 7 days of scheduled feeding were compared. All data are presented as mean ± SEM. Statistical differences between groups were determined by unpaired one-tailed Student's *t*-tests or two-way ANOVA followed by Bonferroni *post hoc* tests. Differences between genotypes over days were analysed using a mixed design ANOVA with genotype (KO vs. WT) as the between-groups variable and days as the within-groups variable.

Results

GHSR-KO mice were more active than WT mice under LL conditions

KO animals showed greater daily activity (expressed as wheel revolutions per day) than WT mice in LL (KO = 4371 ± 1204, WT = 2868 ± 476, t_{29} = 2.3, $P < 0.05$). Genotypes did not differ in terms of running-wheel activity in DD (KO = 14 752 ± 1472, WT = 11 918 ± 1287, t_{29} = 1.5, $P > 0.05$; see Fig. 1).

An analysis of tau and acrophases showed no significant differences between KO and WT mice, using independent *t*-tests (see Fig. 2).

GHSR-KO mice displayed rhythmic wheel-running activity under LD lighting cycles

On an LD cycle, GHSR-KO and WT mice did not differ in terms of circadian period or acrophase (t_8 = 0.3, $P > 0.05$; t_8 = 1.0, $P > 0.05$). Both GHSR-KO and WT mice showed a circadian period of ≈ 24 h and a time of acrophase ≈ 18:00, ≈ 4 h into the dark cycle (see Fig. 3 and Table S1). Furthermore, as can be seen in Fig. 4, GHSR-KO mice showed greater average daily activity overall than WT mice in LD (t_{26} = 9.7; $P < 0.0001$).

GHSR-KO mice showed circadian alterations and were more active than WT mice under LL conditions

GHSR-KO and WT mice were switched from a regular LD cycle to LL, and this produced different responses between these two groups of mice. In the days following the switch, GHSR-KO mice showed an average period that was ≈ 30 min longer than that of WT animals (t_8 = 2.1; $P < 0.05$). Similarly, acrophases occurred ≈ 2 h later in GHSR-KO mice compared to WTs (t_8 = 2.8; $P < 0.05$; see Fig. 3 and Table S1). This difference was no longer significant after > 1 month in LL ($P > 0.05$; see Table S1). Interestingly, after a month of LL conditions, nearly all of the WT animals (four of five) were arrhythmic while three of the five KO animals still had significant periods and acrophases. However, a χ^2 analysis did not reveal a significant difference in the probability of rhythmicity between these two groups (χ^2_1 = 0.7292, n = 14, P = 0.39). It is important to note that locomotor activity was higher in GHSR-KO mice than in their WT littermates throughout the duration of the LL manipulation. While locomotor activity decreased overall in both groups throughout the 30-day LL period, voluntary activity continued to be higher in GHSR-KO mice. *T*-tests of the total activity for the first 10 days in LL (t_{18} = 5.5, $P < 0.0001$) and after 30 days in LL (t_{18} = 9.6, $P < 0.0001$) show that KO animals were significantly more active that WT animals throughout LL exposure (see Fig. 4).

KO animals anticipated a restricted feeding schedule in LL

Both GHSR-KO and WT mice entrained to a 24-h feeding schedule under conditions of LL (see Fig. 5 and Table S1). In terms of circadian variables, the genotypes did not differ (t_7 = 0.25; $P > 0.05$);

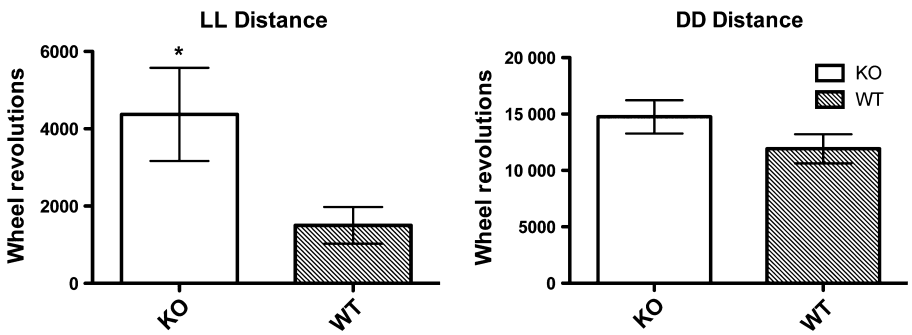

FIG. 1. Average ± SEM total daily running wheel activity for KO (white bars) and WT (shaded bars) animals during 10 days in LL (left panel), or DD (right panel). *T*-tests reveal that KO animals had greater total running wheel activity than WT animals in LL (*$P < 0.05$) but not DD. See text for details.

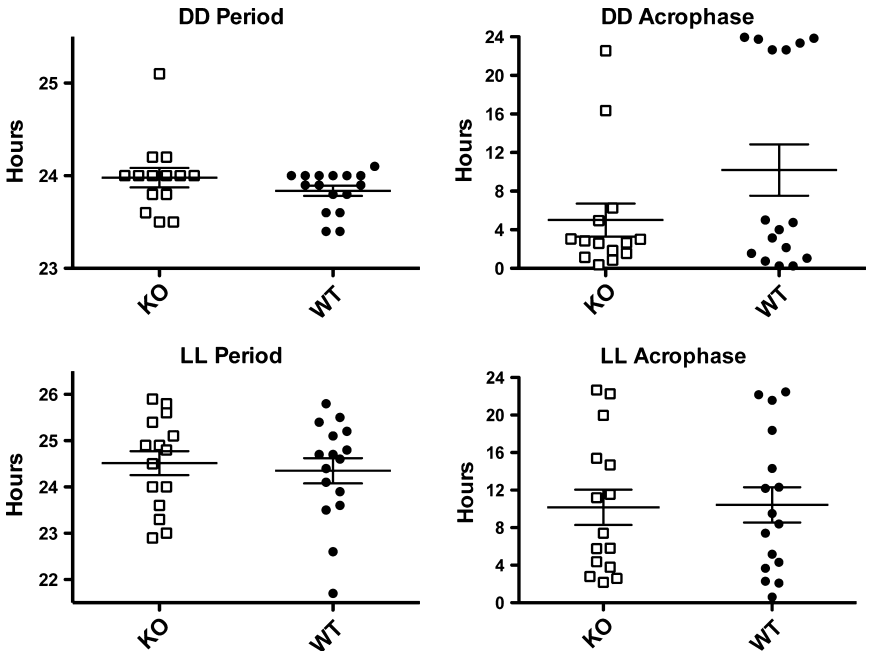

FIG. 2. Graphs showing average ± SEM period length in h (top panels) and time of acrophase ± SEM in 24-h clock time (bottom panels) of KO (left panels, open squares) and WT (black circles) mice during 10 days exposure to LL (left panels), or DD. Period and acrophase did not differ between KO and WT animals in either lighting condition.

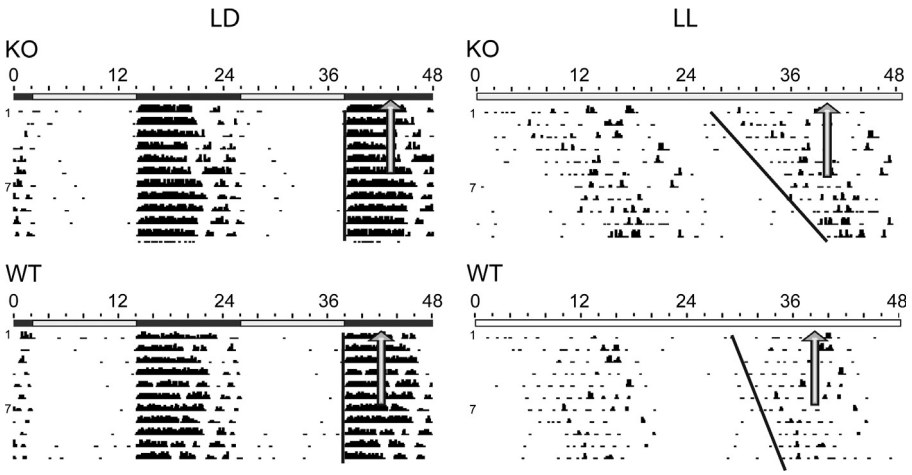

FIG. 3. Representative actograms from KO (top panels) and WT (bottom panels) mice during exposure to LD (left panels) and during the first 10 days of exposure to LL. Black lines represent the approximate circadian period, and approximate acrophases are indicated by arrows. Period and acrophase did not differ between WT and KO animals in LD, but did differ significantly in the first 10 days of exposure to LL.

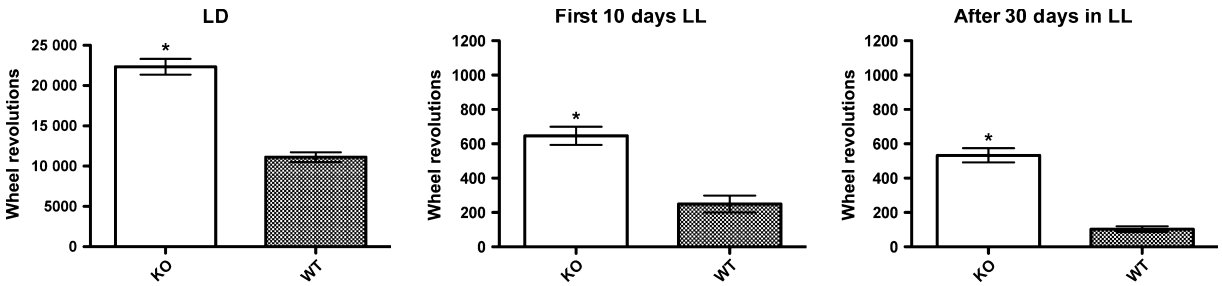

FIG. 4. Average ± SEM total daily running wheel activity KO (white bars) and WT (shaded bars) animals during 14 days of LD, during the first 10 days in LL, and after 30 days in LL. KO animals showed greater total activity in LD and LL (*$P < 0.05$), although all animals showed decreased activity in LL relative to LD. See text for details.

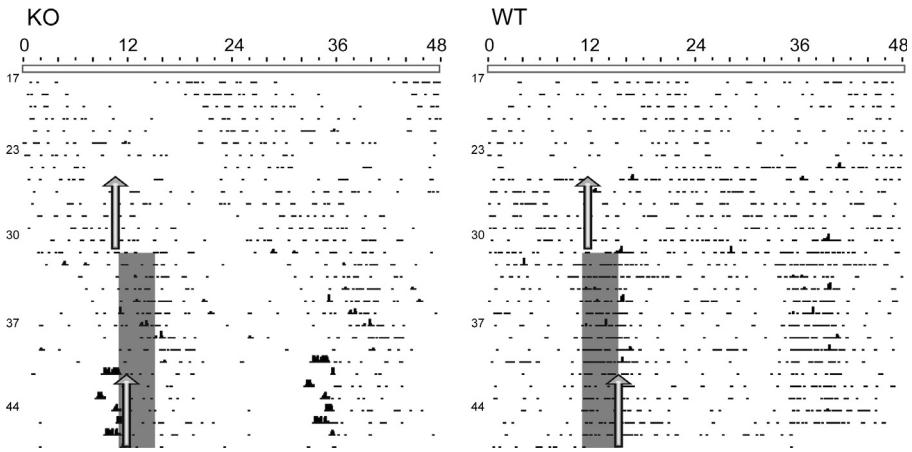

FIG. 5. Representative actograms from KO (left) and WT (right) mice after ≈ 30 days of exposure to LL under *ad libitum* feeding conditions, followed by LL on an RF schedule. Grey boxes indicate the time during which food was available. Approximate acrophases during the last 10 days of LL under *ad libitum* feeding and during the last 10 days of RF are indicated by arrows. Period and acrophase did not differ between WT and KO animals during the last 10 days in LL. Acrophase (but not period) differed significantly during the final 10 days of exposure to RF. See text for details.

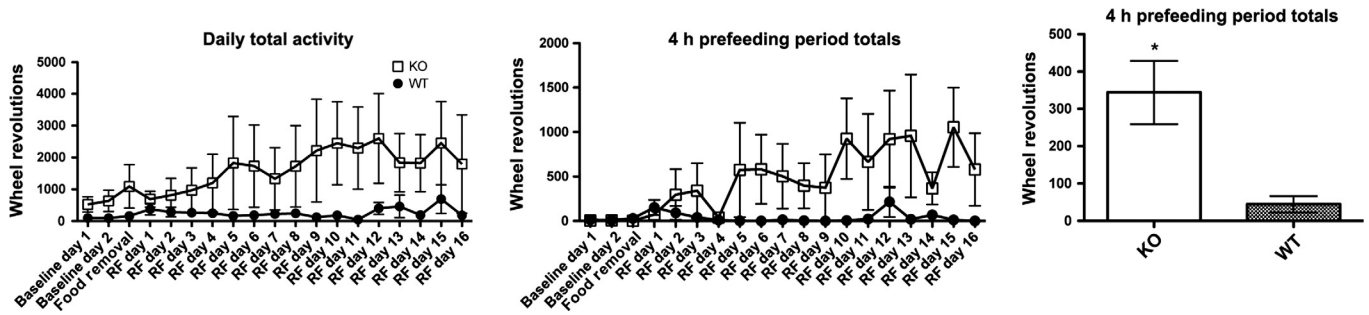

FIG. 6. Average ± SEM total daily running wheel activity (left panel) and activity in the 4-h period immediately prior to food access (center panel) for WT (black circles) and KO (white squares) animals during exposure to LL on an RF schedule (LLRF). Baseline days 1 and 2 are the final 2 days of *ad libitum* access to food. The following day (RF1), food was removed, then presented at the same time and available for 4 h each day for 16 days. KO animals showed greater activity than WTs throughout the RF period (right panel, $*P < 0.05$). See text for details.

both showed periods that were almost exactly 24 h during the last 10 days of the 16-day scheduled feeding period (see Table S1). However, as Fig. 5 shows, acrophases did significantly differ between the two groups ($t_7 = 4.1$; $P < 0.001$), with GHSR-KO animals showing peak activity ≈ 1 h (11.47 h) into the feeding period, while WT animals did not show peak activity until several hours later, near the time of food removal (14.24 h). Values do not include data from one KO animal, due to equipment failure during the last 10 days of recording (see Table S1).

Total daily running activity in KO animals continued to be greater than WTs during the LLRF period (see Fig. 6). ANOVA revealed a main effect of genotype ($F_{1,152}$=28.02, $P < 0.0001$), with greater total activity in the KO group, but no main effect of day or day × genotype interaction. Bonferonni analysis showed no significant differences between KO and WT animals on any individual day of RF.

An analysis of the running-wheel activity in the 4 h immediately before food access also showed much greater activity in KO animals, with ANOVA showing a main effect of genotype ($F_{1,152}$=23.64, $P < 0.0001$) but no main effect of day, day × genotype interaction, nor any differences in *post hoc* analyses (see Fig. 11). A *t*-test of the first 7 days of activity during this anticipatory period shows greater activity in KO animals ($t_{12} = 3.4$; $P < 0.01$).

This increase in energy expenditure in KO animals was not compensated for in terms of food intake, as there were no differences between KOs and WTs in terms of body weight (KO, 33 + 0.96; WT, 34 + 0.90 g; $t_{16} = 1.1$, $P > 0.05$) or amount of food eaten (KO=5.1 g + 0.21; WT=5.1 g + 0.19; $t_{28} = 0.095$, $P > 0.05$) over the course of the experiment in LL.

WT animals were more active and developed anticipatory activity more quickly in DD than did KO mice

In the first phase of the experiment in DD, WT animals showed greater activity in DD than did KOs. Averages of daily number of wheel revolutions were 16 482 ± 1049 for WT mice vs. 12 607 ± 771 for KO mice ($t_{22} = 3.0$, $P < .05$). In DD, the free-running periods of GHSR-KO and WT animals, 23.95 and 23.75 h respectively, did not differ ($t_{10} = 0.48$, $P > 0.05$), nor did the acrophases ($t_{10} = 1.2$, $P > 0.05$)., which were 24.22 h for KO animals and 23.12 h for WT animals (see Table S2).

Over the course of the feeding experiment, the genotypes did not differ in body weight (KO, 28 + 0.19; WT, 28 + 0.19 g; $t_{30} = 0.16$, $P > 0.05$), nor daily food intake (KO, 5.0 + 0.20; WT, 5.1 + 0.18 g; $t_{30} = 0.23$, $P > 0.05$). As can be seen in Fig. 12, both GHSR-KO and WT mice entrained to a 24-h feeling schedule while in DD. Both genotypes showed periods that were nearly 24 h ($t_{10} = 1.2$, $P > 0.05$) during the last 10 days of the scheduled feeding period (see Fig. 7 and Table S2). Acrophases occurred shortly before the beginning of the feeding period in KO animals (KO,

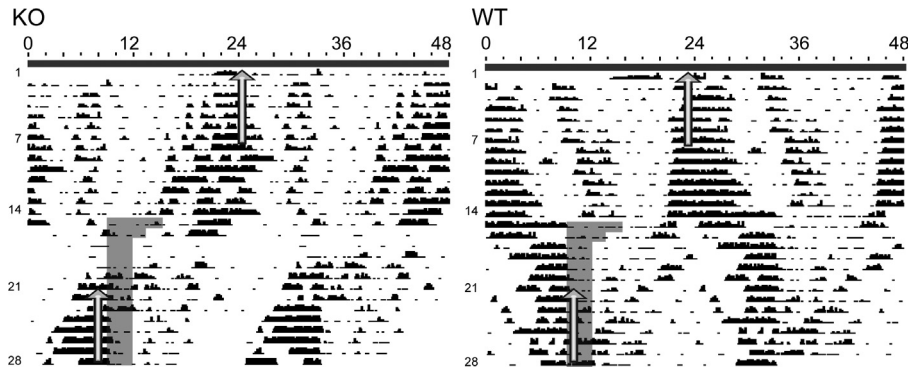

FIG. 7. Representative actograms from KO (left) and WT (right) mice after ≈ 14 days of exposure to DD under *ad libitum* feeding conditions, followed by 14 days on an RF schedule. Grey boxes indicate the time during which food was available. Approximate acrophases during the last 10 days of DD under *ad libitum* feeding and during the last 10 days of RF are indicated by arrows. Period and acrophase did not differ between WT and KO animals. See text for details.

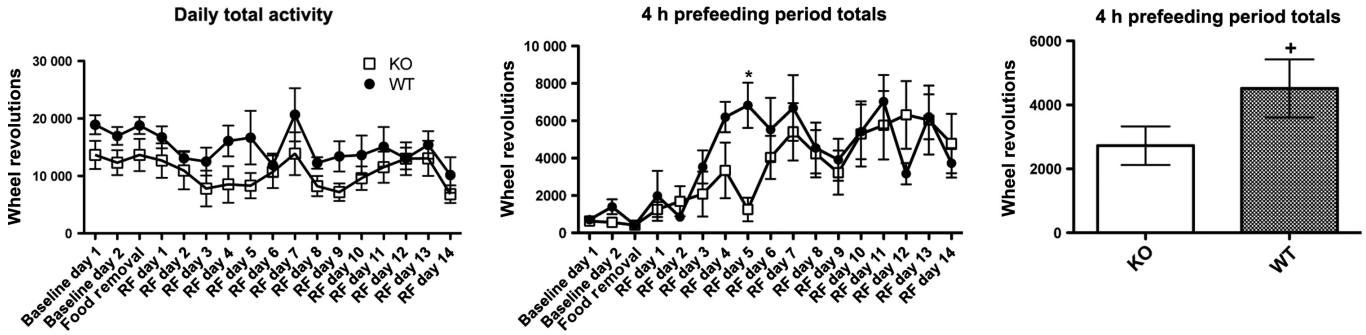

FIG. 8. Average ± SEM total daily running wheel activity in DD (left panel) and activity in the 4-h period immediately prior to food access (central panel) for WT (black circles) and KO (white squares) animals during exposure to DD on an RF schedule (DDRF). Baseline days 1 and 2 are the final 2 days of *ad libitum* access to food. The following day, food was removed, then presented at the same time and available for 4 h each day for 16 days. WT animals showed greater food anticipatory activity on day 5 of RF (*$P < 0.05$). WT animals showed a trend toward greater total activity throughout the RF period (right panel; †$P < 0.1$). See text for details.

07.51 h) and ≈ 1 h after food availability in WT animals (WT, 09.55 h), but did not differ statistically significantly ($t_{10} = 0.99$, $P > 0.05$; see Fig. 7).

Total daily running activity during the RF period in DD (see Fig. 8) showed the opposite effect to that seen in LL, with a main effect of genotype ($F_{1,170}$ =21.90, $P < 0.0001$), revealing greater total activity in the WT group, but *post hoc* tests were not significant. There was a trend for a main effect of day ($F_{16,170} = 1.67$, $P = 0.058$), but no day × genotype interaction for total activity (see Fig. 8, left panel). An analysis of the running-wheel activity in the 4 h immediately before food access also showed greater anticipatory activity in WT animals for a couple of days before KO animals reached the same level. ANOVA revealed a main effect of day ($F_{16,160} = 7.64$, $P < 0.0001$), no effect of genotype interaction, but a trend for a day × genotype interaction ($F_{16,160} = 6.55$, $P = 0.088$). *Post hoc* analyses showed a significant difference between WT and KO animals on day 5 of the restricted feeding schedule (see Fig. 8, central panel). A visual inspection of the data suggested that the difference between the two genotypes occurred only within the first week after beginning scheduled feeding, so this analysis was rerun with only the first 7 days. Under these conditions, the interaction between day and genotype achieved significance ($F_{9,90} = 2.11$, $P = 0.037$). A *t*-test of the first 7 days of activity during the 4-h pre-meal period showed a strong trend towards greater activity in WT animals than in KOs ($t_{12} = 1.6$, $P = 0.06$; see Fig. 8).

Ghrelin receptors were found throughout outputs of the SCN

Figure 9 shows histochemical expression of the LacZ reporter gene on the GHSR promoter, indicating the location of the ghrelin receptor. Staining was seen in hypothalamic outputs of the SCN such as the subparaventricular zone (SPVZ) (Fig. 9A), DMH (Fig. 9E and G), paraventricular nucleus of the hypothalamus (PVN; Fig. 9C and D) and arcuate nucleus (ARC; Fig. 9E and H), while the SCN (Fig. 9A), ventromedial hypothalamus (VMH) (Fig. 9E and G) and lateral hypothalamus (LH; Fig. 9E and F) had staining that was discernable but less robust. The paraventricular nucleus of the thalamus (PVT; Fig. 9B) also showed intense staining. X-gal staining was found throughout the extent of the brain, in fore-, mid- and hindbrain regions (data not shown) described previously using mice of the same transgenic line (Abizaid *et al.*, 2006; Diano *et al.*, 2006).

GHSR-KO mice displayed rhythmic Per 1 and Per2 expression in SCN, *and Fos expression in the hypothalamus, under LD*

There were no differences between GHSR-KO and their WT littermates in their circadian patterns of PER1 and PER2 protein expres-

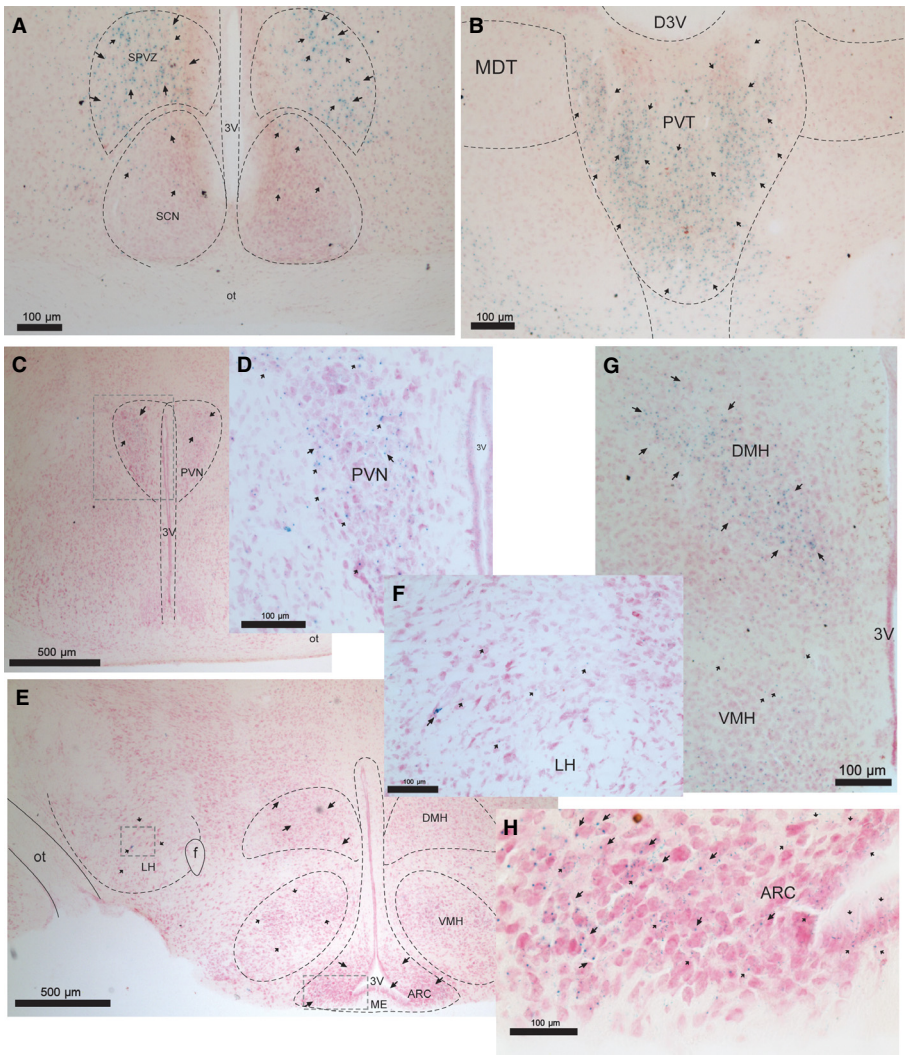

FIG. 9. Photomicrographs of the brains of GHSR-KO mice (n = 2) stained using the beta-galactocidase staining procedure. Teal blue spots show the presence of beta-galactocidase as the result of activation of the GHSR promoter, indicating the location of the ghrelin receptor (indicated by black arrows). (A) SCN and SPVZ; (B) PVT; (C) PVN; (D) PVN at higher magnification; (E) LH, DMH, VMH, and ARC; (F) LH; (G) DMH and VMH; and (H) ARC. Scale bars as indicated in the Fig.

sion in the SCN ($P > 0.05$), nor in the circadian patterns of Fos expression in different hypothalamic regions (see Figs 10 and 11).

GHSR-KO and WT mice displayed rhythmic Fos expression in the SCN under LL and DD

Quantification of the cFos protein immunoreactivity in the hypothalamic nuclei and the PVT showed rhythmic expression in many brain regions studied. A two-way ANOVA of cFos expession in the SCN showed a significant effect of CT ($F_{3,23}$ = 3.2, $P < 0.05$), but no effect of genotype and no CT × genotype interaction. Similarly, two-way ANOVAS showed significant effects of CT for the PVN ($F_{3,23}$ = 4.6 $P < 0.05$), LH ($F_{3,23}$ = 5.5, $P < 0.05$), DMH ($F_{3,23}$ = 4.7, $P < 0.05$) and PVT ($F_{3,23}$ = 3.8, $P < 0.05$), but no effects of genotype or genotype × CT interactions. Significant rhythms were not observed for the SPVZ, VMH or ARC. There were no differences between genotypes, nor any genotype × time interaction (see Fig. 12).

Quantification of the cFos protein immunoreactivity under DD in the hypothalamic nuclei and the PVT showed rhythmic expression in the SCN and LH, but not in other brain areas studied (Fig. 13). A two-way ANOVA of cFos expression showed a significant effect of CT in the in the SCN ($F_{3,22}$ = 12, $P < 0.05$), and LH ($F_{3,22}$ = 3.3, $P < 0.05$), but no effect of genotype or CT × genotype interaction. Significant effects of CT were not observed for the other areas, nor were there differences between genotypes, or any genotype × time interaction (see Fig. 5).

Discussion

The results of these experiments support the idea that GHSR-KO mice have subtle differences in their circadian rhythms, particularly under conditions that uncouple or dysregulate the master circadian clock, such as LL and food entrainment. In DD, when the master clock is free to run according to its own endogenous period, circadian rhythms of cFos expression in the SCN and wheel-running activity periods were very similar for both GHSR-KO and WT mice. Both genotypes showed entrainment to temporally limited food access in DD, as has been shown before under LD conditions (Blum *et al.*, 2009; LeSauter *et al.*, 2009). Interestingly, GHRS-KO mice do seem to show the same delay to food entrainment and reduced anticipatory locomotor activity that was seen previously in animals

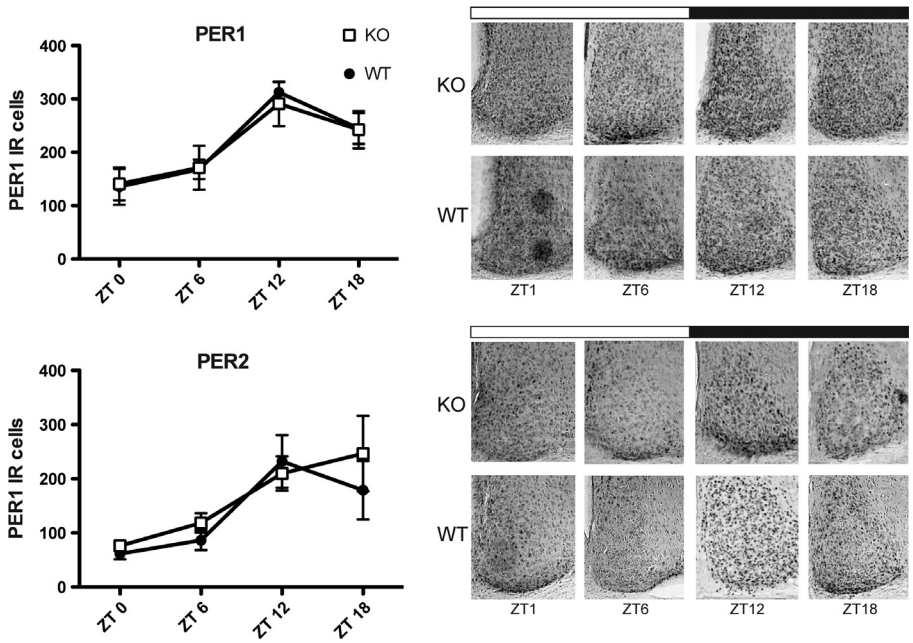

FIG. 10. Graphs and photomicrographs showing immunocytochemistry for PER1 and PER2 proteins in the SCN of GHSR-KO and WT mice. Analysis of the PER1 and PER2 proteins showed rhythmic expression of both PER1 and PER2 in these mice but no difference between genotypes nor any genotype × time interaction.

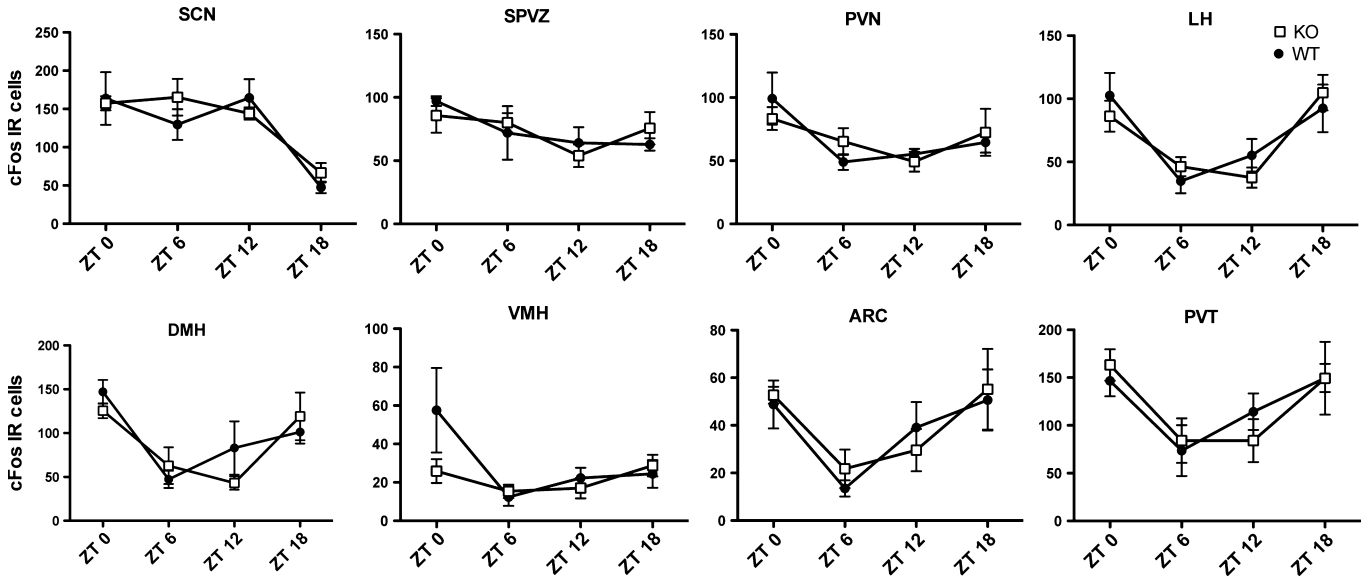

FIG. 11. Graphs showing numbers of cFOS-immunoreactive cells for GHSR-KO and WT mice housed on an LD cycle. Analysis of the cFos protein quantification in the hypothalamic nuclei and the PVT showed rhythmic expression in all brain regions studied except for the SPVZ, which showed a trend for rhythmic expression. There were no differences between genotypes nor any genotype × time interaction.

on an LD schedule. In the present study, as in our previous experiment in LD (Blum *et al.*, 2009), WT animals housed in DD began to show high levels of anticipatory activity soon after beginning the scheduled feeding paradigm. By day 4 and particularly day 5 of scheduled feeding of this experiment, WT animals show high levels of activity in the 4 h prior to food availability while KO animals matched this only on day 6. However, previous experiments have also shown that the amount of anticipatory activity is reduced in GHSR-KO animals on an LD cycle (LeSauter *et al.*, 2009). In DD, this was supported at the trend level.

The real surprises in this study were the differences between GHSR-KO and WT animals that emerged under LL. In terms of cFOS activation, they did not differ. The SCN and several other brain areas showed circadian rhythms of immunoreactivity that did not differ between groups. Where striking differences did emerge was in the differential effect of LL on the amount of running-wheel activity. In experiment 1, KO animals showed greater activity than WT mice in LL but not in DD. After 10 days in LL, KOs ran ≈ 4300 wheel revolutions per day vs. 1500 revolutions per day in WT mice. In contrast, after 10 days in DD, KO and WT mice did not differ, with KO mice running ≈ 14 000 revolutions per day compared to WTs that ran ≈ 12 000 per day (see Fig. 1). In experiment 2, a separate group of KO animals were more active overall, showing greater activity levels in both LD and LL (see Fig. 4). WT

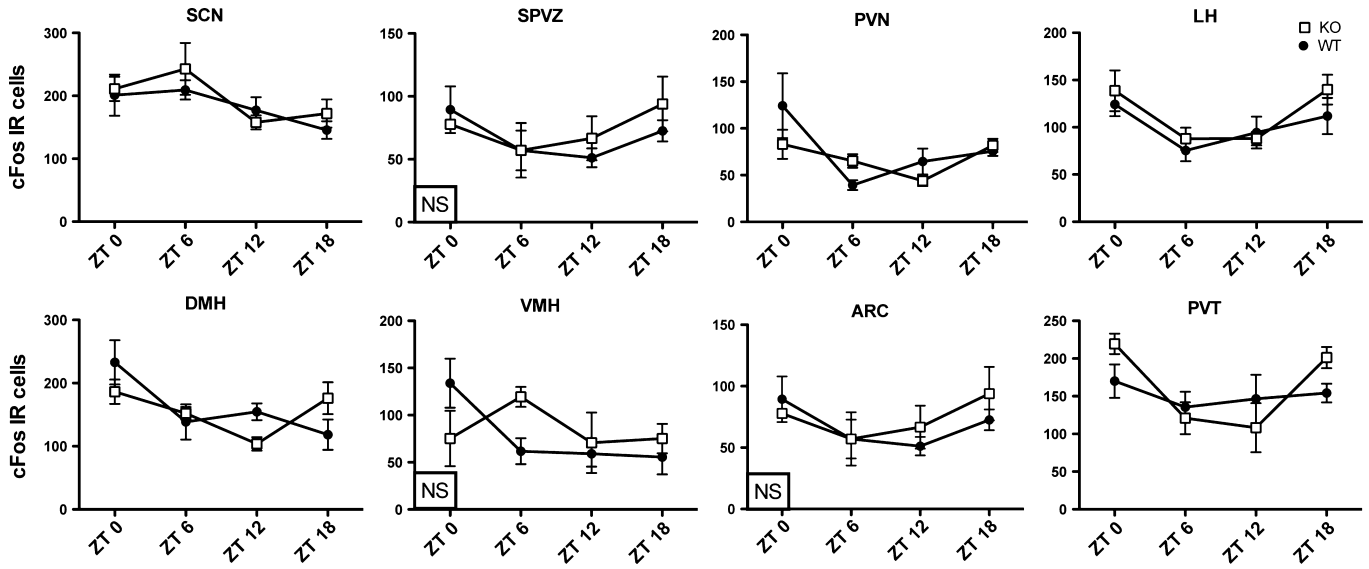

FIG. 12. Graphs showing numbers of cFOS-immunoreactive cells for GHSR-KO and WT mice housed on an LL cycle for a minimum of 10 days. Analysis of the cFos protein immunoreactivity in the hypothalamic nuclei and the PVT showed rhythmic expression in many brain regions studied. Significant rhythms were not observed for the SPVZ, VMH or ARC. There were no differences between genotypes nor any genotype × time interaction.

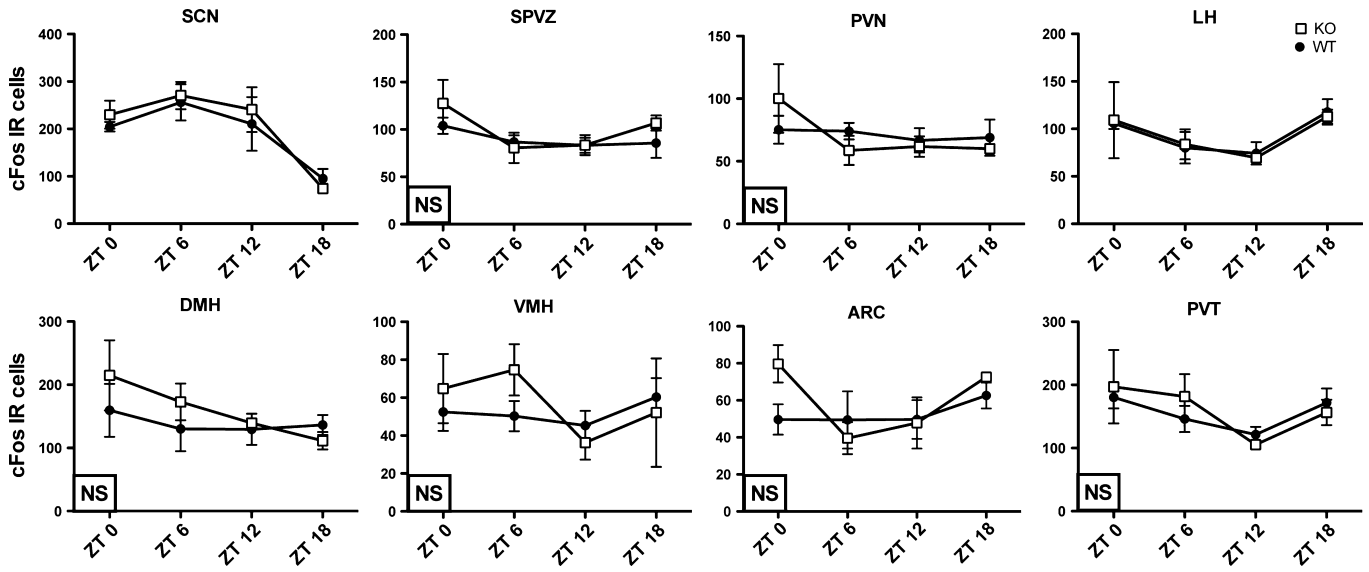

FIG. 13. Graphs showing numbers of cFOS-immunoreactive cells for GHSR-KO and WT mice housed on a DD cycle for a minimum of 10 days. Analysis of the cFos protein immunoreactivity in the hypothalamic nuclei and the PVT showed rhythmic expression in only the SCN and LH. Significant rhythms were not observed for the SPVZ, PVN, DMH, VMH, ARC or PVT. There were no differences between genotypes, nor any genotype × time interaction.

animals showed very little activity under LL, dropping from ≈ 10 000 wheel revolutions per day in LD down to ≈ 200 in LL. KO animals were more active but showed the same dramatic decrease in amount of activity, falling from 20 000 wheel revolutions per day to ≈ 200–800 after 30 days in LL (see Fig. 9). In a separate group of animals exposed to DD this effect was reversed, with WTs showing more wheel revolutions than KOs.

This difference in the amount of overall activity in KO mice between LD and LL may be accounted for, in part, by the inhibitory effects of ghrelin on spontaneous locomotor activity. High activity levels in ghrelin-KO and GHSR-KO mice have been reported previously, and this has been linked to increased energy expenditure in animals from the same strain that we used in the current study (Wortley *et al.*, 2005; Pfluger *et al.*, 2008). Conversely, GHSR-KO animals on a high-fat diet actually showed reduced activity compared to their WT littermates (Zigman *et al.*, 2005), but these animals were on a different genetic background than our own, which may account for the difference in activity levels. In fact, GHSR-KO mice on the purely C57BL/6J background failed to show any anticipatory activity after 2 weeks on a restricted feeling schedule (Davis *et al.*, 2011), whereas our animals on the mixed C57BL/6J-DBA background do develop anticipatory behavior under a variety of lighting conditions, but at a slower rate than WT animals in LD (Blum *et al.*, 2009) and DD (present study). This suggests that these strain effects may have a profound effect on circadian phenotype.

This raises the question of what role ghrelin ordinarily plays in the circadian system that could account for this accentuation of activity in LL. Ghrelin receptors are expressed in thalamic and hypothalamic

nuclei that are major outputs of the SCN master clock, such as the PVT, SPVZ, DMH and LH. Their absence in GHSR-KO animals may alter any modulatory influences ghrelin ordinarily has on the behavioral consequences of changes to the outgoing signals from the SCN (Watts & Swanson, 1987; Watts *et al.*, 1987; Moga *et al.*, 1995; Leak & Moore, 2001). Certainly a role for the DMH in food entrainment has been proposed (Gooley *et al.*, 2006; Mieda *et al.*, 2006) and debated (Landry *et al.*, 2006, 2007; Moriya *et al.*, 2009), and new evidence for a strong interaction between the DMH and SCN is emerging (Acosta-Galvan *et al.*, 2011). However, the hyperactivity in LL emerged in the GHSR-KO animals even before restricted feeding began, suggesting other brain areas my also be important. The PVT, for instance, is a major relay for circadian information, receiving information not only from the SCN but also from the SPVZ, intergeniculate leaflet and retina (Watts *et al.*, 1987; Moga *et al.*, 1995; Moore *et al.*, 2000). Thus, the absence of ghrelin action in the PVT could potentially change the normally inhibitory effects of light on behavior.

The second place where there was a differential effect of LL was on circadian period. This was not a consistent effect. In experiment 1, where wheel-running activity was measured in order to select an appropriate CT time for killing, animals were taken from their home cage in the animal colony and placed in LL or DD for only 10 days. Under these conditions, the taus for LL and DD did not differ between KO and WT animals. Although periods were slightly longer for KOs than WTs in LL, this was not significant.

The situation in experiment 2 was quite different. In this experiment, 10 mice were placed in running wheels for a period of several months and studied under several different lighting conditions, including a few days on a 25-day cycle. Thus after a brief exposure to 25-h days, followed by LL, both KO and WT mice showed a lengthening of their circadian period. However, GHSR-KOs showed an average period that was $\approx$ 30 min longer than that of WTs after 10 days in LL. This effect was no longer apparent after 30 days in LL, but by that time circadian behavior rhythms had become less coherent for KO animals and especially for WT animals, the majority of which were arrhythmic. This is consistent with studies showing that long-term exposure to LL disrupts the synchrony among SCN clock cells (Ohta *et al.*, 2005).

Although both groups do show robust entrainment to food that is able to reestablish a significant 24-h circadian period, and also a significant acrophase, the timing of the acrophase is not consistent between WTs and KOs after food entrainment, with KOs showing peak activity during the time when food is available, while WTs show peak activity near the end of the time of food access. The timing of the acrophase of activity was also later in WT animals in DD, although not significantly so. This suggests that KO animals have an alteration in the phase relationship between the timing cue provided by food availability and the behavioral activation that results in anticipatory running behavior. The ghrelin-mimetic drug growth-hormone releasing peptide 6 (GHRP-6) has been shown to inhibit light-induced cFos expression in the SCN and attenuate a light induced phase shift (Yi *et al.*, 2006; Yi *et al.*, 2008), suggesting that ghrelin can act as a non-photic stimulus to alter the timing of light-signaled behaviour. Therefore, it is not surprising that the absence of ghrelin could alter the timing of activity, especially in LL, where photic Zeitgebers are also absent. In this situation, the absence of ghrelin activity at the GHRS receptor did not have a significant effect on comsummatory behaviour, as the two groups ate the same amount of food and there were no differences in body weight.

One question that must be addressed is the surprising lack of food anticipatory activity in WT mice housed in LL. Indeed, food anticipatory activity has been previously demonstrated in rats housed in LL (Bolles & Stokes, 1965; Edmonds & Adler, 1977a,b; Lamont *et al.*, 2005). In Lamont *et al.* (2005), no attempt was made to quantify the amount of anticipatory activity, but certainly overall activity levels were very low after an extended period in LL, as can be seen in the actograms presented in that article. Species differences may account for the lack of food anticipatory activity observed in the present study in WT mice. In one study using spiny mice, *Acomys cahirinus*, wheel-running activity was reduced dramatically in LL compared to LD and only two of the 11 mice studied actually showed entrainment to a restricted feeding schedule under LL, although all 11 had shown significant food anticipatory activity on an LD schedule prior to exposure to LL (Chabot *et al.*, 2012). In the current experiment, 30 days in LL reduced daily activity levels in WT mice to fewer than 200 wheel revolutions per day, as compared to 600 in KO mice. With such a low level of activity in WT mice, it may simply be difficult to detect food anticipatory activity in these animals. Sampling of brain and peripheral tissues for clock gene protein and RNA at different time points during the temporal feeding period would have demonstrated whether central and peripheral circadian oscillators were entrained to the time of food availability, although the large number of animals required for this type of study was prohibitive. Alternately, a circadian-controlled measurement that is suppressed by light to a lesser degree, such as body temperature, may have been useful in detecting food anticipation in these mice. Regrettably, these data were not collected.

Together, these data provide further support for the hypothesis that ghrelin plays a role in the food-entrainable clock, but also suggest that there may be an interaction between the effect of light and ghrelin that extends beyond a simple deficit in the ability of GHSR-KO animals to entrain to scheduled feeding. In conclusion, life-long insensitivity to ghrelin has subtle but demonstrable effects on behavioural activation and the power of food to synchronise the circadian organisation of voluntary activity.

Supporting Information

Additional supporting information can be found in the online version of this article:

Table S1. Summary data of period and acrophase (in hours) for individual animals under LD, LL with *ad libitum* feeding and 10 and 30 days, and LL with temporally restricted access to food (LLRF).

Table S2. Summary data of period and acrophase (hours) for individual animals under DD with *ad libitum* feeding and DD with temporally restricted access to food (DDRF).

Acknowledgements

PERIOD1 antibody was the generous gift of Shimon Amir. This project was funded by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Funds for innovation (CFI) awarded to A.A. E.W.L. was supported by postdoctoral fellowship from the Fonds de la recherche en santé du Québec (FRSQ).

Abbreviations

ARC, arcuate nucleus; CT, circadian time; DD, constant darkness; DMH, dorsomedial nucleus of the hypothalamus; GHSR, growth hormone secretagogue receptor; KO, knockout; LD, 12 h of light and 12 h of darkness; LH, lateral hypothalamus; LL, constant light; PER1, PERIOD1; PER2, PERIOD2; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; RF, restricted feeding; SCN, suprachiasmatic nucleus; SPVZ, subparaventricular zone; VMH, ventromedial hypothalamus; ZT, zeitgeber time.

References

Abizaid, A., Mezei, G., Thanarajasingam, G. & Horvath, T.L. (2005) Estrogen enhances light-induced activation of dorsal raphe serotonergic neurons. *Eur. J. Neurosci.*, **21**, 1536–1546.

Abizaid, A., Liu, Z.W., Andrews, Z.B., Shanabrough, M., Borok, E., Elsworth, J.D., Roth, R.H., Sleeman, M.W., Picciotto, M.R., Tschop, M.H., Gao, X.B. & Horvath, T.L. (2006) Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *J. Clin. Invest.*, **116**, 3229–3239.

Acosta-Galvan, G., Yi, C.X., van der Vliet, J., Jhamandas, J.H., Panula, P., Angeles-Castellanos, M., Del Carmen Basualdo, M., Escobar, C. & Buijs, R.M. (2011) Interaction between hypothalamic dorsomedial nucleus and the suprachiasmatic nucleus determines intensity of food anticipatory behavior. *Proc. Natl. Acad. Sci. USA*, **108**, 5813–5818.

Amir, S., Lamont, E.W., Robinson, B. & Stewart, J. (2004) A circadian rhythm in the expression of PERIOD2 protein reveals a novel SCN-controlled oscillator in the oval nucleus of the bed nucleus of the stria terminalis. *J. Neurosci.*, **24**, 781–790.

Angeles-Castellanos, M., Aguilar-Roblero, R. & Escobar, C. (2004) c-Fos expression in hypothalamic nuclei of food-entrained rats. *Am. J. Physiol.-Reg. I.*, **286**, R158–R165.

Blum, I.D., Patterson, Z., Khazall, R., Lamont, E.W., Sleeman, M.W., Horvath, T.L. & Abizaid, A. (2009) Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice. *Neuroscience*, **164**, 351–359.

Bolles, R.C. & Stokes, L.W. (1965) Rat's anticipation of diurnal and a-diurnal feeding. *J. Comp. Physiol. Psych.*, **60**, 290–294.

Chabot, C.C., Connolly, D.M. & Waring, B.B. (2012) The effects of lighting conditions and food restriction paradigms on locomotor activity of common spiny mice, Acomys cahirinus. *J. Circadian Rhythms*, **10**, 6.

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E. & Weigle, D.S. (2001) A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes*, **50**, 1714–1719.

Davis, J.F., Choi, D.L., Clegg, D.J. & Benoit, S.C. (2011) Signaling through the ghrelin receptor modulates hippocampal function and meal anticipation in mice. *Physiol. Behav.*, **103**, 39–43.

Diano, S., Farr, S.A., Benoit, S.C., McNay, E.C., da Silva, I., Horvath, B., Gaskin, F.S., Nonaka, N., Jaeger, L.B., Banks, W.A., Morley, J.E., Pinto, S., Sherwin, R.S., Xu, L., Yamada, K.A., Sleeman, M.W., Tschop, M.H. & Horvath, T.L. (2006) Ghrelin controls hippocampal spine synapse density and memory performance. *Nat. Neurosci.*, **9**, 381–388.

Drazen, D.L., Vahl, T.P., D'Alessio, D.A., Seeley, R.J. & Woods, S.C. (2006) Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology*, **147**, 23–30.

Edmonds, S.C. & Adler, N.T. (1977a) Food and light as entrainers of circadian running activity in the rat. *Physiol. Behav.*, **18**, 915–919.

Edmonds, S.C. & Adler, N.T. (1977b) The multiplicity of biological oscillators in the control of circadian running activity in the rat. *Physiol. Behav.*, **18**, 921–930.

Gooley, J.J., Schomer, A. & Saper, C.B. (2006) The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. *Nat. Neurosci.*, **9**, 398–407.

Guan, X.M., Yu, H., Palyha, O.C., McKee, K.K., Feighner, S.D., Sirinathsinghji, D.J., Smith, R.G., Van der Ploeg, L.H. & Howard, A.D. (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res. Mol. Brain Res.*, **48**, 23–29.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H. & Kangawa, K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, **402**, 656–660.

Lamont, E.W., Diaz, L.R., Barry-Shaw, J., Stewart, J. & Amir, S. (2005) Daily restricted feeding rescues a rhythm of period2 expression in the arrhythmic suprachiasmatic nucleus. *Neuroscience*, **132**, 245–248.

Lamont, E.W., Patterson, Z., Rodrigues, T., Vallejos, O., Blum, I.D. & Abizaid, A. (2012) Ghrelin-deficient mice have fewer orexin cells and reduced cFOS expression in the mesolimbic dopamine pathway under a restricted feeding paradigm. *Neuroscience*, **218**, 12–19.

Landry, G.J., Simon, M.M., Webb, I.C. & Mistlberger, R.E. (2006) Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats. *Am. J. Physiol.-Reg. I.*, **290**, R1527–R1534.

Landry, G.J., Yamakawa, G.R. & Mistlberger, R.E. (2007) Robust food anticipatory circadian rhythms in rats with complete ablation of the thalamic paraventricular nucleus. *Brain Res.*, **1141**, 108–118.

Leak, R.K. & Moore, R.Y. (2001) Topographic organization of suprachiasmatic nucleus projection neurons. *J. Comp. Neurol.*, **433**, 312–334.

LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D.W. & Silver, R. (2009) Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proc. Natl. Acad. Sci. USA*, **106**, 13582–13587.

Mieda, M., Williams, S.C., Richardson, J.A., Tanaka, K. & Yanagisawa, M. (2006) The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker. *Proc. Natl. Acad. Sci. USA*, **103**, 12150–12155.

Mitchell, V., Bouret, S., Beauvillain, J.C., Schilling, A., Perret, M., Kordon, C. & Epelbaum, J. (2001) Comparative distribution of mRNA encoding the growth hormone secretagogue-receptor (GHS-R) in Microcebus murinus (Primate, lemurian) and rat forebrain and pituitary. *J. Comp. Neurol.*, **429**, 469–489.

Moga, M.M., Weis, R.P. & Moore, R.Y. (1995) Efferent projections of the paraventricular thalamic nucleus in the rat. *J. Comp. Neurol.*, **359**, 221–238.

Moore, R.Y., Weis, R. & Moga, M.M. (2000) Efferent projections of the intergeniculate leaflet and the ventral lateral geniculate nucleus in the rat. *J. Comp. Neurol.*, **420**, 398–418.

Moriya, T., Aida, R., Kudo, T., Akiyama, M., Doi, M., Hayasaka, N., Nakahata, N., Mistlberger, R., Okamura, H. & Shibata, S. (2009) The dorsomedial hypothalamic nucleus is not necessary for food-anticipatory circadian rhythms of behavior, temperature or clock gene expression in mice. *Eur. J. Neurosci.*, **29**, 1447–1460.

Ohta, H., Yamazaki, S. & McMahon, D.G. (2005) Constant light desynchronizes mammalian clock neurons. *Nat. Neurosci.*, **8**, 267–269.

Pfluger, P.T., Kirchner, H., Gunnel, S., Schrott, B., Perez-Tilve, D., Fu, S., Benoit, S.C., Horvath, T., Joost, H.G., Wortley, K.E., Sleeman, M.W. & Tschop, M.H. (2008) Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure. *Am. J. Physiol.-Gastr. L.*, **294**, G610–G618.

Reppert, S.M. & Weaver, D.R. (2002) Coordination of circadian timing in mammals. *Nature*, **418**, 935–941.

Sanchez, J., Oliver, P., Pico, C. & Palou, A. (2004) Diurnal rhythms of leptin and ghrelin in the systemic circulation and in the gastric mucosa are related to food intake in rats. *Pflug. Arch.*, **448**, 500–506.

Stephan, F.K. (2002) The "other" circadian system: food as a Zeitgeber. *J. Biol. Rhythm.*, **17**, 284–292.

Toshinai, K., Mondal, M.S., Nakazato, M., Date, Y., Murakami, N., Kojima, M., Kangawa, K. & Matsukura, S. (2001) Upregulation of Ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochem. Bioph. Res. Co.*, **281**, 1220–1225.

Tschop, M., Smiley, D.L. & Heiman, M.L. (2000) Ghrelin induces adiposity in rodents. *Nature*, **407**, 908–913.

Verwey, M., Khoja, Z., Stewart, J. & Amir, S. (2007) Differential regulation of the expression of Period2 protein in the limbic forebrain and dorsomedial hypothalamus by daily limited access to highly palatable food in food-deprived and free-fed rats. *Neuroscience*, **147**, 277–285.

Waddington Lamont, E., Harbour, V.L., Barry-Shaw, J., Renteria Diaz, L., Robinson, B., Stewart, J. & Amir, S. (2007) Restricted access to food, but not sucrose, saccharine, or salt, synchronizes the expression of Period2 protein in the limbic forebrain. *Neuroscience*, **144**, 402–411.

Watts, A.G. & Swanson, L.W. (1987) Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. *J. Comp. Neurol.*, **258**, 230–252.

Watts, A.G., Swanson, L.W. & Sanchez-Watts, G. (1987) Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport of Phaseolus vulgaris leucoagglutinin in the rat. *J. Comp. Neurol.*, **258**, 204–229.

Wortley, K.E., del Rincon, J.P., Murray, J.D., Garcia, K., Iida, K., Thorner, M.O. & Sleeman, M.W. (2005) Absence of ghrelin protects against early-onset obesity. *J. Clin. Invest.*, **115**, 3573–3578.

Yi, C.X., van der Vliet, J., Dai, J., Yin, G., Ru, L. & Buijs, R.M. (2006) Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. *Endocrinology*, **147**, 283–294.

Yi, C.X., Challet, E., Pévet, P., Kalsbeek, A., Escobar, C. & Buijs, R.M. (2008) A circulating ghrelin mimetic attenuates light-induced phase delay of mice and light-induced Fos expression in the suprachiasmatic nucleus of rats. *Eur. J. Neurosci.*, **27**, 1965–1972.

Zigman, J.M., Nakano, Y., Coppari, R., Balthasar, N., Marcus, J.N., Lee, C.E., Jones, J.E., Deysher, A.E., Waxman, A.R., White, R.D., Williams, T.D., Lachey, J.L., Seeley, R.J., Lowell, B.B. & Elmquist, J.K. (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J. Clin. Invest.*, **115**, 3564–3572.

Zigman, J.M., Jones, J.E., Lee, C.E., Saper, C.B. & Elmquist, J.K. (2006) Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J. Comp. Neurol.*, **494**, 528–548.