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Original Article

Effects of chronic scheduled–caloric dietary on ghrelin secretion and food intake in adult male rats

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Abstract

Introduction: Effects of chronic scheduled dietary on plasma ghrelin and food intake in adult male rats were assessed.

Methods: Forty male Wistar rats (180-200g) were distributed into four groups (n=10), freely fed rats (control) and three scheduled-fed groups with different caloric intakes: high fat, standard and restricted diet. Then, plasma ghrelin and food intake were measured on days 0, 7 and 14.

Results: Plasma ghrelin was significantly different among all groups, the scheduled-standard rats having the lowest ghrelin on day 7 and the restricted rats having the highest ghrelin on day 14. Noteworthy, fasted ghrelin in controls was as much as that of schedule groups exception restricted diet at the end of experiment. Controls consumed stable food over the time, while schedule groups showed time and caloric-dependency of food intake. Schedule-standard and restricted groups on feeding time consumed high level of food. Schedule-high fat group displayed a time dependent reduction in food intake. A positive correlation was found between plasma ghrelin and food intake in fasting status for freely fed rats and anticipating status for standard and restricted-schedule groups.

Conclusion: A component of ghrelin secretion can be entrained or learned for time feeding as well as long last-fasting and more is affected by quantitative and qualitative of caloric intake. Elevated ghrelin levels in restricted model are subjective both by low energy levels and learning. Also, caloric intake amount can be controlled by learning; we observed a reduction in meal size in scheduled–high fat diet.

Keywords:
Ghrelin;
Food intake;
High-fat diet;
Caloric restriction

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Introduction

Ghrelin is a gastrointestinal hormone that is implicated in a variety of physiological actions including growth hormone secretion, appetite and food intake, energy homeostasis, cardiovascular functions, reproduction, etc. Among these, ghrelin effects on food intake are the most potent. Ghrelin is the only known circulating hormone that increases food intake (Tschöp et al., 2000; Cowley et al., 2003; Müller et al., 2015) and has prominent stimulatory roles in modulating meal size, meal frequency, bodyweight and food-motivated reward behaviors

(Perello et al., 2015; Wren et al., 2001; Faulconbridge et al., 2003). Ghrelin is commonly referred to as a “hunger” hormone and food deprivation index that stimulates hunger and initiates meals. Although less is known about mechanisms underlying ghrelin regulation of feeding behavior, the hypothalamic arcuate nucleus (ARC) appears to play a critical role in the ghrelin effects on food intake and satiety (Lee et al., 2016). The ARC contains different groups of neurons such as neuropeptide Y (NPY)/ agouti-related protein (AgRP) neurons and pro-opiomelanocortin (POMC)/ amphetamine-regulated transcript (CART) neurons whose activity is differentially affected by ghrelin, such that ghrelin enhances the activity of NPY and AgRP neurons while suppressing the activity of POMC/CART neurons (Cowley et al., 2003). The effects of ghrelin on the activity of ARC neurons are critical for eating behavior, since ghrelin is unable to enhance food intake in mice lacking NPY/AgRP neurons (Chen et al., 2004).

Ghrelin levels are flexible to changes in macronutrient composition and/or the type of diet of that is being consumed, such that modified sham feeding of different types of diet (e.g. high-fat, carbohydrate or protein) all induced a ghrelin response; however, there was a markedly greater increase in circulating ghrelin concentrations with high-protein foods (Zhu et al., 2014). In addition to the regulation of ghrelin levels by nutrient content, a growing body of literature in both human and animal models indicate that ghrelin is more appropriately described as a signal for the anticipation of food intake. For example, animals that learn to anticipate meals when placed on a meal entrainment schedule exhibit increasing levels of circulating ghrelin prior to the ingestion of the meal (Drazen et al., 2006; Hsu et al., 2016). Similarly, a robust increase in ghrelin levels immediately before each scheduled meal (breakfast, lunch and dinner) is demonstrated in humans with meals provided on a fixed schedule throughout a 24- hour period (Frecka et al., 2008; Verbaeys et al., 2011). Human studies have also demonstrated that varying habitual feeding times (e.g., short or long inter-meal intervals) potently influences temporal patterns of circulating ghrelin levels, where peak ghrelin concentrations occur immediately prior to an individual's habitual meal time. These results suggest that ghrelin is not exclusively a “hunger signal” whose release is just affected by the magnitude of food deprivation, but ghrelin secretion can elevate in anticipation of food intake based on learned and habitual feeding patterns. However, whether palatability of the diet (for example high caloric content), habitual feeding patterns and the magnitude of food restriction modulate ghrelin release independently of each other, require further investigation. Changes induced in the ghrelin system potentially contribute to the inter-individual differences in body fat mass and thereby may play an important role in the obesity development. By modulating the response of ghrelin secretion before each meal, we may be able to minimize the effects of eating large meals on weight gain. Therefore, we designed the present study to test the hypothesis that a combination of anticipation of a meal and type of caloric intake could differentially affect ghrelin secretion response compared with long-last fasting.

Materials and methods

All experimental protocols used in this study were approved by the Ethical Committee of Isfahan University of Medical Sciences (Isfahan, Iran) in accordance with the Guide for Care and Use of Laboratory Animals (National Institute of Health Publication No.80-23, revised 1996). Forty male Wistar rats (180-200g) were housed in 12:12-h lighting cycle with *ad libitum* access to food and water and 23±2°C ambient temperature and 45–65% humidity (n=10). Rat entrained to a 12:12 DL cycle (reverse cycle) for a week prior to further operations. In reverse cycle enabled us to provide access to food during specific times of dark phase.

Experimental procedure

Rats were assigned to one of four weight-matched groups: a freely-fed group with 24h access to standard chow and 3 schedule-fed groups that were given access to the food 3 times during the dark phase aligned to their active phase with 3 different calorie intake: high fat, standard and restricted diet. In a high-fat diet, cholesterol (35% fat from lard, 60% kcal from fat) was provided into 3 meals (Gajda et al., 2008). While in the standard diet, amount of standard chow was free just at 3 meals for rats and in the restricted group, the food was well-defined and restricted (4g/time/rat; 60%) for animals (Suckow

et al., 2005). Rats were maintained on either diet for 14d. Food consumption and body weight was measured on days 0, 7 and 14 in 12-14 fasted condition when they were free to food access for 1 hour (group of freely-fed rats was food deprived for the same amount of time as meal-fed rats before their usual meal, dark phase). For determination of hormone secretion output, tail blood sampling technique was used to collect blood (at the amount of 500µl) from the rats around 9 am on days 0, 7 and 14 (group of freely-fed rats was food deprived for the same amount of time as meal-fed rats before their usual meal, dark phase). Plasma acyl ghrelin was measured using rat ghrelin EISA kit with intra-assay precision CV< 10% (Zellbio Company ELISA kit-Germany).

Statistical analysis

Results are expressed as mean±SEM. Statistical analysis was performed using SPSS 16 (SPSS Inc., Chicago, IL, USA). Statistical evaluation was carried out using a one-way analysis of variance, followed by Tukey test for comparison of means. Correlation between changes in ghrelin level and food intake was performed using Pearson correlation at the 5% error term. The $P<0.05$ was considered statistically significant.

Results

Plasma ghrelin level before food availability

Our results showed that plasma ghrelin started at a baseline of 0.3917±0.006 ng/ml for four groups after 10-12 hours food deprivation (Fig. 1). It then remained approximately stable for freely fed rats for the remainder of the study. Plasma ghrelin levels were significantly different among all groups over the time of 2 weeks, significant points were observed on 7 and 14 days. Schedule- standard fed rats showed the lowest ghrelin level on day 7 ($P<0.05$), while, the schedule-restricted feeding rats represented the highest ghrelin level on day14 ($P<0.05$) compared to freely fed rats (control). As shown in Figure 1, decreased ghrelin values in schedule-standard feeding groups on day 7 reached to fasted level as control group on day 14. It was worthy to mention that fasted ghrelin value in control was as much as that of anticipated chow groups exception restricted diet in the end of experiment. The amount of restricted feeding-induced ghrelin peak reached to a statistical significance after 2 weeks than control group or itself time trend ($P<0.05$). Final significant finding, the plasma ghrelin level was similar to that of fasting status in control group for 2 week in scheduled-high fat diet rats.

Food intake

Data in Figures 2A and B show the pattern of food intake and body weight over time. Rats assigned to freely food intake (control) consumed stable food after 10-12h of deprivation on days 0, 7 and 14. The d1 food intake was 4±0.08g compared with 3.3±0.2g on d14 ($P>0.05$). However the animals adapted to the regimen on scheduled program showed time and caloric -dependency of food consumption when they anticipated food. As Figure 2A revealed, more variations of food intake occurred on day 7, although the significance held in reserve until day14 in schedule-fed animals than control ($P<0.01$). As shown in Figure 2A, it can be seen that food intake was approximately 1.5-fold higher in the restricted -treated animals than the control group ($P<0.01$) on days 7 and 14. Interestingly, schedule-standard diet group consumed high level of food pellet as well as restricted group on feeding time ($P<0.05$) in comparison to control group. Rats on the imposed schedule-high fat diet displayed a faster growth on fixed-scheduled feeding rate compared freely-fed control while a time dependent reduction of amount of standard chow consumption significantly was observed ($P<0.05$). Schedule-restricted feeding showed lowest body weight while high fat and standard feeding schedule groups had high level of body weight compared with control group ($P<0.05$, Fig. 2B). Baseline body weight did not differ between scheduled-fed and freely -fed animals (scheduled – fed: 204.9±5g; control: 208.6±9g).

Correlation between food intake and plasma ghrelin concentration following scheduled–caloric dietary

The analysis revealed a significant positive correlation between concentration of the plasma ghrelin and food intake in fasting status for freely fed rats and anticipating status for standard and restricted-scheduled fed groups (Pearson's correlation; r=0.84, r=0.98 and r= 0.95, $P<0.05$, $P<0.01$ and $P<0.001$; Fig. 3) while a significant

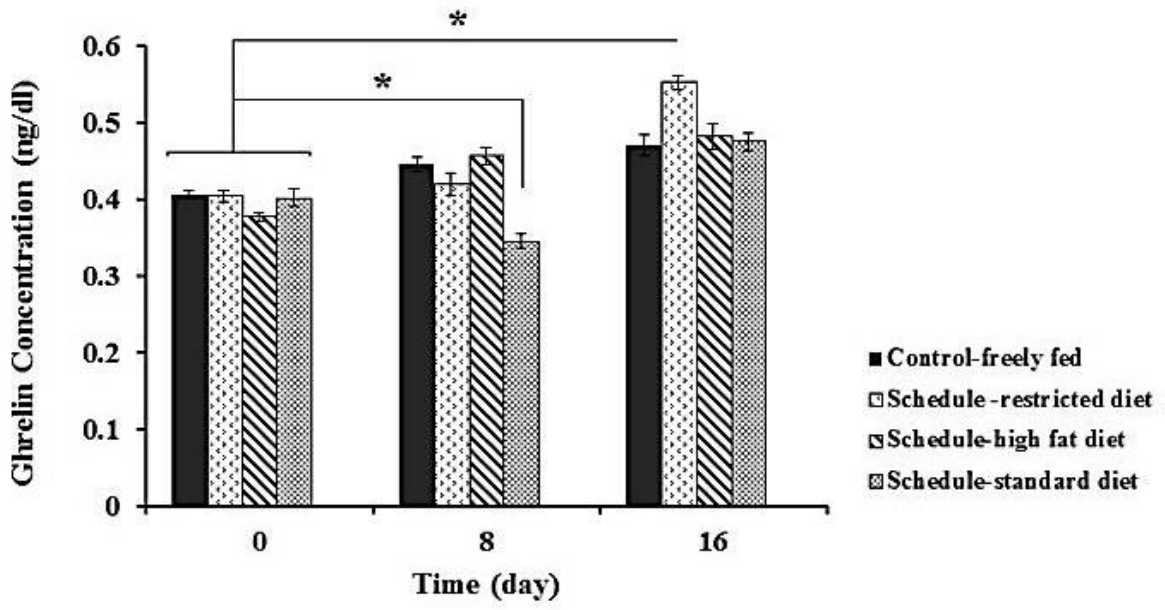

Fig.1. Plasma ghrelin levels (ng/ml) before food availability in freely- and schedule-fed groups on days 0, 7 and 14. Each point represents mean±SEM. $^{*}P<0.05$ compared with freely fed group or control.

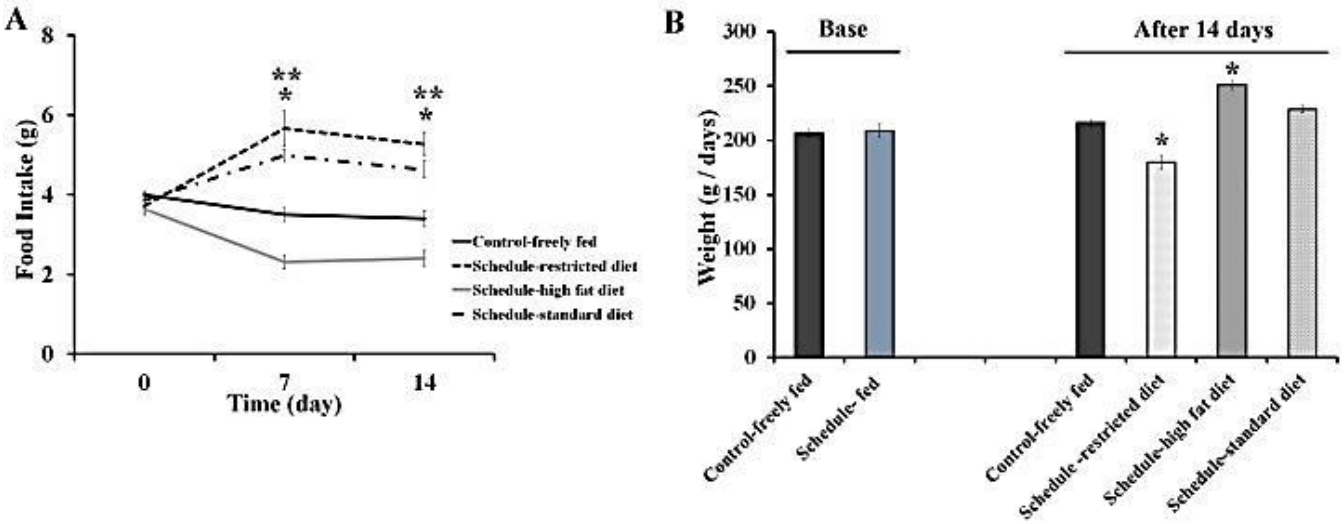

Fig.2. Pattern of food intake (A) and body weight (B) over time. Each point represents mean±SEM. $^{**}P<0.01$ and $^{*}P<0.05$ compared with freely fed group control.

correlation was not observed in scheduled-high fat group. These findings supported the mentioned idea that ghrelin levels were affected by the magnitude of food restriction, scheduled feeding behavior and caloric content.

Discussion

The results of this study showed that ghrelin level rise in anticipation of food based on learned feeding pattern were as much as hunger ghrelin whose release was linked to the magnitude of food deprivation. Moreover, the meal-standard feeding and fasted condition affected ghrelin response equally after 2 weeks. Nevertheless, rats trained to expect their daily foods, showed significantly differences in plasma ghrelin dependent on calorie intake. Rats with restricted meal-fed (3 meal with low food 60%) showed high levels of ghrelin secretion before anticipated food. While in rats with standard and high fat food habituated to large (unlimited) food into 3 meals, plasma ghrelin elevated before anticipated food in a similar magnitude with that in rats fasted for 12-14 hours before dark phase. Another significant finding was a persistent increase in plasma ghrelin levels as fasting status for 2 week in scheduled-high

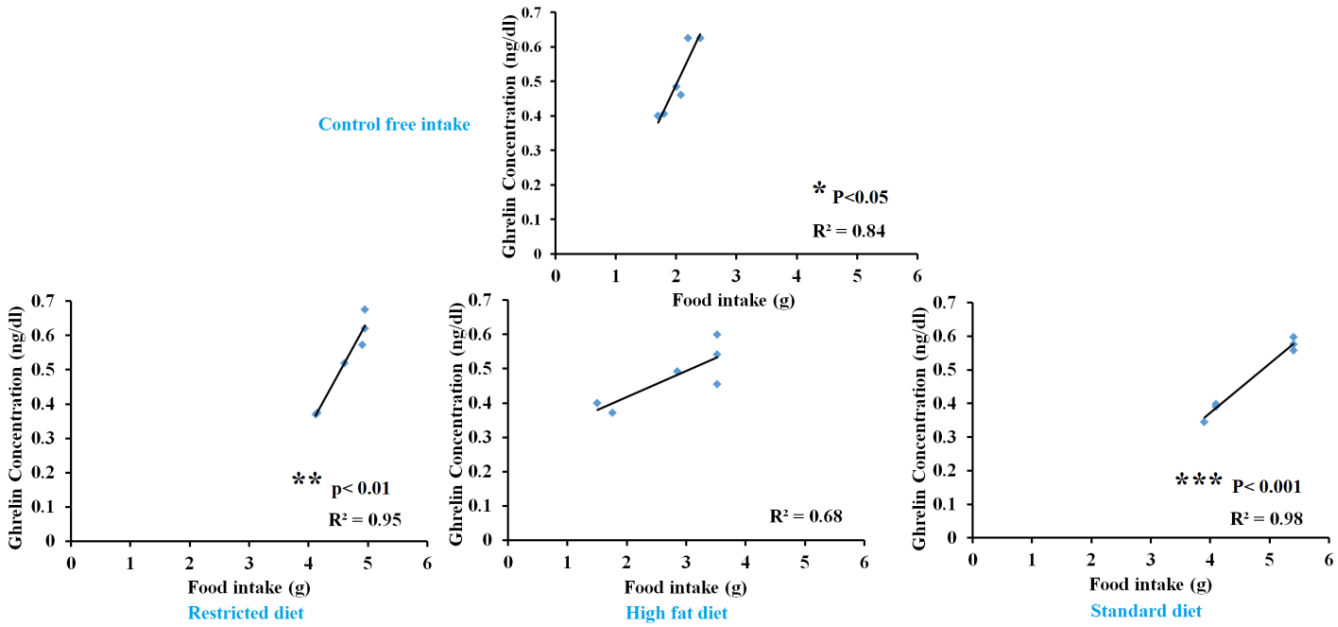

Fig.3. Correlation analysis between the plasma concentration of ghrelin and food intake in different groups (n=6). Results are expressed as mean±SEM (Pearson's correlation test; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$) in fasting status for freely fed rats and anticipating status for standard and restricted-scheduled fed groups.

fat diet rats, a condition that is favorable for obesity development. Comparable results have been found in human studies that are instructed to anticipate a large meal following a 14-hour fast and then exposed to the presentation of a breakfast. There was no difference in pre-meal ghrelin levels compared to equally fasted-controls (Cummings et al., 2001). Importantly, human and animal that learns to anticipate meals when placed on a meal entrainment schedule (fixed or varying habitual feeding times, short or long inter-meal intervals) exhibits increasing levels of circulating ghrelin prior to the meal in comparison to animals that were equally food-restricted for short time (Drazen et al., 2006; Frecka et al., 2008). These results suggest that duration of fasting is a critical factor to induce ghrelin secretion, while meal anticipation even with different patterns (one or more meal times per day, fixed or variable) can strongly stimulate peripheral ghrelin secretion.

In respect of caloric outputs, several lines of evidence show that ghrelin secretory response is influenced by energy levels and that this response is sensitive to energy manipulation, acting in a compensatory fashion when imposed to an energy deficit by dietary restriction. The effects of long-term undernutrition on circulating ghrelin levels and gastric ghrelin mRNA expression show that ghrelin levels and ghrelin gastric mRNA are up-modulated during under nutrition in female rats (Gualillo et al., 2002). We also found, by 2 week scheduled food restriction, the acylated ghrelin concentration was 30% higher than the scheduled standard and freely fed rats. It seems that increased ghrelin levels represent an adaptive response to prevent long-lasting alterations in energy balance and body weight homeostasis. Moreover, our data showed that excessive calorie intake in rats with high fat diet induced an increase in plasma ghrelin levels in a similar magnitude with those in fasted freely fed rats. In a study by Gomez et al. (2012) incremental changes of fasting ghrelin were studied under high-fat diet and was observed that plasma ghrelin (total) elevation to overnight fasting was not altered in rats fed a high fat diet on a long term basis (10 or 60 week). Results from the recent studies demonstrated that high fat diet in mice induces obesogenic changes in the ghrelin system, including increased numbers of ghrelin precursor-expressing cells in the stomach and ghrelin resistance in NPY/AgRP neurons (François et al., 2016; Briggs et al., 2014). In humans, high fat diet was found to increase feeling of hunger (Perello et al., 2010). Although, some studies showed that high fat diet-fed mice had normal levels of active ghrelin and decreased plasma levels of deacylated ghrelin. A study showed that postnatal overfeeding associated with maternal high fat diet increased plasma levels of acylated ghrelin in mice (Du et al., 2015). Consistent with other studies, our data showed that the ghrelin

response is differentially regulated, depending on calorie content. Collectively, it is concluded that a component of ghrelin secretion can be entrained or learned for feeding time as well as long last-fasting, which more is affected by quantitative and qualitative of caloric intake. Indeed, low caloric intake can potentiate the effect of food anticipating on ghrelin secretion and standard chow and that high fat diet had the same effect as long last fasting.

Similar changes are expected to occur in food intake following the endogenous ghrelin fluctuations. Hence, we measured amount of food consumption on days 0, 7 and 14 when all groups had free access to standard chow for 1 hour. Based on ghrelin outputs, it was predicted that restricted-scheduled feeding group with more ghrelin response consume more food than other groups, but there was no significant change in food consumption in groups with equal levels of ghrelin (freely fed, scheduled- normal and high fat rats). Food intake measurements showed that food consumption in restricted and normal schedule feeding groups significantly increased, while high fat diet-fed rats displayed a decreased meal pattern for standard chow eating on days 0, 7 and 14 compared with fasted freely fed rats. It is now recognized that pre-meal ghrelin elevation is determined by two factors: food deprivation, to keep energy homeostasis and habitual food behavior which may override physiological regulatory mechanisms that set food intake by the state of body energy balance (Drazen et al., 2006). In previous studies that feeding behavior was entrained based on limited food availability and rats were only allowed to have access to food at a fixed time, the animals rapidly consumed a large quantity of food to meet their metabolic requirements (Mistlberger and Rusak, 1987; Abe and Rusak, 1992). Moreover, ghrelin signaling has an important role in habitual-driven feeding as shown in our study. Thus elevated ghrelin secretory response in our restricted model is subjective both by low energy levels and learning, acting in a compensatory fashion to reverse energy deficit by higher food consumption if it was available. Also, it has been indicated that high ghrelin levels in anorexia nervosa are due to compensatory mechanisms to increase calorie intake and induce a positive energy balance state (Tanaka et al., 2004; Troisi et al., 2005; Janas-Kozik et al., 2007). In our study, food intake pattern analysis of scheduled-restricted diet revealed rats that anticipated food arrival by learned ghrelin surge before scheduled meal had a nonstop eating behavior and maximum consumption when they had free access to food, whereas a chronic imposed scheduled feeding in rats with calorie restriction on daily basis, despite elevated compensatory response of ghrelin secretion, was associated with low body weight. While ghrelin levels were similar in schedule-normal, high fat and freely fed groups, food intake differentially was affected. This discrepancy in effects may be explained by the fact that the preprandial rise in plasma ghrelin is only due to a learned anticipatory response but not energy deficit, and thus the animals display a nonstop eating behavior when have access to food. However, an increased food intake in freely fed rats just on fasted days was proportional to food deprivation time. Also, the increased ghrelin levels in the schedule normal-fed animals as fasting can be interpreted as a biological feedback mechanism to cope with the forced feeding schedule; since it prepares the body for even scarcer food periods by increasing energy efficiency and investing in fat deposition (Verbaeys et al., 2011). Studies also show that ghrelin receptor blockade (Dailey et al., 2016) or genetic deletion (Walker et al., 2012) in rodents suppresses the ability of conditioned stimuli to produce hyperphagia. Hence, the schedule normal-fed animals meet higher body weight than freely fed rats during the lifespan.

Comparison of food intake between groups with scheduled–normal and-high fat diets revealed a decreased meal pattern for standard chow on days 7 and 14, with the same anticipated ghrelin surge. In this regard, Bake et al. reported when mice even were schedule-fed on palatable high fat diet for 2h a day, they consumed nearly 80% of their daily calorie intake and showed a large reduction in control diet (Bake et al., 2013; Bake et al., 2014).

Conclusion

In general, our results suggest while ghrelin receptor signaling might be at least necessary to anticipate feeding behavior (Davis et al., 2011), the observed behavioral profile is not critically dependent on ghrelin or other specific hormones and neuropeptides including NPY, orexin or leptin (Gunapala et al., 2011), but may require functional dopaminergic, serotonergic or other effective signaling systems (Liu

et al., 2012; Hsu et al., 2010). Furthermore, although scheduled feeding entrains ghrelin, appetitive behaviors control total energy balance. As the ghrelin secretion can be learned, amount of calorie intake can be controlled by learning, hence, we observed a reduction in standard meal size in animals with scheduled–high fat diet. This suggests the presence of an association between the mechanisms underlying binge like eating on a palatable diet and those responsible for anticipated feeding behavior.

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Conflict of interest

The authors declare that no competing interests exist.

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