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Ghrelin activates hypophysiotropic corticotropin-releasing factor neurons independently of the arcuate nucleus

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ABSTRACT

Previous work has established that the hormone ghrelin engages the hypothalamic–pituitary–adrenal neuroendocrine axis via activation of corticotropin-releasing factor (CRF) neurons of the hypothalamic paraventricular nucleus (PVN). The neuronal circuitry that mediates this effect of ghrelin is currently unknown. Here, we show that ghrelin-induced activation of PVN CRF neurons involved inhibition of γ-aminobutyric acid (GABA) inputs, likely via ghrelin binding sites that were localized at GABAergic terminals within the PVN. While ghrelin activated PVN CRF neurons in the presence of neuropeptide Y (NPY) receptor antagonists or in arcuate nucleus (ARC)-ablated mice, it failed to do it so in mice with ghrelin receptor expression limited to ARC agouti gene related protein (AgRP)/NPY neurons. These data support the notion that ghrelin activates PVN CRF neurons via inhibition of local GABAergic tone, in an ARC-independent manner. Furthermore, these data suggest that the neuronal circuits mediating ghrelin's orexigenic action *vs.* its role as a stress signal are anatomically dissociated.

1. Introduction

Ghrelin is a stomach-derived hormone that potently increases food intake (Kojima and Kangawa, 2005). In addition, ghrelin regulates a variety of stress-related behavioral and neuroendocrine responses that include the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Spencer et al., 2015). In healthy humans, ghrelin administration increases ACTH and cortisol plasma concentrations (Arvat et al., 2001). In mice, ghrelin also increases plasma glucocorticoid levels via activation of the corticotropin-releasing factor (CRF) neurons of the hypothalamic paraventricular nucleus (PVN) (Cabral et al., 2012). Interestingly, intra-PVN ghrelin administration also activates hypophysiotropic CRF neurons and the HPA axis; however, CRF neurons lack expression of ghrelin receptors (GHSRs; growth hormone secretagogue receptors), suggesting that ghrelin action on these neurons is indirect (Cabral et al., 2012). The neuronal circuits through which ghrelin engages neuroendocrine stress responses have yet to be elucidated.

Several pieces of evidence place the hypothalamic arcuate nucleus (ARC) as a leading candidate to mediate ghrelin-induced activation of the PVN CRF neurons. The ARC is strategically located to sense peripheral ghrelin and contains a set of key neurons that co-express not only high levels of GHSR but also the orexigenic neuropeptides agouti-gene-related protein (AgRP) and neuropeptide Y (NPY) as well as γ-aminobutyric acid (GABA) (Schaeffer et al., 2013; Willesen et al., 1999; Zigman et al., 2006). Intact ARC and NPY signaling are required for the orexigenic effects of peripheral ghrelin (Cabral et al., 2014; Nakazato et al., 2001), and selective expression of GHSR in AgRP/NPY/GABA neurons is sufficient to partially mediate ghrelin-induced food intake. NPY neurons strongly innervate PVN CRF neurons (Li et al., 2000), which in turn are activated by NPY (Dimitrov et al., 2007; Sarkar and Lechan, 2003). Thus, NPY released from GHSR-expressing ARC neurons

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could potentially mediate ghrelin-induced activation of the CRF neurons.

In addition, PVN CRF neurons are heavily innervated and regulated by GABA inputs that provide a substantial inhibitory tone (Cole and Sawchenko, 2002; Park et al., 2007; Ulrich-Lai and Herman, 2009). GABA inputs to the PVN are provided by extrahypothalamic areas, such as the bed nucleus of the stria terminalis (BNST), as well as numerous hypothalamic nuclei, including the ARC, the anterior hypothalamic area (AHA), the medial preoptic area (mPOA), the dorsomedial hypothalamus (DMH), the periventricular nucleus (Pe) and the PVN itself (Herman et al., 2003). Some of these brain nuclei express GHSR (Cabral et al., 2013; Zigman et al., 2006); however, it is currently unknown whether ghrelin can directly engage these GABA neurons.

The current study was designed to dissect the neuronal components mediating ghrelin-induced activation of PVN CRF neurons. This knowledge is essential in order to understand the role of this hormone as a stress signal.

2. Material and methods

2.1. Animals

Male mice were housed in a 12-h light/dark cycle with regular chow (4 g% fat) and water available *ad libitum*. All experiments were performed using 3–4 month old mice (21–27 g body weight) on a pure C57BL/6J background and received approval from the Institutional Animal Care and Use Committees of UTSW (ID:1090-06-06-1) or the IMBICE (ID:10-0112). In order to generate the ARC-ablated mice, 4-day old pups were subcutaneously (SC) injected with either monosodium glutamate (2 mg/g body weight (BW), Sigma–Aldrich, cat. G1626) or saline (ARC-ablated or ARC-intact mice, respectively). In order to study mice with GHSR expression only in AgRP neurons, we crossed AgRP-CreER^{T2} transgenic and GHSR-null mice (Wang et al., 2014). Study mice included: wild-type (2 wild-type GHSR alleles without AgRP-CreER^{T2}), wild-type/AgRP-CreER^{T2} (2 wild-type GHSR alleles with 1 copy of AgRP-CreER^{T2}), GHSR-null/AgRP-CreER^{T2} (2 GHSR-null alleles and 1 copy of AgRP-CreER^{T2}) and GHSR-null (2 GHSR-null alleles without AgRP-CreER^{T2}). These genetically modified mice were treated with tamoxifen (150 mg/kg body weight, i.p., Sigma–Aldrich, cat.T5648) between the ages of 5–7 weeks, for 5 consecutive days, and then used for experiments 10 days after the final tamoxifen administration (Wang et al., 2014).

2.2. In vivo *experimental procedures*

All surgeries were performed as previousy reported (Cabral et al., 2012). First, mice were intracerebroventricularly (ICV)-injected with phosphate-buffered saline (PBS) alone or containing ghrelin (0.3 nmol/mouse) and perfused 2-h after treatment ($n = 5$ per group). In an independent experiment, ICV ghrelin- or vehicle-treated animals were sacrificed by decapitation 30-min after treatment ($n = 6$ per group). Blood samples were collected and circular PVN "punches" were excised using a 15 g needle (see below).

To block NPY signaling, mice were ICV-injected with vehicle 1 (4% DMSO in PBS) or a combination of Y1 and Y5 NPY receptor antagonists (BIBO3304 and CGP71683-Tocris Biosicence Cat. 2412 and 2199, respectively, 1 μg each/mouse). After 30-min, mice of each group were ICV-treated with either vehicle 2 (PBS) or ghrelin (0.3 nmol/mouse) and perfused 2-h later ($n = 4$–5 per group). Pilot studies, based on a previous study (Polidori et al., 2000), showed that this dose of the Y1 and Y5 antagonists mix fell within the lower range of doses that had the capacity to decrease rebound food intake in a fast-refeeding paradigm without affecting basal c-Fos levels in the PVN; the timing of the study was chosen because a food intake inhibition is detected in this time window. Mice were perfused 2-h after treatment.

To block GABA signaling, mice were intra-PVN administered with saline or muscimol (250 ng/side, Sigma–Aldrich, cat. 2763-96-4). After 3 min, mice of each group were ICV-treated with either PBS or ghrelin (0.3 nmol/mouse, $n = 6$ per group). The pretreatment conditions were chosen based on a previous study (Cullinan et al., 2008). Additional mice were intra-PVN injected with muscimol, as described above, and 3-min later intra-PVN treated with MTII (1 μg/side, Phoenix Pharmaceutical, cat. 043-23, $n = 3$). Mice were perfused 2-h after treatment.

ARC-intact and ARC-ablated mice were injected with saline or ghrelin (0.6 nmol/g BW, SC) and perfused 2-h later ($n = 6$–9 per group). A different set of ARC-intact and ARC-ablated mice ($n = 4$ per group) were used for the ARC lesion validation.

Adult tamoxifen-treated wild-type/wild-type, wild-type/AgRP-CreER^{T2}, GHSR-null/AgRP-CreER^{T2} and GHSR-null/wild-type mice were also injected with PBS or ghrelin (0.6 nmol/g BW, SC) and perfused 2-h later ($n = 4$–5 per group) (Wang et al., 2014).

All experiments were performed in the morning, between 0830 h and 1130 h. In all experiments, singly-housed mice were exposed to a pre-weighed amount of chow before treatment and food intake was calculated by subtracting the weight of added food from the weight of food remaining at the end of the experiment. Cannula position was verified at the end of all the experiments by visualization of the injection cannula tracts.

2.3. Assessment of plasma corticosterone and CRF mRNA in PVN punches

These procedures have been described in detail before (Cabral et al., 2012). Corticosterone plasma concentration was measured using an ELISA kit according to the manufacturer's protocol (Assay Designs). For the CRF mRNA levels quantification, total RNA from PVN punches was isolated and quantified by absorbance at 260 nm. Total RNA was reverse-transcribed into cDNA with random hexamer primers and SuperScript II reagents (Invitrogen). Quantitative PCR was performed using SYBR-green chemistry (Applied Biosystems). The CRF mRNA levels are calculated by the comparative threshold cycle method and expressed relative to the housekeeping gene Cyclophilin A. Standard curves for CRF and Cyclophilin A transcript levels were generated using hypothalamic cDNA of mouse. Primer sequences for CRF: Sense: 5′-TCTGGATCTCACCTTCCACCT-3′, Antisense: 5′-CCATCAGTTTCCTGTTGCTGT-3′. Primer sequences for Cyclophilin A: Sense: 5′-TGGTCTTTGGGAAGGTGAAAG-3′, Antisense: 5′-TGTCCACAGTCGGAAATGGT-3′. Averaged levels of CRF normalized to Cyclophilin A in each experimental group were compared with similar values obtained from vehicle-treated mice to determine relative expression levels.

2.4. Assessment of c-Fos and c-Fos/CRF co-localization

As previously described in detail (Cabral et al., 2012), brains were removed from perfused mice, and coronally cut at 25 μm. For immunostaining, sections were pretreated with H_2O_2, treated with blocking solution and incubated with anti-c-Fos antibody (Calbiochem, cat. PC38, 1:15,000) for 2 days at 4 °C. Then, sections were incubated with biotinylated anti-rabbit antibody (Vector Laboratories, cat. BA-1000, 1:1,500) for 1 h and with Vectastain Elite ABC kit (Vector Laboratories, cat. PK-6200) according to manufacturer's protocols. Finally, visible signal was developed with diaminobenzidine (DAB)/Nickel solution (Sigma–Aldrich, cat. 32750), which generated a purple-black precipitate. Double c-Fos and CRF immunostaining was performed on independent brain