

Enhanced Food-Anticipatory Circadian Rhythms in the Genetically Obese Zucker Rat

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MISTLBERGER, R. E. AND E. G. MARCHANT. *Enhanced food-anticipatory circadian rhythms in genetically obese Zucker rats.* *PHYSIOL BEHAV* **66**(2) 329–335, 1999.—This study examines the effects of the leptin receptor mutation in obese Zucker rats on entrainment of food-anticipatory rhythms to daily feeding schedules. Leptin is secreted by adipocytes in proportion to fat content, exhibits a daily rhythm in plasma that is synchronized to feeding time, and inhibits activity of arcuate neuropeptide Y neurons that stimulate feeding behavior and regulate metabolism. Activity within this neuropeptide Y system is enhanced by food deprivation and attenuated by overfeeding and diet-induced obesity. Diet-induced obesity, in turn, attenuates food-anticipatory rhythms. If the effects of obesity on food-entrained rhythms are mediated by leptin inhibition of neuropeptide Y neurons, then these rhythms may be enhanced in leptin-insensitive Zucker obese rats. Alternatively, if daily rhythms of leptin mediate the generation or entrainment of these rhythms, Zucker rats may fail to anticipate daily feedings. Zucker obese and lean rats received food for 3 h/day during the midlight period. Both groups exhibited significant food-anticipatory activity that persisted during three cycles of food deprivation, but this rhythm was significantly more robust in obese rats, when expressed as anticipation and persistence ratios, and as peak values. Anticipatory rhythms did not persist in either group when food was provided ad lib. These results indicate that central actions of leptin may mediate the inhibitory effects of obesity on the expression of food-anticipatory rhythms in rats, but do not mediate the inhibitory effects of ad lib food access, and do not serve as necessary internal entrainment cues or clock components for the food-entrainable circadian system. © 1999 Elsevier Science Inc.

Circadian rhythms Food entrainment Food restriction Zucker rats Obesity

CIRCADIAN rhythms in mammals are regulated by a multioscillator system. The hypothalamic suprachiasmatic nuclei (SCN) are the site of one collection of cell autonomous circadian oscillators that function together as the master pacemaker for entrainment of circadian rhythms to light–dark (LD) cycles (27,60). This pacemaker can also be entrained by a variety of nonphotic stimuli that induce locomotor activity or arousal [e.g., (23,30,45,59)]. However, it does not appear to be necessary for entrainment of behavioral, autonomic, and endocrine rhythms to a scheduled daily meal. Food-entrained activity rhythms are characteristically expressed as consolidated bouts of running or foraging-related activities that begin 1–3 h prior to scheduled feeding, and that increase in intensity until feeding time [reviewed in (35)]. This anticipatory activity to a daily feeding event exhibits the circadian properties of persistence during constant conditions (i.e., when food is withheld for several days, activity reoccurs at its usual

phase) and circadian limits to entrainment (i.e., anticipation develops only to feeding schedules with intermeal intervals within the circadian range of ~22–31 h), indicating regulation by a self-sustaining circadian pacemaker (7,46,54). However, food-anticipatory rhythms in behavior or physiology are neither disrupted nor prevented by complete ablation of the SCN in rats (6,55), hamsters (1,34), and mice (30).

At present, neural components of the food-entrainable pacemaker system are unknown (35). One region that has not been directly evaluated for a role in food entrainment is the arcuate nucleus (ARC)–neuropeptide Y (NPY) system that has been implicated in the regulation of feeding behavior and energy homeostasis. Several observations indicate that activity within this system correlates with and may drive or modulate circadian food anticipatory activity. The ARC includes NPY-containing neurons that innervate several hypothalamic nuclei, including the paraventricular and dorsomedial hypo-

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thalamus (3). NPY is the most potent orexigenic compound yet discovered; intrahypothalamic injections, at widely distributed sites, but particularly in the vicinity of the perifornical area, powerfully stimulate eating in sated or food-deprived rats at any time of day (52,53). NPY-induced eating appears to reflect a true "hunger" state, because exogenous NPY also increases learned instrumental responding for food (25), and if food is withheld, activity is directed at the usual feeding site (41). NPY activity is elevated in food-deprived animals, and is maximal prior to a daily feeding time in food-restricted rats (26,62). NPY synthesis and/or release are increased by serotonin antagonists (16,18) and decreased by serotonin agonists, including fluoxetine (17,19). Food-anticipatory rhythms are also attenuated by chronic treatment with fluoxetine (2), and by obesity induced by prior overfeeding, which is likely associated with reduced NPY activity (43). Collectively, these findings establish the ARC as an important integrative site for mobilization of behavioral and physiological responses in the service of weight regulation, and suggest a close relationship with the food-entrainable circadian system.

In the present study, we examined the role of the ARC–NPY system in food entrainment by measuring food-anticipatory activity in lean and obese Zucker rats. Obesity in the Zucker rat is caused by a point mutation in the gene encoding the receptor for the protein hormone leptin (24,44). Leptin is secreted by adipose cells in proportion to the level of body fat (20,29). Plasma leptin levels express a robust daily rhythm that is directly regulated by the timing of food intake, and rapidly shifts to follow large phase displacements of the feeding cycle (28,31,48,51). Exogenous leptin administration attenuates food intake, increases energy expenditure, and reduces body weight in several animal models (50,57,61). A primary site of action for leptin is the ARC–NPY system (57,61); ARC–NPY cells express mRNA for leptin receptors (33), exogenous leptin inhibits NPY synthesis and release (49,56), and ARC–NPY lesions block the effects of leptin on body weight (13). The leptin receptor mutation in Zucker rats causes reduced binding and reduced sensitivity or insensitivity to leptin (9–11,22) and ARC–NPY expression is consequently upregulated, resulting in increased food intake, decreased energy expenditure, and obesity (4,5,15,32,57,61). Food-anticipatory rhythms in rats diminish rapidly during ad lib access to food [but reappear if food is withheld; (7,46)], and are attenuated in previously overfed obese rats (43), conditions under which NPY activity is likely reduced.

These data raise the possibility that leptin–ARC–NPY system may play a mediating or modulating role in the expression of food-anticipatory rhythms. One extreme possibility is that daily rhythms of plasma leptin or leptin sensitivity may serve as an internal entrainment cue or as a "gear" in the pacemaker mechanism driving food-anticipatory circadian rhythms. If so, then the leptin mutation in homozygous Zucker rats may impair or prevent the generation of food-anticipatory rhythms by compromising central responses to cycling leptin. Alternatively, leptin may modulate the output of the food-entrainable circadian system, and account for apparent "gating" of food-anticipatory rhythms by corpulence status and food availability. High levels of leptin, and low NPY, may normally inhibit the expression of food-anticipatory rhythms, and may be responsible for low levels of food anticipation in obese animals, and rapid attenuation of food anticipatory rhythms during ad lib food access. If so, then upregulation of NPY due to leptin insensitivity in Zucker rats might be associated with a more robust expression of food-anticipatory rhythms, despite the animals' great obesity, and enhanced

persistence of these rhythms during several cycles of complete food deprivation and ad lib feeding.

METHODS

Procedures

Seven obese (543 ± 8 g) and six lean female Zucker rats (230 ± 6 g) (Charles River, Montreal) were transferred at 4.5 months age to individual sawdust lined plastic cages ($45 \times 25 \times 20$ cm) in ventilated isolation cabinets. Each cage was equipped with a food hopper for rat chow pellets (Richmond Standard 5001 rat chow, PMI Feeds, BC) and an external food bin, accessible through a window, for powdered 5001 chow. Activity at the feeding window was detected by photocells positioned over the feeding cup and monitored continuously by computer using the Activity Counting System interface and software (Simon Fraser University).

Food-bin activity was recorded for 34 days, during which rat chow pellets and water were available ad lib (LD 12:12, 20 lx during lights on). Food was removed for 20 h and then provided in powdered form for 3 h each day beginning 5 h after lights on. After 22 days, food was removed for 80 h, returned for 72 h, then removed again for 68 h, and provided ad lib for a final week. Deprivation tests served to assess whether food-anticipatory activity rhythms persisted during constant conditions. All animals were weighed before and after each food deprivation test.

Data Analysis

Activity data were transferred to a Macintosh computer and displayed in the form of actograms and average waveforms for visual inspection of food-anticipatory rhythms. Anticipation of feeding time was quantified in several ways. The 22 days of restricted feeding were first divided into four blocks of days [1–5, 6–10, 11–16, and 17–22]. Inspection of activity charts indicated that food anticipatory activity began within 3 h of mealtime. Therefore, mean levels of activity during this 3-h time block were calculated for each rat for each of the four blocks of days, and this activity was expressed as an "anticipation ratio" with respect to the mean level of activity occurring during the rest of the day (excluding the 3-h mealtime). For comparative purposes, the same calculations were made for a block of 5 days of ad lib feeding prior to food restriction. The duration of anticipation was quantified by defining activity onset as the first of three consecutive 10-min bins within 3 h of feeding time that exceeded the 24-h mean level of activity for that block of days. The peak level of anticipatory activity was defined as the maximal value observed in any 10-min bin within a bout of anticipatory activity.

Persistence of food-anticipatory rhythms in the absence of a daily feeding stimulus was evaluated to determine whether anticipatory rhythms were self-sustaining in both lean and obese rats. Activity counts were averaged across the deprivation days within each test. The mean levels of activity during the 3 h corresponding to prior mealtime (i.e., hours 5–8 of lights on) and the 3-h time blocks immediately preceding and following were calculated. These three means were then expressed as ratios with respect to total daily activity. For comparative purposes, the same calculations were made on the 3 days of ad lib feeding between deprivation tests.

Differences between lean and obese rats were evaluated by repeated measures ANOVA and *t*-tests (Bonferroni corrected). All means are expressed $\pm$ SE.

RESULTS

Circadian Rhythms of Zucker Rats During Restricted Daily Feeding

Prior to restricted feeding, when food was provided in the form of chow pellets and the food cup for powdered chow was empty, activity at the food cup window was sporadic, primarily nocturnal, and significantly lower in the obese rats (mean counts/day: obese 115 ± 14 , lean 216 ± 18 , $t = 4.37$, $p = 0.001$). During restricted feeding, lean and obese rats exhibited prominent increases in activity at the feeding cup in anticipation of food access (e.g., Fig. 1a and b). However, this anticipatory response appeared to be stronger in the obese rats, in several respects. First, the peak level of anticipatory activity evident in the group average waveform for the last 6 days of restricted feeding was greater in the obese rats (Fig. 2). Second, there was a significant effect of group on the magnitude of the anticipation ratios, $F(1, 11) = 16.5$, $p = 0.002$. Obese rats had higher ratios in day blocks 2, 3, and 4 of restricted feeding (i.e., days 6–11, 12–17, 18–24), compared to lean rats ($p < 0.01$; Fig. 3a). Third, in obese rats, anticipation ratios for day blocks 2–4 of restricted feeding were significantly greater than for the ad lib food access day block ($p < 0.002$). In lean rats, the anticipation ratios were significantly different from ad lib food access days only for the last block of food restriction days ($p < 0.01$). By this measure, anticipation was evident, on average, within 6–11 days in the obese rats, and within 17–22 days in the lean rats. The lean and obese rats did not differ in mean activity levels during the rest of the day (Fig. 2), indicating that enhanced anticipation ratios in obese

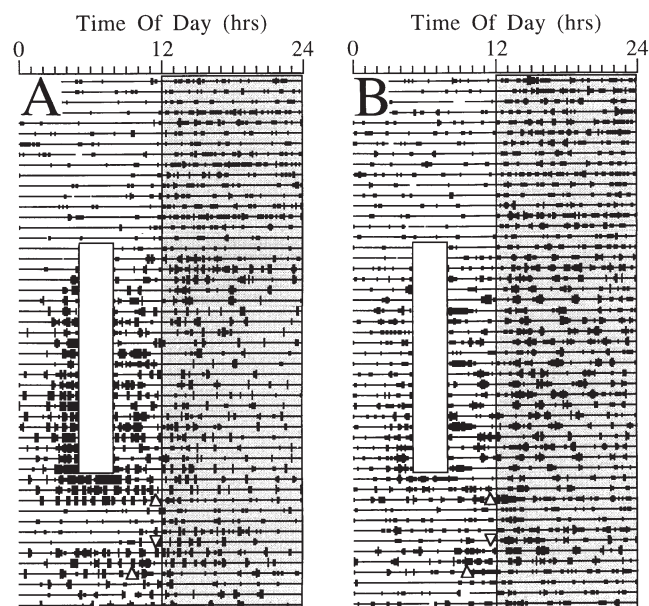


FIG. 1. Activity charts illustrating daily rhythms of activity directed at a feeding bin in representative obese (A) and lean (B) Zucker rats. Each line represents 24 h, with time in 10-min bins plotted left to right. Vertical deflections indicate bins during which activity at the food bin was recorded. Time of food availability during restriction is indicated by the hollow vertical bar (the food bins were left empty prior to the restricted feeding schedule). The inverted triangle indicates the beginning of 3 days of food deprivation. Triangles indicate the resumption of ad lib food access after 3 days of food deprivation.

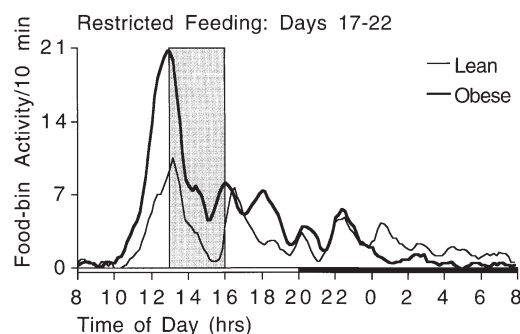


FIG. 2. Group average waveforms of activity at the food bin during the last 6 days of restricted feeding in the obese (heavy line) and lean Zucker rats. The data are plotted in 10-min bins and were smoothed once using a running average. The shaded column indicates feeding time, and the heavy bar along the abscissa indicates the daily dark period.

rats were not an artifact of relative hypoactivity at other times of day.

The average duration of anticipation (Fig. 3b) differed marginally between groups, $F(1, 11) = 4.74$, $p = 0.06$, but strongly across day blocks, $F(3, 33) = 9.06$, $p < 0.001$. Within the obese group, anticipation duration during day blocks 2–4 of restricted feeding was significantly greater than in the first day block ($p < 0.01$) of restricted feeding. In the lean group, only day blocks 3 and 4 differed from the first ($p < 0.01$).

The peak level of food anticipatory activity/10 min bin (Fig. 3c) also differed marginally between groups, $F(1, 8) = 5.01$, $p = 0.055$, but did not differ significantly across day blocks.

During restricted feeding, body weights declined by $7.3 \pm 1.2\%$ in the obese group, and $6.1 \pm 4.2\%$ in the lean group ($p > 0.1$).

Circadian Rhythms During Food Deprivation

Visual inspection of actograms and average waveforms indicated that anticipatory activity persisted strongly during each day of the first 3-day food deprivation test in all seven obese rats. Persistence was comparatively weaker in the lean rats (e.g., Fig. 1a and b). This is evident in the group mean “persistence” ratios (Fig. 3d), which were significantly higher in the obese rats during the second 3-h time block ($p < 0.001$). During the 3-day period of ad lib feeding, these ratios were substantially reduced in both groups. However, the ratios increased again during the second deprivation test in the obese rats, but this increase in diurnal activity was weak or absent in the lean rats.

During the two deprivation tests, body weights declined by about 5% in both groups, but recovered within 3 days of ad lib feeding.

DISCUSSION

Food anticipatory activity is strongly attenuated in rats made obese by high fat diets (43). The leptin receptor mutation in homozygous Zucker rats results in a more severe obesity, but this was associated with stronger, rather than weaker anticipatory activity, by comparison of their anticipation ratios, peaks, and latencies with those of lean, age-matched heterozygous

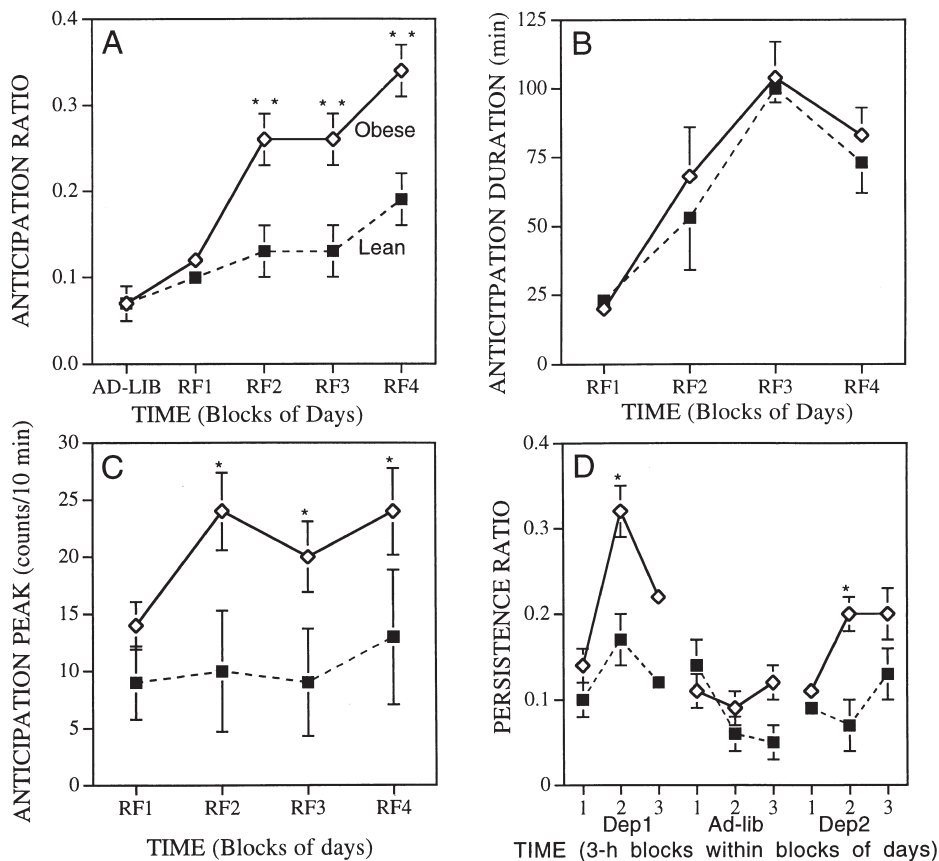


FIG. 3. (A) Group mean anticipation ratios in lean (dashed lines) and obese (solid lines) Zucker rats during ad lib feeding, and four consecutive blocks of days of restricted feeding. (B) Duration of anticipatory activity in lean and obese rats. (C) Peak level of anticipatory activity per 10-min bin in lean and obese rats. (D) Persistence ratios during the first (dep1) and second (dep2) 3-day blocks of food deprivation after restricted feeding, and the intervening 3-day block of ad lib feeding (ad lib). Within each of these 3-day blocks, the ratios of activity as a percentage of total daily activity were calculated for hours (2–4 [1], 5–7 [2], and 8–11 [3] of the light period (* $p < 0.03$, ** $p < 0.01$).

Zucker rats. This suggests that the leptin–ARC–NPY system may modulate the behavioral output of the food-entrainable circadian system; low levels of ARC–NPY activity in diet-induced obesity may attenuate output, while high levels of ARC–NPY release associated with impaired leptin-mediated inhibition may amplify output. Leptin feedback from adipose tissue may be an important signal by which corpulence status gates the expression of food-anticipatory circadian rhythms.

When food is made available ad lib, food-anticipatory rhythms in rats generally disappear immediately or damp out within a few days. However, if food is removed for several days, activity reappears at the previous restricted feeding time (7,46). This apparent memory for feeding time is not dependent on the light-entrainable SCN pacemaker, and it suggests that the food-entrainable pacemaker for anticipatory rhythms is self-sustaining (i.e., does not require a discrete daily feeding event to persist), but that its output is strongly gated by factors associated with ad lib food access. Leptin levels should increase during ad lib food access, as rats regain lost body weight. If leptin feedback was responsible for inhibiting activity related to prior feeding time, then we would expect to see sustained anticipatory rhythms in obese Zucker rats during ad lib food access. However, obese and lean rats in this study did

not differ in persistence of anticipatory rhythms during ad lib food access. This indicates that although leptin feedback may mediate the effects of obesity on food-anticipatory activity, it evidently does not serve as the gating signal by which ad lib food access blocks the expression of food-anticipatory rhythms.

Given that Zucker fatty rats do express strong food-anticipatory rhythms, despite impaired leptin signaling, it is likely that daily variations in leptin levels or cellular responses to leptin do not directly drive daily rhythms of food-anticipatory activity, and are not necessary for entrainment of the pacemaker. However, two limitations of the Zucker rat model are worth noting. First, homozygous Zucker rats develop in the presence of the leptin receptor mutation, and it is conceivable that alternate mechanisms may be adapted developmentally to compensate for some lost functions, thereby masking a role for leptin in the circadian system. Second, the leptin receptor mutation greatly reduces binding and eliminates cellular responses to binding, but it cannot be ruled out that residual binding activates some other intracellular pathway that mediates circadian functions of leptin. Convergent evidence from other approaches will be necessary to strengthen conclusions from the leptin receptor mutation model. We have recently found that neurotoxic ablation of the ARC in neonatal rats

does not impair food-anticipatory rhythms examined after weaning, supporting the conclusion that the leptin-ARC-NPY system may modulate, but does not mediate this circadian function (Mistlberger, Antle, and Marchant, unpublished observations).

Previous studies show that circadian rhythms of temperature, activity and ad lib feeding in Zucker obese rats are attenuated in amplitude and advanced in phase with respect to the light-dark cycle, by comparison with lean rats (21,40,42). If phase-advanced feeding is prevented by limiting food access to the night, body weight gain is attenuated by about 10% compared to calorie matched controls with ad lib food access (40). The mechanism underlying this small but significant improvement in corpulence status by nocturnal feeding is unknown. One possibility is that when food is available ad lib, high levels of diurnal feeding occur out of phase with metabolic rhythms regulating energy expenditure and fat deposition. Normally feeding time entrains behavioral and metabolic rhythms, ensuring their optimal coordination. If this food-entrainment function were to fail, then ingestive and metabolic rhythms might not be optimally synchronized. The results of the present study suggest that this is probably not the case in Zucker fatties, because the obese rats did show a strong synchronizing influence of scheduled feeding, at least with respect to behavioral rhythms. If there is a dysfunction in the food-entrainable component of the circadian system in Zucker rats, this must be confined to the specific efferent

pathways by which the food-entrained pacemaker is coupled to physiological variables other than those that drive food-seeking activity.

The literature reviewed and the new data presented in this study identify leptin and possibly its ARC-NPY target cells as modulators of food-anticipatory rhythms in Zucker rats. However, little is known of the site of mechanisms that mediate the generation and entrainment of these rhythms. Lesions of hypothalamic (anterior, suprachiasmatic, preoptic, periventricular, subparaventricular, paraventricular, lateral, ventromedial, and dorsomedial nuclei), extrahypothalamic (neocortex, accumbens, hippocampus, amygdala, thalamic intergeniculate leaflet), and endocrine structures (median eminence, hypophysis, adrenal gland) and autonomic nerves (parasympathetic, sympathetic) have failed to identify central sites and peripheral pathways necessary for food entrainment (8,12,35-39). Conceivably, the food-entrainable system may be constructed of a population of widely distributed cells that are difficult to eliminate by experimental ablation (e.g., the recently characterized orexin/hypocretin hypothalamic cell population (14,47,58). If so, selective gene deletion approaches may play an important role in their localization.

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