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Signaling through the ghrelin receptor modulates hippocampal function and meal anticipation in mice

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ABSTRACT

The ability to predict a particular meal is achieved in part by learned associations with stimuli that predict nutrient availability. Ghrelin is an orexigenic peptide produced by both the gut and brain that rises before anticipated meals and it has been suggested that pre-prandial ghrelin increases may act as a signal to predict meal delivery. Here, we used wild type and ghrelin receptor deficient mice to test the hypothesis that ghrelin signaling is necessary for the processing of emotionally relevant stimuli, spatial learning and habituated feeding responses. We tested spatial and fear-related memory with the Morris water maze and step through passive avoidance tests, respectively and utilized food anticipatory activity to monitor habituated feeding responses following two weeks of a meal feeding paradigm. Our results indicate that ghrelin signaling modulates spatial memory performance and is necessary for the development of food anticipatory activity. Collectively, these results suggest that ghrelin receptor signaling is necessary for adaptations in the anticipatory responses that accompany restricted feeding.

1. Introduction

An organism's ability to predict the onset of a meal is a vital function modified by nutrient status and environmental stimuli. The ability to predict a particular meal is achieved in part by learned associations with stimuli that predict nutrient availability [24,30,40] thus ensuring adequate restoration of energy homeostasis. In some situations, environmental cues associated with palatable foods induce feeding in calorically replete animals [12,23] referred to herein as "non-homeostatic" feeding. Taken together, these observations underscore the impact of learning on both homeostatic and non-homeostatic feeding behaviors.

Restricted feeding schedules, in which food is present for only a few hours per day, are often used to induce food anticipatory activity (FAA) in rodents. Restricted feeding increases stress hormones [26] and palatable foods can induce FAA in the absence of caloric need [20]; suggesting that restricted feeding can be an emotionally relevant experience. Environmental cues associated with the delivery of a meal induce neuronal activation within both hypothalamic and extra-hypothalamic brain regions including the hippocampus, amygdala and frontal cortex [11,20,26] suggesting that meal anticipation may be influenced by cognitive processing.

Ghrelin is a 28 amino acid peptide that increases food intake [21,34,35] and gut motility [19]. Plasma ghrelin levels peak before the expected meals in both humans and animals [5,8,33] and drop postprandially [36], suggesting that ghrelin may act as a meal initiation signal. Endogenous ghrelin signals through a seven transmembrane G coupled protein receptor (GHSR) that is constitutively active [13] and present in many brain regions including the hypothalamus, hippocampus, thalamus, and ventral tegmental area (VTA) [9,43]. Interestingly, ghrelin cell bodies have been identified within the hypothalamus [4] and projections from these neurons are found within the septum and amygdala. In addition, ghrelin signaling within the hippocampus has been reported to modulate spine density and spatial learning [7]. Thus, it is possible that ghrelin may initiate feeding through its actions on circuits which mediate cognition and reward.

In the current manuscript we tested the hypothesis that ghrelin signaling is necessary for the processing of emotionally relevant stimuli, and hippocampal-dependent learning. We further hypothesized that ghrelin mediates habituated feeding responses. This was achieved by investigating the performance of wild type or ghrelin receptor deficient (GHSR−/−) mice in step through passive avoidance and Morris water maze paradigms. In addition, we utilized FAA to measure habituated feeding responses in wild type and GHSR−/− mice. Our results suggest that ghrelin receptor signaling is necessary for hippocampal-dependent learning and habituated responding for food.

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2. General methods

2.1. Subjects

Ghrelin receptor null mice (GHSR−/−) and their wild type littermates (n = 8/group) weighing 25–30 g were housed individually in a vivarium with a 12:12 light/dark schedule. The temperature of the room was maintained at 25 °C. All animals had *ad libitum* access to standard chow diet (Teklad, 3.41 kcal/gm, 0.51 kcal/gm from fat) and water throughout the study unless otherwise noted. The GHSR−/− mice used in this study were a gift from Jeffery Zigman and Joel Elmquist at the University of Texas Southwestern Medical Center in Dallas, TX and were generated as previously described [43]. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati and are in accordance with the guidelines set forth by the American Psychological Association.

2.2. Body composition analysis

Body composition was evaluated using a whole body NMR instrument (Echo-MRI, Waco, TX). Body composition analysis was obtained by placing each mouse into a clear Plexiglas tube and subsequently scanning them for 45 s. All mice were measured prior to the meal feeding protocol to determine baseline composition.

3. Method

3.1. Fear potentiated learning

One-trial learning, step through passive avoidance behavior was measured according to Adler et al. [1]. Briefly, the mice were placed into a two sided chamber, one side was illuminated and the other side was dark. Upon being placed into the illuminated chamber the mice were allowed to enter into the dark compartment. Since mice prefer dark to light, they normally entered within 5 s. Two additional trials were delivered on the following day. After the second trial, unavoidable mild electric footshocks (0.75 mA, 2 s) were delivered through the grid floor. After entering into the dark side of the chamber, the mice could not escape the footshock. After this single trial, the mice were immediately removed from the apparatus and placed into their home cage. The consolidation of passive avoidance behavior was tested 24 h later. In that testing session each mouse was placed into the illuminated chamber and the latency to enter the dark compartment was measured up to a maximum of 900 s.

3.2. Spatial learning

Morris water maze: The MWM consisted of a circular fiberglass pool (122 cm diameter, 75 cm height; Rowland Fiberglass Inc., Ingleside, TX) filled with water (17–19 °C, 43 cm deep). A clear glass platform (10.5 cm × 10.5 cm; square) was submerged 1 cm below the water surface. The pool was situated in a room that contained extramaze cues visible to the mice during testing (42 cm × 76 cm posters printed with contrasting patterns and shapes). Latency to escape the water was calculated for each trial by overhanging digital video camera and computer controlled TopScan software (Cleversystem Inc., Reston, VA) and used as index of spatial learning and memory ability.

3.2.1. Fixed position platform

At the onset of each trial, an individual mouse (Wild type or GHSR −/−) was placed into the water at one of four possible starting points (N, S, W, and E). The starting location for each trial was varied and all start locations were used in a given day. A trial was terminated and the latency was recorded when the mouse found and climbed onto the platform for 5 s. If the mouse did not reach the platform within 1 min, the trial was terminated, and the mouse was placed on the platform for 5 s. Each mouse received three trials per day, 30 min apart, for four consecutive days. The training started each day 1 h into the dark phase and was performed in a well-lit room. Each trial was digitally recorded for subsequent path analysis utilizing the Clever-system TopScan software (Reston, VA). On the 4th day a probe trial was performed where the hidden escape platform was removed from the pool and each animal was allowed to swim for 60 s. The amount of time spent in the quadrant of the pool where the platform had been located was quantified.

3.3. Conditioned locomotor activity

To determine if GHSR−/− mice were able to acquire feeding-induced increases in locomotor activity, GHSR−/− mice and their wild type littermates underwent a meal feeding regimen in which each animal was given chow plus water from 1200 to 1600 h and water alone for the remainder of the day. Food intake was monitored daily in meal-fed mice and after 14 d, the meal-fed mice consumed the same amount of chow during the 4-h access period as they did in 24 h prior to testing. The point being that the mice had learned to adjust their intakes to account for 24 h of calories in 4 h. On test day (day 14) feeding responses were measured every 30 min for 2 h of the 4 h feeding session to observe differences in the initiation of feeding responses in each group. Each mouse had a home cage fitted with a locomotor activity monitoring unit (Lafayette Instruments, Lafayette, IN). The total activity for each animal was collected over a 24 h period on days 1 and 14 beginning at 1000 h. Conditioned locomotor activity was monitored between 1000 and 1200 h on day 1 and day 14.

3.4. Statistical analysis

Data were analyzed using STATISTICA version 6.0 for PCs. All data were analyzed using analysis of variance (ANOVA) and LSD post-hoc comparisons were used to asses the source of significant main effects.

4. Results

4.1. Body weight

Mice lacking a functional ghrelin receptor (GHSR−/−) were leaner ($F(1,13) = 9.43$, $p<0.008$) and weighed significantly less ($F(1,12) = 15.34$, $p<0.002$) (Fig. 1A–B) compared to their wild type littermates, suggesting that deletion of functional GHSRs has long term metabolic consequences.

4.2. Fear potentiated learning

Next, we assessed the ability of GHSR−/− and wild type mice to acquire fear potentiated learning utilizing a step through passive avoidance procedure. Both GHSR−/− and wild type mice displayed significant delays in step through latencies ($F(3,20) = 15.22$, $p<0.01$) Fig. 2, suggesting that both groups learned to avoid the foot shock contingency.

4.3. Hippocampal-dependent learning

We then addressed hippocampal-dependent learning in GHSR−/− mice. Three days of training, in which each mouse was allowed to independently navigate to a submerged platform within the MWM, induced significant decreases in escape latencies in wild type mice ($p<0.01$); however this effect was absent in GHSR−/− mice (Fig. 3A). After training GHSR−/− mice were unable to efficiently locate the position where the platform had been as evidenced by a reduced number of entrees into the paired quadrant (Fig. 3B), suggesting

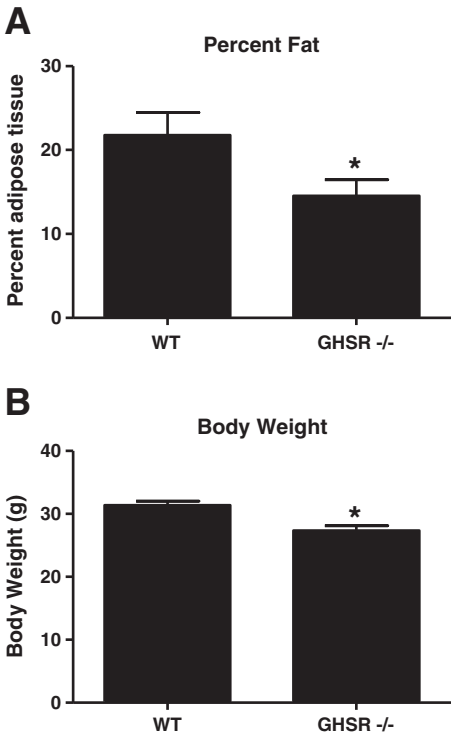

Fig. 1. A) Body composition and B) body weight in wild type and GHSR−/− mice after 16 weeks on standard chow diet * = p<0.05.

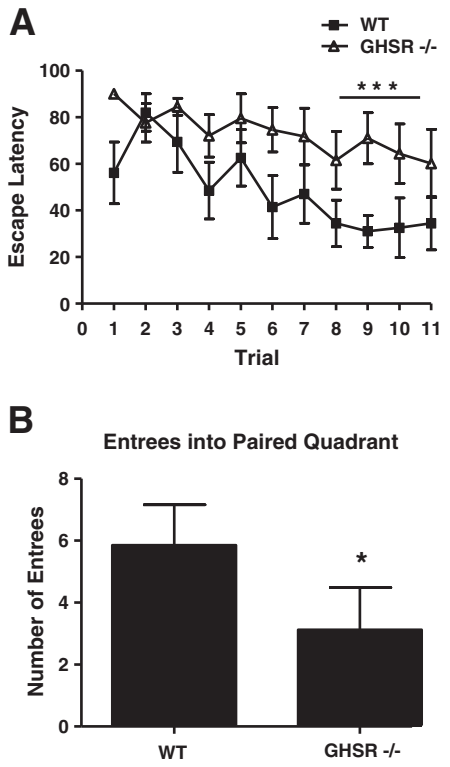

Fig. 3. A) Acquisition of spatial learning across eleven trials of training and B) expression of spatial learning on test day only represented as total number of entrees into paired quadrant in wild type and GHSR−/− mice * = p<0.01.

that GHSR−/− mice display deficiencies in the expression of spatial learning.

4.4. Food anticipatory behavior

The meal feeding protocol employed here significantly increased anticipatory locomotor activity in wild type mice (p<0.01); however this effect was absent in GHSR−/− mice (Fig. 4A). After training wild type and GHSR−/− mice consumed equal amounts of food. Specifically both groups consumed their daily ration of chow in the first 2 h of the 4 h feeding period (Fig. 4B), suggesting that each group was capable of adapting intake levels to cope with the restricted feeding schedule.

5. Discussion

The goal of the current experiments was to test the hypothesis that ghrelin receptor signaling is necessary for the processing of emotionally relevant stimuli, hippocampal-dependent learning and food anticipation. Here, we report that mice lacking functional ghrelin receptors (GHSR−/−) display impaired hippocampal function and are unable to acquire anticipatory locomotor activity associated with restricted feeding. Importantly, the ability to anticipate meals

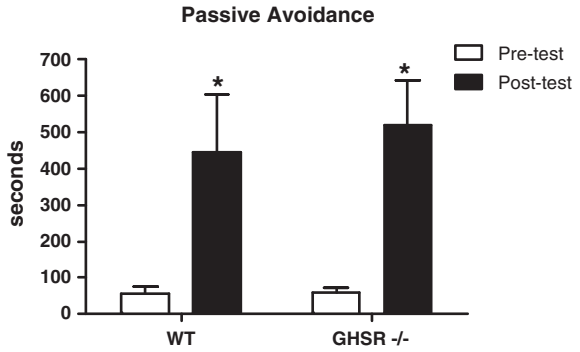

Fig. 2. Passive avoidance learning represented by average step through latencies in wild type and GHSR−/− mice after 4 days of spatial learning training * = p<0.01.

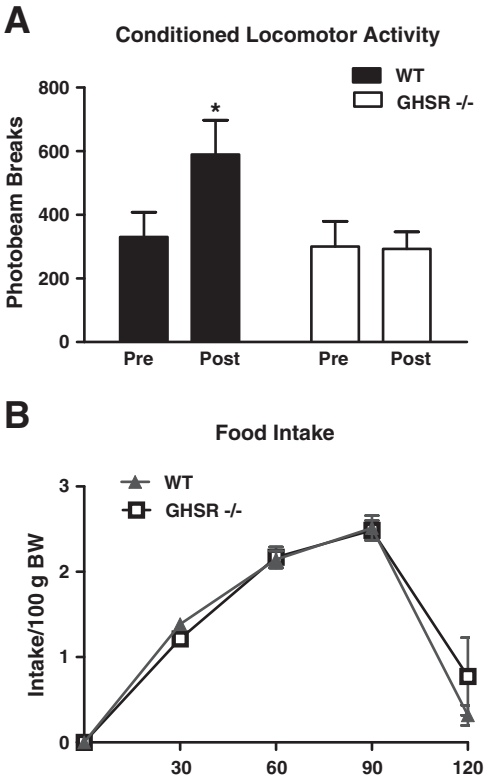

Fig. 4. A) Food anticipatory activity and B) feeding responses in wild type and GHSR−/− mice after 14 days of meal feeding * = p<0.01.

prepares an organism for the consumption, absorption and metabolism of a caloric load [41]. This concept assumes that peripheral hormones which convey information relating to energy balance can access central circuitry responsible for eliciting feeding. Here, we assume that gastric ghrelin accessing central circuitry is responsible for the behavioral effects observed. However, future studies are required to rule out any contribution from the central ghrelin system. Meal onset is controlled by many factors including, pre-meal surges in metabolic hormones [8], time of day, and learned associations with stimuli that predict meal delivery [23,30,40]. In the context of learning, meal anticipation alone can drive food consumption independent of metabolic need, and this is exemplified by the ability of learned environmental cues to stimulate feeding in sated animals ([39], for review see [12]). Moreover, environmental cues that signal the availability of palatable foods activate brain reward circuitry [28,29], an effect that presumably occurs through learning. Furthermore, the propensity of cues to induce feeding in periods of satiation suggests that learning may also be a significant contributing factor to the current rise in obesity in humans [42]. Taken together, the ability to predict food availability can be modified by learning and yields both adaptive and maladaptive effects on food intake.

The hippocampus is one brain structure implicated in the regulation of learning and memory which also mediates feeding. For example, humans with hippocampal damage will initiate feeding only minutes after completing a meal ([10,27]). In addition, food sated animals with hippocampal lesions show increased appetitive responding relative to controls [6], suggesting that intact hippocampal function is necessary for the inhibitory control of food intake. Ghrelin receptors are present within the hippocampus, raising the possibility that ghrelin receptor signaling within hippocampal neurons may mediate hippocampal function. This possibility was examined by investigating spatial learning in GHSR−/− mice. Here, we report that mice lacking a functional ghrelin receptor were unable to acquire normal spatial learning. Moreover, when tested after training, GHSR−/− mice displayed decreased retention of the spatial learning task. This finding is in agreement with previous studies which report that ghrelin binds to hippocampal neurons where it promotes long term potentiation, dendritic spine formation and enhanced spatial memory performance using a spontaneous alternation task [7]. Our observations support these findings and in addition, suggest that signaling through the ghrelin receptor is necessary for hippocampal mediated behaviors.

It has been suggested that the hippocampus and amygdala represent two functionally distinct neuronal substrates in regards to their ability to modify learning and memory systems [14,15,17,22,25]. The amygdala is hypothesized to integrate emotional experience with memory to gain control of future behaviors [14]. Restricted feeding induces neuronal activation within the stress-associated brain regions and leads to an increased release of stress hormones at the predicted time of meal [26] suggesting that reducing food availability can be an emotionally salient event. Ghrelin positive neurons exist within the hypothalamus, and these neurons project to the amygdala [4]. When administered directly into the amygdala, ghrelin modulates anxiolytic behavior [3] suggesting that this hypothalamic–amygdalar projection is functionally relevant. Collectively, these observations led us to examine the possibility that ghrelin receptor signaling may regulate amygdala-dependent function. The GHSR−/− mice used here displayed normal step through latencies in the passive avoidance paradigm, suggesting that deletion of the ghrelin receptor had no effect on amygdalar-dependent learning. It is of interest to note here that both intracranial [7] and intra-amygdalar [3] ghrelin administration augments step down passive avoidance latencies. One interpretation of these data is that ghrelin acts upon a yet to be identified receptor within the amygdala, thus deletion of this specific GHSR was without affect. It is also possible that in these studies, ghrelin spread to adjacent brain regions or in the case on third ventricular injections, acted synaptically through hypothalamic circuits which express GHSR and project to the amygdala [4] to exert its effects of anxiety-like behavior. In either case, our results suggest that signaling through the GHSR does not alter amygdalar-dependent learning.

Food anticipatory activity (FAA) is one way to measure habituated feeding responses. When food availability is restricted to a few hours a day, rodents develop food anticipatory activity patterns in which increases in activity are detected within 1–3 h prior to meal delivery. This behavior is thought to recapitulate foraging strategies that might be employed in the wild to procure a meal [32]. Functional anatomical studies suggest that FAA is controlled by a distributed set of nuclei comprised of the hippocampus, periventricular thalamic nucleus and the dorsomedial nucleus of the hypothalamus [26]. Ghrelin receptors are present within this septohippocampal-thalamo-hypothalamic circuitry hypothesized to mediate FAA [9] suggesting that ghrelin is capable of signaling within these regions. In the present study, wild type and GHSR−/− mice habituated to the meal feeding regimen as evidenced by their ability to become calorically replete within a 4 h timeframe. Additionally, anticipatory increases in locomotor activity were antecedent to the habituated feeding response. However, this behavioral anticipatory response was absent in GHSR−/− mice despite the fact that these mice initiated feeding normally thus suggesting that intact ghrelin signaling is necessary for FAA but not meal initiation. In terms of meal initiation, the only endogenous neural peptide which reliably initiates feeding is neuropeptide Y (NPY). Rodents are nocturnal animals and thus typically consume their largest meal at the onset of the dark period. Functional deletion of the NPY gene attenuates meal initiation during this period indicating that endogenous NPY signaling is required for meal onset under normal circumstances [31]. Both ghrelin and centrally produced orexin activate NPY containing neurons in the arcuate nucleus [37,38] thus it is possible that in the absence of the functional ghrelin receptor orexin may be capable of activating NPY neurons and thus initiating the feeding response.

The finding that ghrelin signaling modulates meal anticipation is consistent with recently published work which reports that GHSR-KO mice displayed attenuated FAA [2,18]. In these studies, GHSR-KO mice were capable of eliciting food anticipatory responding, but not to the same degree observed in wild type control mice. One difference between these studies is that the GHSR−/− mice and GHSR-KO mice were generated in different laboratories and were backcrossed to somewhat different strains to maintain the colony. Moreover, the GHSR−/− mice used here did not display FAA, that is, the locomotor pattern after fourteen days of meal feeding was not significantly different from activity levels prior to meal feeding, whereas in the former study the FAA activity levels were merely attenuated. It is possible that these differences are due to the strain differences present in the two different mouse models, or the method of monitoring FAA. However, it is clear that both studies confirm that ghrelin receptor signaling modulates FAA. Consistent with this notion is the observation that central ghrelin administration increases locomotor activity in ad libitum fed rodents [16]. Thus it is possible that apart from its effects on food consumption, pre-prandial rises in plasma ghrelin facilitate meal seeking behavior.

It is important to mention here that each of these findings could be source specific as we used a single genetic mouse model to examine each function. Thus, future studies are needed to confirm each of these findings in isolation as well as in combination with different methodologies to alter ghrelin receptor function.

In summary, the studies presented here suggest that ghrelin receptor signaling is required for hippocampal but not amygdalar dependent learning. In addition, we report that ghrelin receptor signaling mediates the increases in locomotor activity that precede meal delivery, but not habituated feeding under a restricted access regimen. It has been suggested that the initiation of feeding is

controlled by indicators that reliably signal the presence of a meal [40] which can be nutrient signals or environmental stimuli as well as stimuli associated with food itself [11]. Thus, cues which are learned and associated with the delivery of a particular meal represent an important aspect of feeding behavior, especially when the availability of food is limited.

Collectively, these results suggest that ghrelin receptor signaling is necessary for adaptations in the anticipatory responses that accompany restricted feeding; perhaps through its ability to regulate learning.

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