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Entrainment of temperature and activity rhythms to restricted feeding in orexin knock out mice

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Abstract

Ablation of the SCN, an established circadian clock, does not abolish food entrainment, suggesting that the food-entrainable oscillator (FEO) must lie outside the SCN. Typically, animals show anticipatory locomotor activity and rise in core body temperature under the influence of the FEO. Signals from the FEO would, therefore, converge onto arousal neurons so that the animal might forage for food. In the present study, we investigate whether the neuropeptide orexin, which has been linked to arousal, might transduce the arousal signal.

Orexin-knockout (orexin-KO) and wildtype (WT) mice (both C57BL/6J derived) were implanted with MiniMitter transmitters that recorded core body temperature and activity (12h LD cycle). After a week of ad-libitum feeding, the mice were given access to food for 4h (ZT 4–8) for nine days followed by 2-days of fasting.

When orexin-KO mice were placed in a restricted feeding schedule, both core body temperature and activity entrained to the feeding schedule. In these mice gross locomotor activity was severely blunted during the nine day period of restricted feeding ($-79.4 \pm 6.3\%$) from the WT, but they showed an increase in core body temperature in anticipation to the meal time similar to the WT mice. There was no difference in the amount of food intake between the genotypes.

We conclude that orexin is not required for entrainment of activity and temperature to a restricted feeding schedule, but is required for the robust expression of gross locomotor activity in anticipation of the scheduled feeding.

Keywords

Orexin; transgenic mice; food anticipatory rhythms; core body temperature; activity; restricted feeding

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1. Introduction

When rodents are given food at a fixed time of day (food restriction) they begin to develop robust food anticipatory activity (FAA). The increase in activity begins to occur 1–3 hours before mealtime and it continues to occur even on the days when no food is given. The FAA is not abolished by lesions of the suprachiasmatic nucleus (SCN), an established circadian clock (Stephan, 1989), suggesting that the food- entrainable oscillator (FEO) must lie outside the SCN. Recent studies have suggested that the FEO may have a distributed organization (Landry et al., 2006) or it may possibly reside within the dorsomedial hypothalamus (DMH) since neurotoxic lesion of the DMH in rats blocks the entrainment of arousal, locomotor activity, and body temperature in response to the restricted period of feeding (Gooley et al., 2006). Moreover, there is a robust expression of a clock gene, *mPER*, in DMH in response to both the regular food and palatable food during restricted feeding (Mieda et al., 2006; Verwey et al., 2007). Therefore, area(s) which possibly act as food- entrainable oscillator(s) convey their signal to downstream arousal neurons, which then awaken the animal in anticipation of food.

One potential target is the lateral hypothalamus, where neurons containing the neuropeptide orexin are located. Orexin has been implicated in arousal because the loss of the neurons in humans, mice and rats leads to the sleep disorder narcolepsy (Lin et al., 1999; Chemelli et al., 1999; Thannickal et al., 2000; Peyron et al., 2000; Gerashchenko et al., 2001; Beuckmann et al., 2004;). Orexin is known to stimulate feeding by activating the key feeding regulatory sites in the hypothalamus, limbic and mid brain areas (Mullett et al., 2000). Entrainment to restricted feeding has been examined in mice where the orexin neurons die as a result of an orexin promoter-driven accumulation of the cytotoxic gene product poly-Q-ataxin - 3. These mice have severely blunted food anticipatory locomotor activity in response to restricted feeding (Akiyama et al., 2004; Mieda et al., 2004). In the orexin/ataxin-3 transgenic mice, because of the loss of orexin neurons in the LH, other neuropeptides that are coexpressed in these neurons, such as galanin and dynorphins, are also lost (Hakansson et al., 1999; Chou et al., 2001). Therefore, it remains to be determined if in the presence of the other coexpressed peptides, the input signals from the FEO are relayed to these neurons and are sufficient to produce the anticipatory behaviors in response to the food restriction. In this study we tested the hypothesis that orexin transmits the signal to downstream arousal neurons by monitoring both temperature and activity in orexin knockout mice in a limited food availability paradigm similar to that adopted in earlier studies (Akiyama et al., 2004; Mieda et al., 2004; Gooley et al., 2006).

2. Results

During ad lib feeding, mean temperature of the wildtype (WT) and orexin-KO mice were not significantly different during the light phase, but during the late dark phase (ZT20-ZT21) the core body temperatures of orexin-KO mice were significantly higher compared to the WT ($P < 0.01$) (Fig.2, upper left panel). During a similar period (ZT19 –ZT-22) an increase in activity was observed in the orexin-KO mice compared to the WT ($P < 0.001$) (Fig.1, upper left panel).

In response to the restricted feeding, WT mice showed a significant increase in activity (Fig. 1) and core body temperature (Fig. 2) three hours prior to the meal time (ZT4). This increase was seen throughout the period of restricted feeding (day 1–9) and also during fasting (day 11). The orexin-KO ($n = 7$) mice entrained to the restricted feeding schedule but compared to WT mice ($n = 10$) had significantly lower activity (Fig. 1, Fig. 3 A&B) throughout the restricted feeding schedule ($P < 0.001$). The anticipatory activity for the three hours prior to the feeding time at 11:00am (ZT-4) was lower by 90.16% during the RF1 (days 1–3), by 84.13% during RF2 (days 4–6) and by 67.16% during RF3 (days 7–9), in the orexin-KO mice compared to the WT.

During fasting day 2 (day 11) following the restricted feeding period, the orexin-KO mice still had significant lower activity for a period of 3–4 hours before and after the scheduled feeding time. However, the activity tended to become comparable to the WT mice during ZT4-ZT6 (scheduled meal time), which indicates that the mice have entrained to the scheduled meal time but there is a lack of robust expression of the locomotor activity in the orexin-KO mice.

As for core body temperature, during the first three days of the RF schedule, the orexin-KO mice ($n = 7$), compared to WT mice ($n = 11$), had significantly lower (-8.94%) core body temperature ($P < 0.001$) (Fig 2) in the first three hours (anticipatory) prior to scheduled feeding time (ZT-4). However, the rise in temperature during this anticipatory period in the orexin-KO mice (28.96 to 36.60°C ; by 24.5%) was almost two times as the WT mice (32.67 to 36.86°C ; by 12.85%). This could be because of significantly lower core body temperature in orexin-KO (28.9°C) than the wildtype (32.7°C) mice, four hours before the scheduled feeding time. During this early period of entrainment, the temperature rhythm reached its nadir in the WT mice at $\sim 7:37$ am while in orexin-KO mice it was at $8:24$ am, indicating a phase delay of 47 mins ($P < 0.05$). However, on restricted feeding days 4–9 (later period of entrainment) the orexin-KO mice had as robust an increase in temperature as the WT mice (Fig 2). During this period, the orexin-KO mice did not show a significant phase delay in the nadir of the temperature rhythm.

Activity and body temperature before the scheduled meals during the restriction feeding did not differ significantly in the male and female mice within the genotypes and across time.

In both genotypes there was a significant increase ($P < 0.001$) in food intake measured across days during restricted feeding but there was no significant difference in the food intake between the two genotypes (Fig. 4).

3. Discussion

The primary results of this study are that in the absence of orexin, activity and core body temperature rhythms entrain to a restricted feeding schedule. In a previous study, activity rhythms were assessed in mice that lacked orexin neurons (orexin/ataxin-3 transgenic mice), and entrainment of the activity rhythms was found to be intact (Akiyama et al., 2004; Mieda et al., 2004) during the restricted feeding schedule, but the activity of the orexin-ataxin transgenic mice was severely blunted. In the present study in orexin-KO mice we also found blunted activity, which is consistent with the previous study. Therefore, it is unlikely that the coexpressed peptides like dynorphin and galanin may have some role in FAA related activity. Taken together, the results also suggest that orexin neurons are not required for transducing the signal from the food entrainable oscillator, although orexin is still necessary for the robust expression of gross locomotor activity in anticipation of feeding.

During the ad-lib feeding, the night-time body temperature of the orexin-KO mice was higher than that of the WT mice, which is consistent with previous reports (Mochizuki et al., 2006). In our study, activity also increased at night. However, in the previous study (Mochizuki et al., 2006) during the same period of the night, activity was found to be lower compared to WT. This discrepancy might be due to the mice being tethered to an EEG recording cable in the previous study (Mochizuki et al., 2006), whereas mice in the present study were not tethered. Increase in the incidence of cataplectic episodes in orexin-KO mice during the dark phase has been reported in previous studies (Chemelli et al., 1999; Mochizuki et al., 2004). During a cataplectic episode the mice are not active. However, our results show either no change in activity of orexin-KO mice during first half or increased activity during the later half of the dark phase compared to WT, suggesting that the increased episodes of cataplexy during the night do not result in lowering the locomotor activity. This is because the mice typically have on average 17 cataplexy episodes in the first four hours of the dark phase with each episode

lasting on average 65.5 sec (Chemelli et al., 1999). Overall, throughout the night orexin-KO mice spend only 3.8% of their time in cataplexy (Mochizuki et al., 2004). Such a small number of episodes are unlikely to change the overall activity.

Several studies have observed the response to restricted feeding schedules by monitoring both core body temperature and activity in hamsters (Mistlberger, 1993; Masuda and Oishi, 1995), rats (Mistlberger and Mumby, 1992; Davidson et al., 2000; Recabarren et al., 2005; Mendoza et al., 2005) and mice (Akiyama et al., 2004; Mieda et al., 2004). In rats, lesions of various brain areas were found to produce a dissociation between core body temperature and activity (Mistlberger and Mumby, 1992; Davidson and Stephan, 1999; Davidson et al., 2000; Mendoza et al., 2005; Recabarren et al., 2005). This suggests that the food anticipation induced signals from the FEO are transmitted downstream to different brain nuclei which then manifest the related behavioral changes before the meal time. Dysfunction of the brain circuitry afferent to SCN leads to abnormal behavioral and molecular circadian rhythms (Pallier et al., 2007).

The FEO is hypothesized to be located in the DMH since neurotoxin induced lesions of the DMH blocks the pre-prandial rise of body temperature, locomotor activity and wakefulness in anticipation of meal time (Gooley et al., 2006). However, a separate study by Landry et al., 2006, suggested a number of these oscillators are distributed in various regions of the brain, and that lesions of one region did not eliminate all of the manifestations of food entrainment (Landry et al., 2006). Gooley et al., hypothesized that the neural and endocrine signals about the circadian phase and energy metabolism possibly converge in the DMH (Gooley et al., 2006; Saper et al., 2005). Therefore, DMH neurons in association with other nuclei entrained to the scheduled meal time possibly convey the signals to the areas related to arousal, and those regulating the core body temperature. DMH neurons project to the arousal related areas, such as, orexin neurons in perifornical area (PeF), tuberomammillary nucleus (TMN), locus coeruleus and the dorsal raphe (Deurveilher and Semba, 2005). The orexin neurons are part of the 'approach-exploratory' system for the regulation of the muscle tone and motor behavior (Milevskiy et al., 2005; Lee et al., 2005). Thus, it is not surprising that without orexin the animals were less active prior to their meal time.

Our results indicate that the signal from the FEO is transmitted in the absence of orexin. Among likely candidates to receive such a signal and arouse the animal include the histaminergic neurons of the TMN. These neurons show activation of c-Fos protein and mRNA during the anticipatory phase, and it occurs ahead of the perifornical area (Inzunza et al., 2000; Huang et al., 2001; Meynard et al., 2005; Valdes et al., 2005). C-Fos expression was not seen in other aminergic neurons related to arousal (Meynard et al., 2005). TMN receives direct projections from the orexinergic neurons in PeF and may help regulate arousal (Huang et al., 2001). However, the role of the TMN in regulating core body temperature is not fully explored. Therefore, we hypothesize that the signals for FAA originate in DMH and are transduced in TMN, and that the PeF provides the nonspecific arousal related input for the robust expression of the FAA related activity. Our results show an initial lag to entrain and a phase delay in the anticipatory temperature rhythm in the orexin-KO mice, which could possibly be due to the lost input from the PeF neurons in the orexin-KO mice. The lost orexin input may also explain the reduced activity prior to the scheduled meal time. The orexin-KO mice do show residual anticipatory activity and this may be due to the intact entrainment process in these mice.

We did not observe any difference in food intake between the orexin-KO and WT mice. This is consistent with previous studies (Landry et al., 2006; Gooley et al., 2006) and in the orexin/ataxin-3 transgenic mice (Akiyama et al., 2004; Mieda et al., 2004). This may also suggest that in response to the restricted feeding, the gastrointestinal rhythms had shifted to the diurnal phase and that the entrainment of the peripheral oscillators was intact and remained unaffected by loss of either DMH neurons or the orexin neurons in the perifornical area. Therefore, it may

be implied that DMH and the PeF orexin neurons may be situated downstream, i.e., on the output side of the FEO and are important for the manifestation of some of the anticipatory behaviors.

We conclude that neither orexin nor the orexin neuron (Akiyama et al., 2004; Mieda et al., 2004) is required for entrainment to a restricted feeding schedule. The signals from the FEO are likely to be received by other arousal neurons, such as the TMN, which awaken the animal. However, upon awakening orexin is required for robust expression of locomotor activity but not for rise in core body temperature.

4. Experimental Procedures

Orexin knockout mice (orexin-KO, $n = 7$) and their wildtype (WT, $n = 11$) littermates were used in the study. Due to successive generations of backcrossing to the C57BL/6J line a congenic orexin-KO line has emerged. Mice were genotyped at weaning by polymerase chain reaction amplification of the genomic DNA extracted from tail biopsies. The probe hybridization assay for the orexin gene was negative for the orexin-KO mice. The immunoreactivity for orexin using the goat anti orexin antibody (SC-8070, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was also checked in some brain sections from the orexin-KO mice, which were found negative for orexin immunoreactivity.

All animals were raised and maintained on a 12:12-h light-dark cycle. Food (pelleted Purina rodent chow) was available ad libitum except during the period of restricted feeding. Animals had ad libitum access to water including the periods of food restriction.

Under surgical anesthesia (Ketamine 80mg/kg/ml and Xylazine 10mg/kg/ml) male (WT $n = 6$ and KO $n = 3$) and female mice (WT $n = 5$ and KO $n = 4$) were implanted with transmitters (E-mitters, Minimitter, Bend, OR) in the intraperitoneal cavity. We chose to include both male and female mice, because in our earlier study (Zhang et al., 2007) we did not observe any difference between male and female mice in terms of the release of orexin as measured in their cerebrospinal fluid, in response to spontaneous and sleep deprivation induced activity. After surgery, animals were housed singly in plastic cages (16"×8"×8") with corn bedding, placed on three tier shelves (each tier 45"×15"×18"), and each tier housed 2 cages. The plastic cages were placed on a receiving platform (ER-4000 receiver, Bend, OR) that transmitted the signal for both the temperature and activity every 2 mins, which was then acquired by the Vital View program (Minimitter, Bend, OR). The temperature and activity signals from the implanted transmitters were detected by a receiver (ER-4000 receiver via telemetry) and the dedicated software (Vital View software, MiniMitter, Bend, OR) created a longitudinal record of movement and temperature.

4.1 Food restriction protocol

Mice (WT and KO) were kept on a 12h light/dark cycle [ZT0 defined as lights on time (7:00am) and ZT12 as lights off (7:00pm)]. Mice were individually housed in a Plexiglas cage, and a week before the restriction feeding experiment they were placed in fresh cages to avoid any handling during the course of the experiment. The mice were given food ad libitum for a week. During this period the food pellets were briefly handled at ZT4 (11:00 am) and ZT8 (3:00pm), without physically handling the animals, to mimic the conditions which may also awaken the mice during the restricted schedule. Then the food was withdrawn at ZT4 and was presented to them at the same time the following day. This day was considered as fasting day 0. Thereafter, from day 1 to day 9 during the restricted feeding regime, the food (~ 8–10gm) was given at ZT 4 for a period of 4 hours and withdrawn at ZT 8. Animals were weighed along with their cages (to minimize direct handling of animals) at ZT8 everyday during the period of restricted feeding (RF). The restricted feeding period was followed by a 2-day food deprivation period

(fasting- days 10 & 11) to assess activity and temperature rhythms in the absence of the daily feeding stimulus.

4.2 Statistical analysis

The activity and temperature data that were collected every 2 minutes were averaged every 30 mins, for the adlib days, fasting days (day 0 and day 10 and 11) before and after the restricted feeding and for the restricted feeding period in bins of 3 days each (RF1 days 1–3; RF2 days 4–6; RF3 days 7–9). A two way repeated measures ANOVA (genotype $\times$ time with repeated measures on time), followed by Holm-Sidak post hoc test was used for statistical analysis. The data sets with probability values of $P < 0.05$ were considered significant. One of the wild type mice could only be recorded for temperature and not activity; therefore, it was excluded from the activity data set. A three way ANOVA (genotype $\times$ gender $\times$ time) followed by Holm-Sidak post hoc test was also done for both the temperature and activity.

In order to estimate the phase differences in the rise of the temperature rhythms between the orexin-KO and WT mice, temperature nadirs for each mouse were defined as the time point before the feeding when the average temperature was at its minimum. The temperature nadirs were statistically compared between the two genotypes for each bin of 3 days.

The food intake between the genotypes across the restricted feeding days was also compared using two way repeated measures ANOVA (genotype $\times$ time with repeated measures on time), followed by Holm-Sidak post hoc test.

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Fig. 1.

Blunted food anticipation activity in response to the restriction feeding in orexin-KO mice. During the restriction feeding, the wildtype (WT, n=10) and orexin-KO mice (n=7) were allowed access to the food only during 4 hours (ZT4-ZT8) for a period of 9 days. The activity is measured every two minutes and then averaged in 30 minute bins. Each data point represents average activity counts ($\pm$ SEM) of the WT and orexin-KO mice over a week of ad-lib feeding (A), fasting on day 0 and day11, and 9 days of the restricted feeding (B – F). Activity during the restricted feeding days is summarized in blocks of 3 days [day1–3 (C); day4–6 (D); day7–9 (E)]. The grey shaded portion in each graph refers to the scheduled meal time (ZT4-ZT8)

during the restricted feeding days (days1–9). * significance $P < 0.01$ between wildtype (WT) and orexin-KO.

Fig. 2.

Anticipatory temperature rhythms in response to the restriction feeding in the WT and orexin-KO mice. The average temperature in degrees Celsius (mean $\pm$ SEM) of the WT (n=11) and orexin-KO mice (n=7) during a week of ad-lib feeding (A), fasting (days 0 and 11) before and after the restricted feeding in both the genotypes for a period of 9 days (B-F). The data for the restricted feeding is represented in 3 blocks, each showing an average of 3 days (C-E). The grey shaded portion in each graph refers to the scheduled meal time (ZT4-ZT8) during restricted feeding days (days 1-9). The orexin-KO mice showed blunted amplitude during the anticipatory periods to the meal time, but after day 4, the increase in amplitude was similar to the WT (D and E) mice. A phase delay in the nadir of body temperature was observed in the

orexin-KO mice during the early light phase. The comparison of the temperature nadirs in the orexin-KO and WT during the restricted feeding (in similar blocks of 3days each, as shown in C–E) and fasting day10 are shown in G. The grey shaded portion in each graph refers to the scheduled meal time (ZT4–ZT8) during the restricted feeding days (days1–9).^{*} significance $P < 0.01$ between wildtype (WT) and orexin-KO.

A. WILD-TYPE ACTIVITY RHYTHM

B. OREXIN-KO ACTIVITY RHYTHM

C. WILD -TYPE TEMPERATURE RHYTHM

D. OREXIN-KO TEMPERATURE RHYTHM

Fig. 3.

Representative double-plotted actograms of locomotor activity (A&B) and temperature (C&D) records of the WT and orexin-KO mice, which were subjected to the restricted feeding paradigm (cf. methods). The grey rectangle in each actogram represents the period during which the animals were allowed access to the food (ZT4-ZT8). Orexin-KO mice showed a decrease in the food anticipatory locomotor activity (B). The temperature records of the orexin-KO mice showed significantly lower temperatures compared to WT during the days 1–3, but by day4 the anticipatory increase in temperature is similar to as seen in WT mice (C).

Fig. 4.

Food intake (in grams) was measured in the orexin-KO and WT mice during the restricted feeding period (days 1–9) without handling the animals so as to avoid confounding effects. Both genotypes show an increase in food intake during RF, but amount of food intake between the genotypes is not significantly different (two way ANOVA, genotype $\times$ time, followed by Holm-Sidak post hoc test).