Psychoneuroendocrinology 110 (2019) 104430

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Effects of disrupted ghrelin receptor function on fear processing, anxiety and saccharin preference in mice

A. Pierre[a], Y. Regin[a], A. Van Schuerbeek[a], Fritz E.M.[b], K. Muylle[a], T. Beckers[c], I.J. Smolders[a], N. Singewald[b], D. De Bundel[a,*]

[a] Department of Pharmaceutical Sciences, Research Group Experimental Pharmacology, Center for Neurosciences (C4N), Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium
[b] Department of Pharmacology and Toxicology, Institute of Pharmacy and CMBI, University of Innsbruck, Innrain 80/82, Innsbruck, Austria
[c] Departement of Psychology and Leuven Brain Institute, KU Leuven, Tiensestraat 102 box 3712, 3000, Leuven, Belgium

ARTICLE INFO

Keywords:
Ghrelin resistance
High-fat diet
Fear processing
Anxiety-like behavior
Saccharin preference

ABSTRACT

Background: Obesity is a risk factor for stress-related mental disorders such as post-traumatic stress disorder. The underlying mechanism through which obesity affects mental health remains poorly understood but dysregulation of the ghrelin system may be involved. Stress increases plasma ghrelin levels, which stimulates food intake as a potential stress-coping mechanism. However, diet-induced obesity induces ghrelin resistance which in turn may have deleterious effects on stress-coping. In our study, we explored whether disruption of ghrelin receptor function though high-fat diet or genetic ablation affects fear processing, anxiety-like behavior and saccharin preference in mice.

Methods: Adult male C57BL6/J mice were placed on a standard diet or high-fat diet for a total period of 8 weeks. We first established that high-fat diet exposure for 4 weeks elicits ghrelin resistance, evidenced by a blunted hyperphagic response following administration of a ghrelin receptor agonist. We then carried out an experiment in which we subjected mice to auditory fear conditioning after 4 weeks of diet exposure and evaluated effects on fear extinction, anxiety-like behavior and saccharin preference. To explore whether fear conditioning as such may influence the effect of diet exposure, we also subjected mice to auditory fear conditioning prior to diet onset and 4 weeks later we investigated auditory fear extinction, anxiety-like behavior and saccharin preference. In a final experiment, we further assessed lack of ghrelin receptor function by investigating auditory fear processing, anxiety-like behavior and saccharin preference in ghrelin receptor knockout mice and their wild-type littermates.

Results: High-fat diet exposure had no significant effect on auditory fear conditioning and its subsequent extinction or on anxiety-like behavior but significantly lowered saccharin preference. Similarly, ghrelin receptor knockout mice did not differ significantly from their wild-type littermates for auditory fear processing or anxiety-like behavior but showed significantly lower saccharin preference compared to wild-type littermates.

Conclusion: Taken together, our data suggest that disruption of ghrelin receptor function *per se* does not affect fear or anxiety-like behavior but may decrease saccharin preference in mice.

1. Introduction

Ghrelin is a 28-amino acid hormone which is synthetized by X/A-like cells of the gastrointestinal tract and released into the bloodstream in response to a negative energy balance (Kojima et al., 1999; Stengel and Taché, 2012). Post-translational modification on the serine-3 residue by ghrelin-o-acyltransferase converts desacyl-ghrelin to acyl-ghrelin, typically referred to as ghrelin, that freely diffuses through

Abbreviations: AgRP, agouti-related peptide; ARC, arcuate nucleus; cDNA, copy desoxyribonucleic acid; CBT, cognitive behavior therapy; CS, conditioned stimulus; CSDS, chronic social defeat stress; EBT, exposure-based behavioral therapy; EPM, elevated plus maze; FC-HFD, fear conditioning prior to diet onset; GHSR, ghrelin receptor; HAB, habituation; HFD, high-fat diet; HFD-FC, fear conditioning 4 weeks after diet onset; HPA, hypothalamic-pituitary-adrenal; i.p., intraperitoneally; ITI, inter-trial interval; KO, knockout; NPY, neuropeptide Y; OF, open field; PTSD, post-traumatic stress disorder; qPCR, real-time polymerase chain reaction; SEM, standard error of the mean; US, unconditioned stimulus; WT, wild-type

* Corresponding author.
E-mail address: dimitri.de.bundel@vub.be (D. De Bundel).

https://doi.org/10.1016/j.psyneuen.2019.104430
Received 12 July 2019; Received in revised form 26 August 2019; Accepted 3 September 2019

fenestrated capillaries of the median eminence into the arcuate nucleus (ARC) of the hypothalamus (Gutierrez et al., 2008; Cabral et al., 2015). There ghrelin binds to the G-protein coupled ghrelin receptor (also known as growth hormone secretagogue receptor, GHSR), expressed on neuropeptide Y (NPY) / agouti-related peptide (AgRP) neurons. Excitation of these orexigenic neurons stimulates food intake (Nakazato et al., 2001). Intact NPY signaling is required for the orexigenic effects of peripheral ghrelin and selective re-expression of GHSR in NPY/AgRP neurons of GHSR knockout (KO) mice partially mediates ghrelin-induced food intake (Wang et al., 2014). Besides its orexigenic effect through activation of ARC NPY/AgRP neurons, ghrelin has been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis by disinhibition of corticotropin-releasing factor neurons in the paraventricular nucleus (Cabral et al., 2012). Consequently, plasma adrenocorticotropic hormone and cortisol concentrations have been demonstrated to increase significantly following peripheral administration of ghrelin (Schmid et al., 2005). Conversely, plasma ghrelin concentrations have been shown to increase in response to various types of stressors such as chronic restraint stress and chronic social defeat stress (CSDS) (Yousufzai et al., 2018; Kristenssson et al., 2006; Chuang et al., 2011). Given the orexigenic properties of ghrelin, it is not surprising that stress has been associated with an increase in food intake. Mice subjected to the CSDS procedure displayed hyperphagia and increased intake of high-calorie food (Chuang et al., 2011). In GHSR KO mice, stress-induced hyperphagia was absent and depressive-like symptoms were more pronounced compared to wild-type (WT) littermates (Chuang et al., 2011; Guo et al., 2019). This suggests that GHSR activation may protect against some of the behavioral consequences of stress exposure. Nevertheless, pharmacological interventions using ghrelin or a GHSR agonist failed to establish antidepressant effects in the CSDS (Gupta et al., 2019). One potential explanation is that the excessive activation of GHSR may result in its desensitization. In line with the notion of deleterious effects of desensitization of GHSR, chronic restraint stress in rats which was associated with reduced binding of acyl-ghrelin to its receptor in the basolateral amygdala enhanced auditory fear, while chronic administration of a GHSR antagonist during stress exposure was sufficient to reverse these effects of stress on auditory fear processing (Yousufzai et al., 2018; Harmatz et al., 2017). Altogether, decreased functionality of the GHSR or ghrelin resistance may hamper the protective effects of circulating ghrelin (Gupta et al., 2019; Lockie et al., 2015).

Post-traumatic stress disorder (PTSD) is a stress-related mental disorder that is typically characterized by emotional distress after exposure to traumatic reminders, avoidance of trauma-related reminders, negative affect and decreased interest in activities (Association AP, 2013). Treatment strategies are available, but more than one quarter of patients do not respond to therapy or rapidly relapse (Koek et al., 2016). First-line treatment for trauma-and stressor-related disorders comprises cognitive behavior therapy (CBT) (Carpenter et al., 2018). A key component of CBT is exposure-based treatment (EBT), a procedure that aims to gradually decrease fear for the traumatic event. However, patients have difficulties to bare this long, exhausting procedure or relapse easily over time (Singewald et al., 2015). Given the high social and economic impact of stress-related mental disorders, a better understanding of their pathophysiology is critical for the development of novel approaches. Interestingly, stress-related mental disorders such as PTSD have a high comorbidity with obesity (Pagoto et al., 2012; Buta et al., 2018; Kubzansky et al., 2014; Vieweg et al., 2006). The pathophysiological underpinnings of this comorbidity remain unknown but there is evidence that a dysregulation of the ghrelin system might be a common underlying risk factor for symptoms (Meyer et al., 2014; Spencer et al., 2012). In line with this hypothesis, interactions of genetic polymorphisms in ghrelin and orexin genes were shown to predict symptom severity in patients with PTSD (Li et al., 2019).

Altogether, GHSR signaling appears to be an important factor for initial stress-coping. However, ghrelin resistance, or the reduced effectiveness of the GHSR, following a prolonged increase in ghrelin signaling may have deleterious effects. Our study is a first attempt using two complementary approaches to investigate whether interventions that decrease GHSR function cause changes in behavior that may be relevant for PTSD: auditory fear processing, anxiety-like behavior and saccharin preference. We studied two models of ghrelin resistance. The first model was based on exposure of adult male C57BL/6 J mice to a high-fat diet (HFD), which was previously shown to induce ghrelin resistance characterized by an impaired hyperphagic response to ghrelin administration (Briggs et al., 2010). The second model was based on genetic ablation of the GHSR.

2. Material & methods

2.1. Animals

Mice were single-housed (1264C Eurostandard type II cages, Tecniplast, Buguggiate, Italy) in a temperature (21 ± 3 °C) and humidity (30–70 %) regulated environment with a 12/12 h light/dark cycle and received food and water ad libitum. All experiments were carried out on adult male mice. C57BL/6 J mice (8 weeks at the start of experimental procedures) were purchased from Janvier (La Genest-Saint-Isle, France) and were habituated to the new environment for at least one week following transport. Mice were maintained on a standard diet (A04, Safe Diets, Augy, France) or HFD (235 H F providing 45% of energy from fat, Safe Diets) as indicated in the experimental procedures and results. Fresh food pellets were provided weekly. A small amount of peanut butter (Calvé, Unilever, Belgium) was applied around two HFD pellets when fresh food was provided to ensure HFD food intake. Adult male GHSR KO mice and WT littermates (8–12 weeks old at the start of experimental procedures, developed by Janssen Pharmaceutica, Beerse, Belgium (Verhulst et al., 2008)) were bred in-house as offspring of heterozygous GHSR couples that were backcrossed on a C57BL/6 J background (Janvier) for at least nine generations. The following primers were used to verify the genotype of the animals: GHSR WT mice: 5′-TGGGGGTGCGAACATTAGC-3′ and 5′-CTGAAGGC ATCTTTCACTACG-3′; GHSR KO mice: 5′-ACATATTCTATGTGAGGC ACC-3′ and 5′-CTGAAGGCATCTTTCACTACG-3′ (Eurogentec, Seraing, Liège, Belgium).

All experiments were approved by the ethical committee of the Vrije Universiteit Brussel (ECD 17.213.1) and complied with the European Community Council Directives (2010/63/EU) and KB 2013-05-29/12. All efforts were made to minimize the number of animals and to reduce suffering of the animals.

2.2. Experimental design

2.2.1. Experiment 1: diet-induced ghrelin resistance

Mice were exposed to a standard diet or HFD for a total duration of 4 weeks. Body weight of all mice was measured weekly. After 4 weeks, food intake in response to a GHSR agonist (2.4) was evaluated. At the end of the experiment, all mice were euthanized by an overdose of barbiturates (250 mg/kg intraperitoneally (i.p.) pentobarbital, Dolethal®, Vétoquinol, Aartselaar, Belgium). Abdominal fat from all animals was removed and weighed by a scientist blinded to treatment. The timeline of the experiment is outlined in Fig. 1A.

2.2.2. Experiment 2: fear conditioning after diet onset

Mice were maintained on a standard diet or HFD for a total duration of 8 weeks. Auditory fear conditioning, fear extinction and a fear extinction retention test (2.3.1) were performed on consecutive days 4 weeks after diet onset. Anxiety-like behavior was tested in the open field (OF) (2.3.2) and elevated plus maze (EPM) (2.3.3.) 5 weeks after diet onset. A two-bottle choice saccharin test (2.3.4) to assess saccharin preference was performed at 7 weeks. Food intake in response to a GHSR agonist (2.4) was evaluated 8 weeks after diet onset. At the end

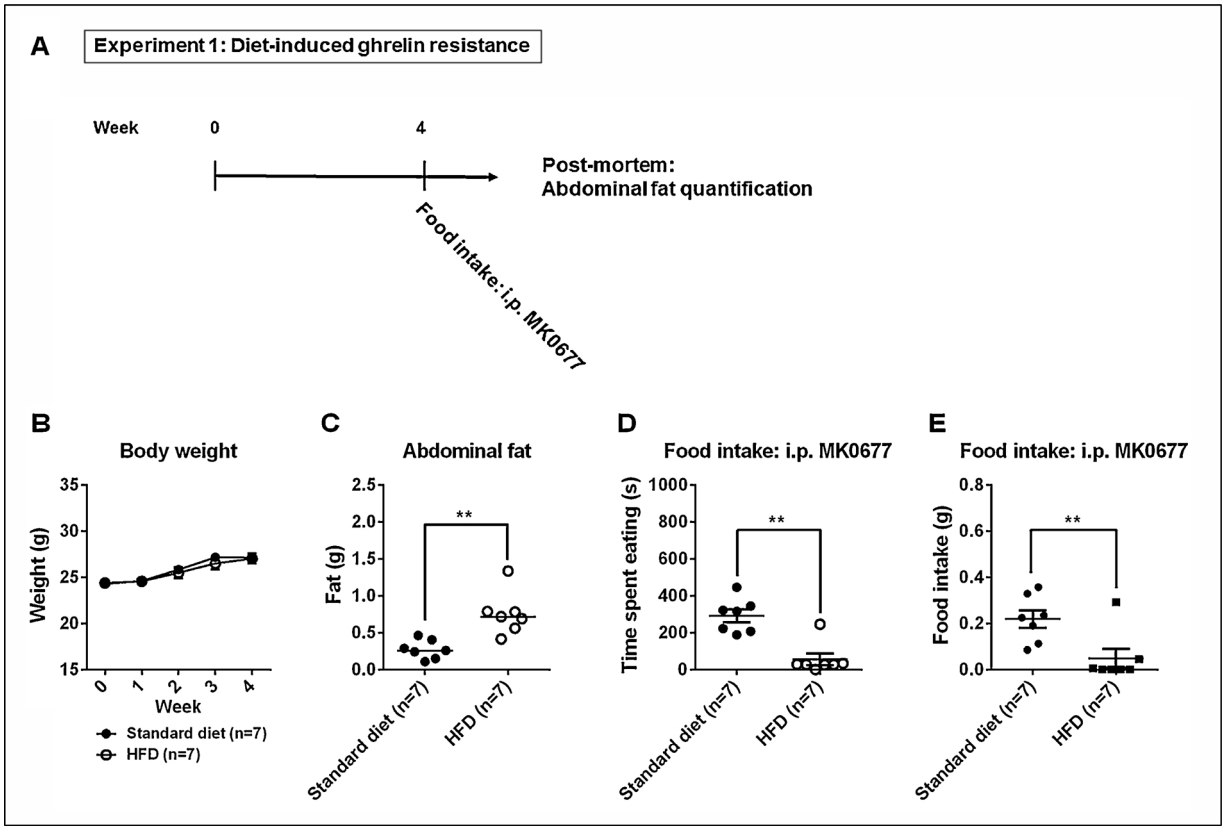


Fig. 1. Experiment 1: Diet-induced ghrelin resistance. (A) Timeline (B) Body weight (Interaction factor $F(4,48) = 0.9975$, $p > 0.05$; Time factor $F(4,48) = 70.81$, $p < 0.0001$; Treatment factor $F(1,12) = 0.1631$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (C) Abdominal fat 4 weeks after diet onset ($p = 0.0012$, Mann-Whitney $U = 1.000$, Mann-Whitney test) (D) Time spent eating after administration of 0.5 mg/kg MK0677 4 weeks after diet onset ($p = 0.0041$, Mann-Whitney $U = 1.000$, Mann-Whitney test) (E) Food intake after administration of 0.5 mg/kg MK0677 4 weeks after diet onset ($p = 0.0093$, Mann-Whitney $U = 1.000$, Mann-Whitney test). Dots represent individual data points; horizontal lines represent mean ± SEM. ** $p < 0.01$. $n = 7$ per experimental group. HFD: high-fat diet. i.p.: intraperitoneally.

of the experiment, all mice were euthanized by an overdose of barbiturates. Abdominal fat from all animals was removed and weighed by a scientist blinded to treatment. The timeline of the experiment is outlined in Fig. 2A.

2.2.3. Experiment 3: fear conditioning prior to diet onset

Mice were subjected to auditory fear conditioning and were subsequently placed on a standard diet or HFD for a total period of 8 weeks. Body weight of all mice was measured weekly. Mice were subjected to fear extinction and a fear extinction retention test in the fear conditioning paradigm (2.3.1) 4 weeks after diet onset. Anxiety-like behavior in the OF (2.3.2) and EPM test (2.3.3) was tested 5 weeks after diet onset. Saccharin preference was studied in a two-bottle choice saccharin test (2.3.4) 7 weeks after diet onset. Food intake in response to a GHSR agonist (2.4) was evaluated 8 weeks after diet onset. At the end of the experiment, all mice were euthanized by an overdose of barbiturates. In these animals, blood was collected through cardiac puncture and brains were removed for further analysis. Abdominal fat from all animals was removed and weighed by a scientist blinded to treatment. The timeline of the experiment is outlined in Fig. 3A.

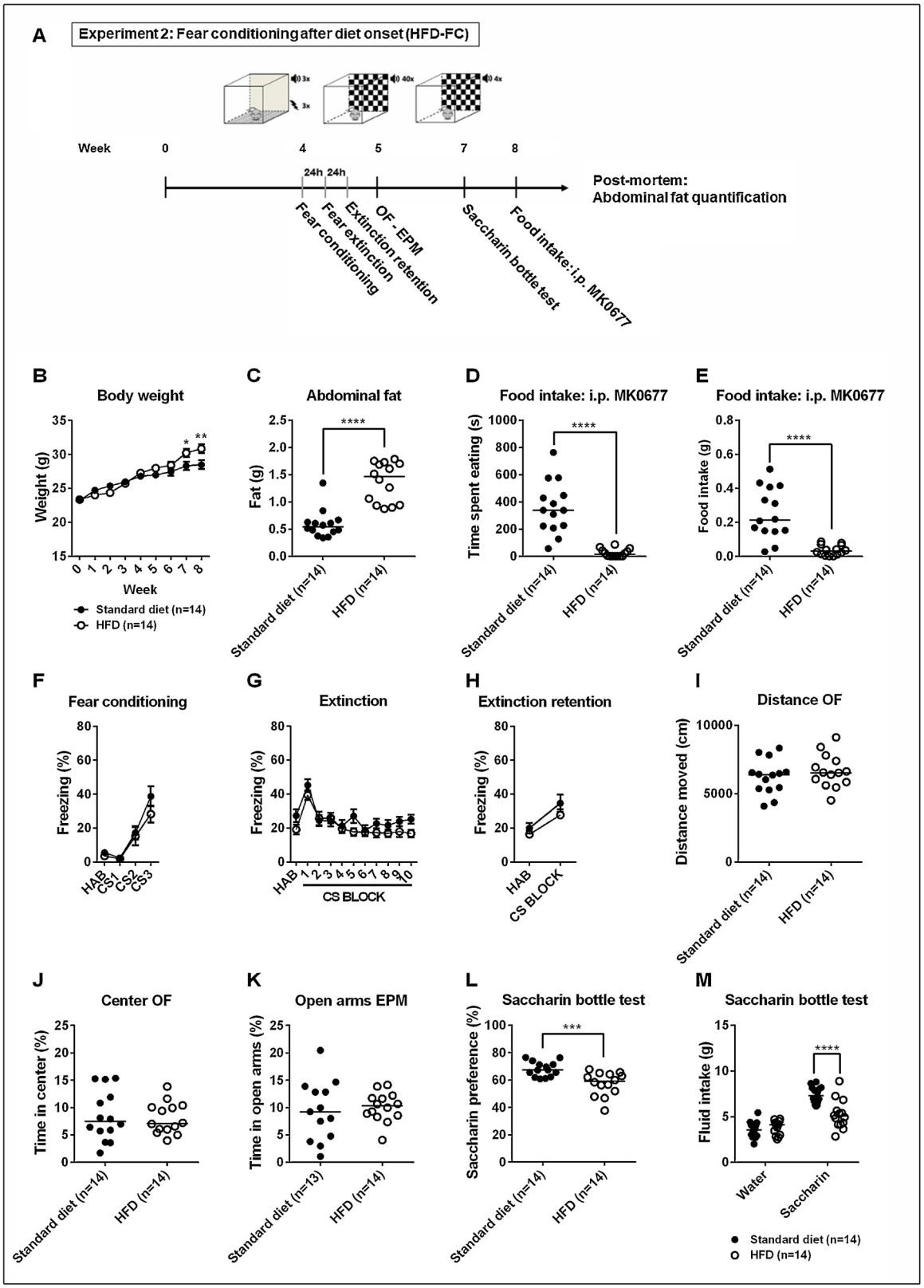
2.2.4. Behavioral testing in GHSR WT and GHSR KO mice

GHSR WT and GHSR KO mice were subjected to the following behavioral tests: auditory fear conditioning, fear extinction and a fear extinction retention test in the fear conditioning paradigm (2.3.1), anxiety-like behavior in the OF (2.3.2) and EPM test (2.3.3) and saccharin preference in a two-bottle choice saccharin preference test (2.3.4). As a positive control for the effect of the GHSR agonist MK0677 (2.4), food intake was measured in GHSR WT and GHSR KO mice. The timeline of the experiment is outlined in Fig. 5A.

2.3. Behavioral tests

2.3.1. Auditory fear conditioning

Mice were exposed to three tone presentations (80 dB, 4 kHz tone, stimulus duration 30 s, inter-trial interval (ITI) 1 min) serving as the conditioned stimulus (CS) co-terminating with an electric shock (0.6 mA shock, 2 s duration) as unconditioned stimulus (US) in context A (17 × 17 x 20 cm chamber with three transparent and one grey wall and a metal floor linked to a current source, 125 lx, cleaned with 1% acetic acid). Fear extinction training was performed in context B (chamber with three black and white chequered walls and one transparent wall, a solid white floor, 15 lx and cleaned with 1–3 % hospital antiseptic concentrate, Regent Medical Overseas, Manchester, UK) either one day or one month after conditioning as specified in the experimental design (Section 2.2) and consisted of forty CS presentations (5 s ITI). Twenty-four hours following fear extinction training, extinction retention was tested by exposing the animals to 4 CS presentations (1 min ITI) in context B. The conditioning chamber was thoroughly cleaned before and after each trial with the context-specific cleaning solution. Ethovision software (Noldus, Wageningen, The Netherlands, inactivity threshold of 0.3%, 1 s) was used to analyze the conditioned reaction (freezing: complete immobility except for breathing). Additionally, integrated data were manually corrected for false positives by an observer blinded to genotype or treatment. Time frames during tone presentation that were erroneously considered by the Ethovision software as freezing were subtracted from the total freezing time.



(caption on next page)

2.3.2. Open field (OF)

An OF test was performed to assess locomotor activity and anxiety-like behavior. Mice were placed in a rectangular arena (60 × 60 x 60 cm) with white opaque ground floor and black opaque surrounding plastic walls to prevent observation of visual cues outside the arena. Mice were left undisturbed for 10 min. The center of the OF was defined as a square of 40 x 40 cm. An illuminance of 50 lx was created in the center of the OF. The distance moved and the time spent in the center of the OF were recorded and tracked by an automatic video tracking system (Ethovision).

Fig. 2. Experiment 2: Fear conditioning after diet onset. (A) Timeline (B) Body weight (Interaction factor $F(8,208) = 9.834$, $p < 0.0001$; Time factor $F(8,208) = 149.6$, $p < 0.0001$; Treatment factor $F(1,26) = 0.8846$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (C) Abdominal fat 8 weeks after diet onset ($p < 0.0001$, Mann-Whitney U = 6.000, Mann-Whitney test) (D) Time spent eating after administration of 0.5 mg/kg MK0677 8 weeks after diet onset ($p < 0.0001$, Mann-Whitney U = 3.000, Mann-Whitney test) (E) Food intake after administration of 0.5 mg/kg MK0677 8 weeks after diet onset ($p < 0.0001$, Mann-Whitney U = 11.00, Mann-Whitney test) (F) Auditory fear conditioning (Interaction factor $F(3,78) = 1.087$, $p > 0.05$; Time factor $F(3,78) = 42.79$, $p < 0.0001$; Treatment $F(1,26) = 1.127$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (G) Fear extinction (10 blocks of 4 CS presentations, Interaction factor $F(10,260) = 1.331$, $p > 0.05$; Time factor $F(10,260) = 16.02$, $p < 0.0001$; Treatment factor $F(1,26) = 2.273$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (H) Extinction retention (4 CS presentations, Interaction factor $F(1,26) = 0.8312$, $p > 0.05$; Time factor $F(1,26) = 48.59$, $p < 0.0001$; Treatment factor $F(1,26) = 1.271$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (I) Distance moved in the OF ($p > 0.05$, Mann-Whitney U = 75.00, Mann-Whitney test) (J) Time spent in the center of the OF ($p > 0.05$, Mann-Whitney U = 96.00, Mann-Whitney test) (K) Time spent in the open arms of the EPM ($p > 0.05$, Mann-Whitney U = 81.00, Mann-Whitney test) (L) Saccharin preference ($p = 0.0010$, Mann-Whitney U = 29.00, Mann-Whitney test) (M) Water and saccharin fluid intake (Interaction factor $F(1,52) = 17.02$, $p = 0.0001$; Row factor $F(1,52) = 83.48$, $p < 0.0001$; Treatment factor $F(1,52) = 10.93$, $p = 0.0017$; Two-Way ANOVA with Bonferroni's post-hoc test). Dots represent individual data points; horizontal lines represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n = 13–14 per experimental group. CS: conditioned stimulus. EPM: elevated plus maze. HFD: high-fat diet. i.p.: intraperitoneally. OF: open field.

2.3.3. Elevated plus maze (EPM)

An EPM test was performed to assess anxiety-like behavior. Mice could freely explore an elevated (1 m from the ground) plus-shaped arena (50 × 5 x 15 cm arms, white opaque ground floor) constructed with two open arms (0.5 cm border), two closed arms (black opaque walls) and a 6 x 6 cm center for a duration of 10 min. An illuminance of 100 lx was created in the brightest part of the EPM. Time spent in the open arms was recorded and analyzed by an automatic video tracking system (Ethovision). One mouse (experiment 2, standard diet) jumped off the maze and was excluded from analysis.

2.3.4. Two-bottle choice saccharin preference test

A two-bottle choice procedure was used to test saccharin preference. On the first day, mice received two drinking bottles in their home cage filled with tap water to get used to the two bottles. The following day, one of the two drinking bottles was filled with a 0.1% saccharin solution (Sigma-Aldrich, Darmstadt, Germany). Twenty-four hours later, the two bottles were weighed, refilled with water or saccharin solution and placed in reversed order. Twenty-four hours later, bottles were weighed again. Saccharin preference was expressed as the average daily intake of water and saccharin (in g) or as the average daily intake of saccharin solution to the total daily fluid intake (as a percentage).

2.4. Feeding response following administration of a GHSR agonist

Ghrelin resistance was evidenced by lower food intake following administration of the GHSR agonist MK0677. Mice were injected i.p. with 0.5 mg/kg of the GHSR agonist MK0677 (ibatumoren mesylate, Tocris, Bristol, UK). MK0677 was dissolved in 1% dimethylsulfoxide (Fluka, Sigma-Aldrich) and 0.9% NaCl (Baxter, Diegem, Belgium). Food intake and time spent eating were evaluated during the following ninety minutes. Three food pellets (A03, Safe Diets) were provided in the home cage at the start of the experiment. Time spent eating was timed manually by an observer blinded to treatment. Food pellets were removed after ninety minutes and weighed by an observer blinded to treatment.

2.5. Plasma ghrelin and corticosterone concentrations

In a subset of animals (mice from experiment 3, exposed for 8 weeks to a standard diet or HFD, three days following MK0677 administration), blood was collected from the heart following respiratory arrest. Pefabloc (1 mg/ml, Sigma-Aldrich) was added immediately following blood collection. Plasma was subsequently obtained through centrifugation at 2500 *g* for 15 min at 4 °C. Samples for ghrelin analysis were acidified with hydrochloric acid (final concentration of 0.05 N, VWR, International, Radnor, Pennsylvania, US). Enzyme-linked immunosorbent assays were performed for acylated ghrelin (EZRGRA-90 K, Millipore, Missouri, USA), total ghrelin (EZRGRT-91 K, Millipore) and corticosterone (ab108821, Abcam, Cambridge, UK) concentrations according to the manufacturer's instructions.

2.6. Real-time polymerase chain reaction (qPCR)

In a subset of animals (mice from experiment 3, exposed for 8 weeks to a standard diet or HFD, three days following MK0677 administration, RNA concentrations of two samples were below the analytical threshold), total brain tissue was rapidly removed, snap-frozen in 2-methylbutane (J.T. Baker, Giwice, Poland) and stored at - 80 °C. Next, a punch of the hypothalamus was taken using a sample corer with 1 mm internal diameter (Agntho's, Lidingö, Sweden). RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA was quantified at 260/280 nm using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). Following extraction, copy desoxyribonucleic acid (cDNA) synthesis (iScript cDNA synthesis kit, Biorad, Temse, Belgium) and cDNA purification (Genelute PCR clean-up kit, Sigma-Aldrich) was carried out. qPCR was performed using Taqman gene expression Master Mix (Applied Biosystems, Thermofischer, Watlham, USA) and Taqman primers for the GHSR (gene of interest), B2M and HPRT (housekeeping genes, Thermofischer, Waltham, USA). All samples were loaded in duplicate. Amplifications were performed using the StepOne Plus system (Life Technologies, Merelbeke, Belgium). qBase + software (Biogazelle, Gent, Belgium) was used to identify stable housekeeping genes and subsequently for reference gene normalization.

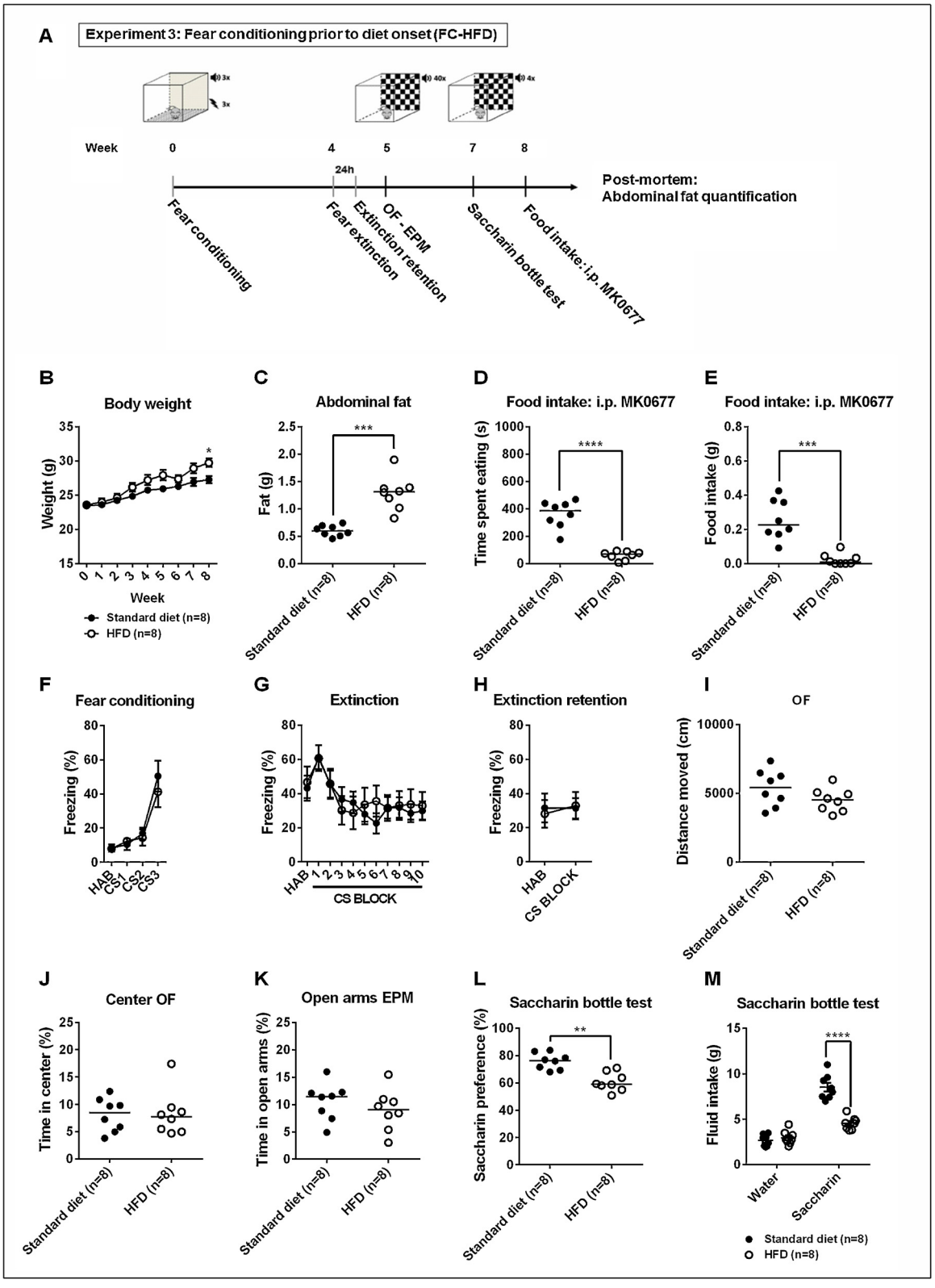
2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.01. Data are expressed as mean ± standard error of the mean (SEM) or median (dotplots). For experiments with one variable, data were analyzed using a Mann-Whitney test. Two-Way ANOVA or Two-Way repeated measures ANOVA followed by a Bonferroni post-hoc test was used to analyze data with two independent variables. Significance threshold was set at alpha = 0.05.

3. Results

3.1. Experiment 1: diet-induced ghrelin resistance

Mice were exposed to a standard diet or HFD for 4 weeks. Mice on the HFD did not show an enhanced increase in body weight relative to standard diet controls (Fig. 1B) but abdominal fat was significantly increased after 4 weeks (Fig. 1C). In addition, mice on a standard diet showed a hyperphagic response to the GHSR agonist MK0677, which was significantly reduced in mice on HFD (Fig. 1D,E).



(caption on next page)

3.2. Experiment 2: fear conditioning after diet onset

Mice were exposed to a standard diet or HFD for a total duration of 8 weeks. Fear conditioning was carried out 4 weeks after diet onset. Mice exposed to HFD showed an enhanced increase in body weight 7 and 8 weeks after diet onset compared to mice on a standard diet (Fig. 2B). Moreover, mice exposed to HFD also showed a significant increase in abdominal fat compared to control mice at the end of the experiment (Fig. 2C). Additionally, mice exposed to a standard diet showed a hyperphagic response to MK0677 administration, which was significantly lower in mice exposed to an HFD (Fig. 2D,E).

Mice that underwent fear conditioning 4 weeks after HFD exposure

Fig. 3. Experiment 3: Fear conditioning prior to diet onset. (A) Timeline (B) Body weight (Interaction factor $F(8,112) = 6.907$, $p < 0.0001$; Time factor $F(8,112) = 132.1$, $p < 0.0001$; Treatment factor $F(1,14) = 3.167$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (C) Abdominal fat 8 weeks after diet onset ($p = 0.0002$, Mann-Whitney U = 0.0, Mann-Whitney test) (D) Time spent eating after administration of 0.5 mg/kg MK0677 8 weeks after diet onset ($p = 0.0002$, Mann-Whitney U = 0.0, Mann-Whitney test) (E) Food intake after administration of 0.5 mg/kg MK0677 8 weeks after diet onset ($p = 0.0003$, Mann-Whitney U = 1.000, Mann-Whitney test) (F) Auditory fear conditioning (Interaction factor $F(3,42) = 0.5803$, $p > 0.05$; Time factor $F(3,42) = 30.45$, $p < 0.0001$; Treatment factor $F(1,14) = 0.2534$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (G) Fear extinction (10 blocks of 4 CS presentations, Interaction factor $F(10,140) = 0.3612$, $p > 0.05$; Time factor $F(10,140) = 14.60$, $p < 0.0001$; Treatment factor $F(1,14) = 0.8660$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (H) Extinction retention (4 CS presentations, Interaction factor $F(1,14) = 0.4476$; $p > 0.05$, Time factor $F(1,14) = 0.3785$, $p > 0.05$; Treatment factor $F(1,14) = 0.009319$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (I) Distance moved in the OF ($p > 0.05$, Mann-Whitney U = 18.50, Mann-Whitney test) (J) Time spent in the center of the OF ($p > 0.05$, Mann-Whitney U = 28.50, Mann-Whitney test) (K) Time spent in the open arms of the EPM ($p > 0.05$, Mann-Whitney U = 21.00, Mann-Whitney test) (L) Saccharin preference ($p = 0.0011$, Mann-Whitney U = 3.000, Mann-Whitney test) (M) Water and saccharin fluid intake (Interaction factor $F(1,28) = 32.59$, $p < 0.0001$; Row factor $F(1,28) = 33.75$, $p < 0.0001$; Treatment factor $F(1,28) = 83.13$, $p < 0.0001$; Two-Way ANOVA with Bonferroni's post-hoc test). Dots represent individual data points; horizontal lines represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n = 8 per experimental group. CS: conditioned stimulus. EPM: elevated plus maze. HFD: high-fat diet. i.p.: intraperitoneally. OF: open field.

did not show alterations in fear acquisition (Fig. 2F), fear extinction (Fig. 2G) or extinction retention (Fig. 2H) compared to control mice receiving a standard diet. An OF and EPM test were performed to examine locomotor activity and anxiety-like behavior. Distance moved and time spent in the center of the OF were not significantly affected in the HFD group compared to control mice (Fig. 2I,J). Additionally, time spent in the open arms of the EPM was not significantly altered (Fig. 2K) after exposure to HFD. In the saccharin preference test, mice on HFD showed significantly lower saccharin preference compared to control mice (Fig. 2L,M).

3.3. Experiment 3: fear conditioning prior to diet onset

Mice were subjected to fear conditioning and subsequently exposed to a standard diet or HFD for a total duration of 8 weeks. Similar to 3.2, mice on the HFD showed a significant increase in body weight (Fig. 3B), abdominal fat (Fig. 3C) and an impaired hyperphagic response to MK0677 administration (Fig. 3D,E).

In this experiment we performed fear conditioning prior to diet onset to investigate whether fear conditioning as such might affect diet outcome and whether once a fear memory is established diet would specifically affect fear extinction. There were no significant differences in fear acquisition between the experimental groups (Fig. 3F). Fear extinction and extinction retention were tested 4 weeks later. Our data demonstrate that neither fear extinction (Fig. 3G), nor the retention of fear extinction (Fig. 3H) were affected by HFD exposure. Also in this experiment, distance moved and time spent in the center of the OF were not significantly affected in the HFD group compared to control mice (Fig. 3I,J). Similarly, time spent in the open arms of the EPM was not significantly altered (Fig. 3K) after exposure to HFD. In the saccharin preference test, mice exposed to an HFD showed significantly lower saccharin preference compared to control mice (Fig. 3L,M).

3.4. Effects of HFD exposure on plasma ghrelin concentration, hypothalamic GHSR expression and plasma corticosterone concentrations

We measured both acyl- and total ghrelin (containing acyl- and desacyl-ghrelin) concentrations in plasma from mice exposed to a standard diet or HFD for 8 weeks. We found no significant effect on plasma acylated (Fig. 4A) and total ghrelin concentrations (Fig. 4B). A qPCR was used to determine GHSR expression in the hypothalamus. No significant differences in GHSR mRNA level expression were found between experimental conditions (Fig. 4C). To test whether ghrelin activates the HPA axis, we evaluated the effect of HFD exposure on plasma corticosterone concentrations. Again, our data showed no significant alterations in corticosterone concentrations after HFD exposure (Fig. 4D). It should be noted however that plasma was collected following barbiturate anaesthesia, which can affect baseline corticosterone levels (Vahl et al., 2005) and may have masked subtle effects on circulating corticosterone.

3.5. Auditory fear, anxiety-like behavior and saccharin preference in GHSR KO mice

No significant difference in body weight was observed between GHSR WT and GHSR KO mice (Fig. 5 B). However, GHSR KO mice showed a significantly blunted hyperphagic response to MK0677 compared to WT mice (Fig. 5C,D). We used GHSR WT and GHSR KO mice to study how auditory fear processing, anxiety-like behavior and saccharin preference would be affected by lack of GHSR function. GHSR KO mice did not display significant abnormalities in fear acquisition (Fig. 5E), fear extinction (Fig. 5F) or the retention of fear extinction (Fig. 5G). Additionally, locomotor activity (Fig. 5H) and time spent in the center of the OF (Fig. 5I) were not significantly different between GHSR WT and GHSR KO mice. Consistent with this notion, time spent in the open arms of the EPM (Fig. 5J) was unaltered among both groups. Saccharin preference (%) was significantly decreased in GHSR KO mice compared to WT littermates (Fig. 5K). However, no significant differences in water or saccharin intake (g) were found (Fig. 5L). These data reveal that GHSR KO mice display a preference for saccharin, but this preference is slightly less pronounced compared to WT littermates.

4. Discussion

In recent years, fear extinction and its pharmacological modulation have become a focus of interest, because extinction is considered to be one of the underlying mechanisms of EBT, which is commonly employed in stress-related disorders such as PTSD (Singewald et al., 2015; Scheveneels et al., 2016). However, this mechanism seems to be impaired in a considerable number of patients, reducing the effectiveness of EBT interventions and leaving patients with refractory symptoms and a high risk of relapse (Milad and Quirk, 2012). In the present study, we aimed to provide some mechanistic insights into whether and how abnormal GHSR signaling may contribute to symptom severity in stress-related disorders. Hereto, we studied the effect of HFD on auditory fear processing, anxiety-like behavior and saccharin preference in mice. Our experiments confirm previous observations that HFD exposure leads to functional ghrelin resistance (Briggs et al., 2010) as evidenced by a blunted hyperphagic response to a GHSR agonist. However, we did not find evidence for lowered GHSR expression in the hypothalamus or alterations in circulating acylated or total ghrelin concentrations. The observed changes in GHSR sensitivity may result from posttranslational modifications of the GHSR. Alternatively, GHSR activity may be impaired due to altered GHSR heteromerization (Schellekens et al., 2013) with other G-protein coupled receptors such as dopamine D2, serotonin 2C or melanocortin 3 receptors (Kern et al., 2012; Schellekens et al., 2015; Rediger et al., 2011) or due to increased expression of liver-expressed antimicrobial peptide 2 (LEAP2), which was recently recognized as an endogenous GHSR antagonist (Ge et al., 2018; M'Kadmi et al., 2019; Mani et al., 2019). However, we were unable to uncover the mechanism through which HFD exposure alters GHSR sensitivity.

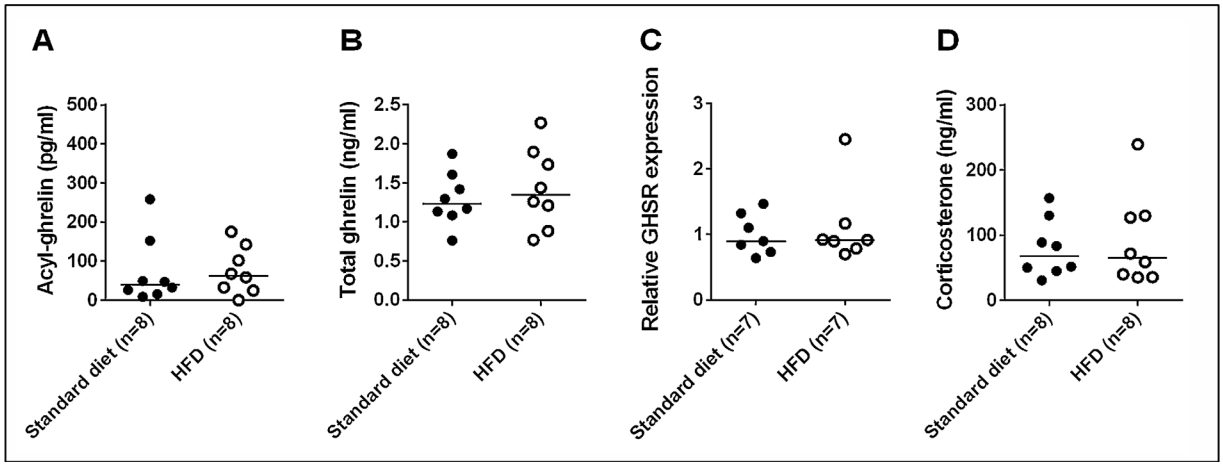


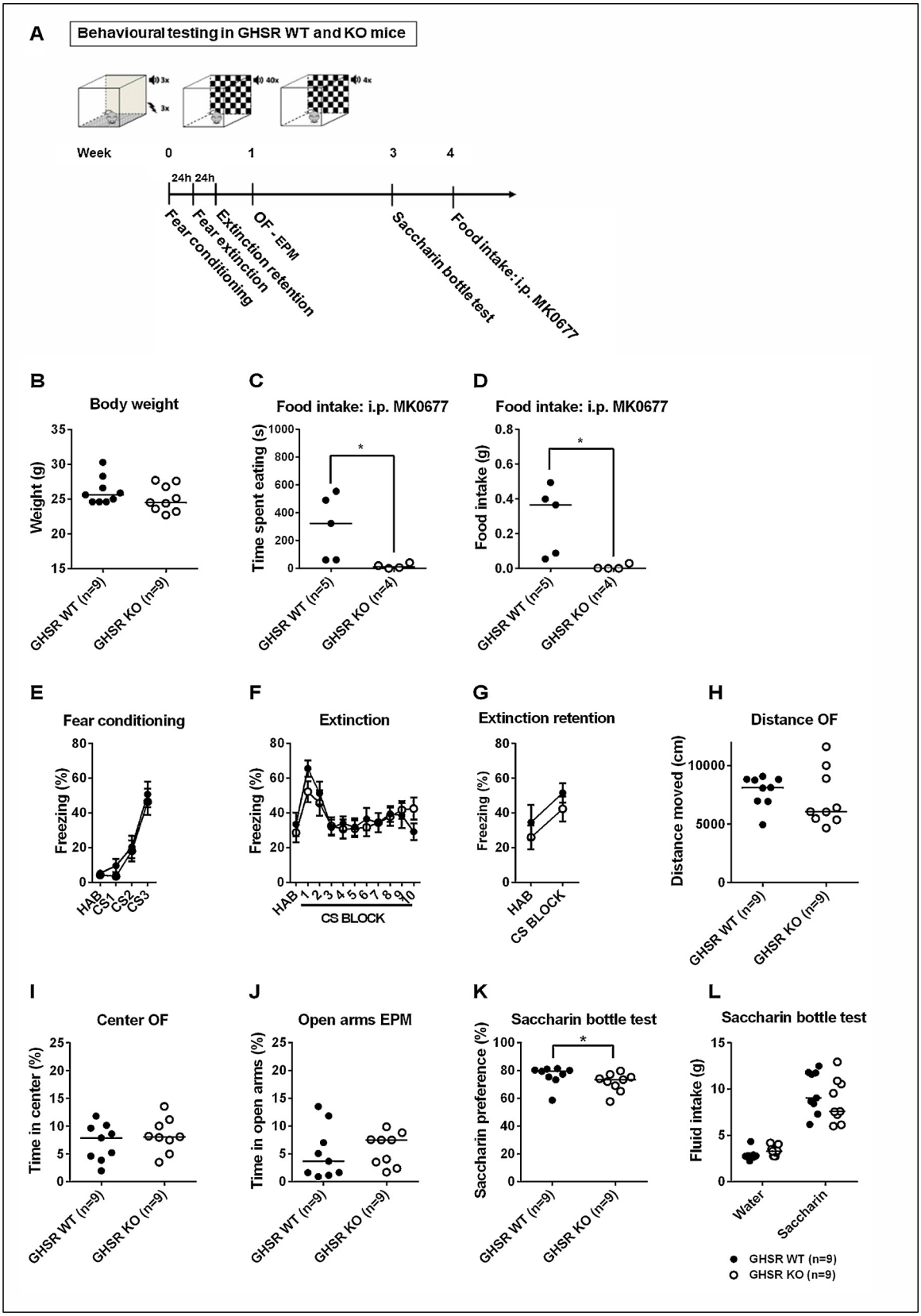
Fig. 4. Effects of HFD exposure on GHSR expression, plasma ghrelin concentration and plasma corticosterone concentrations. (A) Plasma acylated ghrelin concentrations ($p > 0.05$, Mann-Whitney U = 28.00, Mann-Whitney test) (B) Plasma total ghrelin concentrations ($p > 0.05$, Mann-Whitney U = 25.00, Mann-Whitney test) (C) GHSR expression in the hypothalamus ($p > 0.05$, Mann-Whitney U = 23.00, Mann-Whitney test) and (D) Plasma corticosterone concentrations ($p > 0.05$, Mann-Whitney U = 31.00, Mann-Whitney test). Dots represent individual data points. n = 7–8 per experimental group. GHSR: ghrelin receptor. HFD: high-fat diet.

Fear conditioning, fear extinction and extinction retention were unaffected after 4 weeks of HFD exposure, a time point where the hyperphagic response to administration of a GHSR agonist was already significantly lower. While some studies have shown reduced auditory fear recall following chronic HFD exposure (Johnson et al., 2016; Cordner and Tamashiro, 2015), our data are in line with a previous study showing no effect of HFD exposure on fear acquisition or expression in mice (Boitard et al., 2015). Moreover, we provide novel evidence for a lack of significant effects of HFD exposure on fear extinction. Interestingly, prolonged exposure to an HFD (more than 4 months) was previously found to induce global memory impairments, assessed in tests of auditory fear memory, spatial learning and memory, and object recognition memory (Johnson et al., 2016; Cordner and Tamashiro, 2015). Consequently, effects of chronic HFD exposure on fear processing may not be fear-specific and rely on impairments in memory performance. Moreover, these effects may result from brain inflammation rather than ghrelin resistance (Marsland et al., 2015). However, chronic restraint stress was previously shown to be associated with ghrelin resistance, characterized by lower ghrelin binding to the GHSR in the amygdala (Yousufzai et al., 2018; Harmatz et al., 2017), and was shown to increase auditory fear expression in rats (Yousufzai et al., 2018; Harmatz et al., 2017). Whether this reduced binding of ghrelin in the amygdala is sufficient to cause these effects on fear processing is not completely clear. Moreover, it is possible that ghrelin resistance caused by restraint stress affects different brain regions compared to ghrelin resistance caused by HFD (Harmatz et al., 2017; Briggs et al., 2010). Indeed, circulating ghrelin induces neuronal activity (c-fos) in the hypothalamus without affecting deeper brain regions such as the amygdala (Cornejo et al., 2018). It has been reported that ghrelin freely diffuses through fenestrated capillaries of the median eminence into the ARC, however, it remains presently unknown how ghrelin could reach brain regions such as the amygdala and hippocampus (Cabral et al., 2015). Moreover, GHSR mRNA expression in the hypothalamus is high, whereas expression in the amygdala is low (Mani et al., 2014).

Negative feelings and difficulties in experiencing pleasure from enjoyable activities are also typical symptoms in stress-related disorders such as PTSD (Association AP, 2013). Interestingly, ghrelin has been proposed to play a key role in stress-induced food-reward (Chuang et al., 2011). However, our results suggest that this system may be self-limiting given that exposure to high-calorie HFD results in ghrelin resistance and lowers food-reward sensitivity. Indeed, mice exposed to HFD showed a significant decrease in saccharin preference. Moreover, total fluid intake of mice exposed to HFD is comparable with previously reported findings describing daily water intake in C57BL/6 J mice (Bachmanov et al., 2002). Fluid intake and more specifically saccharin fluid intake was significantly increased in mice receiving a standard diet. This effect is consistent with previously reported data in which exposure to HFD for three weeks lowered saccharin preference (Lockie et al., 2015). One limitation to this observation is that HFD may affect gustatory reward systems without affecting other reward systems (Lockie et al., 2015). Moreover, we were unable to demonstrate that the observed effect on saccharine preference was caused by altered GHSR sensitivity. We observed no behavioral effects in the OF or EPM following HFD exposure. Our data are in line with findings of other studies (Zuloaga et al., 2016; Del Rio et al., 2016), but some groups reported anxiogenic anxiety-like behavior 1 (but not 3 weeks), 12 and 16 weeks following HFD exposure (Zemdegs et al., 2016; Kaczmarczyk et al., 2013). The duration of HFD exposure may explain such differences.

Finally, in our study, HFD exposure did not alter acyl or total ghrelin concentrations in the plasma or GHSR expression in the hypothalamus. Literature is ambiguous regarding the effect of HFD exposure on plasma ghrelin concentrations and GHSR expression with one study showing a decrease in plasma ghrelin and GHSR expression while others report no changes (Briggs et al., 2010; Francois et al., 2016; Naznin et al., 2015). Given that ghrelin has been shown to drive activity in the HPA axis, we measured plasma corticosterone concentrations following HFD exposure, but we found no significant differences compared to mice receiving standard diet. This finding is consistent with previously reported data demonstrating no effect on plasma corticosterone concentrations in mice following exposure to an HFD for twelve weeks (Sharma et al., 2013; Naznin et al., 2018).

Taken together, our data show that exposure to an HFD does not affect auditory fear processing or anxiety-like behavior but significantly decreases saccharin preference. To further explore the effect of reduced GHSR function, we subjected GHSR KO mice to the same behavioral tests. Confirming the functional GHSR KO (Zigman et al., 2005), the hyperphagic response to MK0677 was abolished in GHSR KO mice. We confirmed that locomotor activity and anxiety-like behavior is unaltered in GHSR KO mice (Albarran-Zeckler et al., 2012, 2011; Mahbod et al., 2018). However, our study is the first to report that lack of a GHSR does not significantly affect auditory fear learning or extinction in mice. In addition, a saccharin preference test was also performed. These data reveal that absence of the GHSR significantly decreases saccharin preference, but it should be noted that the effect is small and was not observed when comparing the total amount of fluid intake. Consequently, we believe that the pronounced decrease in saccharin preference following HFD exposure may only partly related to ghrelin



(caption on next page)

Fig. 5. Auditory fear, anxiety-like behavior and saccharin preference in GHSR KO mice. (A) Timeline (B) Body weight (Mann-Whitney test, Mann-Whitney U = 25.00, $p > 0.05$) (C) Time spent eating after administration of 0.5 mg/kg MK0677 (Mann-Whitney test, Mann-Whitney U = 0.0, $p = 0.0159$) (D) Food intake after administration of 0.5 mg/kg MK0677 (Mann-Whitney test, Mann-Whitney U = 0.0, $p = 0.0159$) (E) Fear conditioning (Interaction factor $F(3,48) = 0.1176$, $p > 0.05$; Time factor $F(3,48) = 36.56$, $p < 0.0001$; Treatment factor $F(1,16) = 0.6164$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (F) Fear extinction (10 blocks of 4 CS presentations, Interaction factor $F(10,160) = 1.521$, $p > 0.05$; Time factor $F(10,160) = 10.53$, $p < 0.0001$; Treatment factor $F(1,16) = 0.06192$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (G) Extinction retention (4 CS presentations, Interaction factor $F(1,16) = 0.003942$, $p > 0.05$; Time factor $F(1,16) = 14.17$, $p = 0.0017$; Treatment factor $F(1,16) = 0.8251$, $p > 0.05$; Two-Way repeated measures ANOVA with Bonferroni's post-hoc test) (H) Distance moved in the OF ($p > 0.05$, Mann-Whitney U = 31.00, Mann-Whitney test) (I) Time spent in the center of the OF ($p > 0.05$, Mann-Whitney U = 34.00, Mann-Whitney test) (J) Time spent in the open arms of the EPM ($p > 0.05$, Mann-Whitney U = 29.00, Mann-Whitney test) (K) Saccharin preference (Mann-Whitney test, Mann-Whitney U = 17.00, $p = 0.0399$) (L) Water and saccharin fluid intake (Interaction factor $F(1,32) = 1.603$, $p > 0.05$; Row factor $F(1,32) = 117.8$, $p < 0.0001$; Treatment factor $F(1,32) = 0.6360$, $p > 0.05$; Two-Way ANOVA with Bonferroni's post-hoc test). Dots represent individual data points; horizontal lines represent mean ± SEM. * $p < 0.05$. n = 4–9 per experimental group. EPM: elevated plus maze. GHSR: ghrelin receptor. i.p.: intraperitoneally. KO: knockout. OF: open field. WT: wild-type.

resistance. Indeed, we were unable to demonstrate the causal effect of HFD exposure on saccharine preference and other factors such as leptin resistance might be implicated (Guo and Lu, 2014).

Our data suggest that changed GHSR function associated with HFD exposure or following its genetic ablation does not cause alterations in fear processing or anxiety-like behavior *per se* but decreases saccharin preference in mice. This finding might have important clinical implications given that PTSD is associated with obesity and emotional distress, negative affect and decreased interest in activities. It is important that our experiments were not carried out in a model for PTSD. Moreover, such animals may not readily translate to human pathology. Nevertheless, it may be interesting to better understand how obesity may aggravate PTSD symptoms.

Author contributions

Experiments were designed by A.P. and D.D.B. with input from I.J.S., T.B. and N.S.. Experiments were performed by A.P., Y.R. and A.V.S.. Experiments were analyzed by A.P., Y.R. and K.M. The manuscript was written by A.P. and D.D.B. with contribution from all other listed authors.

Declaration of Competing Interest

We have no conflict of interest to declare.

Acknowledgments

The authors would like to thank Giulia Albertini, Wissal Allaoui, An Buckinx, Anke De Smet and Gino de Smet for their technical support. Furthermore, we thank the Department of Toxicology, Dermato-Cosmetology and Pharmacognosy (Vrije Universiteit Brussel) to make it possible to perform qPCR analysis. This study was financially supported by the Fund for Scientific Research Flanders (FWO-G001816N and FWO-11ZL616N), the Strategic Research Programme of the Vrije Universiteit Brussel (SRP49) and the Austrian Science FundFWF-I2433-B26.

References

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402 (6762), 656–660.
Stengel, A., Taché, Y., 2012. Yin and Yang - the gastric X/A-like cell as possible dual regulator of food intake. J. Neurogastroenterol. Motil. 18 (2), 138–149.
Gutierrez, J.A., Solenberg, P.J., Perkins, D.R., Willency, J.A., Knierman, M.D., Jin, Z., et al., 2008. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc. Natl. Acad. Sci. U. S. A. 105, 6320–6325.
Cabral, A., De Francesco, P.N., Perello, M., 2015. Brain circuits mediating the orexigenic action of peripheral ghrelin: narrow gates for a vast kingdom. Front. Endocrinol. (Lausanne) 6.
Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., et al., 2001. A role for ghrelin in the central regulation of feeding. Nature 409 (6817), 194–198.
Wang, Q., Liu, C., Uchida, A., Chuang, J.C., Walker, A., Liu, T., et al., 2014. Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. Mol. Metab. 3 (1), 64–72.
Cabral, A., Suescun, O., Zigman, J.M., Perello, M., 2012. Ghrelin indirectly activates hypophysiotropic CRF neurons in rodents. PLoS One 7.
Schmid, D.A., Held, K., Ising, M., Uhr, M., Weikel, J.C., Steiger, A., 2005. Ghrelin stimulates appetite, imagination of food, GH, ACTH, and cortisol, but does not affect leptin in normal controls. Neuropsychopharmacology 30 (6), 1187–1192.
Yousufzai, M., Harmatz, E.S., Shah, M., Malik, M.O., Goosens, K.A., 2018. Ghrelin is a persistent biomarker for chronic stress exposure in adolescent rats and humans. Transl. Psychiatry 8 (1), 74.
Kristenssson, E., Sundqvist, M., Astin, M., Kjerling, M., Mattsson, H., Dornonville de la Cour, C., et al., 2006. Acute psychological stress raises plasma ghrelin in the rat. Regul. Pept. 134 (2–3), 114–117.
Chuang, J.-C., Perello, M., Sakata, I., Osborne-Lawrence, S., Savitt, J.M., Lutter, M., et al., 2011. Ghrelin mediates stress-induced food-reward behavior in mice. J. Clin. Invest. 121 (7), 2684–2692.
Guo, L., Niu, M., Yang, J., Li, L., Liu, S., Sun, Y., et al., 2019. GHS-R1a deficiency alleviates depression-related behaviors after chronic social defeat stress. Front. Neurosci. 13, 364.
Gupta, D., Chuang, J.C., Mani, B.K., Shankar, K., Rodriguez, J.A., Osborne-Lawrence, S., et al., 2019. beta1-adrenergic receptors mediate plasma acyl-ghrelin elevation and depressive-like behavior induced by chronic psychosocial stress. Neuropsychopharmacology.
Harmatz, E.S., Stone, L., Lim, S.H., Lee, G., McGrath, A., Gisabella, B., et al., 2017. Central ghrelin resistance permits the overconsolidation of fear memory. Biol. Psychiatry 81 (12), 1003–1013.
Lockie, S.H., Dinan, T., Lawrence, A.J., Spencer, S.J., Andrews, Z.B., 2015. Diet-induced obesity causes ghrelin resistance in reward processing tasks. Psychoneuroendocrinology 62, 114–120.
Association AP, 2013. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association, Washington, DC.
Koek, R.J., Schwartz, H.N., Scully, S., Langevin, J.P., Spangler, S., Korotinsky, A., et al., 2016. Treatment-refractory posttraumatic stress disorder (TRPTSD): a review and framework for the future. Prog. Neuropsychopharmacol. Biol. Psychiatry 70, 170–218.
Carpenter, J.K., Andrews, L.A., Witcraft, S.M., Powers, M.B., Smits, J.A.J., Hofmann, S.G., 2018. Cognitive behavioral therapy for anxiety and related disorders: a meta-analysis of randomized placebo-controlled trials. Depress. Anxiety 35 (6), 502–514.
Singewald, N., Schmuckermair, C., Whittle, N., Holmes, A., Ressler, K.J., 2015. Pharmacology of cognitive enhancers for exposure-based therapy of fear, anxiety and trauma-related disorders. Pharmacol. Ther. 149, 150–190.
Pagoto, S.L., Schneider, K.L., Bodenlos, J.S., Appelhans, B.M., Whited, M.C., Ma, Y., et al., 2012. Association of post-traumatic stress disorder and obesity in a nationally representative sample. Obesity 20 (1), 200–205.
Buta, E., Masheb, R., Gueorguieva, R., Bathulapalli, H., Brandt, C.A., Goulet, J.L., 2018. Posttraumatic stress disorder diagnosis and gender are associated with accelerated weight gain trajectories in veterans during the post-deployment period. Eat Behav. 29, 8–13.
Kubzansky, L.D., Bordelois, P., Jun, H.J., Roberts, A.L., Cerda, M., Bluestone, N., et al., 2014. The weight of traumatic stress a prospective study of posttraumatic stress disorder symptoms and weight status in women. JAMA Psychiatry 71 (1), 44–51.
Vieweg, W.V.R., Julius, D.A., Benesek, J., Satterwhite, L., Fernandez, A., Feuer, S.J., et al., 2006. Posttraumatic stress disorder and body mass index in military veterans - Preliminary findings. Prog. Neuropsychopharmacol. Biol. Psychiatry 30 (6), 1150–1154.
Meyer, R.M., Burgos-Robles, A., Liu, E., Correia, S.S., Goosens, K.A., 2014. A ghrelin-growth hormone axis drives stress-induced vulnerability to enhanced fear. Mol. Psychiatry 19 (12), 1284–1294.
Spencer, S.J., Xu, L., Clarke, M.A., Lemus, M., Reichenbach, A., Geenen, B., et al., 2012. Ghrelin regulates the hypothalamic-pituitary-Adrenal Axis and restricts anxiety after acute stress. Biol. Psychiatry 72 (6), 457–465.
Li, G., Zhang, K., Wang, L., Cao, C., Fang, R., Liu, P., et al., 2019. The preliminary investigation of orexigenic hormone gene polymorphisms on posttraumatic stress disorder symptoms. Psychoneuroendocrinology 100, 131–136.
Briggs, D.I., Enriori, P.J., Lemus, M.B., Cowley, M.A., Andrews, Z.B., 2010. Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. Endocrinology 151 (10), 4745–4755.
Vahl, T.P., Ulrich-Lai, Y.M., Ostrander, M.M., Dolgas, C.M., Elfers, E.E., Seeley, R.J., et al., 2005. Comparative analysis of ACTH and corticosterone sampling methods in rats.

Am. J. Physiol. Endocrinol. Metab. 289 (5), E823–E828.
Verhulst, P.J., De Smet, B., Saels, I., Thijs, T., Ver Donck, L., Moechars, D., et al., 2008. Role of ghrelin in the relationship between hyperphagia and accelerated gastric emptying in diabetic mice. Gastroenterology 135 (4), 1267–1276.
Scheveneels, S., Boddez, Y., Vervliet, B., Hermans, D., 2016. The validity of laboratory-based treatment research: bridging the gap between fear extinction and exposure treatment. Behav. Res. Ther. 86, 87–94.
Milad, M.R., Quirk, G.J., 2012. Fear extinction as a model for translational neuroscience: ten years of progress. Annu. Rev. Psychol. 63, 129–151.
Schellekens, H., Dinan, T.G., Cryan, J.F., 2013. Taking two to tango: a role for ghrelin receptor heterodimerization in stress and reward. Front. Neurosci. 7, 148.
Kern, A., Albarran-Zeckler, R., Walsh, H.E., Smith, R.G., 2012. Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. Neuron 73 (2), 317–332.
Schellekens, H., De Francesco, P.N., Kandil, D., Theeuwes, W.F., McCarthy, T., van Oeffelen, W.E., et al., 2015. Ghrelin's orexigenic effect is modulated via a serotonin 2C receptor interaction. ACS Chem. Neurosci. 6 (7), 1186–1197.
Rediger, A., Piechowski, C.L., Yi, C.X., Tarnow, P., Strotmann, R., Gruters, A., et al., 2011. Mutually opposite signal modulation by hypothalamic heterodimerization of ghrelin and melanocortin-3 receptors. J. Biol. Chem. 286 (45), 39623–39631.
Ge, X., Yang, H., Bednarek, M.A., Galon-Tilleman, H., Chen, P., Chen, M., et al., 2018. LEAP2 is an endogenous antagonist of the ghrelin receptor. Cell Metab. 27 (2), 461–469 e6.
M'Kadmi, C., Cabral, A., Barrile, F., Giribaldi, J., Cantel, S., Damian, M., et al., 2019. N-terminal liver-expressed antimicrobial peptide 2 (LEAP2) region exhibits inverse agonist activity toward the ghrelin receptor. J. Med. Chem. 62 (2), 965–973.
Mani, B.K., Puzziferri, N., He, Z., Rodriguez, J.A., Osborne-Lawrence, S., Metzger, N.P., et al., 2019. LEAP2 changes with body mass and food intake in humans and mice. J. Clin. Invest.
Johnson, L.A., Zuloaga, K.L., Kugelman, T.L., Mader, K.S., Morre, J.T., Zuloaga, D.G., et al., 2016. Amelioration of metabolic syndrome-associated cognitive impairments in mice via a reduction in dietary fat content or infusion of non-diabetic plasma. EBioMedicine 3, 26–42.
Cordner, Z.A., Tamashiro, K.L., 2015. Effects of high-fat diet exposure on learning & memory. Physiol. Behav. 152 (Pt B), 363–371.
Boitard, C., Maroun, M., Tantot, F., Cavaroc, A., Sauvant, J., Marchand, A., et al., 2015. Juvenile obesity enhances emotional memory and amygdala plasticity through glucocorticoids. J. Neurosci. 35 (9), 4092–4103.
Marsland, A.L., Gianaros, P.J., Kuan, D.C., Sheu, L.K., Krajina, K., Manuck, S.B., 2015. Brain morphology links systemic inflammation to cognitive function in midlife adults. Brain Behav. Immun. 48, 195–204.
Cornejo, M.P., Barrile, F., De Francesco, P.N., Portiansky, E.L., Reynaldo, M., Perello, M., 2018. Ghrelin recruits specific subsets of dopamine and GABA neurons of different ventral tegmental area sub-nuclei. Neuroscience 392, 107–120.
Mani, B.K., Walker, A.K., Lopez Soto, E.J., Raingo, J., Lee, C.E., Perello, M., et al., 2014. Neuroanatomical characterization of a growth hormone secretagogue receptor-green fluorescent protein reporter mouse. J. Comp. Neurol. 522 (16), 3644–3666.
Bachmanov, A.A., Reed, D.R., Beauchamp, G.K., Tordoff, M.G., 2002. Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behav. Genet. 32 (6), 435–443.
Zuloaga, K.L., Johnson, L.A., Roese, N.E., Marzulla, T., Zhang, W., Nie, X., et al., 2016. High fat diet-induced diabetes in mice exacerbates cognitive deficit due to chronic hypoperfusion. J. Cereb. Blood Flow Metab. 36 (7), 1257–1270.
Del Rio, D., Morales, L., Ruiz-Gayo, M., Del Olmo, N., 2016. Effect of high-fat diets on mood and learning performance in adolescent mice. Behav. Brain Res. 311, 167–172.
Zemdegs, J., Quesseveur, G., Jarriault, D., Penicaud, L., Fioramonti, X., Guiard, B.P., 2016. High-fat diet-induced metabolic disorders impairs 5-HT function and anxiety-like behavior in mice. Br. J. Pharmacol. 173 (13), 2095–2110.
Kaczmarczyk, M.M., Machaj, A.S., Chiu, G.S., Lawson, M.A., Gainey, S.J., York, J.M., et al., 2013. Methylphenidate prevents high-fat diet (HFD)-induced learning/memory impairment in juvenile mice. Psychoneuroendocrinology 38 (9), 1553–1564.
Francois, M., Barde, S., Legrand, R., Lucas, N., Azhar, S., El Dhaybi, M., et al., 2016. High-fat diet increases ghrelin-expressing cells in stomach, contributing to obesity. Nutrition 32 (6), 709–715.
Naznin, F., Toshinai, K., Waise, T.M., NamKoong, C., Md Moin, A.S., Sakoda, H., et al., 2015. Diet-induced obesity causes peripheral and central ghrelin resistance by promoting inflammation. J. Endocrinol. 226 (1), 81–92.
Sharma, S., Fernandes, M.F., Fulton, S., 2013. Adaptations in brain reward circuitry underlie palatable food cravings and anxiety induced by high-fat diet withdrawal. Int. J. Obes. 37 (9), 1183–1191.
Naznin, F., Toshinai, K., Waise, T.M.Z., Okada, T., Sakoda, H., Nakazato, M., 2018. Restoration of metabolic inflammation-related ghrelin resistance by weight loss. J. Mol. Endocrinol. 60 (2), 109–118.
Zigman, J.M., Nakano, Y., Coppari, R., Balthasar, N., Marcus, J.N., Lee, C.E., et al., 2005. Mice lacking ghrelin receptors resist the development of diet-induced obesity. J. Clin. Invest. 115 (12), 3564–3572.
Albarran-Zeckler, R.G., Brantley, A.F., Smith, R.G., 2012. Growth hormone secretagogue receptor (GHS-R1a) knockout mice exhibit improved spatial memory and deficits in contextual memory. Behav. Brain Res. 232 (1), 13–19.
Albarran-Zeckler, R.G., Sun, Y., Smith, R.G., 2011. Physiological roles revealed by ghrelin and ghrelin receptor deficient mice. Peptides 32 (11), 2229–2235.
Mahbod, P., Smith, E.P., Fitzgerald, M.E., Morano, R.L., Packard, B.A., Ghosal, S., et al., 2018. Desacyl ghrelin decreases anxiety-like behavior in male mice. Endocrinology 159 (1), 388–399.
Guo, M., Lu, X.Y., 2014. Leptin receptor deficiency confers resistance to behavioral effects of fluoxetine and desipramine via separable substrates. Transl. Psychiatry 4, e486.