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Research report

The antagonism of ghrelin alters the appetitive response to learned cues associated with food

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HIGHLIGHTS

- The gastric peptide, ghrelin, is known to influence feeding and reward learning.
- The ghrelin receptor antagonist, GHRP-6 [D-Lys3], was administered systemically to rats.
- Rats also received a Pavlovian-to-instrumental transfer (PIT) or a cue potentiated feeding (CPF) test.
- GHRP-6 [D-Lys3] led to an increase in PIT but had no effect on CPF.
- GHRP-6 [D-Lys3] also led to increased *c-fos* activity in brain reward circuitry in PIT-, but not CPF-tested rats.

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ABSTRACT

The rapid increase in obesity may be partly mediated by an increase in the exposure to cues for food. Food-paired cues play a role in food procurement and intake under conditions of satiety. The mechanism by which this occurs requires characterization, but may involve ghrelin. This orexigenic peptide alters the response to food-paired conditioned stimuli, and neural responses to food images in reward nuclei. Therefore, we tested whether a ghrelin receptor antagonist alters the influence of food-paired cues on the performance of instrumental responses that earn food and the consumption of food itself using tests of Pavlovian-to-instrumental transfer (PIT) and cue potentiated feeding (CPF), respectively. Food-deprived rats received Pavlovian conditioning where an auditory cue was paired with delivery of sucrose solution followed by instrumental conditioning to lever press for sucrose. Following training, rats were given *ad libitum* access to chow. On test day, rats were injected with the ghrelin receptor antagonist GHRP-6 [D-Lys3] and then tested for PIT or CPF. Disrupting ghrelin signaling enhanced expression of PIT. In addition, GHRP-6 [D-Lys3] impaired the initiation of feeding behavior in CPF without influencing overall intake of sucrose. Finally, in PIT tested rats, enhanced FOS immunoreactivity was revealed following the antagonist in regions thought to underlie PIT; however, the antagonist had no effect on FOS immunoreactivity in CPF tested rats.

1. Introduction

Obesity is a public health problem affecting millions of people worldwide [1,2]. An increasing proportion of human food consumption may reflect an influence of environmental food-associated cues (*e.g.*, television advertisements, radio jingles etc.), which are known to drive food preference and intake [3,4]. In the obesogenic environment it is likely that various discrete, contextual and temporal food-associated cues can acquire motivational and behavioral responses that lead to greater food intake than is necessary to maintain energy balance. Food-associated cues can take the form of a learned attribute of the food (*e.g.*, smell or sight of pizza; [5]) or an originally unrelated stimulus (*e.g.*, rotating red light, music, physical location; [6]). After repeated cue + food pairings, the cue alone has the ability to increase appetite [7,8], initiate cephalic phase responses [9] and increase planned and actual food intake even when satiated [10–13]. These cue + food associations appear to be more reactive in certain individuals [14] and can be acquired in children [6], adolescents [15] and adults [10,16]. Understand-

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ing physiological mechanisms driving these learned responses may lead to pharmacological or other manipulations that will inhibit the conditioned response to acquire and eat more food than is needed to maintain energy balance—this may have significant implications for curtailing obesity rates.

Two rodent models used to study the impact of food cues on feeding behavior are cue potentiated feeding (CPF) and Pavlovian-instrumental transfer (PIT). CPF models the mechanisms underlying food-paired cues on the consumption of food itself in sated animals. In this preparation, food-restricted rodents are trained in a Pavlovian conditioning procedure, in which a conditioned stimulus (CS) (*e.g.*, auditory tone) is paired with food delivery (the unconditioned stimulus; US). After training, rats are allowed to eat *ad libitum* for many days and then are presented with the CS under a non-deprived state, and food consumption is measured. Many studies have shown that rodents consume more food in the presence of the CS, relative to periods of no stimulus presentation, or when a control cue that was previously unpaired with food is presented [12,17–19]. PIT, on the other hand, tests the effect of food cues on food-procurement responses (*e.g.*, instrumental lever press responding). PIT also involves a CS-US Pavlovian conditioning phase, but has an additional instrumental conditioning phase in which animals learn to press a lever that is associated with the delivery of food. At test, CS presentation enhances the rate of lever responding, which is thought to reflect the evocation of incentive motivation conditioned to the CS [20,21].

Ghrelin, an orexigenic hormone produced by the stomach and other tissues, is a potential mediator of cue + food associations that lead to increases in food procurement and intake independent of an energy demand [22–24]. In non-human animals that are *ad libitum* fed, peripheral treatment with ghrelin mimics food deprivation-induced discriminative responding [25], results in elevations in food hoarding and intake [26], and facilitates cue-induced reinstatement of lever responding for food [27]. In humans, ghrelin levels after a fast are correlated with perceived hunger levels [28]. Furthermore, intravenous administration of ghrelin to humans 3 h after a meal increases subjective feelings of hunger, and the neural response to food images in the amygdala, orbitofrontal cortex, hippocampus and the mesolimbic dopamine system [29]. These are the same brain areas that have been implicated in ghrelin-mediated changes in associative learning in a number of studies [27,30,31]. PIT also depends on the intact function of various components of the mesolimbic system [32–34], and CPF has been shown to depend on corticolimbic circuitry that includes the lateral hypothalamus (LH) [35], basolateral amygdala (BLA) [11], ventral hippocampus (VH) [22] and the ventromedial prefrontal cortex (vmPFC) [36]. Except for the vmPFC, dense GHSR expression has been revealed in all of these areas [18,37].

Consistent with the distribution of GHSR, disruptions in signaling accomplished by either genetic deletion in GHSR-deficient mice, or via peripheral administration of a GHSR antagonist, Compound 26, lead to a reduction in the capacity for a CS to evoke overeating when tested under *ad libitum* conditions (*i.e.*, CPF) [18]. Interestingly, we have shown that systemic administration of the GHSR antagonist, GHRP-6 [D-Lys3], can enhance PIT in food restricted mice [38]. Although a high dose of the antagonist depressed behavior generally, lower doses led to a significant augmentation in PIT without affecting baseline responding [38]. From these contrasting results it is tempting to suggest that GHSRs may have different effects on the ability of food cues to enhance consummatory and appetitive (*e.g.*, food procurement) behaviors. Notably, previous studies have revealed double dissociations between brain lesions that affect PIT and CPF [17]. However, whereas CPF studies are conducted after *ad libitum* feeding, PIT studies have been almost exclusively conducted under food restricted conditions. Thus, different effects of GHSR antagonists on CPF and PIT may reflect differences in the role of GHSRs under different motivational states.

Accordingly, in this study we sought to examine the effects of GHRP-6 [D-Lys3] administration on PIT and CPF when rats are tested under *ad libitum* feeding conditions. If the effects of GHSRs depend primarily on motivational state, we might expect the antagonist to depress both CPF and PIT under these conditions. For example, in freely-fed rats, learned food cues may encourage ghrelin secretion, which may induce hunger and encourage both eating [39] and ongoing lever-pressing for food. In that case, administration of a GHSR antagonist would depress both CPF and PIT by preventing such a "cephalic phase" (*e.g.*, [9]) hunger response. By contrast, if GHSRs have dissociable effects on consummatory and appetitive aspects of responding regardless of motivational state, we might expect GHSR antagonist administration to depress CPF while still enhancing PIT, as in our previous observations [38]. Our results suggest a dissociable role of GHRP-6 [D-Lys3], on the one hand enhancing the modulation of ongoing lever responding during PIT, and also disrupting the initiation of CPF. Finally, in order to begin an analysis of brain function in the various test conditions, following tests of PIT or CPF conducted after injections of either the antagonist or saline, we examined FOS expression in several brain regions known to express GHSR.

2. Methods

2.1. Subjects

The subjects were 64 male Long-Evans rats (Charles River Laboratories, Raleigh, NC, USA), which weighed 300–325 g when they arrived in the laboratory vivarium. After 1 week acclimation to food (Harlan 2018) and water *ad libitum*, the rats were then reduced to 85% of their *ad libitum* weights by restricting their access to food. This food restriction continued throughout training sessions. Rats were fed immediately after the completion of each training session. Upon completion of all training sessions, the rats were then returned to *ad libitum* food and water for 2 weeks prior to the final test session. Throughout the study, the rats were housed in individual tub cages in a 12:12 light:dark cycle with the lights on at 0700 h. Behavioral testing was carried out between the hours of 9AM and 5PM.

2.2. Apparatus

Behavioral training took place in eight identical chambers ($22.9 \times 20.3 \times 20.3$ cm), with aluminum front and back walls, clear polycarbonate sides and ceiling, and floor comprised of parallel, 0.48 cm stainless steel rods spaced 1.9 cm apart. Each chamber was enclosed in a double-walled sound-attenuating shell. An illuminated clear acrylic food cup (approximately 3 cm in diameter and 0.3 cm deep, with a capacity of about 1.9 ml) was recessed in the center of one of the end walls. An infrared photocell placed just inside the food cup was polled (1 kHz) by computer circuitry to detect head entries, time spent in, and latency to the food cup. Tubing entered into the food cup from the outside of the chamber, allowing for 0.2 M sucrose solution to be administered directly into the food cup. The tubing was attached to a syringe that was placed on a syringe pump that was connected to a computer system to allow for precise dispensing of solutions when needed. An aluminum lever (2×2 cm) was mounted on each side of the food cup, centered between the cup and the side walls. Throughout the Pavlovian training sessions and cue-potentiated feeding tests, the levers were covered with aluminum boxes ($3 \times 2 \times 3$ cm). A speaker, used to present the auditory CSs, was mounted on the inside wall of the sound-attenuating chamber. Ventilation fans provided mask-