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Central administration of ghrelin induces conditioned avoidance in rodents

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Abstract
Feelings of hunger carry a negative-valence (emotion) signal that appears to be conveyed through agouti-related peptide (AgRP) neurons in the hypothalamic arcuate nucleus. The circulating hunger hormone, ghrelin, activates these neurons although it remains unclear whether it also carries a negative-valence signal. Given that ghrelin also activates pathways in the midbrain that are important for reward, it remains possible that ghrelin could act as a positive reinforcer and hence, carry a positive-valence signal. Here we used condition preference/avoidance tests to explore the reinforcing/aversive properties of ghrelin, delivered by intracerebroventricular (ICV) injection (2 μg/injection once a day for 4 days). We found that ICV ghrelin produces conditioned avoidance, both in a conditioned place preference/avoidance test (CPP/CPA, in which the animals avoid a chamber previously paired to ghrelin injection) and in a conditioned flavor preference/avoidance test (CFP/CFA, in which the animals consume/avoid a taste previously paired to ghrelin injection). These effects of ghrelin to induce a CPA were observed when conditioning to ghrelin occurred in the absence or presence of food. We did not find evidence, however, that brain ghrelin delivery to rats induces malaise (in the pica test). Our data indicate that ICV ghrelin carries a negative-valence signal consistent with its role as a circulating hunger hormone and with its effects to activate AgRP neurones.

1. Introduction

Recent studies suggest that appetitive and food seeking behaviors that occur when hungry and that precede consumption can be driven by unpleasant negative-emotion/valence signals, and that the motivation to seek food and the initiation

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of its consumption is generated by the learned alleviation of negative valence that occurs once the food has been eaten (Betley et al., 2015; Sternson and Eiselt, 2016). One key neuronal substrate transmitting this negative-valence signal is the agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC) of the hypothalamus (Betley et al., 2015). AgRP neurons (that, in addition, contain neuropeptide Y (NPY) and gamma aminobutyric acid (GABA)) are an important component in the pathways controlling homeostatic feeding; they receive information about peripheral energy status through endocrine signals such as leptin or ghrelin (that inhibit or activate these cells, respectively) (Cowley et al., 2003; Takahashi and Cone, 2005). The activation of AgRP neurons increases food consumption, while inhibition or ablation suppresses feeding (Aponte et al., 2011; Atasoy et al., 2012; Krashes et al., 2011; Luquet et al., 2005).

The circulating, stomach-derived, hunger hormone ghrelin is secreted preprandially (Cummings et al., 2001). Ghrelin receptor agonists increase food intake and activate ARC AgRP/NPY neurons (Cowley et al., 2003; Dickson and Luckman, 1997; Lawrence et al., 2002). In mice that lack AgRP, ghrelin fails to increase food intake suggesting a critical role of AgRP in ghrelin-induced food consumption (Chen et al., 2004). The ghrelin receptor, the growth hormone secretagogue receptor 1 A (GHS-R1A) is highly expressed in many hypothalamic nuclei including the ARC (Zigman et al., 2006), and co-localizes in almost all AgRP/NPY neurons in this area (Willesen et al., 1999). GHS-R1A is also abundant in reward-linked brain regions such as the ventral tegmental area (VTA) (Abizaid et al., 2006; Zigman et al., 2006), and has been found to be co-localized with a subpopulation of dopaminergic neurons in this area (Abizaid et al., 2006). Systemically administered ghrelin targets the VTA dopamine neurons, including those that project to the nucleus accumbens (NAc) and that confer reward. Thus, ghrelin has been shown to increase NAc dopamine release in mice (Jerlhag et al., 2006) and to increase the neuronal response in brain areas linked to reward in humans (Malik et al., 2008). Thus, ghrelin could influence reward-linked appetitive and consummatory behaviours by engaging this pathway. Consistent with this, ghrelin microinjected into the VTA increases food consumption and motivated behavior for food (Skibicka et al., 2011; Skibicka et al., 2013).

It seems clear that ghrelin may increase food intake through discrete neuronal circuits with divergent reinforcing properties. Some of these neuronal circuits may induce food consumption by motivated behaviors arisen from learning that consuming food will alleviate negative valence (e.g. AgRP/NPY neurons in the ARC), or by motivated behaviors linked to anticipation of positive valence and reward once food is consumed (e.g. through the mesolimbic dopamine pathway). Here, we sought to elucidate the overall reinforcing properties of ghrelin. Specifically, we explored whether ICV ghrelin is able to condition preference or avoidance in place and flavor preference paradigms.

2. Experimental procedures

2.1. Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany) were used. Body weight at the time of surgery was 220–310 g. Animals were kept under standardized non-barrier conditions on a 12/12 h light/dark cycle at 20–22 °C and 50% humidity. On arrival at the animal facility, animals had *ad libitum* access to standard maintenance chow (SDS RM1 diet, Special Diet Services, Witham, Essex, UK). Water was available *ad libitum*. The animal procedures were approved by the local ethics committee for animal care in Gothenburg, Sweden (Göteborgs djurförsöksetiska nämnd; permit number 45-2014) and were conducted in accordance with guidelines.

Adult male C57Bl/6 J mice (Envigo, Bicester, UK) were used, with body weight between 25–32 g at the time of surgery. Mice were maintained in standardized non-barrier conditions, on a 12/12 h light/dark cycle, at 22 °C ±1 °C and 45% ±10% humidity. Standard maintenance chow (SDS RM1 diet) and water were available *ad libitum*. Procedures on mice were carried out in accordance with the Animal (Scientific Procedures) Act 1986 (UK) and approved by the University of Manchester Animal Welfare and Ethics Review Board.

2.2. Guide cannula implantation

Rats were anaesthetized with a combination of Rompun® vet. 10 mg/kg (Bayer, Leverkusen, Germany) and Ketaminol® vet. 75 mg/kg (Intervet, Boxmeer, Netherlands) and placed in a stereotaxic frame. The skull bone was exposed and the skull sutures were identified. Holes for guide cannulae and anchoring screws were drilled through the skull. A 26-gauge cannula was positioned according to stereotactic coordinates and fixed in place with anchoring screws and dental cement (Dentalon, Heraeus Kulzer, Hanau, Germany). The guide cannula was placed 2 mm dorsal to the lateral ventricle and the following coordinates were used: 0.9 mm posterior to bregma, ± 1.6 mm lateral to the midline and 2.5 mm ventral of the skull surface. At injection, the injector used was 2 mm longer than the guide cannula to target the lateral ventricle. The injection volume was always 2 μl. After surgery the rats received an analgesic (Rimadyl® vet. 5 mg/kg, Orion Pharma Animal Health, Sollentuna, Sweden) and were singly housed during >4 day recovery period. To validate cannula placement in the lateral ventricle, conscious rats were injected with 20 ng angiotensin II (1158, Tocris, Bristol, U.K) for which correct placement is indicated by a dipsogenic (immediate water drinking) response (Epstein et al., 1970).

Mice were anaesthetized with 3% isoflurane (Abbot Abbvie Ltd, Maidenhead, UK) in oxygen (1500 ml/min) and placed in a stereotaxic frame. Throughout surgery, mice were maintained at the correct depth of anaesthesia using between 1-2% isoflurane in oxygen (800 ml/min). The skull bone was exposed and the skull sutures identified. A small screw was inserted into the left parietal plate of the skull. A hole was drilled 0.4 mm posterior to bregma, 1 mm lateral to the midline, through which a sterile guide cannula was implanted at a depth of 1.2 mm ventral to the skull surface (made in house from a 23-gauge needle cut to length). The guide cannula was fixed in place with the anchoring screw and dental cement (Simplex Rapid Powder, Kemdent, Swindon, UK / methyl methacrylate, Metrodent, Huddersfield, UK). At injection, the injector used was 0.5 mm longer than the guide cannula, to target the lateral ventricle. Injection volume was 2 μl.

2.3. Conditioned place preference/avoidance (CPP/CPA)

CPP/CPA testing in rats was performed using an apparatus composed of two chambers with distinct visual and tactile qualities and separated by a guillotine door (Med Associates Inc, Fairfax, VT, USA). One chamber was white and had a smooth-surface plastic floor while the other was black and had a rugged-surface plastic floor. Time spent in each chamber was recorded using infrared