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Sex-related difference in food-anticipatory activity of mice

Zhigang Li [a,b,1], Yan Wang [b,1], Kevin Kai Sun [c,1], Kangli Wang [d], Zhong Sheng Sun [b,d,*], Mei Zhao [e,**], Jundong Wang [a,***]

[a] Shanxi Key Laboratory of Ecological Animal Science and Environmental Medicine, Shanxi Agricultural University, Taigu 030801, China
[b] Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing 100101, China
[c] University of CA, San Diego, CA 92093, USA
[d] Institute of Genomic Medicine, Wenzhou Medical College, Wenzhou 325000, China
[e] Institute of Psychology, Chinese Academy of Sciences, Beijing 100101, China

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ABSTRACT

The expression of food-anticipatory activity (FAA) is induced by restricted feeding (RF), and its entrainment requires food-entrainable oscillators, the neuroanatomical basis of which is currently unclear. Although RF impacts various hormones, sex-related differences in FAA are unclear. 'Here, we report significantly more food-anticipatory wheel-running activity in male than in female mice during RF. In parallel with the sex-related difference in FAA, male and female mice display different food intake and body weight in response to RF. Since gonadal hormones could be involved in the sex-specific difference in FAA, we compared sham and gonadectomized male and female wild-type mice. In gonadectomized mice, the sex difference in FAA was abolished, indicating a role for gonadal hormones in FAA. Further, plasma concentrations of the hormone ghrelin were higher in female than in male mice during *ad libitum* (AL) feeding, and RF induced a temporal advance in its peak in both sexes. RF also shifted the expression peak of the circadian gene *mPer1* in the hippocampus and liver, although no sex difference was found in either the level or the cyclic phase of its expression. $Per1^{Brdm1}$ mutant mice were still sexually dimorphic for FAA, but diminished FAA was noted in both male and female $Per2^{Brdm1}$ mutant mice. In summary, our results imply that gonadal hormones contribute to the sex difference in FAA, possibly through modulating ghrelin activity.

Introduction

The time of feeding is a potent zeitgeber for synchronizing behavioral and physiological adaptations (Feillet et al., 2006a; Stephan, 2002). Under restricted feeding (RF) conditions, mice exhibit increased locomotor activity in the hours preceding the presentation of food, a behavior dubbed food-anticipatory activity (FAA). It is believed that FAA is an output of food-entrainable oscillators (FEOs) utilizing an unknown neural substrate (Silver et al., 2011). Anticipation of food leads to alterations in behavior, metabolism, hormones, and gene expression in the central and peripheral nervous systems, enabling animals to optimize use of limited food resources (Challet et al., 2009; Johnston, 2014).

Changes in food availability display a tight association with body weight, food intake, and energy balance, all of which regulate the anticipatory behavior (Johnston, 2014). Under *ad libitum* (AL) feeding, the animals' body weight increases gradually during adulthood, whereas under RF conditions, both the amount of ingested food and their body weight decrease (Hampstead et al., 2003). Animals display a variable intensity of FAA in association with different feeding cycles (Mistlberger and Marchant, 1995; Stephan and Becker, 1989). In addition, a high-fat diet and diabetes result in dampened FAA in anticipation of daily meals under RF conditions, suggesting that the intensity of FAA is closely related to energy storage and expenditure (Gallardo et al., 2012; Persons et al., 1993).

Previous studies have reported that gonadal hormones modulate the expression of locomotor rhythms (Mong et al., 2011). In humans, young women go to bed earlier than men, whereas menopausal women display a daily rhythm similar to men (Roenneberg and Merrow, 2007). In rodents, females begin their daily activities earlier than males. However, gonadectomized females delay the start of their daily activities relative to sham females (Davis et al., 1983). Moreover, gonadectomized males display reduced precision and duration, and lengthened period, in free-running activity. These effects are suppressed by testosterone propionate treatment (Iwahana et al., 2008).

* Correspondence to: Z.S. Sun, Beijing Institutes of Life Science, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China. Fax: +86 10 64880586.
** Correspondence to: M. Zhao, 16 Lincui Road, Chaoyang District, Beijing 100101, China. Fax: +86 1064872070.
*** Corresponding author. Fax: +86 3546222942.
E-mail addresses: sunzs@mail.biols.ac.cn (Z.S. Sun), zhaomei@psych.ac.cn (M. Zhao), wangjd@sxau.edu.cn (J. Wang).
[1] These authors contributed equally to this work.

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The orexigenic hormone ghrelin is an acylated peptide that increases food intake (Nakazato et al., 2001). Previous reports indicate that plasma ghrelin level peaks in anticipation of food (Cummings et al., 2001; Drazen et al., 2006; LeSauter et al., 2009). In humans, the serum level of ghrelin is positively correlated with that of testosterone (Greenman et al., 2009; Kozakowski et al., 2008). However, serum ghrelin level is lower in men than in women (Kozakowski et al., 2008). Ghrelin receptor-knockout mice display attenuated anticipatory activity under RF conditions (LeSauter et al., 2009), indicating that ghrelin may be involved in the regulation of FAA in mice.

Although it is generally accepted that FAA is mediated by a self-sustained circadian mechanism, with the FEOs as its principal components (Mistlberger, 2009), its relationship with the circadian clock remains elusive. FAA is altered in mice lacking certain circadian genes essential for timekeeping. For instance, *Per2*^{Brdm1} mutant mice express no obvious FAA (Feillet et al., 2006b), while *Npas2*$^{-/-}$ mice display a delayed FAA (Dudley et al., 2003) and *Cry1*/*Cry2* double-knockout mice exhibit both delayed and unstable FAA (Iijima et al., 2005). On the contrary, *mPer1*, *Bmal1*, and *Clock* mutant mice retain normal FAA (Feillet et al., 2006b; Pendergast et al., 2009; Pitts et al., 2003). Furthermore, lesion studies of the central circadian pacemaker in mammals, the suprachiasmatic nucleus (SCN), indicate that the entrainment of FAA is controlled by the FEOs outside the SCN (Davidson and Stephan, 1999). Sex differences in several circadian features have been observed in both humans and non-human animals (Davis et al., 1983; Duffy et al., 2011; Schull et al., 1989). For example, males differ from females in the timing of activity onset, lengths of circadian periods, and circadian rhythms of melatonin expression and body temperature (Duffy et al., 2011). Since the oscillator driving FAA is separate from the central pacemaker and utilizes different molecular components, whether there are sex-based differences in FAA between male and female mice still needs to be clarified.

The liver is a major participant in energy homeostasis (Muoio and Newgard, 2006) and has been used to examine RF-related shifting of clock gene expression (Stokkan et al., 2001). In addition, central nervous regulation of FAA is thought to occur through a distributed set of nuclei including the hippocampus, the periventricular thalamic nucleus, and the dorsomedial nucleus of the hypothalamus (Poulin and Timofeeva, 2008). Thus, we selected the liver and hippocampus as target tissues for this study.

In this study, we report on a difference in FAA between the sexes and examine several potential regulators of the phenomenon. Given the relevance of energy demand to FAA, we compared food intake and body weight between male and female mice under RF conditions. To investigate the putative role of gonadal hormones, we compared levels of plasma ghrelin in both male and female mice before and after gonadectomy. To determine the role of the circadian system in the difference in FAA, we examined gene expression of *mouse Per1* (*mPer1*) in the liver and hippocampus of male and female wild-type mice, under both AL and RF conditions. We also compared sex differences in the expression of FAA in *Per1*^{Brdm1} and *Per2*^{Brdm1}mutant mice.

Materials and methods

Animals

Male and female C57BL/6 J mice aged 4 weeks were purchased from the Animal Center of Vital River Laboratories (Beijing, China) and kept on a normal 12:12 h light–dark cycle (light intensity 200 lx; light off at 21:00). All mice were given *ad libitum* access to standard chow and water, except during food restriction periods as described below. *Per1*^{Brdm1} and *Per2*^{Brdm1} mutant mice were generated and characterized as previously described (Albrecht et al., 2001; Zheng et al., 1999). Our lab imported the *Per1*^{Brdm1} and *Per2*^{Brdm1} mutant mice in 2005. Because the mutants were of mixed background (C57BL/6Tyr$^{c\text{-}Brd}$ × 129S7), we backcrossed them to C57BL/6 J over 12 generations. In normal light–dark conditions, *Per1*^{Brdm1} and *Per2*^{Brdm1} mutant mice could be entrained to a 12:12 h light–dark cycle similarly to the wild-type controls. In constant darkness, *Per1*^{Brdm1} mutants display a shorter circadian period, with reduced precision and stability (Zheng et al., 2001), and *Per2*^{Brdm1}mutants lose their circadian rhythm entirely (Zheng et al., 1999). All experimental procedures and protocols were in accordance with the Guidelines for Care and Use of Laboratory Animals, as adopted by the NIH, and performed with the approval of the Institutional Animal Care and Use Committee at the Institutes of Psychology, Chinese Academy of Sciences.

Feeding schedules and behavioral recordings

Eight-week-old male and female mice were individually housed in Plexiglas cages with clean sawdust bedding covering the bottoms. Groups of four of these cages (26 cm × 46.5 cm × 19.5 cm) were placed in a light-tight chamber (140 cm × 70 cm × 40 cm), with separate ventilation and lighting systems. On each day under RF conditions, we measured the food weight before zeitgeber time 4 (ZT4) and after ZT8, and the difference was calculated as food intake. To prevent animals from hiding food in the floor bedding, we used an overhead food bin 12 cm high. To prevent food spillage, we selected longer, cylindrically shaped food (3–4 cm) weighing 4–5 g and placed new food each day. The mice were given 24-h access to running wheels (diameter 18 cm) for 7 days before food restriction. After overnight food deprivation (Day 0), the period of food availability was restricted to 4 h (ZT4–ZT8) for 10 consecutive days. Daily food intake, wheel-running activity, body weight, and anticipatory wheel-running activity were recorded under RF conditions. Wheel-running data were collected in 5-min intervals using the VitalView system (MiniMitter, Bend, OR) 24 h a day throughout the experiments and were analyzed by Actiview software (MiniMitter, Bend, OR).

To investigate the expression pattern of the *mPer1* gene under RF conditions, male and female mice were fed at ZT4–ZT8 on Day 9 as per the feeding schedule described above, and then killed by CO_2 inhalation quickly at ZT18, ZT22 (Day 9), ZT2, ZT6, ZT10, or ZT14 (Day 10, fasting, $n = 4$ at each time point). To investigate the correlation between FAA intensity and *mPer1* gene expression level, male and female mice with no food available on Day 10 were killed by CO_2 inhalation quickly at ZT2, ZT3, and ZT4 ($n = 3$ at each time point). Mice that acquired food at ZT4 were killed at ZT5 or ZT6 on Day 10 ($n = 3$ at each time point).

Tissue collection, RNA extraction, and gene expression analysis

Hippocampus and liver samples of male and female mice were collected, and total RNA was extracted by using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. Two microgram of total RNA was reverse-transcribed into single-stranded cDNA using SuperScript® III Reverse Transcriptase (Invitrogen, USA). Real-time PCR was then performed on an Opticon 2 Real-Time Cycler (Bio-Rad, USA) using Real Master Mix (SYBR Green, Tiangen, China) with the following program: 95 °C for 10 min; 40 cycles of 95 °C for 25 s, 60 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. 18S ribosomal RNA (18S rRNA) was used as an internal control for normalization, and each sample was run in triplicate. Primers were the following: *mPer1*: Forward primer 5′-CAGCAGTGGAGTCTGGAGGA-3′, Reverse primer 5′-TAGGAGCTCTGAGAAGCGGG-3′; 18S rRNA: Forward primer 5′-GTAACCCGTTGAACCCCATT-3′, Reverse primer 5′-CCATCCAATCGG TAGTAGCG-3′.

Orchidectomy and ovariectomy

Eight-week-old male and female mice were anesthetized by intraperitoneal injection of chloral hydrate (Sigma, USA) and placed in ventral recumbency with the tail towards the surgeon. For male mice,

the skin covering the testis was shaved and swabbed with iodine and alcohol, and a 5.5-mm skin incision was made. In the testis-removed group, the testis of the mice was exteriorized through the skin wall. The male sham operation group (henceforth referred to as sham male mice) underwent all the same procedures except the testis removal.

For ovary-removed mice, the dorsal mid-lumbar area was shaved and swabbed with iodine and alcohol. A single incision 5.5- to 10-mm long was made into the muscle wall on both the right and left sides, approximately one-third of the distance between the spinal cord and the ventral midline. The uterine vasculature between the oviduct and uterus was clamped by a hemostat, and then the ovary and oviduct were removed with a single cut through the muscle wall. Finally, the hemostat was removed and the remaining tissue was replaced into the peritoneal cavity. The same procedures were performed in the female sham operation group (henceforth referred to as sham female mice) except the ovary removal. After surgery, all animals recovered for 2 weeks in their home cages and then were introduced to the cages with running wheels under RF conditions.

Determination of plasma ghrelin levels

Blood of male and female mice was drawn into chilled vacuum blood collection tubes containing K_2EDTA (Xinle Sci &Tech Co., Ltd, China). Plasma ghrelin level was measured by enzyme-linked immunosorbent assay (ELISA) (Yaji Biotechnology, Shanghai, China).

Data analysis

All values are expressed as means ± SEM. For comparing daily FAA, daily food intake, sex-related FAA in sham and gonadectomized male and female wild-type mice, and in *Per1*^{Brdm1} and *Per2*^{Brdm1}mutant mice, data were analyzed by repeated-measures ANOVA. For comparing plasma ghrelin levels between the sexes, data were analyzed by two-way ANOVA. For other measurements, including total FAA and body weight, data were compared using Student's *t*-test. Statistical significance was reached at $p < 0.05$. The effect size was calculated as eta squared (η^2) for ANOVAs and Cohen's d for pair-wise comparisons. According to Cohen (1988), $d = 0.2$ is considered a small effect, $d = 0.5$, a medium effect, and $d = 0.8$, a large effect. For eta squared, $\eta^2 = 0.02$ is considered a small effect; $\eta^2 = 0.13$, a medium effect; and $\eta^2 = 0.26$, a large effect. In all figures, asterisks represent statistical significance where $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; N.S. represents no significance.

Results

Male and female mice exhibit significantly different FAA in anticipation of food

To examine whether there was a difference in FAA between male and female mice, we recorded daily wheel-running activity via the VitalView system. As shown in Fig. 1A, the intensity of FAA in terms of anticipatory wheel-running activity was stronger in male mice than in female mice each day during RF. To exclude the effects of differences in daily activity, we also measured the FAA/total activity ratio, which was defined as the activity in the 3 h before mealtime as a percentage of the total activity in 1 day. Repeated-measures ANOVA revealed a significant difference in the ratio of FAA/total activity between male and female mice during the 10-day RF schedule (Fig. 1B, $F_{1,20} = 18.22$, $p < 0.001$, $\eta^2 = 0.14$, $n = 11$ in each group). By further comparing the average FAA in the 10-day period between male and female mice, we found that both the intensity (Fig. 1C) and ratio of FAA/ total activity (Fig. 1D, $t_{20} = 4.84$, $p < 0.01$, $d = 1.95$) were higher in male than in female mice. Moreover, the FAA of male mice started at ZT1–ZT2, 3 h before mealtime (Fig. 1E, $F_{1,20} = 9.08$, $p = 0.007$, $\eta^2 = 0.18$), whereas no such activity was observed in female mice in that period. Although some FAA was noted in the female mice at ZT2–ZT3, 2 h before mealtime (Fig. 1F, $F_{1,20} = 12.18$, $p = 0.002$, $\eta^2 = 0.14$), its intensity was significantly weaker than the corresponding FAA in males, and the difference between sexes still persisted at ZT3–ZT4 (Fig. 1G, $F_{1,20} = 20.17$, $p < 0.001$, $\eta^2 = 0.12$). To further exclude the possibility that the sex-specific FAA difference was due to the differences in daily activity between male and female mice, we compared the daily activity of these animals under RF conditions and found no significant differences (Fig. 1H). Taken together, these results indicated that there was a significant difference in FAA between male and female mice, and that male mice displayed more anticipatory wheel-running activity.

Male and female mice display different food intake and body weight in response to RF

Restricted feeding induces many metabolic and physiological adaptations, including the reduction in food intake and body weight in anticipation of the reduced availability of food (Gallardo et al., 2012; Hampstead et al., 2003; Szentirmai et al., 2010). Thus, we examined whether there were differences in food consumption and body weight between male and female mice under RF conditions. Compared with their respective AL controls, the food consumption of both male (Fig. 2B) and female (Fig. 2C) mice was decreased more than 50% at the beginning of RF (Day 1). This then gradually recovered to control levels, by Day 7 for female mice and by Day 8 for male mice, respectively, most likely because the animals learned to anticipate the omission of subsequent meals (Booth et al., 2012) (Figs. 2B, C). In accordance with the mentioned sex differences in FAA, the male mice tended to consume greater food than females under RF conditions (Fig. 2A, $F_{1,20} = 4.78$, $p = 0.041$, $\eta^2 = 0.002$), most likely necessitated by the extra FAA-related energy expenditure in the males versus the females. Because food intake influences body weight, we next measured the body weight of male and female mice before (Day 0) and after (Day 10) RF. Under AL conditions, the body weights of male and female mice increased from 22.16 ± 0.5 g to 24.65 ± 0.31 g, and from 19.11 ± 0.35 g to 21.41 ± 0.37 g, respectively, in the 10-day period. However, under RF conditions, the body weights in both sexes decreased, to 20.37 ± 0.45 g in males and 18.42 ± 0.37 g in females on Day 10 (Fig. 2D). Considering the sex difference in response to RF, we measured the net weight loss, which was defined as the actual weight on Day 10 compared to that on Day 0, and the percent weight loss, defined as the percentage of weight lost after 10-days of RF compared to their original weights. The results shown in Fig. 2E revealed that male mice displayed a more pronounced decrease in net weight loss (2.06 ± 0.34 g) than females (1.05 ± 0.32 g, $t_{20} = 2.12$, $p < 0.05$, $d = 0.69$), most likely because of the higher FAA and concomitant energy expenditure. As shown in Fig. 2F, the percent weight loss tended towards being greater in male mice than in females ($t_{20} = 1.90$, $p = 0.07$, $d = 1.47$). These results imply an association between energy demands and the different FAA intensity between the sexes.

Gonadal hormones mediate the difference in FAA between male and female mice

To explore the mechanism underlying the different FAA between males and females, we examined the roles of gonadal hormones in orchidectomized and ovariectomized animals. We compared the daily FAA and average FAA among the wild-type sham male, sham female, ovariectomized female, and orchidectomized male groups under RF conditions. Like the normal male mice, the sham male mice expressed more anticipatory wheel-running activity than the sham female mice (Figs. 3A, B, and E, $t_{14} = 5.57$, $p < 0.001$, $d = 2.87$). Moreover, the 10-day analysis indicated that the average FAA in orchidectomized mice

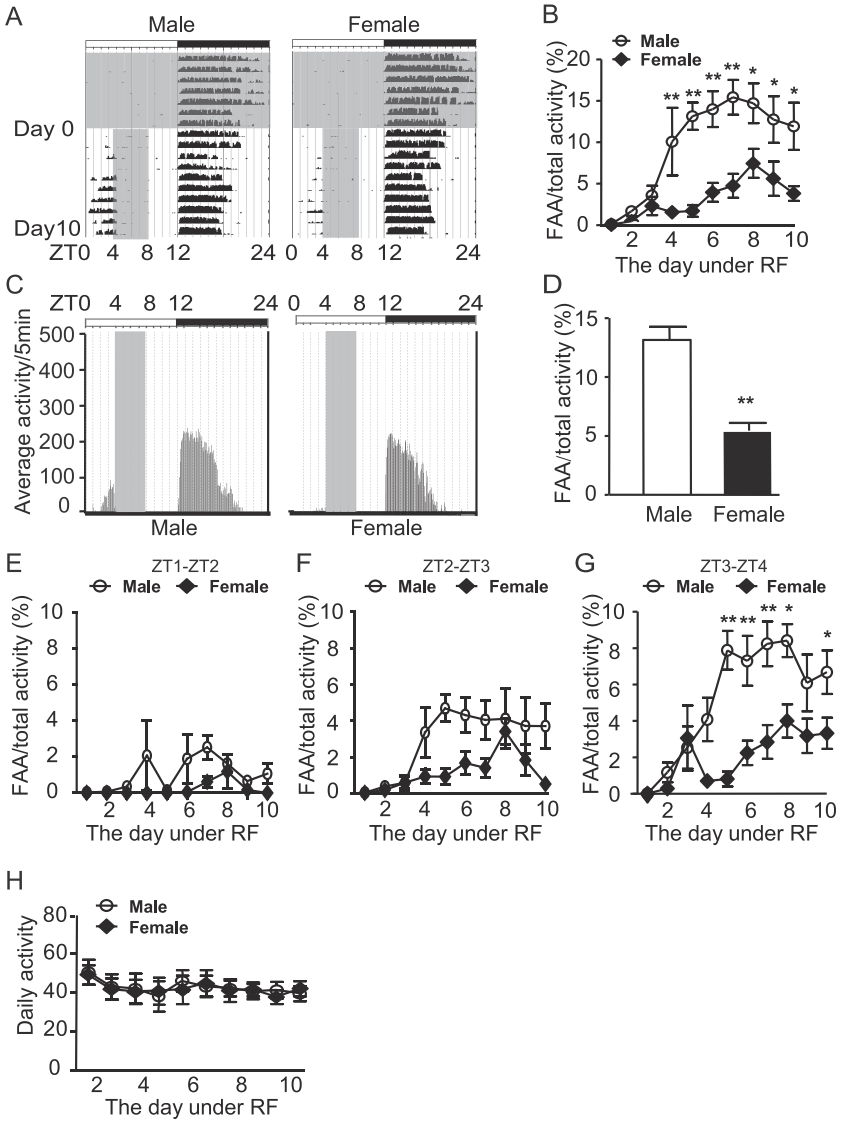

Fig. 1. Sex differences in food-anticipatory activity in the mice. (A) Representative activity of male (left) and female mice (right) under *ad libitum* (AL) and restricted feeding (RF) conditions. The bar above shows the light–dark cycle. The time of food availability is shown in gray shading ($n = 11$ for each sex). (B) The percentage of daily FAA/total activity of male and female mice under RF conditions ($p < 0.01$). (C) The representative average FAA and average activity of one male mouse (left) and one female mouse (right) in the 10-day RF schedule. (D) The percentage of average FAA/total activity of male and female mice in the 10-day RF schedule ($p < 0.01$). (E–G) The percentage of FAA/ total activity at ZT1–ZT2 (E), ZT2–ZT3 (F), and ZT3–ZT4 (G). (H) The daily activity of male and female mice during RF.

was significantly attenuated compared with the sham male mice (Figs. 3A, C, and E, $t_{14} = 4.86$, $p = 0.003$, $d = 2.42$). In contrast, the FAA was significantly increased in ovariectomized females compared to sham females (Figs. 3B, D, and E, $t_{14} = 4.09$, $p = 0.001$, $d = 1.67$), suggesting different roles for estrogens and androgens in the regulation of FAA. Importantly, there was no sex difference when comparing the sham male mice with the ovariectomized females (Figs. 3A, D, and E, $t_{14} = 0.16$, $p = 0.73$, $d = 0.07$) and when comparing the sham female mice with the orchidectomized males (Figs. 3B, C, and E, $t_{14} = 0.36$, $p = 0.72$, $d = 0.18$). These findings suggest that gonadal hormones play crucial roles in the sexual dimorphism of FAA seen in mice. In fact, on every test day, FAA was attenuated in orchidectomized males compared with sham males (Fig. 3F, $F_{1,14} = 7.25$, $p = 0.02$, $\eta^2 = 0.14$) and was elevated in ovariectomized females compared with sham females (Fig. 3F, $F_{1,14} = 5.30$, $p = 0.037$, $\eta^2 = 0.10$), providing further evidence that gonadal hormones mediate the difference in FAA between male and female mice.

Sex differences in ghrelin levels in mouse plasma

Ghrelin is released in anticipation of meals (Verhagen et al., 2011) and displays characteristically different serum levels between the two sexes (Pagotto et al., 2003). We therefore investigated whether there was a difference in the ghrelin levels between male and female mice under AL and RF conditions. The peak level of plasma ghrelin was observed at ZT10 in males, but at ZT14 in females under AL (Fig. 4A). Additionally, ghrelin level was higher in females than in males (Fig. 4A, $F_{1,60} = 82.33$, $p < 0.01$, $\eta^2 = 0.49$), which was consistent with previous studies (Pagotto et al., 2003). Under RF conditions, the peak level of ghrelin was temporally advanced, to ZT2 in male mice and to ZT6 in female mice (Fig. 4B). These results suggest that ghrelin may participate in the regulation of sex differences in FAA. Finally, we compared ghrelin levels between sham and orchidectomized males under RF conditions. The level of ghrelin peaked at ZT2 in sham males and at ZT6 in orchidectomized males (Fig. 4C). These results suggest that gonadal

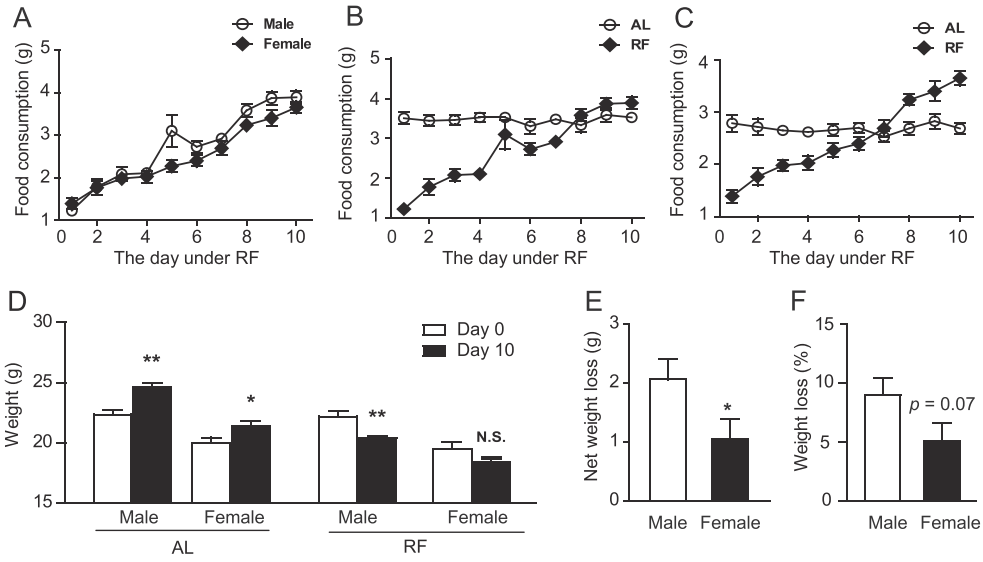

Fig. 2. Sex differences in food intake and body weight under RF conditions. (A) Comparison of the food consumption between male and female mice under RF conditions ($n = 11$, $p = 0.04$). (B) Comparison of the food consumption between AL and RF conditions in the male mice. (C) Comparison of the food consumption between AL and RF conditions in the female mice. (D) Body weight variation of male and female mice in the 10-day AL and RF periods. (E–F) Comparison of the net weight loss (E) and the percent weight loss (F) between male and female mice under RF conditions.

hormones can regulate ghrelin levels, and could, thus, modulate sex-specific FAA.

The clock gene mPer1 is not necessary for the sex-based differences in FAA in mice

To further clarify the role of the circadian clock in the sex-based differences in FAA, we next examined whether the expression pattern of *mPer1* in male mice was different from that in females in response to RF. To achieve this, male and female mice that were maintained on a 12:12 h light–dark cycle and fed *ad libitum* for 2 weeks were deprived of food on Day 0, and subsequently placed on RF for 10 days (Days 1–10, Fig. 5A). As expected, male mice displayed higher FAA than females at ZT1–ZT2, ZT2–ZT3, and ZT3–ZT4 on Day 9 (Fig. 5B). To analyze gene expression, we collected hippocampus and liver samples of male and female mice at ZT18, ZT22 (Day 9), ZT2, ZT6, ZT10, and ZT14

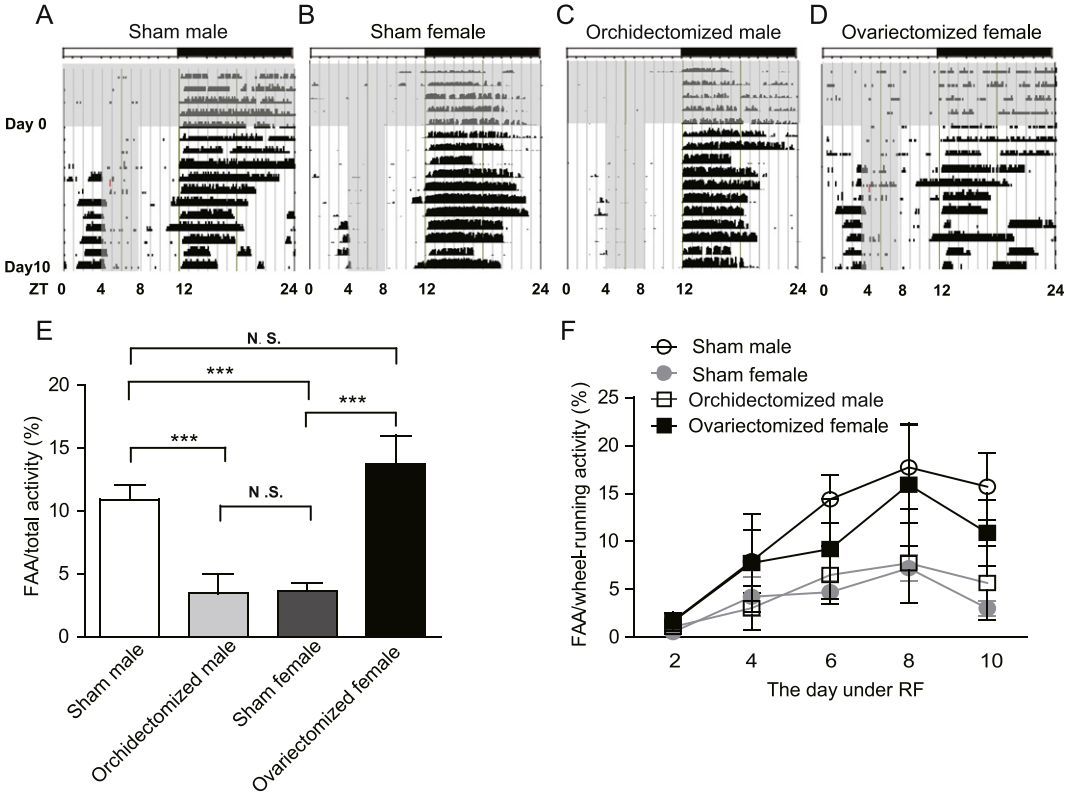

Fig. 3. Gonadal hormones influence the sex-specific difference in anticipatory activity (FAA). (A–D) Representative activities of wild-type sham male (A), sham female (B), orchidectomized male (C), and ovariectomized female mice (D) under RF conditions. (E) The percentage of average FAA/average wheel-running activity among sham male and female, orchidectomized male, and ovariectomized female mice in the 10-day RF schedule. (F) The percentage of daily FAA/total daily activity in male and female mice under RF conditions.

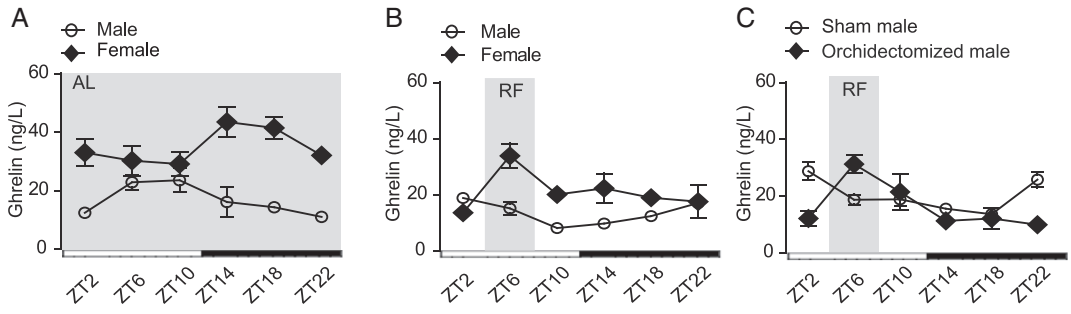

Fig. 4. Restricted feeding influences plasma ghrelin concentrations. (A–B) The plasma ghrelin levels under AL (A) and RF (B) conditions. (C) The plasma ghrelin levels of sham and orchidectomized male mice under RF conditions.

(Day 10) under RF conditions (Fig. 5A, $n = 4$ at each time point). Consistent with previous results (Wakamatsu et al., 2001), the expression peak of *mPer1* in the hippocampus was observed at ZT14 in both sexes under AL feeding, and there was no difference in the expression pattern between males and females (Fig. 5C). Under the RF condition, however, the expression peak of *mPer1* in the hippocampus was temporally advanced to ZT6 in both male and female mice (Fig. 5D), and two-way ANOVA analysis revealed no sex differences either in the level or the cyclic phase of its expression.

RF can entrain the expression of *mPer1* in the liver (Stokkan et al., 2001), so we next examined its expression level in male and female mouse liver. The results indicated that the expression pattern of *mPer1* was indistinguishable between the sexes when feeding was under either AL (Fig. 5E) or RF conditions (Fig. 5F). The mRNA expression of *mPer1* was significantly higher in males than females at ZT2 (2 h before mealtime, 1.4 fold, $p = 0.01$, $d = 1.41$), suggesting that hepatic expression of *mPer1*may be related to regulating FAA. To verify the role of *mPer1* in FAA, we examined the expression of *mPer1* in the liver of mice on the 10th day under food restriction (as described in Fig. 5G). Before mealtime (ZT4), the expression level of *mPer1* in male mice was 2.78-fold higher at ZT2 ($t_4 = 9.14$, $p = 0.008$, $d = 7.46$), and 1.38-fold higher at ZT3 ($t_4 = 2.48$, $p = 0.06$, $d = 2.03$), than that in females. After mealtime, however, *mPer1* expression was lower in males than in females at ZT5, and not different at ZT6. This suggests that *mPer1* expression could be associated with the intensity of FAA.

To further examine the possible impact of *mPer1* on sex-specific FAA, we entrained both male and female mutant mice to RF for 10 days. *Per1*^{Brdm1} mice are null mutants with no recognizable functional domain of mPer1 expressed (Albrecht et al., 2001). Similarly to the normal male mice, male *Per1*^{Brdm1} mutant mice displayed significantly more FAA than did mutant females (Figs. 6A–C, $F_{1,14} = 5.52$, $p = 0.034$, $\eta^2 = 0.12$, $n = 8$). The mutant females demonstrated a similar FAA to the wild-type females. Together with the gene expression analysis, these results indicate that *mPer1* most likely did not contribute to the difference in FAA between male and female mice.

Diminished FAA in both male and female Per2^{Brdm1} *mutant mice*

To examine whether *mPer2* plays a role in regulating the sexual dimorphism of FAA, we measured the FAA of both male and female *Per2*^{Brdm1} mutant mice under RF conditions for 10 days. We found that FAA decreased to the basal level in both male and female mutants. Furthermore, no sex difference could be detected (Figs. 6D–F, $F_{1,14} = 0.17$, $p = 0.688$, $\eta^2 = 0.004$, $n = 8$).

Discussion

Previous studies have provided clear evidence of sex differences in both intrinsic and in light-entrainable circadian rhythms (Davis et al., 1983; Duffy et al., 2011). For instance, the intrinsic period of free-running circadian rhythms is significantly shorter in females than in males (Duffy et al., 2011). Further, females begin their daily activity earlier than both males and gonadectomized females, when living under a 12:12 h light–dark cycle (Davis et al., 1983). In the present study, we identified a sexual dimorphism in FAA in mice, which was characterized by an earlier phase and greater anticipatory wheel-running activity in male mice compared to females.

Because RF induces responses in peripheral organs involved in energy metabolism, the establishment of FAA is associated with digestive processes regulating energy balance (Davidson et al., 2003; Fuller et al., 2008). We found, in parallel with the anticipatory wheel-running activity, that food consumption was also slightly higher in male mice than in females under the RF condition. However, male mice lost more body weight than females in that condition. A previous study has shown that female rats are more capable than males of decreasing energy expenditure during caloric restriction, by protecting metabolically active organs (Valle et al., 2005). Therefore, it is possible that the elevated weight loss in male mice resulted from greater energy expenditure. The delayed and less intense FAA in female mice implies that they have a more efficient energy consumption system, which could promote the survival of both themselves and the species as a whole. Furthermore, homeostatic processes stimulate the arousal system when signals related to energy depletion reach a critical level, inducing the animal to seek food to restore the system to its preferred state (Mistlberger, 2009). Therefore, the greater energy expenditure in male mice under RF conditions could result in energy depletion, and thus FAA may be a reflection of the need to acquire food.

We found that RF strongly entrained the expression of *mPer1* in both sexes, with the expression peak shifting from a diurnal to a nocturnal pattern. Importantly, we found that *mPer1* expression in the liver was associated with the intensity of FAA in both sexes. These results indicate that an output of the FAA oscillator may drive the expression of *mPer1*, suggesting a novel function of the protein. Our study further indicated that mutation of the *mPer1* gene did not abolish the dimorphism in FAA between the male and female mice. We compared FAA in male and female *Per2*^{Brdm1} mice as well, and the results revealed that FAA decreased to the basal level in both groups. Thus, no sex differences could be detected. Nevertheless, our results indicated that, unlike its homologue mPer1, mPer2 might participate in modulating FAA. It should be noted that the *Per2*^{Brdm1}mutation used in this study has an in-frame deletion that removes most of the PAS domain. This particular mutant abrogates expression of FAA (Feillet et al., 2006b; Zheng et al., 1999), but no obvious alteration in FAA has been reported in *Per2*^{lbc} mice, in which the mutation is a null-mutant allele (Bae et al., 2001; Storch and Weitz, 2009). The distinct difference in FAA in the two *Per2* mutant mice might be due to the redundant functions of paralogous genes present in *Per2*^{lbc} mice that are absent in *Per2*^{Brdm1} mice. It could be speculated that the total lack of mPer2 protein in *Per2*^{lbc} mice could elicit compensatory mechanisms during development. In contrast, the mutant mPer2 protein expressed in *Per2*^{Brdm1} mice would be partly functional and therefore not be compensated by paralogous genes, such as mPer1

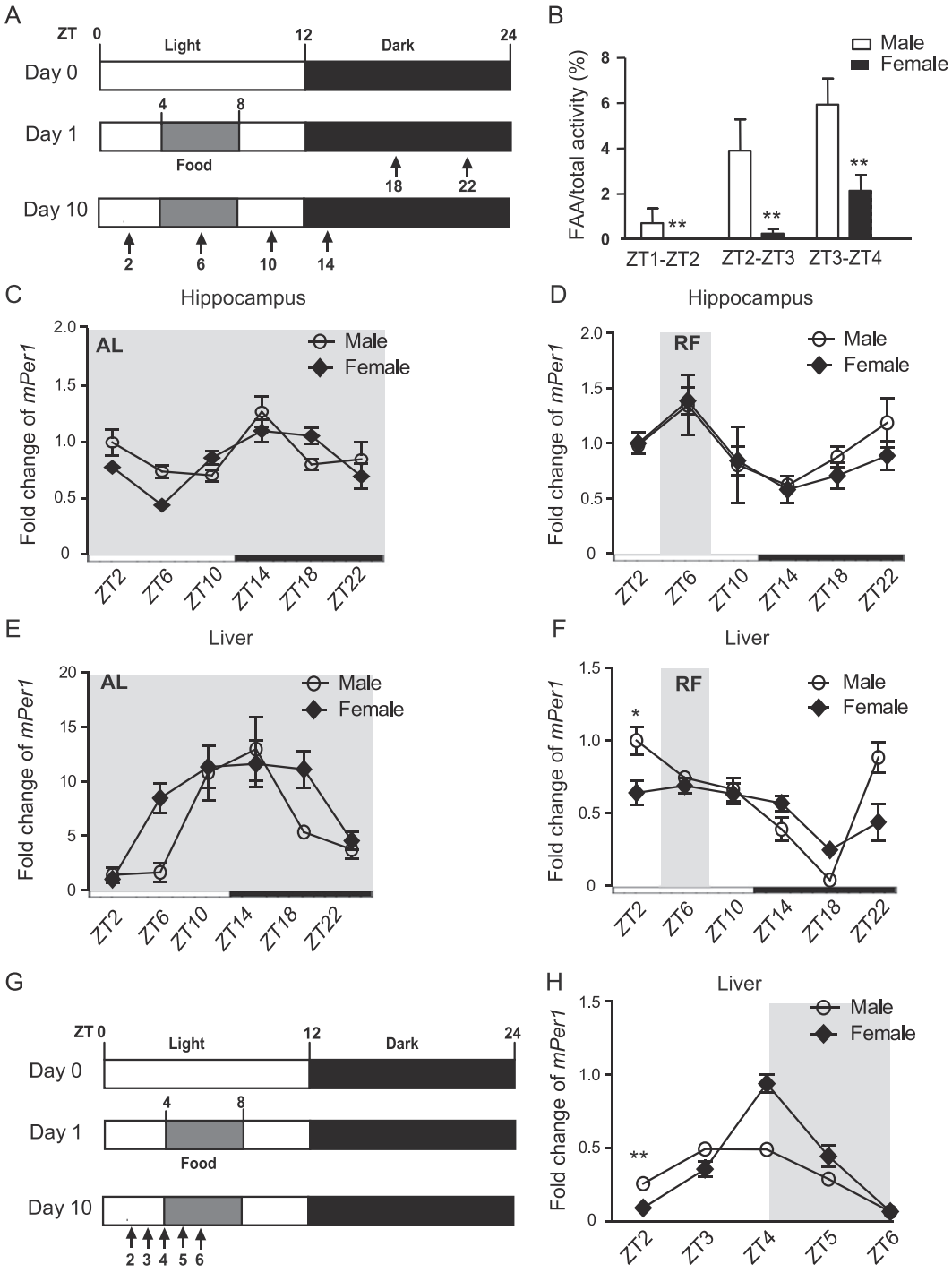

Fig. 5. The expression pattern of *mPer1* in hippocampus and liver of male and female mice under RF conditions. (A) Experimental schedules. The open and solid bars represent the light and dark periods, respectively. The gray-shaded boxes indicate the availability of food. Arrows indicate the time points of sacrificing the animals. (B) The anticipatory wheel-running activity of male and female mice on the 9th day under RF conditions. (C–D) The expression level of *mPer1* in the hippocampus under AL (C) and RF (D) conditions. $n = 4$ at each time point. (E–F) The expression level of *mPer1* in the liver under AL (E) and RF (F) conditions. (G) Experimental schedules. (H) The expression level of *mPer1* in liver of male and female mice under RF conditions. $n = 3$ at each time point.

or mPer3 (Challet et al., 2009; Takahashi et al., 2008), although these hypotheses need to be validated.

We showed that compared with their sham groups, ovariectomized female mice exhibited increased FAA, while orchidectomized male mice displayed significantly attenuated FAA, suggesting that estrogens and androgens might inversely contribute to FAA. The expression level of the orexigenic hormone ghrelin increases remarkably in response to RF and is important for the full display of FAA (LeSauter et al., 2009; Verhagen et al., 2011). Given that (1) estrogen negatively modulates ghrelin expression in rats (Matsubara et al., 2004), (2) the level of testosterone correlates positively with that of ghrelin in humans (Greenman et al., 2009; Pagotto et al., 2003), and (3) serum ghrelin is lower in men than in women (Kozakowski et al., 2008), it is possible that the existing higher level of ghrelin in females suppresses the response to RF. At the same time, the lower level of ghrelin might noticeably augment the response in males. We speculate that gonadal hormones modulate the circulating ghrelin concentration to contribute to the different FAAs between male and female mice, although the

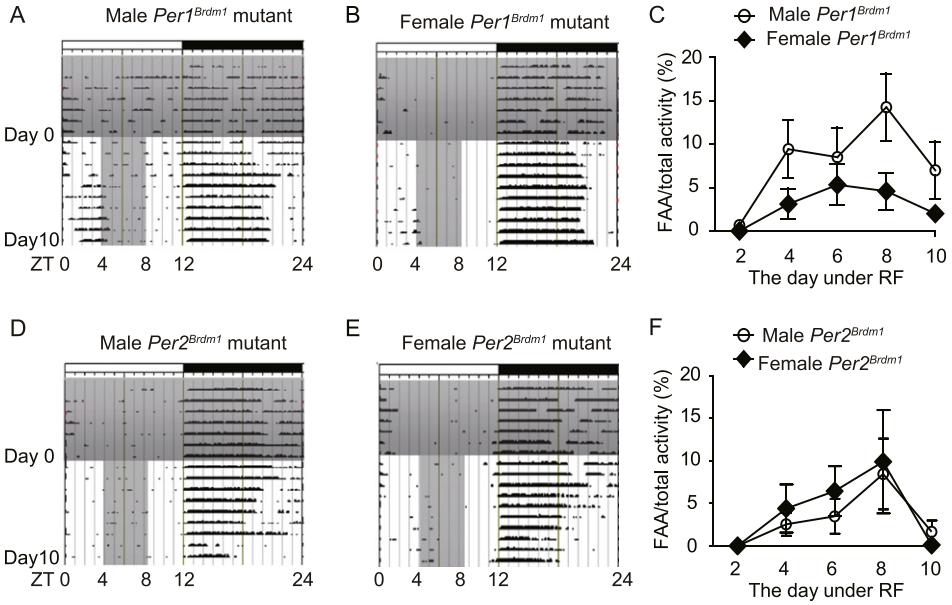

Fig. 6. Food anticipation of male and female *Per1*^{Brdm1} and *Per2*^{Brdm1} mutant mice. (A–B) Representative activities of male (A) and female (B) *Per1*^{Brdm1}mutant mice under RF conditions. (C) The percentage of FAA/daily activity in male and female *Per1* ^{Brdm1} mutant mice under RF condition ($p < 0.01$). (D–E) Representative activities of male (D) and female (E) *Per2* ^{Brdm1} mutant mice under RF conditions. (F) The percentage of FAA/daily activity in male and female *Per2* ^{Brdm1} mutant mice under RF conditions.

possibility of other hormones affecting this regulation cannot be ruled out (Feillet, 2010; Labouebe et al., 2013; Lindheim et al., 1993; Patton and Mistlberger, 2013).

Conclusion

In summary, we report a difference in FAA between male and female mice characterized by more anticipatory wheel-running activity in male mice than that in females. By providing molecular and behavioral evidence with surgical animals and knockout models, we suggest that the sex differences in FAA are not regulated by the circadian clock gene *mPer1*. Rather, estrogens and androgens might inversely contribute to the intensity of FAA by modulating ghrelin, implying essential roles for each of these hormones in regulating the differences in FAA between male and female mice.

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