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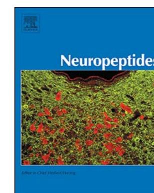

Blocking constitutive activity of GHSR1a in the lateral amygdala facilitates acquisition of conditioned taste aversion

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ABSTRACT

Ghrelin is a circulating peptide hormone promoting feeding and regulating energy metabolism in human and rodents. Ghrelin functions by binding to its receptor, the growth hormone secretagogue receptor 1a (GHSR1a), which are widely distributed throughout the brain including the amygdala, a brain region important for regulating valenced behavior, such as aversion. Interestingly, GHSR1a was once characterized by highly constitutive, ligand-independent activity. However, the physiological importance of such ligand-independent signaling on aversive memory processing has not been tested yet. Here, we applied [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P (D-SP), a full inverse agonist for GHSR1a, into the lateral amygdala (LA) and investigated the effect of blocking GHSR1a constitutive activity on conditioned taste aversion (CTA) in rats. We found that intra-LA infusion of a single low dose of D-SP (8 ng/0.5 μl/side) facilitates CTA acquisition. Moreover, pre-administration of a high dose of D-SP into the LA abolishes the suppressive effect of exogenous ghrelin on CTA acquisition. In contrast, pre-administration of the same dose of D-SP does not affect the suppression of substance P, a potent neurokinin-1 (NK1) receptor ligand, on CTA. Therefore, our data indicated that the spontaneous or basal activity of GHSR1a signaling in the LA might interfere with CTA memory formation. D-SP decreases the constitutive activity of GHSR1a and thus facilitates CTA. Altogether, our present findings along with previous results support the idea that ghrelin/GHSR1a signaling in the LA circuit blocks conditioned taste aversion.

1. Introduction

Ghrelin, also called the “hunger hormone”, is a circulating peptide primarily synthesized by the stomach (Kojima et al., 1999). The acylated form of ghrelin (acyl-ghrelin) can pass across the blood-brain barrier and bind to its central receptor, the growth hormone secretagogue receptor 1a (GHSR1a) (Bednarek et al., 2000). GHSR1a is widely distributed throughout the brain, including not only classic hypothalamic hunger regions (Guan et al., 1997), but also extra-hypothalamic regions not typically associated with hunger, such as the cortex, the hippocampus, the ventral tegmental area and the amygdala (Zigman et al., 2006). In addition, ghrelin is found to be tonically secreted at all time not just when food is expected (Harmatz et al., 2017; Kumar et al., 2013), further suggesting that ghrelin/GHSR1a signaling may have a broader role than simply modulating hunger or appetitive processing. Indeed, an increasing number of studies have reported that beyond feeding control and energy metabolism, ghrelin/GHS-R1a plays complex roles in modulating multiple brain functions, such as reward, mood and memory (Andrews, 2011; Beheshti and Shahrokhi, 2015; Harmatz et al., 2017; Meyer et al., 2014; Muller et al., 2015).

The amygdala is one of the key structures essential for acquisition and storage of multiple types of aversive memory, including auditory fear conditioning (AFC) and conditioned taste aversion (CTA) (LeDoux, 2000; Zhou et al., 2009). In particular, the nuclei of lateral amygdala (LA) receives multimodal sensory input from the thalamus and cortex, and it is considered to serve as an essential site where NMDA receptor-dependent synaptic plasticity are required for associative learning and memory formation (Ehrlich et al., 2009; LeDoux, 2000). The presence of GHSR1a in the basolateral complex of the amygdala (BLA), a brain region important for regulating negative emotional states and valenced behavior, suggests that ghrelin/GHSR1a signaling may modulate aversive processing. To our knowledge, there are many contradictory findings regarding the role of acyl-ghrelin in BLA-dependent fear memory (Alvarez-Crespo et al., 2012; Carlini et al., 2004; Harmatz et al., 2017; Meyer et al., 2014). CTA is another established learning paradigm used to study the molecular, cellular, circuit and systemic mechanisms of non-declarative memory. Our previous studies have demonstrated that micro-infusion of nanograms of ghrelin into the LA before training blocked the acquisition of CTA memory in rats, a remarkable behavioral effect mediated by ghrelin/GHSR1a signaling

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activity and it was completely abolished by pretreatment with GHSR1a antagonist (Song et al., 2013; Zhu et al., 2013).

Interestingly, besides being triggered by endogenous or exogenous ghrelin, GHSR1a was recently found to be strongly, constitutively active which shows a very high basal signaling activity in the absence of ligand or agonist (Holst et al., 2005; Holst et al., 2003). Also, [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-Substance P (D-SP), a low potency GHSR1a antagonist, was actually a potent and highly efficient inverse agonist for GHSR1a. The potency of D-SP to inhibit constitutive, ligand-independent signaling activity of GHSR1a was approximately 50- to 100-fold higher than its potency as an antagonist for ghrelin-induced signaling (Holst et al., 2005; Holst et al., 2003; Holst et al., 2006). Although GHSR1a is widely distributed throughout the brain, ghrelin is undetectable in the central nervous system, with the exception of trace amount in the hypothalamus (Cowley, 2003; Furness et al., 2011; Kern et al., 2015). Therefore, the constitutive activity of GHSR1a could be physiologically important in its role as a regulator of multiple brain functions including GH secretion, food intake and emotional memory formation. Previous studies indicated that exogenous ghrelin modulates aversive memory such as fear through activation of GHSR1a signaling; however the physiological importance of constitutive activity of GHSR1a on aversive memory processing has not been tested yet. Here, we applied D-SP, a full inverse agonist for GHSR1a, into the lateral amygdala (LA) and investigated its effect on conditioned taste aversion (CTA) in rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (300–350 g) used in the experiments were purchased from the Experimental Animal Center at Lukang Pharmaceutical Co (Jining, China). Rats were single-housed under 12 h light/12 h dark cycle and at controlled temperature (20–22 °C). Food and water were accessible ad libitum, except during CTA training and memory retrieval. All behavioral procedures were conducted during the light phase of the cycle. Rats were allowed for acclimation in colony room for at least 2 weeks before the start of any experiments. All procedures were performed in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals* and were approved by the Chancellor's Animal Research Committee of Qingdao University.

2.2. Surgical procedures and micro-infusion

Rats were anesthetized with 8% chloral hydrate (400 mg/kg, i.p.). Twenty-two-gauge stainless steel guide cannulas were implanted bilaterally into the LA with the guidance of a stereotaxic apparatus (RWD Life Science). The coordinates for LA were anteroposterior − 2.65 mm, mediolateral ± 5.2 mm and dorsovenral − 7.5 mm relative to bregma, according to Paxinos and Watson (1998). The cannulas were anchored to the skull with acrylic dental cement and secured with skull screws. To prevent clogging, a 28-gauge dummy cannula was inserted into the guide cannula. Analgesics were given immediately after surgery and antibiotics treatment was continued for three days. Animals were allowed to recover from surgery for 7–10 days before starting behavioral experiments. For micro-infusion, the dummy cannula was removed from the guide cannula and a 28-gauge infusion cannula, extending 0.8 mm beyond the tip of the guide cannula, was inserted. The infusion cannula was connected via PE20 tubing to a Hamilton micro-syringe driven by a micro-infusion pump (Stoelting Co., USA). Micro-infusion was performed bilaterally with an infusion rate of 0.1 μl/min. The infusion cannula was left in position for an additional 5 min before withdrawal.

Ghrelin (6 μM), GHS-R1a antagonist YIL781 (750 μM), [D-Arg[1], D-Phe[5], D-Trp[7, 9], Leu[11]]-Substance P (D-SP, 10 μM or 500 μM), substance P (SP, 15 μM) and neurokinin-1 (NK1) receptor antagonist L760735 (30 μM) were all dissolved in physiological saline to make the stock and the final infusion solution. All those chemicals were purchased from R&D Systems (Minneapolis, MN, USA). Lithium chloride (LiCl) and saccharin were purchased from Sigma (St Louis, MO, USA). Drug dosages used for micro-infusion as well as the timing of drug administration were chosen according to previous reports or our preliminary results (Bassi et al., 2014; Kertes et al., 2009; Song et al., 2013). Chemicals or vehicle saline (pH 7.4) were locally infused into the LA with equal volume (0.5 μl/side).

2.3. Behavioral procedure

CTA training and test were performed according to previous study (Xin et al., 2014). Briefly, saccharin (0.1% w/v) was used as a novel taste (CS) and intra-peritoneal injection of LiCl (0.15 M, 2% body weight) as the malaise-inducing agent (US). Rats were deprived of water for 24 h, and then habituated over 5 days to obtain their daily water supply within 20 min from two serological pipettes each containing 10 ml of water. Rats with comparable total water intake and body weight on the last day of habituation were randomly assigned to either drug or vehicle group. On training day, all animals were presented with 0.1% saccharin instead of water for 10 min. Twenty minutes later, they were injected intraperitoneally with 0.15 M LiCl solution. Twenty-four hours after conditioning, a multiple-choice memory test was performed to evaluate the acquired aversion to saccharin. Rats were presented with an array of 6 pipettes for 20 min, three containing 5 ml saccharin and three containing 5 ml water in a pseudo-random order. The aversive memory to saccharin were quantified by an aversion index (AI), defined as AI = [(ml of water) / (ml of water + ml of saccharin)] × 100% consumed in the test. The higher AI means the better CTA memory.

The cannula locations were checked by brain tissue sectioning and crystal violet staining after finishing behavioral experiments. Only those animals with bilateral cannula tip placements within the basolateral complex of amygdala (BLA) were included in data analysis.

2.4. Statistical analyses

Data were expressed as mean ± SEM. Statistical analysis was performed with one-way or two-way ANOVA followed by multiple comparisons test instructed by GraphPad Prism 6.0 software. The significance level was set to $p < 0.05$.

3. Results

3.1. Intra-LA infusion of D-SP facilitates CTA acquisition but has no effect on retrieval

We first micro-infused a single dose of D-SP, a potent inverse agonist for GHSR1a, into the LA 20 min before CTA training and tested CTA memory 24 h later (Fig. 1A). We found that rats receiving bilateral infusion of D-SP (8 ng/0.5 μl/side) showed significantly higher AI in comparison to the ones receiving vehicle (normal saline, NS) infusion, indicating that intra-LA infusion of D-SP, a full inverse agonist for GHSR1a, facilitates CTA memory acquisition (Fig. 1B; one-way ANOVA, $F_{(3, 25)} = 47.25$, $p < 0.0001$; Dunnett's multiple comparisons test, D-SP vs. NS group, $p < 0.05$, n = 7 for D-SP group and n = 8 for NS group). In the opposite way, our study consistently showed that intra-LA infusion of ghrelin (10 ng/0.5 μl/side) before training remarkably inhibited CTA memory (Fig. 1B; one-way ANOVA, $F_{(3, 25)} = 47.25$, $p < 0.001$; Dunnett's multiple comparisons test, ghrelin vs. NS group, $p < 0.0001$, n = 7 for ghrelin group and n = 8 for NS group). Since GHS-R1a antagonist YIL781 can completely abolish ghrelin's blockage on CTA acquisition (Song et al., 2013), we also checked whether intra-LA infusion of YIL781 before training affects CTA memory. As shown in

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