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The *Ghsr*^{Q343X} allele favors the storage of fat by acting on nutrient partitioning

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ABSTRACT

The Growth Hormone Secretagogue Receptor (GHSR) mediates key properties of the gut hormone ghrelin on metabolism and behavior. Nevertheless, most recent observations also support that the GHSR is a constitutively active G protein-coupled receptor endowed of a sophisticated tuning involving a balance of endogenous ligands. Demonstrating the feasibility of shifting GHSR canonical signaling *in vivo*, we previously reported that a model with enhanced sensitivity to ghrelin (*Ghsr*^{Q343X} mutant rats) developed fat accumulation and glucose intolerance. Herein, we investigated the contribution of energy homeostasis to the onset of this phenotype, as well as behavioral responses to feeding or pharmacological challenges, by comparing *Ghsr*$^{M/M}$ rats to wild-type littermate rats 1) as freely behaving animals and 2) in feeding and locomotor paradigms. Herein, *Ghsr*$^{M/M}$ rats showed enhanced locomotor response to a GHSR agonist while locomotor or anorexigenic responses to amphetamine or cabergoline (dopamine receptor 2 agonist), respectively, were preserved. *Ad libitum* fed *Ghsr*$^{M/M}$ rats consumed and conditioned for sucrose similarly to littermate control rats. In calorie-restricted conditions, *Ghsr*$^{M/M}$ rats retained food anticipatory activity and maintained better their body weight and glycemia. Importantly, prior to fat accumulation, male *Ghsr*$^{M/M}$ rats preferentially used carbohydrates as fuel substrate without alterations of energy intake, energy expenditure or physical activity and showed alterations of the GHSR system (*i.e.* enhanced ratio of GHSR hormones LEAP2:acyl-ghrelin and increased *Ghsr* expression in the hypothalamus). Overall, the present study provides proof of concept that shifted GHSR signaling can specifically alter nutrient partitioning resulting in modified balance of carbohydrate/lipid utilization.

Introduction

The *growth hormone secretagogue receptor* (GHSR) (Howard, et al. 1996) holds a unique interest as the target of the gut hormone ghrelin (Kojima, et al. 1999), a hormone with key pharmacological properties such as GH release (Kojima et al. 1999), enhanced fat storage and enhanced food intake, mediated by its action on brain energy homeostasis centers (Nakazato, et al. 2001; Theander-Carrillo, et al. 2006; Tschop, et al. 2000) and hedonic circuits (Abizaid, et al. 2006; Jerlhag, et al. 2007; Naleid, et al. 2005). While the ablation of the *Ghsr* gene (*Ghsr*$^{-/-}$) or of ghrelin-producing cells in mice reported mitigated results on energy homeostasis (Muller, et al. 2015), *Ghsr*$^{-/-}$ mice failed to enhance locomotor activity similarly to control mice during scheduled feeding (Blum, et al. 2009; LeSauter, et al. 2009) and to preserve glycemic control during severe caloric restriction (Wang, et al. 2014). The GHSR could therefore play key roles in situations of stress while its role in the fed state remains largely unknown (Mani and Zigman 2017).

The GHSR is a G protein-coupled receptor (GPCR) showing high constitutive activity as documented in cellular (Holst, et al. 2003) and acellular systems (Damian, et al. 2012). Recent observations identified the liver hormone LEAP2 as a novel GHSR ligand (Ge, et al. 2018) with inverse agonist properties (M'Kadmi, et al. 2019; Mani, et al. 2019). Therefore, according to its expression pattern in the periphery and in several key brain regions, including the hypothalamus, ventral tegmental area (VTA) or hippocampus (Zigman, et al. 2006), the GHSR could exert its functions according to a complex balance between GHSR ligands, whose accessibility in each GHSR expressing brain structure still needs to be delineated (Perello, et al. 2019). Overall, these observations, that appear as a game changer (Andrews 2019), support an unprecedented refinement for an endocrine system, questioning the therapeutic potential of this GPCR target (Al-Massadi, et al. 2018). We reasoned that a preclinical model with shifted canonical GHSR signaling could provide key insights for future drug discovery.

To this purpose, we used a rat model carrying the *Ghsr*^{Q343X} nonsense mutation that is predicted to cause the deletion of the last 22 amino acids of the GHSR, a domain containing most Ser/Thr phosphorylation sites (Bulbul, et al. 2011). Although some studies initially documented altered responses when ghrelin was injected to homozygous mutant rats carrying this allele (*Ghsr*$^{M/M}$ rats) (Bulbul et al. 2011; Clifford, et al. 2012; MacKay, et al. 2016), we proposed that the mutant receptor encoded by this mutated allele could enhance GHSR sensitivity to ligands (Chebani, et al. 2016). First, in cellular systems, the GHSR-Q343X receptor shows altered β-arrestin recruitment and internalization to the benefit of G protein signaling in response to the agonist. Second, young adult *Ghsr*$^{M/M}$ rats show increased GH release and chow intake at low doses of the agonist. Third, *Ghsr*$^{M/M}$ rats develop fat accumulation and insulin resistance with age, an evolutive phenotype consistent with enhanced ghrelin effects (Chebani et al. 2016). However, the contribution of energy homeostasis to the onset of fat accumulation in *Ghsr*$^{M/M}$ rats remained unexplored, as well as the physiological consequences of this mutation on food-related behaviors.

In this study, we assessed the consequence of shifting GHSR signaling onto 1) feeding and locomotor response to pharmacological manipulations or feeding challenges, and 2) metabolic efficiency, nutrient partitioning by using an automated food and calorimetric recording system.

Material and methods

Animals

The mutant line of FHH-Ghsr^{m1Mcwi} rats carrying the *Ghsr*^{Q343X} allele was obtained from the Medical College of Wisconsin (Milwaukee, WI, USA). The homozygous FHH-*Ghsr*^{m1Mcwi} rats and wild-type littermates are referred to as *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats, respectively. Animals used in this study (187 rats from 7 litters) were obtained from crossing heterozygous rats. Animals were raised four by cages with free access to water and chow diet (A04, SAFE), in a room with controlled temperature (22-24°C) and illumination (12h light/dark schedule with lights on at 7:00 am). The genotype of the rats at the *Ghsr* locus was determined as previously described (Chebani et al. 2016). Experiments were performed with male or female rats with *ad libitum* access to food and water, unless otherwise specified. The procedures involving rats were approved by the ethics committee of animal experimentation of the Université Paris-Descartes.

Analysis of Metabolic efficiency

Calorimetry exploration was performed using 10 week-old male rats at the start of the experiment. Body composition was assessed at the start of the experiment, at the end of baseline, after 24h fasting, and after 48h refeeding (end of the experiment) using an Echo Medical system (EchoMRI 100, Whole Body Composition Analyser, EchoMRI, Houston, USA). Energy expenditure, oxygen consumption and carbon dioxide production, respiratory exchange ratio (RER), food intake and homecage activity were obtained using calorimetric chambers (Labmaster, TSE Systems GmbH, Bad Homburg, Germany) as previously depicted (Joly-Amado, et al. 2012). Rats were individually housed, fed standard chow and acclimated to the chambers for 48h before experimental measurements. A meal was defined as the consumption of > 0.3 g of food, separated from the next feeding episode by at least 10 mins. To assess metabolic flexibility, all RER data were compiled to obtain relative cumulative frequency curves for *Ghsr*$^{M/M}$ and

Ghsr$^{WT/WT}$ rats. Sigmoidal dose-response curves were fitted to determine EC_{50} and Hill slopes (indicative of metabolic rigidity) (Riachi, et al. 2004).

Hormone measurements

Blood samples were collected from the same cohort of rats in a second experiment performed 3 weeks after the calorimetry exploration. Blood samples were obtained by tail bleeding in *ad libitum* fed and 24h fasted conditions. Samples for LEAP2 measurements were collected on EDTA and centribuged, and plasma were stored at -80°C until measurements. Sample preparation for ghrelin measurements were performed as previously reported (Chebani et al. 2016). Rat acyl ghrelin and des-acyl ghrelin were mesured with ELISA kits (Bertin Pharma) and LEAP2 was mesured using an EIA kit (Phoenix Pharmaceuticals).

Statistics

Results are presented as mean ± SEM. Sample size (n) and *p* values are given in the figure captions. Statistical analyses were performed using GraphPad Prism® 5.01, SPSS statistics (IBM Inc.) and R software. Differences between 2 groups were determined using non-parametric Mann-Whitney or Wilcoxon tests, or parametric Student's tests, as appropriate. Comparisons of multiple groups were performed using two-way ANOVA, repeated measure ANOVA or ANOVA on aligned rank transformed data (ARTool package) and *p* value of post-hoc tests were adjusted with the Sidak correction. Covariance analyses (ANCOVA) used the Mouse Metabolic Phenotyping Center web page http://www.mmpc.org/shared/regression.aspx). For all statistical analyses, a *p* value of less than 0.05 was considered as significant.

Results

Pharmacological probing of central dopaminergic circuits in *Ghsr*$^{M/M}$ rats reveals enhanced locomotor response to a GHSR agonist but preserved responses to amphetamine or to a DRD2 agonist

To examine central dopaminergic circuits in *Ghsr*$^{M/M}$ rats, locomotion or refeeding response was examined in *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats in response to pharmacologic modulators of dopamine signaling or GHSR agonist. First, we assessed the locomotor response to amphetamine (AMPH), a psychoactive drug known to reverse dopamine transporter activity at post-synaptic target leading to enhances DA release and action (Fig. 1A, B, C). Both experimental groups displayed similar response to the novel environment (Fig. 1A), and to AMPH-induced hyperlocomotion (Fig. 1B, C). Second, GHSR-DRD2 heteromers can produce behavioral response independent of ghrelin binding and correlated to DA function (Kern, et al. 2012). *Ghsr*$^{M/M}$ rats model offer a great platform to probe how possible interaction of GHSR and DRD2 receptor might contribute to alter response to DRD2 agonist.To this purpose, the anorexigenic effect of the DRD2 agonist cabergoline was evaluated in *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats. Cabergoline mediated a potent anorexigenic response in the refeeding of prefasted *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ animals (Fig. 1D, E). No significant difference was found across genotypes. Third, the pharmacological properties of the GHSR on locomotion were tested in *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats challenged with varying doses of the agonist hexarelin. Repeated measure ANOVA revealed a significant dose x genotype interaction ($p<0.01$) suggestive of a differential dose-response effect between the two groups of rats (Fig. 1F). Indeed, post hoc analyses showed that the locomotor response to the highest tested dose of hexarelin was twice as high in *Ghsr*$^{M/M}$ compared to *Ghsr*$^{WT/WT}$ littermate rats, an observation further confirmed in the analyses of the response to the highest hexarelin dose (Fig. 1G, H). Overall, these observations, while supporting enhanced GHSR responsiveness in *Ghsr*$^{M/M}$ rats, also rule out gross abnormalities in the dopaminergic system of these rats.

$Ghsr^{M/M}$ rats show unaltered spontaneous conditioning and motivation for sucrose but accelerated performance in operant system

The former results indicated that relative to their body weight, chow consumption was comparable between $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ rat, while showing enhanced sensitivity to endogenous ghrelin (Chebani et al. 2016). We hypothesized that these animals might show improved consumption and/or motivation for palatable food. To test this, we took advantage of the spontaneously high preference for sucrose in the fawn hood strain (Tordoff, et al. 2008) to investigate the spontaneous consumption and reinforcing properties of sucrose in $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ rats using free choice and instrumental conditioning paradigms. In a two-bottle choice between a sucrose solution and water, both $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ male littermates displayed similar levels of consumption throughout days, with a strong, sustained preference for the sucrose solution over water (Fig. 2A). For instrumental conditioning paradigms, we focused our interest on female rats who successfully achieved conditioning without requiring calorie restriction, a manipulation known to interfere with ghrelin endogenous tone. During conditioning sessions, *ad libitum* fed $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ rats achieved success criterion during the last 2 sessions of each Fixed Ratio (FR) schedule (Fig. 2B upper panel; repeated measure ANOVA; session effect; $p<0.001$). Total responding levels were similar between $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ rats on the Progressive Ratio (PR) PR schedule, and for both groups, nose-poking activity on the inactive, dummy target was close to zero, indicating specific, goal-oriented responding at the active nose-poking hole. Interestingly, at 5 mins after the beginning of sessions, $Ghsr^{M/M}$ rats had significantly achieved more responses than $Ghsr^{WT/WT}$ rats, starting from the 3rd FR3 session (Fig. 2B lower panel), although the total number of responses was comparable between $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ rats throughout the FR and PR schedules. This significant difference in performing speed was also seen 15 mins after the beginning of the session (Fig. 2B lower panel). Overall, *ad libitum* fed $Ghsr^{M/M}$ rats showed preference or consumption for palatable food similar to $Ghsr^{WT/WT}$ rats, but performed faster to obtain rewarding food in an operant nose-poking task.

$Ghsr^{M/M}$ display preserved food anticipatory activity in response to scheduled feeding.

Previous investigations revealed that $Ghsr^{M/M}$ rats preserved better their body weight and glycemia in scheduled restricted feeding conditions (Chebani et al. 2016), however whether this condition also affected other behavioral response related to food-seeking response remained unexplored. To test this, two paradigms differing on the kind of locomotor activity measured (wheel running or ambulatory locomotion) were used. In the first experiment, $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ littermate rats habituated to a single housing cage with free access to running wheels were put on a restricted feeding schedule. Unexpectedly, during the *ad libitum* feeding period, $Ghsr^{M/M}$ rats showed decreased running activity during the first hour of dark phase (Fig. 3A). At the beginning of caloric restriction, wheel running activity was increased in both groups of rats and the activity of $Ghsr^{M/M}$ rats no longer differed from that of $Ghsr^{WT/WT}$ rats (Fig. 3B). Running activity was further enhanced at the end of the caloric restriction and the activity pattern was re-distributed with maximal activity levels in anticipation to dark phase and meal access (Fig. 3C). $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ rats displayed comparable levels of daily activity, as well as similar food anticipatory behavior (Fig. 3C). Besides, throughout caloric restriction $Ghsr^{M/M}$ rats consumed similar amounts of food compared to $Ghsr^{WT/WT}$ littermates (Fig. 3D) but were better able to maintain their body weight (Fig. 3E) and glycemia (Fig. 3F). To verify that food anticipatory activity was indeed unaltered in $Ghsr^{M/M}$ rats while avoiding the potentially confounding effect of wheel running, which is rewarding by itself, we used an alternative paradigm in which another cohort of rats at same age were put on a 4h restricted feeding schedule and recorded daily in actimetry cages during the 2 hours preceding food access. In this setting, $Ghsr^{M/M}$ and $Ghsr^{WT/WT}$ rats developed food anticipatory activity to comparable levels (Fig. 3G), while chow intake and body weight were similar across genotypes (Fig. 3H, I). Altogether, $Ghsr^{M/M}$ rats therefore show anticipatory activity and food consumption similar to $Ghsr^{WT/WT}$ littermates on a restricted feeding schedule, suggesting that the $Ghsr^{Q343X}$ mutation does not affect behavioral anticipation of food.

$Ghsr^{M/M}$ rats display increased body mass and similar body composition at 12 weeks of age

In order to determine whether alteration in energy homeostasis precede the development of insulin resistance in 6 month-old rats (Chebani et al. 2016), metabolic efficiency was assessed using indirect calorimetry in asymptomatic mutant rats in response to nutritional manipulation. *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats entered in metabolic cages at similar age (12.3±0.2 and 12.0±0.3 week-old, respectively). Upon entry, *Ghsr*$^{M/M}$ rats were on average 7% heavier than *Ghsr*$^{WT/WT}$ littermates, but proportions of fat and lean masses relatively to total body mass were comparable in *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats (Fig. 4A). Across the experiment, *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ animals similarly lost and regained total body mass, fat mass and lean mass upon fasting and refeeding, respectively (Fig. 4B). Thus, in young adult rats, the *Ghsr*^{Q343X} mutation resulted in higher body weight, with increases in both fat and lean masses in proportion to total body mass, suggesting an aggravation of the phenotype later with age, which is in line with similar blood glucose levels between genotypes in all feeding conditions (Supplementary Fig. 1).

As shown Fig. 4C, *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ animals showed comparable 24h patterns of activity across baseline, fasting and refeeding, both in light and dark phases. In both groups of rats, fasting increased homecage activity, while refeeding returned it to basal level (Fig. 4D). As expected, the feeding pattern post-fast significantly differed from the *ad libitum* pre-fast condition for both groups of rats (Fig. 4E), with no differences across genotypes regarding 24h intake (Fig. 4F). Furthermore, the diurnal and nocturnal meal parameters across feeding conditions were comparable between *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats, as measured by mean meal size, total time spent eating, number of meals, meal duration, inter-meal intervals and ingestion rate (Supplementary Fig. 2). Not surprisingly, both groups of rats showed similar diurnal and nocturnal levels of energy expenditure characterized by decreased energy expenditure during fasting, while there was a general tendency to return to its basal level upon refeeding (Fig. 4G). Analyses of 24h energy expenditure revealed no differences between genotypes in the *ad*

libitum feeding condition, but a trend to decreased energy expenditure was observed in *Ghsr*$^{M/M}$ rats during fast and refeeding conditions (Fig. 4H). Estimated resting metabolic rate was also comparable for *Ghsr*$^{M/M}$ rats and their *Ghsr*$^{WT/WT}$ littermates (9.1±0.4 kcal/h/lean mass and 9.4±0.3 kcal/h/lean mass, respectively). Altogether, these results suggest that in young adult rats, the *Ghsr*^{Q343X} mutation does not alter daily locomotor activity, caloric intake, meal patterns or energy expenditure in conditions of *ad libitum* access to food as well as short-term food deprivation.

Ghsr$^{M/M}$ rats exhibit metabolic fuel preference towards carbohydrates

As *Ghsr*$^{M/M}$ rats showed increased body weight without any major changes in energy intake or expenditure compared to their *Ghsr*$^{WT/WT}$ littermates, we then investigated if *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats displayed differences in utilization of metabolic substrates, by exploring RER in baseline, fasted and refed conditions (Fig. 5A, B, C). In all nutritional states, mean 24h RER was higher in *Ghsr*$^{M/M}$ rats compared to *Ghsr*$^{WT/WT}$ littermates (Fig. 5D), indicating a slight but sustained increase in carbohydrate utilization as energy substrate, at the expense of fat, in *Ghsr*$^{M/M}$ rats compared to *Ghsr*$^{WT/WT}$ rats. However, qualitative RER variations across the experiment were similar in *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ animals, with RER decreasing during fasting compared to *ad libitum* basal feeding (Fig. 5A, B, D), and returning to baseline levels during the first 24h of refeeding (Fig. 5C, D). Separate analyses of light and dark phases indicated that, during light phase, *Ghsr*$^{M/M}$ rats had in all conditions a higher RER than *Ghsr*$^{WT/WT}$ rats (~2% in the fed state) (Fig. 5E) which is not the case during the dark phase (Fig. 5F). Thus, *Ghsr*$^{M/M}$ compared to *Ghsr*$^{WT/WT}$ rats presented an overall decreased use of lipids and increased use of carbohydrates as energy substrates illustrated further by decreased cumulative fat oxidation across feeding conditions (Supplementary Fig. 3A, B, C, D). In order to explore metabolic flexibility, RER data were compiled to obtain relative cumulative frequency curves for *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats. Comparison of the fits revealed a right shift of the curve for *Ghsr*$^{M/M}$ compared to *Ghsr*$^{WT/WT}$ rats (Supplementary Fig. 3E), indicating that RER

distribution of the *Ghsr*$^{M/M}$ group is narrower and skewed towards higher values compared to the *Ghsr*$^{WT/WT}$ group. Moreover, 1/Hill slope was lower for *Ghsr*$^{M/M}$ rats compared with *Ghsr*$^{WT/WT}$ rats indicative of decreased metabolic flexibility in *Ghsr*$^{M/M}$ rats.

Ghsr$^{M/M}$ rats show enhanced LEAP2:acyl ghrelin molar ratio but unaltered energy or glucose homeostasis markers centrally or in the periphery

Then, blood concentrations of GHSR hormones (i.e. LEAP2 and acyl-ghrelin) were assessed in satiated and fasted states using a subgroup of these same experimental rats (Fig. 5). The two-way ANOVA performed on circulating LEAP2 levels showed a significant genotype x feeding condition interaction ($p < 0.01$) revealing significantly higher LEAP2 concentrations in *Ghsr*$^{M/M}$ compared to *Ghsr*$^{WT/WT}$ littermates rats in the fed state only (Fig.5G). The same analysis performed on circulating acyl ghrelin levels also showed a significant genotype x feeding condition interaction ($p < 0.05$) revealing this time lower circulating acyl ghrelin levels in *Ghsr*$^{M/M}$ as compared to *Ghsr*$^{WT/WT}$ rats only in the fasting state (Fig.5H). Additionally, des-acyl ghrelin levels showed a pattern of response similar to acyl ghrelin (data not shown). When these same data were analyzed using a three-way ANOVA, a significant genotype x feeding condition x hormone interaction ($p<0.05$) was observed, illustrating a biologically significant interplay between GHSR hormones across feeding conditions in *Ghsr*$^{M/M}$ rats. Furthermore, Fig.5I shows that this interplay benefits LEAP2 over acyl-ghrelin levels in *Ghsr*$^{M/M}$ rats compared to *Ghsr*$^{WT/WT}$ rats as suggested by increased LEAP2:acyl-ghrelin molar ratio in the former. Finally, to rule out energy or glucose homeostasis abnormalities at gene expression level in asymptomatic *Ghsr*$^{M/M}$ rats, the expression of selected genes was compared in *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rat tissues. These investigations essentially revealed no significant differences across the panel of selected genes between groups (Fig. 6). Nevertheless, the *Ghsr* gene showed enhanced expression in the hypothalamus of *Ghsr*$^{M/M}$ compared to *Ghsr*$^{WT/WT}$ rats, an observation that specifically matched with the *Ghsr1a* isoform (Fig.6A). Despite the low

expression level of *Ghsr* in the peripheral tissues tested, the enhanced sensitivity of the digital PCR technique disclosed similar *Ghsr* gene expression across groups in the soleus muscle (Fig.6H). Overall, when still asymptomatic regarding body composition or disordered gene expression markers, *Ghsr*$^{M/M}$ rats may nevertheless show altered GHSR markers (hormones and gene expression).

Discussion

Recent observations suggest that the GHSR is a constitutively active GPCR endowed of a sophisticated tuning involving by a balance of endogenous ligands (Ge et al. 2018; Mani et al. 2019). Therefore, shifting GHSR canonical signaling could provide unique insights for future drug discovery. This is the case of the functionally significant *Ghsr*^{Q343X} mutation, whose homozygous rat carriers display enhanced responsiveness to GHSR agonists, enhanced fat accumulation, and insulin resistance (Chebani et al. 2016). The present study now shows that *Ghsr*$^{M/M}$ rats specifically display an enhanced locomotor response to a GHSR agonist while responses using dopaminergic drugs are seemingly unaltered. Similarly, spontaneous consumption and conditioning for sucrose appeared not to be impacted by the *Ghsr*^{Q343X} mutation, nor did food anticipatory activity. *Ghsr*$^{M/M}$ rats, prior to fat accumulation and insulin resistance, show a shifted fuel preference towards carbohydrates at early adulthood. In contrast, *Ghsr*$^{M/M}$ rats did not show obvious qualitative or quantitative alterations of energy intake, energy expenditure or locomotion. Altogether, the present study supports the feasibility of biasing GHSR signaling to the benefit of the storage of fat by acting preferentially on nutrient partitioning.

The present study provides novel insights into the relationship between GHSR signaling and metabolic fuel preference. Indeed, *Ghsr*$^{M/M}$ rats showed a higher RER than *Ghsr*$^{WT/WT}$ littermates, indicating a shift in metabolic preference towards decreased use of fat. It is noteworthy that increased RER in *Ghsr*$^{M/M}$ rats was essentially observed during light phase in the *ad libitum* condition (physiological fasting) and during

early fasting and early refeeding. These observations are therefore in line with acute and chronic pharmacological studies showing that supraphysiological levels of acyl ghrelin elevates RER, indicating shifted metabolic fuel preference towards enhanced carbohydrate utilization, while energy expenditure and home cage activity are unaffected (Currie, et al. 2005; Theander-Carrillo et al. 2006; Tschop et al. 2000). Overall, based on the assets of the *Ghsr*^{Q343X} model enhancing ghrelin responsiveness (Chebani et al. 2016), the present data support a physiological role for GHSR signaling in regular diet conditions to promote fat storage and preservation by acting on nutrient partitioning, a mechanism that might be, at least in part, centrally mediated (Joly-Amado et al. 2012).

Metabolic characterization of *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ littermates explored herein supports a specific alteration of substrate balance (decreased fat oxidation) associated the *Ghsr*^{Q343X} mutation, rather than a modification of total energy intake or expenditure. Indeed, as measured in freely behaving conditions, *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ littermate rats showed similar chow intake, feeding patterns and energy expenditure. Although the latter did tend to decrease in *Ghsr*$^{M/M}$ rats compared to *Ghsr*$^{WT/WT}$ rats in situations of energy deficit, it did not differ in the fed condition, thus excluding a key role of GHSR signaling in energy expenditure control. Interestingly, young adult *Ghsr*$^{M/M}$ rats showed increased body weight compared to their *Ghsr*$^{WT/WT}$ littermates, but displayed equivalent proportions of fat and lean masses relatively to total body mass, comparable fasting glycemia as well as similar expression of several molecular markers of energy and glucose homeostasis in the hypothalamus and several peripheral tissues. In comparison, as exemplified in our prior study (Chebani et al. 2016), older *Ghsr*$^{M/M}$ rats showed enhanced body weight and adiposity and decreased glucose tolerance, suggesting that the phenotype of these rats worsen with age, which is consistent with studies in *Ghsr*$^{-/-}$ mice involving the GHSR in metabolic aging (Lin, et al. 2011; Ma, et al. 2011). Indeed, as they are aging, *Ghsr*$^{-/-}$ mice fed on a standard diet display reduced obesity and improved insulin sensitivity compared to *Ghsr*$^{+/+}$ littermates, a phenotype mirroring that of adult *Ghsr*$^{M/M}$ rats. In contrast to the mechanism described in *Ghsr*$^{M/M}$ rats

(lower fat oxidation associated with development of adiposity), while RER of 1 year-old mice was similar amongst genotypes, it was increased in 2 year-old *Ghsr*$^{-/-}$ mice compared to *Ghsr*$^{+/+}$ littermates. It was suggested that increased RER in *Ghsr*$^{-/-}$ mice could be related to their lean phenotype and improved insulin sensitivity (Lin et al. 2011). Altogether, the phenotype of *Ghsr*$^{M/M}$ rats and *Ghsr*$^{-/-}$ mice seems to implicate GHSR signaling in age-associated adiposity and insulin resistance. In addition, we speculate that enhanced acyl ghrelin sensitivity in *Ghsr*^{Q343X} rats illustrates how low fat oxidation may contribute to the occurrence of overweight (Galgani and Ravussin 2008).

The analysis of circulating levels of GHSR hormones performed herein provide a new perspective regarding the *Ghsr*^{Q343X} model. The present study confirmed our previous observation that acyl ghrelin levels are similar among groups, but increased less during fasted or negative energy balance states in *Ghsr*$^{M/M}$ rats compared to their *Ghsr*$^{WT/WT}$ littermates (Chebani et al. 2016). Second, the LEAP2 assay disclosed increased levels in *Ghsr*$^{M/M}$ rats over *Ghsr*$^{WT/WT}$ rats in the fed condition while these were similar in fasted rats. Altogether, the LEAP2:acyl-ghrelin molar ratio was significantly enhanced across feeding conditions in *Ghsr*$^{M/M}$ rats compared to *Ghsr*$^{WT/WT}$ rats, consistent with a concerted regulation of these two hormones across feeding conditions. This could be interpreted as an adaptive response to the enhanced GHSR sensitivity in *Ghsr*$^{M/M}$ rats. Interestingly, enhanced LEAP2:acyl-ghrelin molar ratio was recently documented in obesity conditions in mice and human patients (Mani et al. 2019). In our case, however, the 12-weeks old *Ghsr*$^{M/M}$ rats did not show changes in fat mass or glycemia. Therefore further studies are needed regarding the LEAP2-acyl ghrelin balance in rat carriers of the *Ghsr*^{Q343X} allele. Finally, these observations may illustrate an evolutive biological state of so called ghrelin resistance in *Ghsr*$^{M/M}$ rats, a condition documented in obesity (Briggs, et al. 2010; Zigman, et al. 2016). We speculate that this aggravating state could participate in the sometime contradictory pharmacological GHSR responses

documented in adult *Ghsr*$^{M/M}$ rats (Bulbul et al. 2011; Chebani et al. 2016; Clifford et al. 2012; MacKay et al. 2016).

The results obtained herein using pharmacological tools refine and further support previous observations on the mechanism-of-action of the *Ghsr*^{Q343X} mutation in rat. First, enhanced locomotor response to the GHSR agonist hexarelin in *Ghsr*$^{M/M}$ rats may suggest increased dopaminergic responsiveness compared to *Ghsr*$^{WT/WT}$ littermates. Former results using dose-responses of ghrelin or hexarelin already showed improved GH release and 4h chow intake, observations indicative of enhanced GHSR responsivity in the hypothalamus (Chebani et al. 2016). Nevertheless, in contrast to these former results, obtaining a significant locomotor response required the highest dose of the injected agonist suggesting that this response may be non-physiological. In sum, these experiments are consistent with the hypothesis that the *Ghsr*^{Q343X} mutation, that results in G protein biased signaling in response to agonist in cellular systems (Chebani et al. 2016), could recapitulate a gain-of-function mutation in the GHSR. Additionally and as disclosed herein, the enhanced expression of *Ghsr1a* in the hypothalamus but not in a peripheral tissue of young adult *Ghsr*$^{M/M}$ rats, could also be considered as a possible contributing factor, although this requires further investigations. Second, pharmacological challenges designed to probe dopaminergic circuits using a DAT blocker (amphetamine) or a DRD2 agonist (cabergoline) revealed no difference across *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats. These observations therefore suggest that the *Ghsr*^{Q343X} allele has no obvious direct or indirect effect regarding both of these pharmacological responses, involving enhanced extracellular dopamine tone (amphetamine) or DRD2-GHSR heterodimers (cabergoline) (Kern et al. 2012). Interestingly, rare human missense or nonsense *GHSR* variants segregating with short stature or GH deficiency, presumed to be loss-of-function mutations on the basis of their *in vitro* mechanism of action, were documented (Inoue, et al. 2011; Pantel, et al. 2006; Pantel, et al. 2009; Pugliese-Pires, et al. 2011), including the *GHSR*^{A204E} mutation that specifically alters constitutive

activity *in vitro.* Just recently, a knock-in mice model expressing this mutation showed altered GH release, food intake and glycemic control (Torz, et al. 2020), therefore demonstrating that the *GHSR*^{A204E} disease-causing mutation is related to a partial impairment of GHSR functioning. This kind of genetic defect mirrors the mechanism of the present *Ghsr*^{Q343X} mutation in rat, documenting enhanced GHSR function both *in vitro* and *in vivo*. Altogether, *Ghsr* mutant models appear as very relevant tools to probe the significance of GHSR signaling *in vivo*, more especially since this constitutively active GPCR was recently established as the key target of both the agonist hormone ghrelin and LEAP2, an endogenous ligand with inverse agonist properties (M'Kadmi et al. 2019; Mani et al. 2019).

Using the *Ghsr*^{Q343X} model to probe food anticipatory activity, *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats were found to have similar running activity in anticipation of food, suggesting that the *Ghsr*^{Q343X} mutation does not impact the development nor expression of food anticipatory activity (FAA). Acyl ghrelin has been proposed to be a food-entrainable oscillator that participates in food anticipatory activity in rodents put on a restricted feeding schedule (Laermans, et al. 2015; LeSauter et al. 2009). Furthermore, when mice are put on a restricted feeding schedule, *Ghsr* knock-out results in attenuated FAA (Blum et al. 2009; Davis, et al. 2011; LeSauter et al. 2009) and reduced activation of several hypothalamic and midbrain nuclei prior to food access (Lamont, et al. 2012), altogether supporting that GHSR signaling plays a role in food anticipation. FAA was suggested to have a “go, no-go” property (LeSauter et al. 2009), therefore, while complete removal of the GHSR results in delayed onset of FAA, the presence of functional canonical acyl ghrelin-GHSR signaling in *Ghsr*$^{M/M}$ rats, albeit enhanced (Chebani et al. 2016), might produce “go” decisions with probabilities similar to the wild type GHSR without affecting FAA onset. Of note, *Ghsr*$^{M/M}$ rats were previously reported to have decreased FAA as assessed by total number of infrared bream breaks during the 2 hours preceding food access (MacKay et al. 2016), a result that is not supported by present observations using two different paradigms (running wheels and actimetry cages).

However, in this former study, FAA was not normalized to total 24h activity levels, and the observed decrease in FAA is likely to be the result of the general reduction in activity levels related to the metabolic phenotype of *Ghsr*$^{M/M}$ rats, rather than a specific attenuation of food-oriented anticipatory activity.

Taking advantage of the high preference for sucrose of the Fawn hood strain (Tordoff et al. 2008) to probe spontaneous phenotypes in *ad libitum* fed *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats, the present study disclosed 1) similar consumption of sucrose in a two-bottle choice paradigm, suggesting that the hedonic perception of sucrose is not altered, and 2) similar levels of total nose-poke responses for sucrose in an instrumental task, supporting similar motivation to obtain food rewards. Thus far, pharmacological studies support that acyl ghrelin-GHSR signaling modulates the appetitive properties of palatable food. Indeed, peripherally administered acyl ghrelin promotes, whereas GHSR antagonist JMV2959 reduces, consumption and motivation to obtain palatable food in ad libitum-chow fed rodents (Landgren, et al. 2011; Perello, et al. 2010; Skibicka, et al. 2012), effects that are essentially reproduced by i.c.v., intra-VTA or intra-ventral hippocampus (vHPC) administration of acyl ghrelin or JMV2959 (Kanoski, et al. 2013; Skibicka, et al. 2011; Skibicka et al. 2012). However, the physiological significance of these effects is still unclear, as *Ghsr*$^{-/-}$, *Ghrl*$^{-/-}$ and *Goat*$^{-/-}$ mouse models rarely showed results supporting altered hedonic feeding behaviors when animals are explored in the fed state (Davis, et al. 2012; Disse, et al. 2010; Lockie, et al. 2015). In the physiological setting of the present study, it is interesting to note that *Ghsr*$^{M/M}$ rats, considered with enhanced response to endogenous ghrelin, did not produce an increase in the number of responses over *Ghsr*$^{WT/WT}$ rats, but had a subtler effect on the performing speed of the animals, which was increased. This observation may suggest enhanced impulsivity to obtain food rewards. Interestingly, ghrelin injected i.c.v. or into the VTA of rats was indeed recently shown to increase impulsive behavior to obtain palatable food (Anderberg, et al. 2016). Overall, the observation

supporting a possible effect of the *Ghsr*^{Q343X} mutation with qualitative rather than a quantitative modulation of spontaneous operant responding for sucrose, at least in female rats, is of particular interest and needs to be clarified further, keeping in mind possible confounding factor such as the current rat strain as well as the metabolic phenotype of aged *Ghsr*$^{M/M}$ rats.

The present study shows limits regarding putative sex differences associated with the *Ghsr*^{Q343X} mutation. First, the present calorimetry study only focused on male rats. While female *Ghsr*$^{M/M}$ rats developed overweight with age with a timing similar to male rats (our former study), a study specifically investigating the energy intake, energy expenditure or RER parameters in female animals is mandatory in order to delineate possible gender differences. Indeed, female animals in mice or rat are known to gain less weight than males in HFD models, and these observations are known to be contributed, at least in part, to differences in sensitivity to energy homeostasis hormones such as leptin or ghrelin. More specifically, ghrelin sensitivity was shown to be modulated by oestrogens (Clegg, et al. 2007). Second, in the reward experiments using sucrose, while female and male *Ghsr*$^{M/M}$ rats show similar responses in the 2-bottle choice paradigm, only female rats succeeded the operant conditioning steps, precluding the analysis of any possible specific effect of the mutation in male *Ghsr*$^{M/M}$ rats.

Altogether, the present study strengthens the observations that *Ghsr*$^{M/M}$ rats may specifically show enhanced responsivity to GHSR agonist *in vivo*. In the fed or fasted conditions, young adult *Ghsr*$^{M/M}$ rats did not show obvious feeding or locomotor alterations but showed shifted fuel preference towards carbohydrates, therefore providing a possible mechanism to the enhanced fat accumulation documented in these rats later with age. Finally, these data also support the feasibility of tricking GHSR signaling to the benefit of fat storage by acting preferentially on nutrient partitioning.

Supplementary materials

Supplementary figures and table (5)

Supplementary file for Material and methods (1)

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C.M., P.Z., R.G.P.D, T.L.-L., J.P. conceptualized the research, C.M., P.Z., R.G.P.D., R.H., Y.C.,H.D., J.P. conducted the experiments, C.M., P.Z., R.G.P.D., T.L.-L., J.P. analyzed the data, G.L.P, D.C., F.N., S.L., J.P. supervised the experiments, C.M. and J.P. wrote the initial draft of the manuscript. All authors edited and revised the manuscript.

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FIGURE LEGENDS

Figure 1: Pharmacological probing of central dopaminergic circuits in *Ghsr*$^{M/M}$ rats. (A) Time course of the locomotor response to a novel environment and (B-C) to the injection of i.p. amphetamine (2 mg/kg) on two consecutive days in *Ghsr*$^{M/M}$ (n=11) and *Ghsr*$^{WT/WT}$ rats (n=10), *Ghsr* heterozygous rats served as the saline control group (n=7). Anorexigenic effects of a DRD2 agonist in a refeeding paradigm performed in fasted *Ghsr*$^{WT/WT}$ (n=9) (D) and *Ghsr*$^{M/M}$ (n=9) (E) rats injected s.c. with cabergoline (0.5 mg/kg) or with saline before refeeding. (F) Total locomotor response to increasing doses of the GHSR agonist hexarelin s.c. injected in male *Ghsr*$^{WT/WT}$ (n=13) and *Ghsr*$^{M/M}$ (n=13) rats, and (G-H) locomotor responses obtained with the highest injected hexarelin dose or saline across time. Data were analyzed by 2-way repeated measure ANOVA followed by Sidak's post-hoc tests. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ~ non-significant trend ($p<0.1$). Data represent mean ± SEM.

Figure 2: Consumption and motivation for sucrose in *Ghsr*$^{M/M}$ rats. (A) Cumulative intake of sucrose solution (0.75%) (plain lines) and water (dotted line) during daily 1h two-bottle choice in *Ghsr*$^{M/M}$ (n=17) and *Ghsr*$^{WT/WT}$ (n=13) *ad libitum* fed rats. (B) Operant nose-poking responses for sucrose pellets in *Ghsr*$^{M/M}$ (n=16) and *Ghsr*$^{WT/WT}$ (n=15) *ad libitum* fed rats during each session (upper panel) or at intermediate times of the session (lower panel). Data were analyzed by 2-way repeated measure ANOVA.* $p<0.05$; ** $p<0.01$. Data represent mean ± SEM.

Figure 3: Food anticipatory activity is preserved in *Ghsr*$^{M/M}$ rats. Wheel running activity in *Ghsr*$^{M/M}$ (n=7) and *Ghsr*$^{WT/WT}$ (n=8) rats averaged over 10 days of *ad libitum* feeding (A) or at beginning (B) or end of the restricted feeding schedule (C), daily food intake (D), body weight (E) and glycemia (F) of rats across the

protocol. Travelled distance recorded in an open field in the 2 hours preceding food access in *Ghsr*$^{M/M}$ (n=8) and *Ghsr*$^{WT/WT}$ (n=7) rats put on a 4h restricted feeding schedule (initial: averaged on the first 4 days; final: averaged on the last 4 days) (G), daily food intake normalized to body weight (H) and body weight (I). Data were analyzed by 2-way repeated measure ANOVA followed by Sidak's post-hoc tests. * p<0.05; ** p<0.01; *** p<0.001; ~ non-significant trend (p<0.1). Data represent mean ± SEM.

Figure 4: *Ghsr*$^{M/M}$ rats show preserved circadian locomotor, feeding and energy balance rhythms across feeding conditions. (A) Body composition at the start of the calorimetric experiment in young adult (12 week-old) rats (n=12/genotype). (B) Changes in total body weight across *ad libitum* feeding (4 days), fasting (24h) and refeeding (48h). (C) Daily fluctuations of homecage activity and (D) 24h homecage activity. (E) Daily fluctuations of food intake and (F) 24h food intake as a function of body mass in each rat. (G) Daily fluctuations of energy expenditure and (H) 24h energy expenditure as a function of body mass in each rat. Values represent means ± SEM. Data were analyzed by Mann-Whitney test (A), by ANOVA on aligned rank transformed data (B-E; G) or by ANCOVA using body weight as a covariate (F; H). * p<0.05; *** p<0.001; ~ non-significant trend (p<0.1). Data represent mean ± SEM.

Figure 5: Increased respiratory exchange ratio in *Ghsr*$^{M/M}$ rats across feeding conditions. Daily pattern of respiratory exchange ratio (RER) during adlibitum condition (4-day average) (A), 24h fasting (B) and the first 24h of refeeding (C) in 12 week-old male rats (n=12/genotype). Averaged RER over 24h (D), light phase (E) and dark phase (F) for each feeding condition. Plasma concentrations of LEAP2 (G), acyl-ghrelin (H) and the calculated LEAP2:acyl-ghrelin molar ratio (I) in a rat subgroup during ad libitum feeding and fasting conditions. Data were analyzed by ANOVA on aligned rank transformed data (A-F; I) or 2-way ANOVA (G,H). * p<0.05; ** p<0.01; *** p<0.001. Data represent mean ± SEM.

Figure 6: mRNA expression of known markers of energy homeostasis, adiposity and glucose homeostasis in *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats in the hypothalamus and in peripheral tissues. qRTPCR analyses in hypothalamus (A), epididymal white adipose tissue (B), inguinal white adipose tissue (C), brown adipose tissue (D), liver (E), tibialis muscle (F), soleus muscle (G) and ddPCR analysis in the soleus muscle (H). Data represent mean ± SEM. Data were analyzed by multiple Mann-Whitney tests using adjusted p values. ** $p<0.01$; *** $p<0.001$; ND, not detectable.

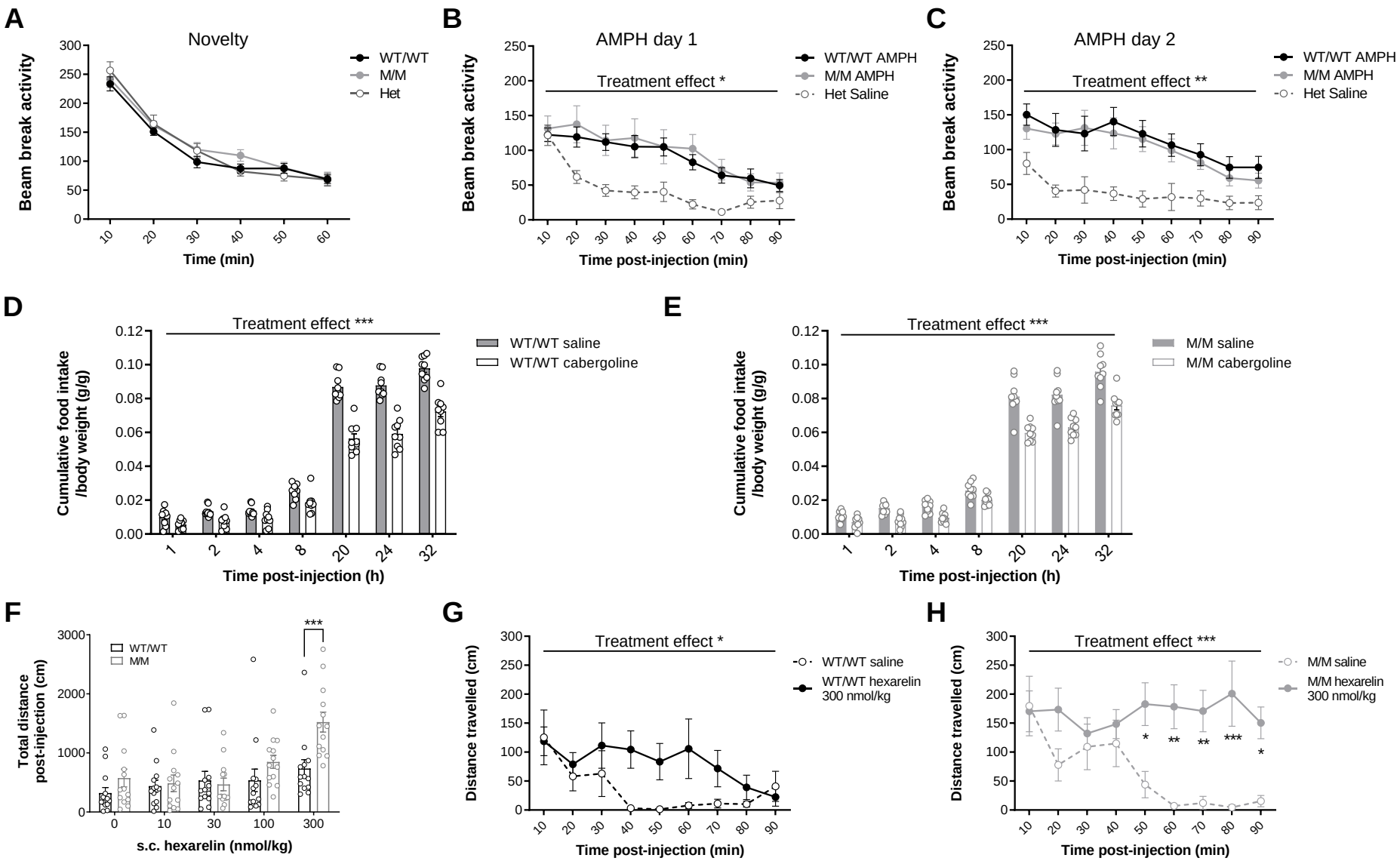

A
Novelty
Beam break activity
Time (min)
WT/WT
M/M
Het
B
AMPH day 1
Treatment effect *
Beam break activity
Time post-injection (min)
WT/WT AMPH
M/M AMPH
Het Saline
C
AMPH day 2
Treatment effect **
Beam break activity
Time post-injection (min)
WT/WT AMPH
M/M AMPH
Het Saline
D
Treatment effect ***
Cumulative food intake /body weight (g/g)
Time post-injection (h)
WT/WT saline
WT/WT cabergoline
E
Treatment effect ***
Cumulative food intake /body weight (g/g)
Time post-injection (h)
M/M saline
M/M cabergoline
F
Total distance post-injection (cm)
s.c. hexarelin (nmol/kg)
WT/WT
M/M
***
G
Treatment effect *
Distance travelled (cm)
Time post-injection (min)
WT/WT saline
WT/WT hexarelin 300 nmol/kg
H
Treatment effect ***
Distance travelled (cm)
Time post-injection (min)
M/M saline
M/M hexarelin 300 nmol/kg

Figure 1

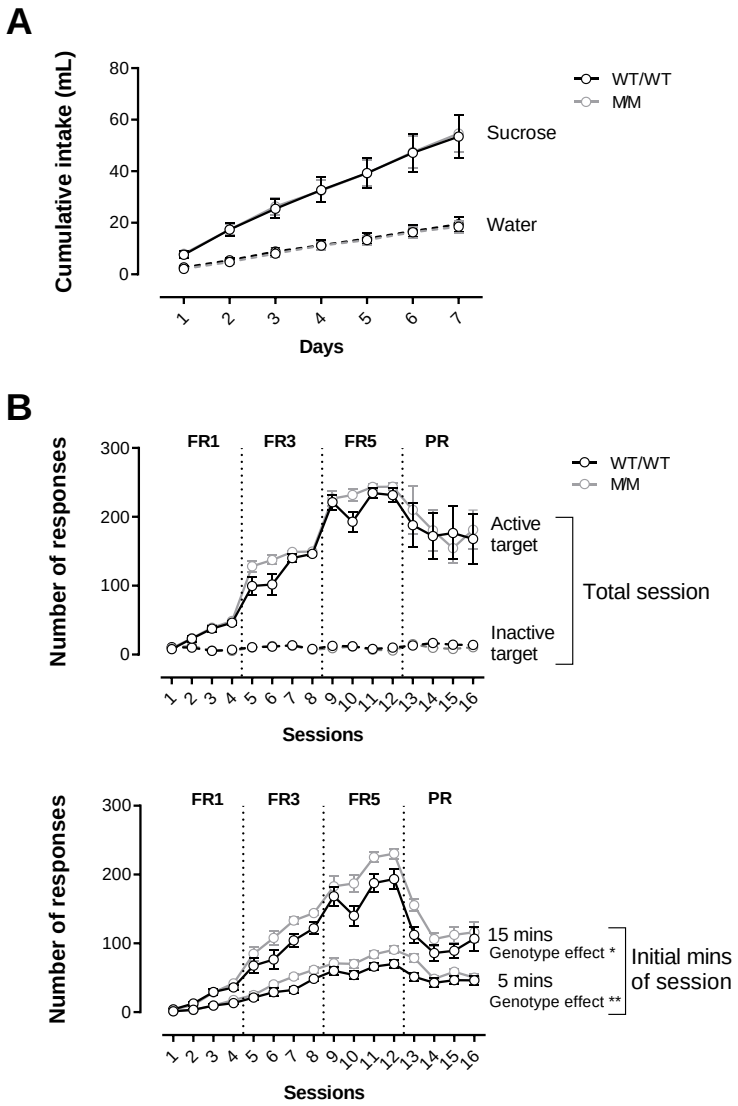

A
Cumulative intake (mL)
80
60
40
20
0
1
2
3
4
5
6
7
Days
WT/WT
M/M
Sucrose
Water
B
Number of responses
300
200
100
0
FR1
FR3
FR5
PR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sessions
WT/WT
M/M
Active target
Inactive target
Total session
Number of responses
300
200
100
0
FR1
FR3
FR5
PR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sessions
15 mins
Genotype effect *
5 mins
Genotype effect **
Initial mins of session

Figure 2

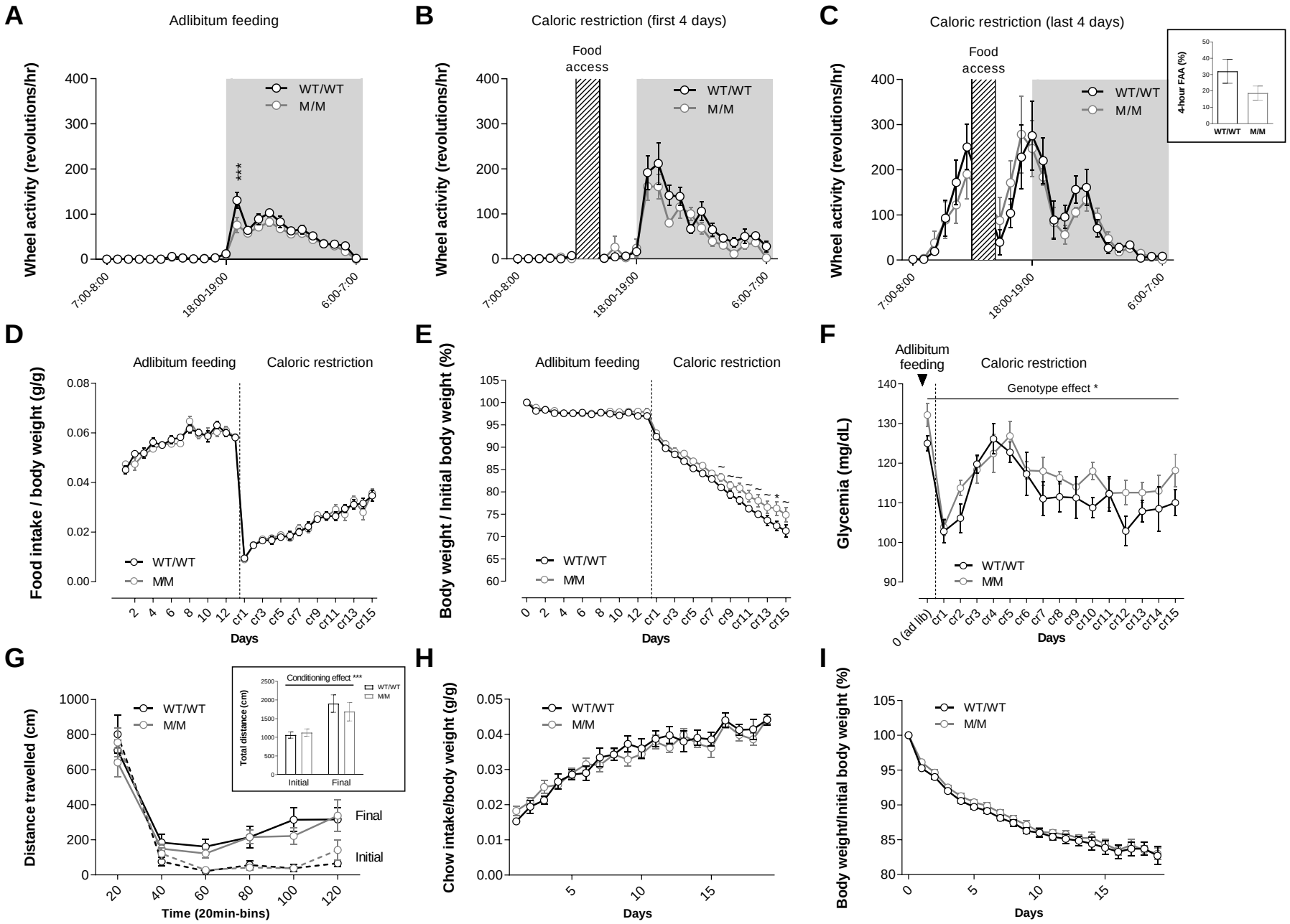

A
Adlibitum feeding
Wheel activity (revolutions/hr)
WT/WT
M/M
***
7:00-8:00
18:00-19:00
6:00-7:00
B
Caloric restriction (first 4 days)
Food access
C
Caloric restriction (last 4 days)
4-hour FAA (%)
D
Food intake / body weight (g/g)
Days
E
Body weight / Initial body weight (%)
F
Adlibitum feeding
Caloric restriction
Genotype effect *
Glycemia (mg/dL)
0 (ad lib)
G
Distance travelled (cm)
Time (20min-bins)
Conditioning effect ***
Total distance (cm)
Initial
Final
H
Chow intake/body weight (g/g)
I
Body weight/Initial body weight (%)

Figure 3

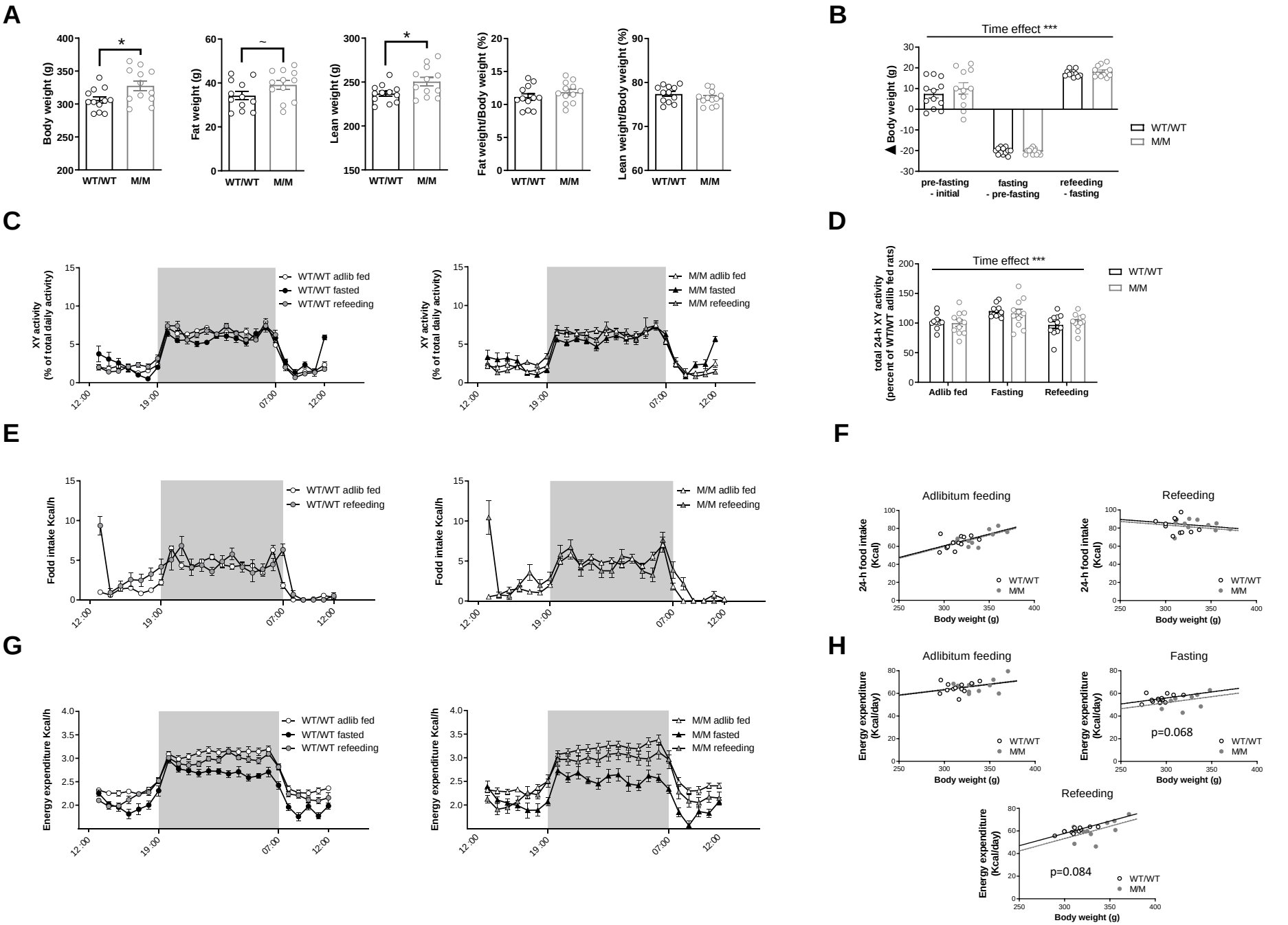

A
Body weight (g)
Fat weight (g)
Lean weight (g)
Fat weight/Body weight (%)
Lean weight/Body weight (%)
WT/WT
M/M
B
Time effect ***
Body weight (g)
pre-fasting - initial
fasting - pre-fasting
refeeding - fasting
C
XY activity (% of total daily activity)
WT/WT adlib fed
WT/WT fasted
WT/WT refeeding
M/M adlib fed
M/M fasted
M/M refeeding
12:00
19:00
07:00
D
Time effect ***
total 24-h XY activity (percent of WT/WT adlib fed rats)
Adlib fed
Fasting
Refeeding
E
Fodd intake Kcal/h
F
Adlibitum feeding
Refeeding
24-h food intake (Kcal)
Body weight (g)
G
Energy expenditure Kcal/h
H
Energy expenditure (Kcal/day)
p=0.068
p=0.084

Figure 4

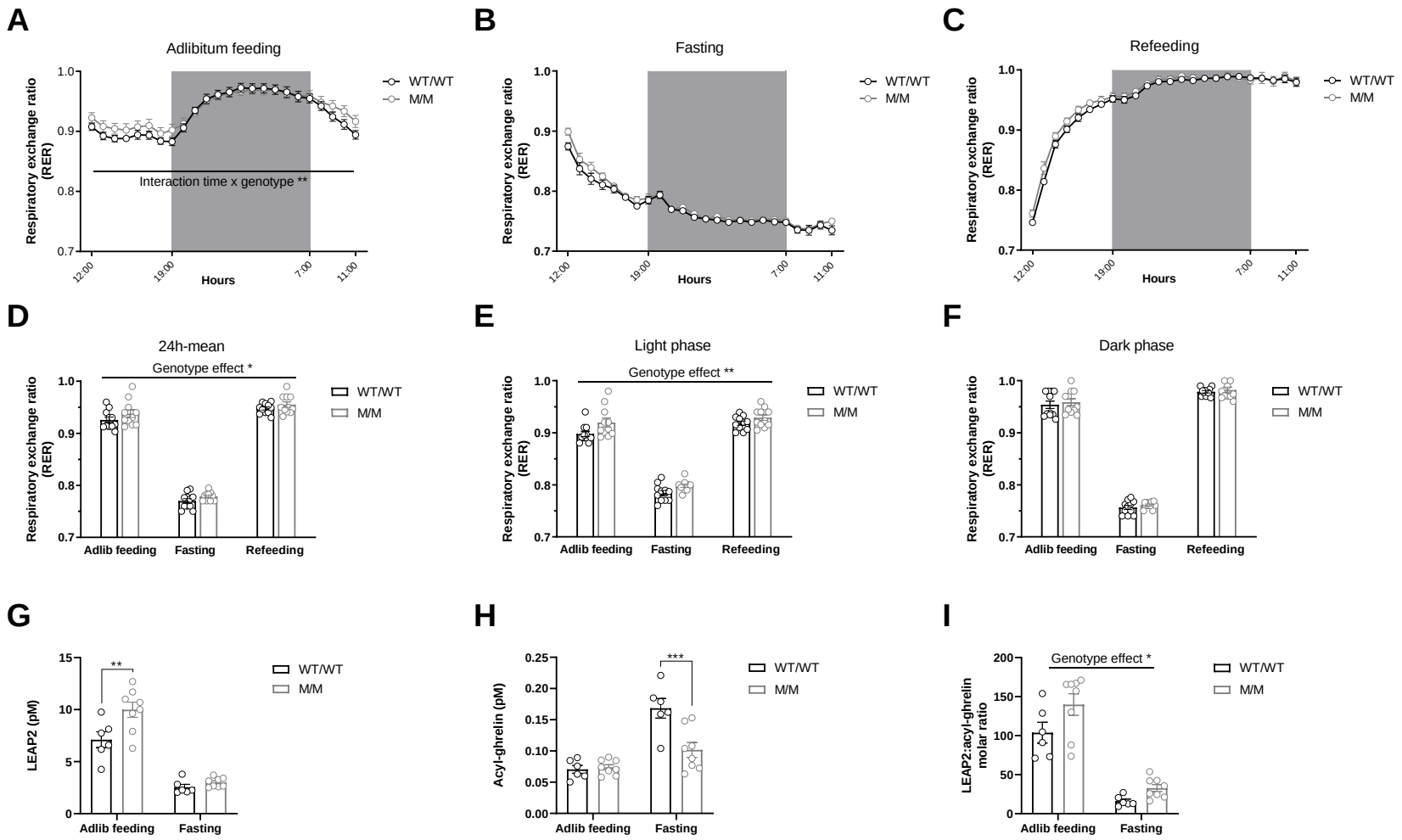

A
Adlibitum feeding
Respiratory exchange ratio (RER)
Interaction time x genotype **
Hours
12:00
19:00
7:00
11:00
WT/WT
M/M
B
Fasting
C
Refeeding
D
24h-mean
Genotype effect *
Adlib feeding
Fasting
Refeeding
E
Light phase
Genotype effect **
F
Dark phase
G
LEAP2 (pM)
**
H
Acyl-ghrelin (pM)
***
I
LEAP2:acyl-ghrelin molar ratio
Genotype effect *

Figure 5

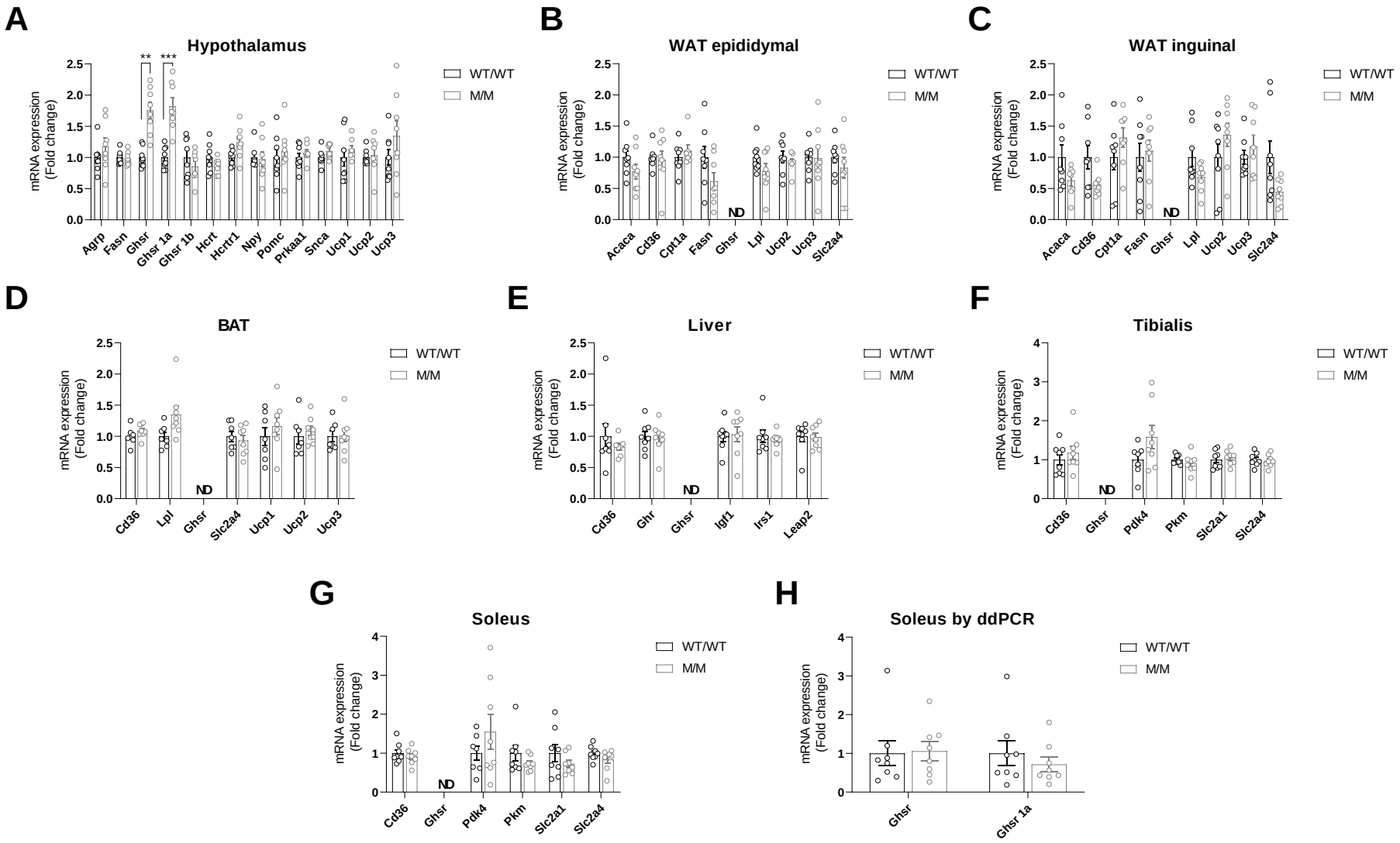
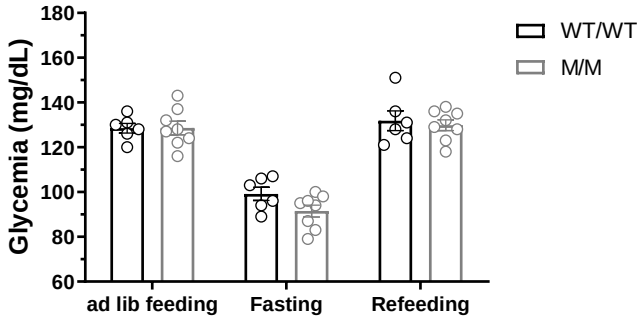
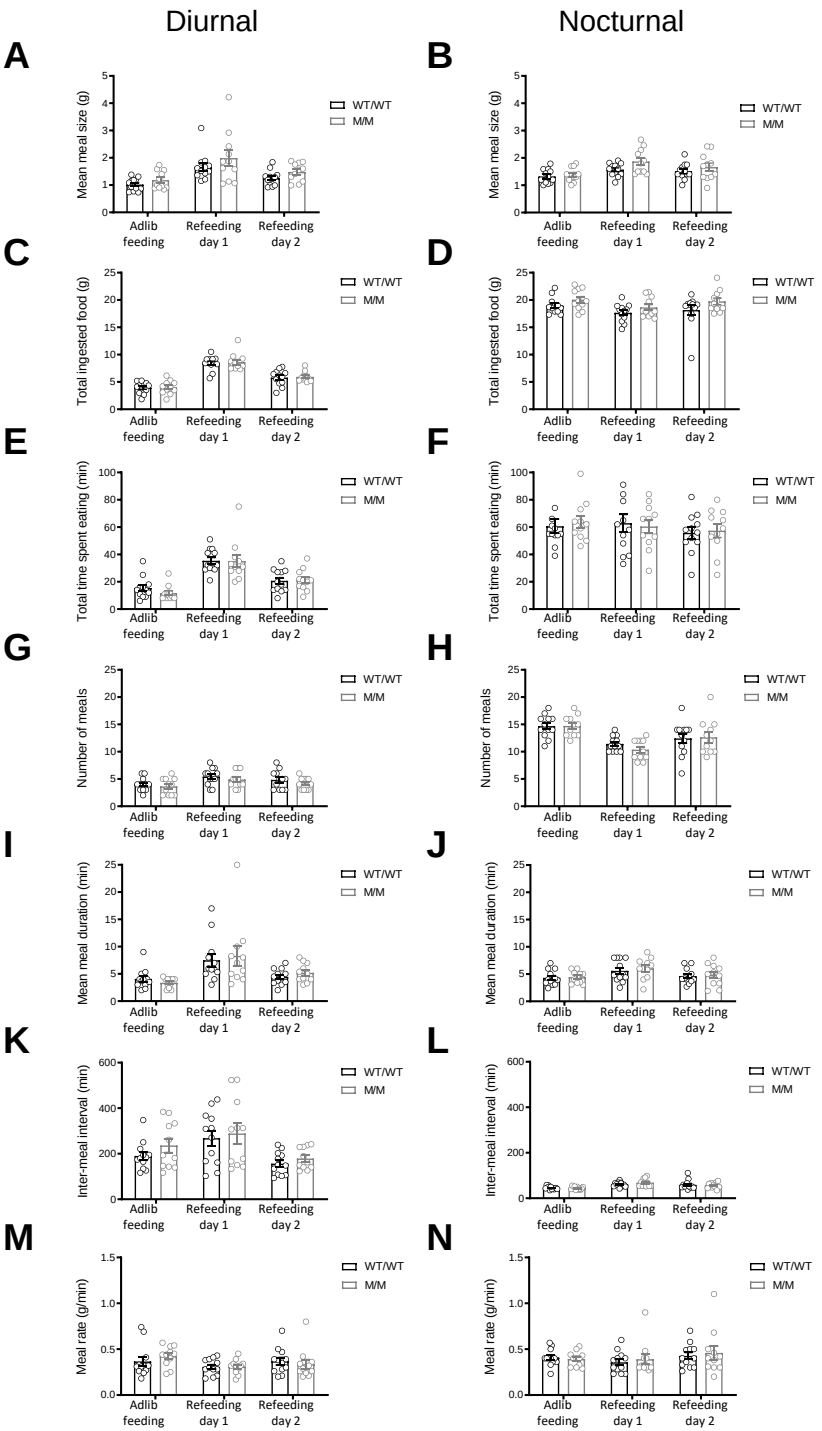

A
Hypothalamus
mRNA expression (Fold change)
WT/WT
M/M
**
***
Agrp
Fasn
Ghsr
Ghsr 1a
Ghsr 1b
Hcrt
Hcrtr1
Npy
Pomc
Prkaa1
Snca
Ucp1
Ucp2
Ucp3
B
WAT epididymal
ND
Acaca
Cd36
Cpt1a
Fasn
Ghsr
Lpl
Ucp2
Ucp3
Slc2a4
C
WAT inguinal
D
BAT
Cd36
Lpl
Ghsr
Slc2a4
Ucp1
Ucp2
Ucp3
E
Liver
Ghr
Igf1
Irs1
Leap2
F
Tibialis
Pdk4
Pkm
Slc2a1
G
Soleus
H
Soleus by ddPCR

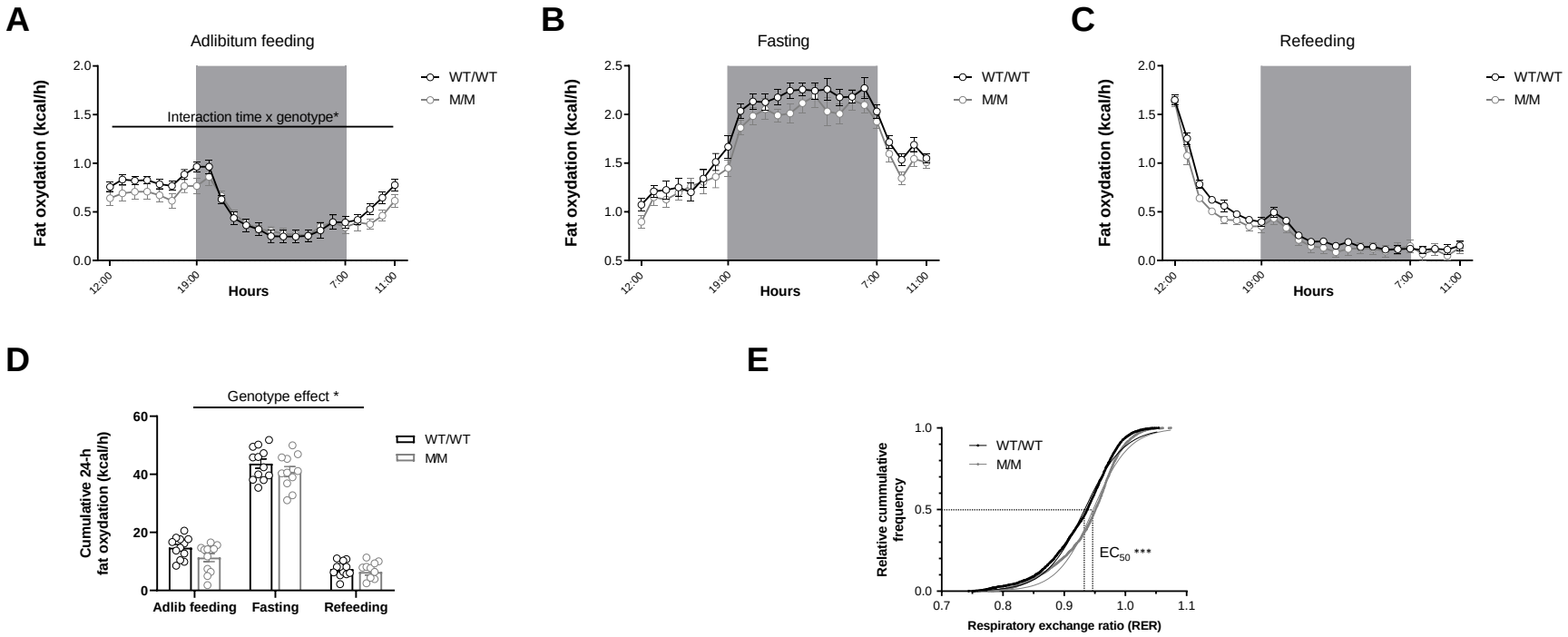
Figure 6



Supplementary Figure 1: Glycemia fluctuations across feeding conditions. *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ male rats at 15 weeks of age (n=6-8/genotype). Data represent mean ± SEM.



Supplementary Figure 2: Diurnal and nocturnal meal patterns of *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats during ad libitum-feeding and refeeding. (A-B) Mean meal size during light (A) and dark (B) phases. (C-D) Total chow ingested during light (C) and dark (D) phases. (E-F) Total time spent eating during light (E) and dark (F) phases. (G-H) Number of meals during light (G) and dark (H) phases. (I-J) Mean meal duration in light (I) and dark (J) phases. (K-L) Inter-meal interval during light (K) and dark (L) phases. (M-N) Meal ingestion rate in light (M) and dark (N) phases (n=12/genotype). Data represent mean ± SEM.



Supplementary Figure 3: Decreased fat oxydation in *Ghsr*$^{M/M}$ rats across feeding conditions. (A-C) Daily pattern of fat oxydation during adlibitum feeding (A), 24h fasting (B) and 24h refeeding (C). Data represent mean ± SEM. (D) 24-h cumulative fat oxydation (n=12/genotype). Data were analyzed by 2-way ANOVA (E) Relative cumulative frequency curves for ad libitum fed *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats (dots) and curve fits (full lines) reveal higher EC_{50} (0.947 vs. 0.933) and a lower 1/Hill slope value (0.067 vs. 0.074) for *Ghsr*$^{M/M}$ rats compared to *Ghsr*$^{WT/WT}$ rats (F-test; p<0.001 for both parameters). * p<0.05; *** p<0.001.

Material and methods – Supplementary file

Amphetamine-induced locomotion

A group of experimentally naive 10 week-old male rats was used to assess the locomotor response to a novel environment and to the injection of the dopamine enhancing drug amphetamine. Rats were habituated to a locomotor cage (Imetronic, Bordeaux, FR) for 60 mins on 2 consecutive days. The next 2 days, groups of *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats were injected i.p. with amphetamine (2 mg/kg) while a control group of *Ghsr* heterozygous rats was injected i.p. with saline.

Cabergoline-induced anorexigenic response

Prior to the food intake experiment, 7 month-old female *Ghsr*$^{M/M}$ and *Ghsr*$^{WT/WT}$ rats were habituated to single housing for at least 1 week. Free access to chow diet was removed at 6:00 pm for a 16h fast. Rats were then s.c. injected with the dopamine receptor D2 (DRD2) agonist cabergoline (0.5 mg/kg, Tocris) or saline at 11:00 am. Each animal received both treatments in a crossover fashion, with a 2-week washout period between treatments.

Hexarelin-induced locomotion

On the first day of each experiment, experimentally naive 5 month-old male rats were individually put in an open field arena (Med Associates Inc., Vermont, USA) with clean bedding. Room illumination was set at 15 lux. Recording sessions began each day at 8:30 am. Rats were habituated to the apparatus on 2 consecutive days for 45 min sessions. The next day, rats were injected by injected s.c. with saline solution (100 µL/100mg). Every other day, rats were challenged with varying doses of hexarelin (10 – 300 nmol/kg, Sigma) in the same setup, using a random crossover design.

Sucrose two-bottle choice

Ad libitum fed 7 month-old male rats were habituated to single housing and drinking from 2 bottles of water for at least 1 week prior to the beginning of the experiment. On 7 consecutive days they had access to a 0.75% sucrose solution and water for 1h, starting at 10:00 am. The position of the sucrose bottle was switched every day to prevent habituation. Bottles were weighed before presentation and again at the end of the 1h test to assess fluid intakes.

Operant responding for sucrose pellets

Operant chambers (Med Associates Inc., Vermont, USA) comprised 2 open nose-pocking niches, one designed as the active target (triggering reward delivery) and the other as the inactive, dummy target. Entry into the active nose-poking hole was rewarded by the delivery of a 45 mg sucrose pellet (Dustless Precision Pellets®). Entries into the inactive nose-poking hole had no consequence. House-light was on throughout the session. A light was switched on in the active nose-poking hole or food magazine to cue target or reward availability, respectively.

Ad libitum fed 7 month-old female rats were trained on fixed ratio (FR) schedules, starting with FR1 (1 nose-poke required to obtain 1 pellet), then on FR3 (3 nose-pokes to obtain 1 pellet) and finally FR5. A training session ended after 30 mins or when the rat had obtained 50 pellets (success criterion). Rats were kept on each FR schedule for 4 consecutive sessions, at the end of which all animals had reached the success criterion. After training, the rats underwent consecutive test sessions on a progressive ratio (PR) schedule to assess their motivation to work for the food reward according to the usual exponential formula ($N = 5 * \exp(0.2N) – 5$). The session ended when rats had failed to obtain a pellet for 30 consecutive minutes.

Running wheels

7 month-old male *Ghsr*$^{WT/WT}$ and *Ghsr*$^{M/M}$ rats matched for body weight were individualized at least 1 week prior to the experiment. Body weight, chow intake and wheel running behavior were monitored daily for 13 days under *ad libitum* feeding conditions until daily running levels had stabilized. On the last baseline day, chow intake was removed at 1:00 pm, and rats were fasted for 24h. On the following days, rats had free access to chow 2h/day, from 1:00 to 3:00 pm. During food access, running wheels were blocked to make sure the rats would feed and not run. The rats had free access to the running wheel for the 22 remaining hours. Every quarter of wheel turn was detected and recorded using a Matlab program. Raw data were converted to numbers of wheel turns in 1h bins using a dedicated R program. Chronic food restriction lasted for 15 days before rats were euthanized.

Food anticipatory activity assessed in open field arena

7 month-old male rats were individualized at least 1 week prior to the experiment. On the first session, locomotor activity was recorded in the open field arena for 2 hours before rats were returned to their homecage where food had been removed. During the 20 following days, rats were recorded daily in the same arena for 2h (bedding left in place) before being returned to their homecage and having access to chow diet for 4h. Body weight and chow intake were assessed daily throughout the experiment.

Quantitative real-time PCR analysis

Analyses were performed on snap frozen tissues stored at -80°C and collected during the light phase in libitum fed rats. Tissues samples (hypothalamus, adipose, muscle and liver) were homogenized in Tri-reagent (Euromedex, France) and RNA was isolated using a standard chloroform/isopropanol protocol. Reverse transcriptase reactions were performed from 2 µg of total RNA using Maxima Reverse Transcriptase (Fisher Scientific), primed with oligo-dT primers and Random Hexamer Primer (Fisher Scientific). Real-time PCR was performed on a LightCycler 480 Real-Time PCR System (Roche Diagnostics) using transcript-specific primers (600 nM), LightCycler 480 SYBR Green I Master (Roche Diagnostics) and cDNA (4 ng) in a final volume of 10 µl. Relative expression of each gene was normalized respect to 2 different endogenous housekeeping control genes depending on the tissue and determined using RefFinder analysis. The relative level of gene expression was calculated using the comparative ($2^{-\Delta\Delta Ct}$) method. See Supplementary table 1 and table 2 for the list of primers used in the study.

Digital PCR analysis

PCRs were prepared with the required QX200 ddPCR EvaGreen Supermix (Bio-Rad) with a final concentration of 150 nM for each primer to a final volume of 20µl. Each reaction was loaded into a sample well of an 8-well disposable cartridge (Bio-Rad) followed by 70 µl of droplet generator oil (Bio-Rad), which was added to the oil wells of the cartridge. Droplets were formed in the QX200 droplet generator (Bio-Rad). Droplets were then transferred to a 96-well PCR plate, heatsealed with foil in a PX1 PCR Plate Sealer Bio-Rad and amplified with an Eppendorf Nexus Gradient master cycler (95°C primary denaturation/activation for 5min, followed by 45 cycles of 95°C for 30 sec and 61°C for 1 min, followed by 4°C 5mn and a final 98°C heat treatment for 5min). PCRs were analyzed with the QX200 droplet reader (Bio-Rad) and data analysis was performed with QuantaSoft software (version 1.7; Bio-Rad).

Supplementary Table 1. Primers sequence for real time PCR analysis

Gene name	Forward primer	Reverse primer
Acaca	GGAAAACATCCCGCACCTT	TTTCTTCTTGACCAGGTCTTCCA
Agrp	AAGCTTTGGCAGAGGTGCTAGA	GTGCAGCCTTACACAGCGAC
CD36	CTGTTCCTCGCCATGAAATGA	GCAGCATGGAACTTGACAACA
Cpt1a	CTTGTTTGGCCTCACCATCA	CACGAGGGTCCGTTTTCCTT
Fasn	TCAGAAGGCCACAGCATTCA	TTCTAAGCTGGCAAGGAGACAAC
Ghr	GAGGCCACGACATGTGTAAAAC	GTGGTAAGGCTTTCTGTGGTGAT
Ghsr	CCGGTCTTCTGCCTCACTGT	GCTGCATCTCCGCGTCTC
Ghsr 1a	CCTCGCTCCGGGACCAGA	AAGCAAACACCACCACAGCAA
Ghsr 1b	AGCAGACAGTGAAGATGCTTG	CTGGGGAAGGGCAAGCAGT
Hcrt	AGAAGACGACGGCCTCAGAC	GGCAATCCGGAGAGATGGT
Hcrtr1	GCTCTGAAGCTAGGTCCACCCT	CACAATGCCAACGAGATCCA
Igf1	GCCGAAAATGCATGGGTG	CGGCTTCAGCAGCCAAAA
Irs1	CAAGACGCTCCAGTGAGGATT	TTGACGGTCCTCTGGTTGCT
Leap2	CTATGCAAAAGACGACGCTGTT	TGGAGGTGACTTCCCCAGTATG
Lpl	CGTATCTGAGCCTGACCAAGAA	AAGGCAGGATGGTTGAGAGTTC
Npy	CTCCGCTCTGCGACACTACA	GTGTCTCAGGGCTGGATCTCTT
Pdk4	TGGCACTTTTACGGGATCAAA	GGAAACAAGAGTCCACACACATTC
Pkm	AACATGCAATAGAGCCCAGCTAT	CAATCCTGCATTCCTCCTTCAC
Pomc	TGTGAAGGTGTACCCCAATGTC	TTCCAGCTCCCTCTTGAACTCTA
Prkaa1	CCTAACTGTAAGTCGGTTGTTGGG	TCAGGTTACTCTGGGCAAACATAC
Slc2a1	ACATGCCTTCTTTGCCAAGCT	CATTGTTCAGTATTCGTGTGTCCTT
Slc2a4	TCCCAAAATCTAGCCTTGCCT	GGATGTACTGTTGTGTTCCTTTGC
Snca	AGGACCAGATGGGCAAGGG	TCCACAGGCATGTCTTCCAG
Ucp1	TCCGGTGGATGTGGTAAAAAC	CACAGCTGGGTACACTTGGGTA
Ucp2	CCGAAATGCCATTGTCAACTG	TGAGGTTGGCTTTCAGGAGAGTA
Ucp3	CCTTTGTTCTACCCGTTAAGCCT	AGAAGTTGGTCCTGAACTTCGC

Supplementary Table 2. House keeping gene primers sequence for real time PCR analysis

Gene name	Forward primer	Reverse primer
Hypothalamus		
Eef1a1	ACCCTCCACTTGGTCGTTTTG	AGCTCCTGCAGCCTTCTTGTC
Actb	CGGCAATGAGCGGTTCC	TGCCACAGGATTCCATACCC
Liver		
Ppia	ACCCCATCTGCTCGCAATAC	GGAATGAGGAAAATATGGAACCC
Rpl13a	TGGAGAAGAAAATCTGCAAGTTCA	TCTTTATTGGGTTCACACCAAGAGT
WAT		
Vcp	GAGGTTTTGGCAGCTTCAGATT	ACCTCCACTGCCCTGACTTG
Ywhaz	CTTTTTGATACTTGCCTAACATGCA	CTAGCCGTCATCTCAAGTTATTTCC
BAT		
Sdha	TGCGGAAGCACGGAAGGAGT	CTTCTGCTGGCCCTCGATGG
Vcp	GAGGTTTTGGCAGCTTCAGATT	ACCTCCACTGCCCTGACTTG
Soleus-Tibialis		
Ppia	ACCCCATCTGCTCGCAATAC	GGAATGAGGAAAATATGGAACCC
Rpl13a	TGGAGAAGAAAATCTGCAAGTTCA	TCTTTATTGGGTTCACACCAAGAGT