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GHRELIN DIRECTLY TARGETS THE VENTRAL TEGMENTAL AREA TO INCREASE FOOD MOTIVATION

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Abstract—Ghrelin, a circulating orexigenic stomach-derived hormone, has recently been implicated in extra-homeostatic feeding, increasing food reward and food-motivated behavior. The precise target site(s) for ghrelin's effects on food reward have yet to be elucidated. The neurocircuitry underpinning food-motivated behavior involves, in particular, the dopamine cells of the ventral tegmental area (VTA) that project to the nucleus accumbens (NAcc). Ghrelin stimulation in both of these mesolimbic reward areas increases chow intake. Here we sought to determine if ghrelin acts directly within these mesolimbic reward areas to increase food reward/motivation in studies that combine feeding behavior, pharmacology, and neuroanatomy. We found that motivated behavior for a sucrose reward, assessed in an operant conditioning paradigm in rats, was increased when ghrelin was microinjected directly into the VTA but not into the NAcc. By contrast, ghrelin administration to both areas increased the free feeding of chow. Importantly, in a state of overnight food restriction, where endogenous levels of ghrelin are increased, ghrelin receptor (GHS-R1A) blockade in the VTA was sufficient to decrease the motivation to work for a sugar reward. Blockade of the GHS-R1A in VTA or NAcc was not sufficient to reduce fasting-induced chow hyperphagia. Taken together our data identify the VTA but not the NAcc as a direct, necessary, and sufficient target site for ghrelin's action on food motivation.

Key words: ghrelin, GHS-R1A, food motivation, operant conditioning, ventral tegmental area, nucleus accumbens.

Rates of obesity and overweight continue to grow at an alarming rate. There is therefore an escalating and urgent need to better understand the underlying pathophysiology of problematic over-eating with a view to identify novel therapeutic targets for this disease area. Homeostatic signals determine food intake that is dictated by the need for nutrient repletion (metabolic hunger) (Saper et al., 2002). It seems clear, however, that a considerable amount of food intake escapes homeostatic control and occurs despite a state of satiation. Moreover, both rewarding and environmental factors likely play a pivotal role for this non-homeostatic food intake. Ghrelin, a circulating hormone produced primarily in the stomach (Kojima et al., 1999; Date et al., 2000), is a potent orexigenic agent with a well-established role in homeostatic feeding (Kojima et al., 1999; Wren et al., 2000). Ghrelin levels are highly correlated with meal initiation and increase during fasting (Cummings et al., 2001). Conversely, blockade of ghrelin receptors (growth hormone secretagogue receptor, GHS-R1A) decreases food intake (Salomé et al., 2009). Ghrelin receptors are abundantly expressed in CNS areas associated with homeostatic feeding, including the hypothalamus and brainstem (Guan et al., 1997; Katayama et al., 2000) and direct ghrelin microinjection in these areas increases food intake (Wren et al., 2001; Faulconbridge et al., 2003). Interestingly, however, ghrelin has recently emerged as one of the major contributing factors to reward-driven feeding that can override the state of satiation (Egecioglu et al., 2010; Perello et al., 2010; Skibicka et al., 2011). The underlying neuroanatomical targets for this novel role of ghrelin in reward-motivated feeding remain unexplored and provide a basis for the present study.

Substances that affect reward-driven behaviors, for example, alcohol, cocaine or food, do so by complex neurobiological mechanisms that result in an altered incentive motivational value of the conditioned reward-predictors in the environment (Wise, 2002) and the reward reinforcer. Operant conditioning is the foremost procedure utilized in addiction research to evaluate the addictive/motivational properties of such agents in animal models (Hodos, 1961). A core element of the underlying neurobiology of the motivated behaviors for reward reinforcers is the mesolimbic reward system, especially the dopamine cells of the ventral tegmental area (VTA) that project to the nucleus accumbens (NAcc). Consistent with a role of the ghrelin system in motivated behavior/food reward, both systemic and central (ventricular) ghrelin injection increases operant behavior for a food reward (Skibicka et al., 2011). Conversely, suppression of central ghrelin signaling by peripheral administration of a GHS-R1A antagonist decreased operant responses for a food reward (Skibicka et al., 2011). Preference for a food reward-paired environment in the conditioned place preference test was reduced by a GHS-R1A antagonist and also in GHS-R1A knockout mice, further evidencing a role for the central ghrelin signaling system in food reward (Egecioglu et al., 2010; Perello et al., 2010). These behavioral expressions of reward that are dependent on central ghrelin signaling are accompanied by molecular and electrophysiological evidence: ghrelin increases dopamine neuron activity in the VTA (Abizaid et al., 2006) and also increases accumbal dopamine release with an associated locomotor response (Jerlhag et al., 2006, 2007). Relevance of these data to food reward

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Abbreviations: NAcc, nucleus accumbens; VTA, ventral tegmental area.

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mechanisms in man is highlighted by the finding that acute ghrelin injection alters the brain response to visual food cues, notably in corresponding reward areas such as the ventral striatum (Malik et al., 2008).

While the importance of the central ghrelin signaling system in reward-motivated feeding is now supported, the ghrelin-responsive neuroanatomical substrates underpinning these effects remain to be elucidated. Ghrelin receptors are expressed in several nuclei with direct or indirect connections to the mesolimbic reward system (Zigman et al., 2006). Strong association of ghrelin's feeding effects with the hypothalamic nuclei and an abundant expression of the GHS-R1A in the hypothalamic nuclei enforces the view that ghrelin might exert its effects on food motivation via its action on the arcuate nucleus or lateral hypothalamus. However, ghrelin microinjection directly into key mesolimbic areas, the VTA and the NAcc, has been shown to increase food intake (Naleid et al., 2005) and also, in the VTA, to increase preference for high calorie food (Egecioglu et al., 2010). Consistent with these findings, GHS-R1A is known to be expressed in the VTA, notably on both dopaminergic and GABAergic neurons (Abizaid et al., 2006). However, GHS-R1A expression in NAcc remains controversial and is evaluated in the current publication (Guan et al., 1997; Naleid et al., 2005; Zigman et al., 2006).

Here we combine behavioral studies, pharmacology and neuroanatomy to investigate ghrelin's potential targets in the mesolimbic pathway. We sought to determine the effects of ghrelin or a GHS-R1A antagonist, applied directly into the VTA or NAcc, on the operant response for sugar pellets and on the free feeding of normal chow.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley rats (200–250 g, Charles River, Germany) were housed in a 12-h light/dark cycle with regular chow and water available *ad libitum*, except when indicated otherwise. All animal procedures were carried out with ethical permission and in accordance with the University of Gothenburg Institutional Animal Care and Use Committee guidelines.

Surgery

All rats in the behavioral studies were implanted with a guide cannula targeting the VTA or the NAcc shell, (26 gauge; Plastics One, Roanoke, VA, USA) under isofluorane anesthesia (2.2% isofluorane content in the air flow into the face mask, placed in the stereotaxic frame for 30 min). Cannulae were placed 1.5 mm above the target site, and an injector extending 1.5 mm from guide cannulae was used for microinjections. To target the VTA, the following coordinates were chosen modified from (Egecioglu et al., 2010): ±0.75 from the midline, 5.7 mm posterior to bregma, and 6.5 mm ventral from the surface of the skull, with injector aimed 8.0 mm ventral to skull. For the NAcc shell, the following coordinates were used (modified from Quarta et al., 2009: ±0.75 from the midline, 1.7 mm anterior to bregma, and 6.0 mm ventral to skull, with injector aimed 7.5 mm ventral). Cannulae were attached to the skull with dental acrylic cement and jeweler's screws and closed with an obturator, as described previously (Skibicka et al., 2009). In all rats, the microinjection site for both VTA and NAcc was verified post mortem, by microinjection of India Ink at the same microinjection volume (0.5 μl) used throughout the study. Only subjects with the correct placement were included in the data analysis.

Operant conditioning procedure

Instrumental conditioning apparatus. Operant conditioning experiments took place in eight rat operant conditioning chambers (30.5×24.1×21.0 cm^2; Med-Associates, Georgia, VT, USA), which were placed in a sound-attenuated, dimly lit cabinet. Each chamber had a metal grid floor, two retractable levers with white light bulbs above them and a food pellet dispenser that delivers 45 mg sucrose pellets (Test Diet, Richmond, IN, USA) to the food tray. Data were collected and processed by MED-PC software.

Training. The procedure used for operant conditioning was adapted from (la Fleur et al., 2007; Tracy et al., 2008; Skibicka et al., 2011). All rats were subjected to a mild food restriction paradigm during which their initial body weight was gradually reduced to 90% over a period of 1 week. Prior to placement in the operant boxes, rats were exposed to the sucrose pellets in the home cage environment on at least two occasions. Next, rats learned to lever press for sucrose pellets under a fixed ratio FR1 schedule, with two sessions/d. In FR1, a single press on the active lever resulted in the delivery of one sucrose pellet. All FR sessions lasted 30 min or until the rats earned 100 pellets, whichever occurred first. Most rats achieved the 100 pellets per session criterion after 5–7 days. Presses on the inactive lever were recorded, but had no programmed consequence. FR1 schedule sessions were followed by FR3 and FR5 (i.e. three and five presses per pellet, respectively). Again, a minimum of 100 responses per session on the active lever was required for the advancement to the next schedule; most rats required only one to two FR3 and FR5 schedule(s) to achieve this level. The FR5 schedule was followed by the progressive ratio (PR) schedule during which the cost of a reward was progressively increased for each following reward, in order to determine the amount of work the rat is willing to put into obtaining the reward. The response requirement increased according to the following equation: response ratio=(5e(0.2×infusion number))−5 through the following series: 1, 2, 4, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328. The PR session ended when the rat had failed to earn a reward within 60 min. Responding was considered stable when the number of food pellets earned per session did not differ more than 15% for three consecutive sessions. In most cases, responding stabilized within five sessions. Those rats that did not reach the required criteria in that amount of time were trained in additional sessions. The PR test was carried out on one session/d. Sessions lasted on average 75 min although all rats stayed in the operant boxes until 120 min to allow for all sessions to end. Rats were subsequently transferred to their home cages for 1 h chow intake measurement. At the end of training and prior to testing, rats were returned to an *ad libitum* feeding schedule.

Experimental design

All rats received intra-parenchymal (VTA or NAcc) microinjections early in the light cycle 10 min prior to the start of operant testing. All conditions were separated by a minimum of 48 h and run in a counterbalanced manner—each rat received all three conditions (vehicle, dose 1 or dose 2 of drug) on separate testing days. On each day each condition was represented equally. All injections were unilateral. Residual effects of acute ghrelin injection past 24 h were unlikely, based on (Faulconbridge et al., 2003) however 24 h food intake was measured to make sure ghrelin does not have longer term effects that would interfere with the current counterbalanced design. After collection of data from all three conditions data were also examined for an interaction of day with treatment, to further eliminate the possibility of repeated injections to interfere with the results.