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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.

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Keywords

stress; ghrelin; orexigenic; γ-aminobutyric acid (GABA); neuropeptide Y (NPY)

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 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 extra-hypothalamic 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 0830h and 1130h. 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 series containing the PVN. In this case, c-Fos immunostained sections were then incubated with a rabbit anti-CRF antibody (1:2,000) for 48 h, and then sequentially incubated with the secondary antibody and the Vectastain Elite ABC kit, as detailed above. Finally, visible signal was developed with DAB solution without nickel, which generates a brown precipitate. Sections were sequentially

mounted, and bright-field images were acquired with a DS-Ri1 Nikon digital camera. To determine the total number c-Fos-immunoreactive (IR) cells in the ARC and the PVN, cells containing distinct nuclear black precipitate were quantified between bregma −1.58 and −1.94 mm for the ARC, and between bregma −0.70 and −0.94 mm for the PVN. Anatomical limits of each brain region were identified using a mouse brain atlas (Paxinos and Franklin, 2001). C-Fos data were expressed as total c-Fos-IR cells per ARC/side coronal section. To estimate the amount of CRF-IR cells positive for c-Fos, all CRF-IR cells positive and negative for c-Fos were counted in the PVN. The results were expressed as a percentage, which represents CRF-IR neurons positive for c-Fos compared to the total number of CRF-IR neurons. Data were corrected for double counting, according to the method of Abercrombie (Abercrombie, 1946) where the ratio of the actual number of neurons to the observed number is represented by T/T+h where T=section thickness, and h=the mean diameter of the neuron along the axis perpendicular to the plane of section. The mean diameter of the neurons was determined with the image analysis software program ImageJ (NIH). Blind quantitative analysis was performed in one series per animal and by two observers.

2.5 Visualization of ghrelin binding sites

Here, a fluorescein-ghrelin[1–18] (hereafter referred to as F-ghrelin) tracer was used. F-ghrelin is an 18 amino acid analog of the hormone with fluorescein attached at its C-terminus. F-ghrelin is fully bioactive *in vivo*, binds to the mouse brain in a similar pattern as seen for the GHSR mRNA distribution and fails to label brain nuclei of GHSR-null mice (Cabral et al., 2013). Here, anesthetized mice were ICV-injected with F-ghrelin (0.3 nmol/mouse) and perfused with fixative 15 min after treatment. Brains were processed in order to generate coronal brain sections, which were then used for double immunostaining against fluorescein and glutamic acid decarboxylase 67 (GAD67). In particular, brain sections were incubated with an anti-fluorescein antibody conjugate to Alexa Fluor 488 (Molecular Probes, cat. A-11096, 1:100) and with a mouse anti-GAD67 antibody (1:1,500, Millipore, Mab 5406, cat. 92590) at 4°C. After 48 h, sections were washed and incubated with an anti-mouse antibody conjugate to Alexa Fluor 594 (Molecular Probes, cat. A-11096, 1:1,000) for 2 h. Then, sections were mounted and coverslipped with a mounting media containing Hoechst dye. Fluorescent photomicrographs were acquired with a laser scanning Olympus confocal microscope (Olympus FV1000) with emission filters of: 490–540 nm, for Alexa488 detection (laser 473 nm), 575–675 nm for Alexa594 detection (laser 559 nm) and 430–455 nm for Hoechst detection (laser 405 nm). In order to estimate the amount of F-ghrelin binding to GAD67-IR terminals, the total numbers of green and double green/red dots surrounding the cell nuclei were counted in the high magnifications images of the PVN. The relationship was expressed as percentages, which represents double green/red dots compared to the total number of green dots observed.

2.6 *Ex-vivo* determination of [^{3}H]-GABA release from PVN explants

Brains were extracted from euthanized mice, placed in cold PBS, and sectioned into 1-mm coronal slices by using a mouse brain matrix. Circular punches containing the PVN (thereafter named PVN explants) were excised using a 15-g needle from the corresponding known location of this nucleus based on a mouse brain atlas (Paxinos and Franklin, 2001).

The PVN explants were incubated in Krebs-Ringer Bicarbonate Buffer (KRBB) saturated with 95% O_2 and 5% CO_2 at 37°C through the whole experiment. Initially, PVN explants were maintained 20 min in KRBB for stabilization. Then, PVN explants were incubated with [^{3}H]-GABA (~400 cpm/mL, 92.1 Ci/mmol, Perkin Elmer) in KRBB for 20 min, in order to fill the synaptic vesicles with the tracer, and washed two times with fresh KRBB. For the study, PVN explants were initially incubated with 200 μL of fresh KRBB for 10 min and the medium was collected. Then, PVN explants were incubated with 200 μL of fresh KRBB containing 56 mM KCl and the medium was also collected. This protocol was performed in the presence or absence of ghrelin (100 nM) during both basal and KCl-stimulated periods (n=5 per group). For radioactivity quantification, collected medium samples were mixed with 150 μl of scintillator (Ecolite) and analyzed in a β-counter (Tracor Analytic).The [^{3}H]-GABA release was expressed as a percentage relative to the average basal release for each condition.

2.7 Assessment of dual GAD67 mRNA expression and ghrelin binding sites

Here, *in situ* hybridization histochemistry (ISHH) for GAD67 and immunostaining for F-ghrelin were performed in brain sections of mice ICV-treated with the tracer. First, mice were ICV-treated with F-ghrelin (0.3 nmol/mouse) and perfused 15 min later, as described above. Then, brain sections were generated and processed for chromogenic immunostaining against fluorescein (Cabral et al., 2013). In particular, sections were pretreated with 0.5% H_2O_2 and blocking solution. Next, sections were incubated with an anti-fluorescein antibody (Molecular Probes, cat. A-11095, 1:1,500) 48 h at 4°C, and then sequentially incubated with a secondary antibody and the Vectastain Elite ABC kit, as detailed above. Finally, visible signal was developed with DAB solution. Then, stained sections were mounted, desiccated overnight and processed for ISHH against GAD67 as described before in detail (Rodriguez-Molina et al., 2014). Briefly, sections were hybridized with a 572 bp single stranded [^{35}S]–UTP (Perkin-Elmer NEG 039H) labeled RNA probe, complementary to the rat GAD67 cDNA [nucleotides 1,265–1,836 of NM_017007.1 sequence], donated by Dr. Petersen (Hays et al., 2002). Tissues were prehybridized in 50% formamide (Invitrogen, cat. 15515-026)/2x SSC. Hybridizations were performed in a buffer containing 50% formamide, 2X SSC, 10% dextran sulfate (D8906, Sigma), 1X Denhardts [0.25% BSA (A4503, Sigma), 0.25% Ficoll 400, 0.25% polyvinylpyrollidone], 0.25% 1M Tris (Trisma base T6066, Sigma)-HCl pH 8.0, 0.5% sodium dodecyl sulfate (Cat. 161-0301, Biorad), 250 μg/mL denatured salmon sperm DNA (D7656, Sigma) and ~500,000 cpm of the radiolabeled probe for 16 h at 54°C. Subsequently, sections were rinsed in 1x SSC, treated with RNase A solution (50 μg/mL, R5503, Sigma) and washed with 1x, 0.5x and 0.1x SSC at 65°C. Finally, sections were dried, exposed to an autoradiographic film for 5 days and then dipped into NTB autoradiography emulsion (Cat. 8895666, Kodak). Autoradiograms were developed after 54 days of exposure at 4°C. Sections were viewed using both bright-field and dark-field illumination; photomicrographs were acquired with a DS-Ri1 Nikon digital camera. The visualization of silver grains overlying the shape of DAB-stained cells was used as a criterium to determine if a fluorescein-IR cell co-expressed GAD67 mRNA. An image editing software program, Adobe PhotoShop 7.0, was used to adjust sharpness, contrast and brightness in the photomicrographs. Analysis was performed in the pBNST, the AHA, the mPOA, the Pe, the DMH, the PVN and the ARC.

2.8 Validation of ARC-ablated mouse model

The ARC lesion was confirmed by ARC cell nuclei counting after thionin staining and NPY immunostaining, as described elsewhere (Cabral et al., 2014). In order to visualize cell nuclei, brain sections were stained with thionin (Sigma, cat. T7029). In order to perform NPY immunostaining, brain sections were incubated with anti-NPY antibody (Abcam, cat. ab30914, 1:10,000). Then, immunoreactive signal was visualized using the chromogenic staining described above.

2.9 Statistical analyses

Data are expressed as the mean±SEM. Equality of variance was analyzed using Bartlett's test. T-test was performed in order to compare data from vehicle- vs. ghrelin-treated mice in the first experiment as well to compare data from naïve ARC-intact vs. ARC-ablated mice. One-way ANOVA followed by the Newman-Keuls test was used to compare *ex vivo* GABA release and ghrelin's effects in experiments with genetically modified mice. Two-way ANOVA followed by the Bonferroni test was used to compare the rest of the studies. Significant differences were considered when $p<0.05$.

3. RESULTS

3.1 Ghrelin-induced activation of PVN CRF neurons does not require NPY signaling and depends on GABA signaling

We first confirmed that ICV ghrelin activates PVN CRF neurons and the HPA axis, choosing a smaller dose than had previously been used (Cabral et al., 2012). We found that ICV ghrelin increased the percentage of CRF-IR neurons positive for c-Fos ($p<0.01$ Figure 1A–B) as well as PVN CRF mRNA levels and plasma corticosterone levels ($p<0.05$, Figure 1C–D).

In order to test whether NPY signaling is required for ghrelin-induced activation of CRF neurons, a combination of Y1 and Y5 antagonists was ICV-administered to mice that were subsequently ICV-treated with ghrelin. Importantly, no significant interactions between pretreatment and treatment factors were found for the food intake, the number of c-Fos-IR cells in the ARC (Figure 2A) and the percentage of PVN CRF-IR cells positive for c-Fos (Figure 2B–C). The NPY receptor antagonists significantly affected ghrelin-induced food intake. In particular, the NPY receptor antagonists failed to affect 2-h food intake in vehicle-treated mice (86±20 vs. 50±30 mg) but significantly reduced the ghrelin-induced food intake (405±40 vs. 232±46 mg, $p<0.05$). In contrast, the NPY receptor antagonists failed to affect ghrelin-induced increase of either ARC c-Fos-IR or the percentage of PVN CRF-IR cells positive for c-Fos.

In order to test the possibility that ghrelin activates PVN CRF neurons via inhibition of the local GABAergic system, we reasoned that the hormone should be unable to activate these neurons if the GABAergic tone is pharmacologically maintained. Thus, mice were intra-PVN injected with the GABA A receptor agonist muscimol, and subsequently ICV-treated with ghrelin. Here, a significant interaction between pretreatment and treatment was found for the food intake factor; muscimol failed to affect 2-h food intake in vehicle-treated mice

(66±19 vs. 38±31 mg) but significantly reduced the ghrelin-induced food intake (322±42 vs. 20±16 mg, $p<0.05$). Importantly, no interaction was found for the number c-Fos-IR cells in the ARC and intra-PVN muscimol failed to affect this ghrelin-induced effect (Figure 2D). In contrast, a significant interaction between pretreatment and treatment was found for the percentage of CRF-IR neurons positive for c-Fos in PVN, where intra-PVN muscimol impaired this ghrelin effect ($p<0.01$, Figure 2E–F). Since local administration of muscimol can result in temporary neuronal inactivation that could unmask the action of ghrelin (Majchrzak and Di Scala, 2000), we tested if CRF neurons were responsive to other postsynaptic inputs. Thus, another set of mice was intra-PVN injected with muscimol and subsequently treated with MTII, a melanocortin-4 receptor agonist known to directly act on CRF neurons. In the presence of muscimol, MTII increased the percentage CRF-IR cells positive for c-Fos as compared to vehicle (50.4±8.0 and 33.8±6.4 %, respectively, $p<0.05$). Thus, these data suggest that ghrelin requires inhibition of GABA signaling in order to disinhibit, or rather, activate the CRF neurons.

3.2 Ghrelin binds to GABAergic terminals within the PVN and inhibits *ex vivo* GABA release

Next, we mapped ghrelin binding sites within the PVN following ICV administration of a fluorescein-labeled ghrelin analog. F-ghrelin labeling was scarcely observed in cell bodies within the PVN and predominantly detected with a presynaptic bouton-shape (Figure 3A). In order to confirm the presynaptic localization of the F-ghrelin binding and also to define the type of terminals, immunostaining against GAD67, a marker of GABA neurons highly enriched in terminals, was also performed. Quantitative estimations indicated that ~100% F-ghrelin binding co-localize with GAD67-IR signal (Figure 3B–E).

In order to directly test if ghrelin affects GABA neurosecretion, the *ex vivo* release of this neurotransmitter from PVN explants was studied (Figure 3F). As expected, depolarization of synaptic terminals induced by high KCl medium significantly increased [^{3}H]-GABA secretion as compared to basal levels ($p<0.01$). The presence of ghrelin in the incubation medium did not significantly affect basal [^{3}H]-GABA release; however, it did reduce KCl-stimulated [^{3}H]-GABA release.

3.3 Several populations of hypothalamic GABA neurons known to innervate the PVN are labeled by F-ghrelin

In order to map which populations of GABA neurons could mediate ghrelin actions in the PVN, we performed double ISHH for GAD67 mRNA and immunostaining for F-ghrelin in brain section of mice previously injected with this tracer. Either GAD67 mRNA-positive or F-ghrelin labeled cells were found in the same brain regions as previously shown (Bowers et al., 1998; Cabral et al., 2013). Among the brain nuclei known to innervate the PVN (Ulrich-Lai and Herman, 2009), cells positive for both GAD67 mRNA signal and F-ghrelin were detected in the mPOA, the Pe, the DMH and the ARC (Figure 4). GAD67 mRNA expression was also detected in the BNST and in cells surrounding the PVN; however, no co-localization with F-ghrelin binding was detected in these areas.

3.4 Peripherally-administered ghrelin activates PVN CRF neurons in ARC-ablated mice

To assess the requirement of the ARC for ghrelin-induced activation of the CRF neurons, ARC-ablated mice were generated (Cabral et al., 2014). Body weights of ARC-ablated and ARC-intact mice were not statistically different (23.7±0.4 and 24.4±0.5 g, respectively). ARC-ablated mice had a significant decrease of the number of thionin-stained cells in the ARC (Cabral et al., 2014) but showed no significant changes in the PVN, as compared to numbers found in ARC-intact mice (193±13 and 209±31, respectively). ARC-ablated mice also had a decrease of the NPY-IR signal intensity in both the ARC and the PVN as compared with values found in the same regions of ARC-intact mice (17.1±7.3 vs. 100±7.1% for the ARC and 38.4±4.9 vs.100±26.9% for the PVN, respectively, $p<0.05$; Figure 5A). In response to ghrelin treatment, significant interactions between group and treatment factors were detected for the food intake and the number c-Fos-IR cells in the ARC. As previously reported, ghrelin failed to increase either food intake (not shown, (Cabral et al., 2014)) or the number of c-Fos-IR cells in the ARC of ARC-ablated mice (Figure 5B and D). Conversely, no significant interactions were detected at the PVN level, where ARC-ablated mice showed a significant increase in the number of c-Fos-IR cells in response to ghrelin (Figure 5C). In particular, ARC-intact and ARC-ablated mice showed similar numbers of c-Fos-IR cells in the PVN in response to vehicle (20±6 and 37±11 cells/side, respectively) and ghrelin treatment (144±35 and 138±18 cells/side, respectively). ARC-ablated mice also remain fully responsive to the ghrelin-induced increase in the percentage of PVN CRF-IR cells positive for c-Fos (Figure 5E).

3.5 Peripherally-administered ghrelin fails to activate PVN CRF neurons in mice expressing GHSR exclusively in ARC AgRP neurons

To investigate the sufficiency of ghrelin signaling on ARC neurons to activate the PVN CRF neurons, we used a mouse model with GHSR expression limited to AgRP neurons (Wang et al., 2014). These mice were generated by crossing GHSR-null mice, which contain a loxP-flanked transcriptional blocking cassette inserted into the *ghsr* gene, to mice in which expression of a tamoxifen-inducible Cre recombinase is driven by the AgRP promoter (Wang et al., 2014). Experimental mice included wild-type mice without the AgRP-CreER^{T2} transgene, wild-type/AgRP-CreER^{T2} mice, GHSR-null mice without the AgRP-CreER^{T2} transgene, and GHSR-null/AgRP-CreER^{T2} mice, which express endogenous levels of GHSR only upon tamoxifen exposure exclusively in AgRP neurons programmed to express GHSR (Figure 6A). We have previously reported that these different genotypes displayed the predicted patterns of GHSR expression and similar average body weights (Wang et al., 2014). Also as reported, while GHSR-null mice failed to increase food intake in response to ghrelin, GHSR-null/AgRP-CreER^{T2} mice increased food intake in response to ghrelin by ~65% of that induced by ghrelin in wild-type mice (Wang et al., 2014). In new analyses, we now demonstrate an increase in the number of c-Fos-IR cells in response to peripheral ghrelin in the ARC of wild-type/AgRP-CreERT2 and GHSR-null/AgRP-CreERT2 mice, but not in GHSR-null mice (Figure 6B–D). However, a different result was observed in the PVN, as GHSR-null/AgRP-CreER^{T2} mice failed to increase the number of c-Fos-IR cells in response to peripheral ghrelin (Figure 6C). In particular, GHSR-null/AgRP-CreER^{T2}mice showed similar numbers of c-Fos-IR cells in the PVN in response to

ghrelin, as compared to ghrelin-treated GHSR-null mice (6±1 and 10±4 cells/section, respectively). GHSR-null/AgRP-CreER^{T2}mice also failed to show a ghrelin-induced increase of the percentage of CRF-IR cells positive for c-Fos in the PVN (Figure 6E).

4. DISCUSSION

Our results indicate that ghrelin-induced activation of CRF neurons does not require NPY signaling but rather involves a decrease of GABA signaling within the PVN due to presynaptic actions of the hormone. Ghrelin sensitive GABA neurons were localized in several hypothalamic nuclei known to provide inputs to the PVN, including the ARC, Pe, mPOA and DMH. However, we found that peripherally-administered ghrelin fully activates PVN CRF neurons in ARC-ablated mice while it fails to activate PVN CRF neurons of mice with GHSR expression limited to ARC AgRP neurons. Thus, we propose that ghrelin activates PVN CRF neurons via inhibition of local PVN GABAergic tone, in an ARC-independent manner.

We had previously shown that intra-PVN ghrelin fully activates hypophysiotropic CRF neurons but fails to activate ARC neurons or induce appetite (Cabral et al., 2012). Now, we provide new evidence indicating that ghrelin-induced activation of PVN CRF neurons occurs independently of ghrelin-engaged ARC neurons controlling food intake. Table 1 summarizes both our current results and those from our previous, related studies. Importantly, some considerations should be acknowledged for each of these experimental models when interpreting the results. For instance, we used a combination of Y1 and Y5 antagonists to achieve NPY signaling blockade because these NPY receptors are highly expressed in the PVN and can mediate the NPY-induced activation of CRF neurons (Cowley et al., 2003; Parker and Herzog, 1999). This pharmacological strategy was successful – as evidenced by its impairment of ghrelin-induced food intake (Lawrence et al., 2002; Shintani et al., 2001) –, and its failure to affect ghrelin-induced activation of PVN CRF neurons suggests that NPY signaling does not mediate this effect of ghrelin. However, the potential involvement of other NPY receptors also expressed in the PVN cannot be discarded (Parker and Herzog, 1999). In another set of experiments, we infused muscimol in the PVN and found that ghrelin was unable to activate CRF neurons when GABA signaling was locally manipulated. We interpreted this result as suggesting that inhibition of GABA release mediates the effect of ghrelin on PVN CRF neurons. However, the unexpected lack of ghrelin-induced food intake in muscimol-treated mice also suggests the possibility that muscimol induces other effects that may secondarily impact CRF neuronal activity. A third example involves the data obtained with the ARC-ablated mice, which we interpreted as suggesting that an intact ARC is not required for ghrelin-induced activation of PVN CRF neurons. The potential exists with the ARC ablation model for a number of off-target effects. In order to minimize potential caveats of this model, we tested young animals that lack confounding metabolic and hormonal abnormalities known to occur in older ARC-ablated animals (Cabral et al., 2014; Perello et al., 2004). Also, it appears as if we avoided any unintended functional alterations of non-ARC regions mediating ghrelin-induced food intake, since the ARC-ablated mice fully responded to the orexigenic effect of ICV ghrelin, and any unintended alterations in ghrelin's accessibility to the brain, since their median eminence remained intact and they showed a full c-Fos induction in the PVN in response to

peripherally administered ghrelin (Cabral et al., 2014). A final example relates to the mice with GHSR re-expression achieved selectively in AgRP neurons, in which no ghrelin-induced PVN CRF neuronal activation was observed. While this observation is most consistent with ghrelin's effects on the PVN not involving GHSR-expressing AgRP ARC neurons, the potential failure to achieve GHSR re-expression in the full complement of usual GHSR-expressing AgRP neurons may have impacted the result (Wang et al., 2014). While these considerations must keep in mind, our data strongly show that neuronal circuits mediating ghrelin's roles as an orexigenic signal and as a stress signal can be dissociated.

Currents study provides a potential mechanism by which ghrelin would activate the PVN CRF neurons. GABAergic terminals within the PVN are known to provide an inhibitory tone that plays a prominent role in the relay and integration of stress-related information (Herman et al., 2004). Intra-PVN administration of the GABA-A antagonist bicuculline activates the HPA axis whereas the intra-PVN administration of muscimol inhibits stress-induced HPA axis activation (Cullinan et al., 2008; Kovacs et al., 2004). We have shown that PVN CRF neurons lack GHSR and that intra-PVN ghrelin activates them (Cabral et al., 2012). In addition, ghrelin has been shown to inhibit inhibitory postsynaptic currents in the PVN (Cowley et al., 2003). Here, we found not only that ghrelin requires an inhibition of local GABA signaling in order to activate the CRF neurons but also that ghrelin binds almost exclusively to GABA terminals. *Ex vivo*, we found that ghrelin inhibits the KCl-stimulated GABA release in PVN explants. High KCl concentrations stimulate neurosecretion by inducing depolarization of the plasma membrane that in turns open voltage-operated calcium channels and allow the influx calcium into the presynaptic terminals (Catterall and Few, 2008). Recently, we have shown that ghrelin inhibits presynaptic calcium channels (Lopez Soto et al., 2015). Thus, our observations support a model in which ghrelin directly inhibits the local GABA tone within the PVN, presumably via inhibition of presynaptic calcium channels, to further activate the CRF neurons.

In order to determine the source of ghrelin-responsive GABA inputs to the PVN, we determined whether brain areas known to innervate the PVN contain GAD67-expressing cells that also bind F-ghrelin. We found that GABA neurons located in several hypothalamic areas, including the ARC, the Pe, the DMH and the MPOA, may provide the ghrelin-responsive terminals present in the PVN. As discussed above, the ARC does not seem to mediate ghrelin-induced activation of PVN CRF neurons. Interestingly, ARC NPY/AgRP neurons are GABAergic, and GABA release by them is required for ghrelin-induced food intake (Tong et al., 2008). In addition, pharmacological blockade of NPY signaling reduced ghrelin-induced food intake but failed to affect ghrelin-induced activation of PVN CRF neurons. Thus, NPY and GABA signaling by NPY/AgRP/GABA neurons seems to mediate the orexigenic, rather than stress-related, actions of ghrelin. This notion was not necessarily expected since the ARC expresses high levels of GHSR and is highly accessible to circulating ghrelin (Cabral et al., 2014; Zigman et al., 2006). Moreover, ARC NPY neurons strongly innervate PVN CRF neurons (Li et al., 2000), which express NPY receptors and are activated by NPY (Dimitrov et al., 2007; Sarkar and Lechan, 2003), and ghrelin induces NPY secretion in the PVN (Cowley et al., 2003). It is interestingly to note, however, that NPY also acts on GABA presynaptic terminals in the PVN and inhibits inhibitory

postsynaptic currents (Cowley et al., 1999; Cowley et al., 2003). Thus, ghrelin-induced NPY release in the PVN could amplify the inhibitory effect of ghrelin on GABA release and the subsequent activation of CRF neurons under physiological conditions. In contrast, NPY may be unable to further amplify ghrelin action on CRF neurons when ghrelin is exogenously administered. Further studies using experimental conditions that induce physiologic increases in ghrelin may help clarify the relevance of the NPY system as a mediator of some ghrelin actions. Independently of these considerations, our results strongly point to non-ARC GABA neurons as key mediators of ghrelin-induced activation of CRF neurons.

The ghrelin system plays a key role signaling stress to the brain (Spencer et al., 2015) Plasma ghrelin levels increase in human beings exposed to psychosocial or standardized trier social stress (Rouach et al., 2007) as well as in rodents exposed to different stress paradigms (Asakawa et al., 2001; Chuang et al., 2011; Ochi et al., 2008). The role of ghrelin signaling on stress responses is complex and seems to differ under basal, stressed or chronic conditions (Chuang and Zigman, 2010; Spencer et al., 2015). In rodents, ghrelin administration activates a variety of stress-related behavioral and neuroendocrine responses. The ghrelin-induced increase of plasma glucocorticoids levels likely helps to cope with stress since glucocorticoids – similarly to ghrelin— increase glycemia, stimulate adiposity and enhance motivated behaviors (Dallman, 2010; Tschop et al., 2000; Tung et al., 2004). Notably, ghrelin signaling seems to be required for full activation of the HPA axis under some stress conditions (Chuang et al., 2011; Spencer et al., 2012). Interestingly, some of the ghrelin-induced anxiety-like behaviors in rodents were shown to be inhibited by a CRF receptor antagonist (Asakawa et al., 2001; Carlini et al., 2002; Currie et al., 2012). However, such behaviors are likely mediated by non-PVN CRF systems since the vast majority of PVN CRF neurons are hypophysiotropic (Wamsteeker Cusulin et al., 2013). In line with this possibility, it has been shown that some stress-related fear-modulating effects of ghrelin do not involve the HPA axis activity but rather the amygdala (Meyer et al., 2014), where CRF neurons are also present (De Francesco et al., 2015). Thus, future studies are required to understand the interplay between the neuronal circuits mediating the effects of ghrelin on stress-related behavioral and neuroendocrine responses.

In summary, our current data support the notion that ghrelin-induced activation of PVN CRF neurons is independent of the ARC nucleus and ghrelin-induced food intake. A dissociation of neuronal circuits mediating orexigenic or stress-related effects of ghrelin is not unexpected since we have already shown that ghrelin's effects on food reward and homeostatic eating are also dissociated (Perello et al., 2010). Although the physiological implications of such dissociations are unclear, it can be hypothesized that engagement of these dissociated neuronal circuits by ghrelin may depend on the levels of plasma ghrelin achieved in different conditions. For instance, we have shown that small increments of circulating ghrelin, similar to those induced by short-term fasting, mainly impact the ARC while higher increments in circulating ghrelin, such as those found after severe caloric restriction, are also able to impact other brain areas including the PVN (Cabral et al., 2015). The pharmacologic implications of these dissociations are perhaps more clear and definitely exciting, especially in terms of the design of future therapies for which targeting of ghrelin's effects on food intake may be desired without impacting ghrelin action on stress responses.

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Highlights

- Ghrelin-induced activation of PVN CRF neurons does not require NPY signaling and depends on GABA signaling.
- Ghrelin receptor is located in GABAergic terminals of the PVN, where ghrelin inhibits GABA release
- Ghrelin activates PVN CRF neurons but does not increase food intake in ARC-ablated mice.
- Ghrelin increases food intake but does not activate PVN CRF neurons in mice with GHSR expression in ARC AgRP neurons only
- Thus, the neuronal circuits mediating ghrelin's role as an orexigenic vs. a stress signal can be anatomically dissociated.

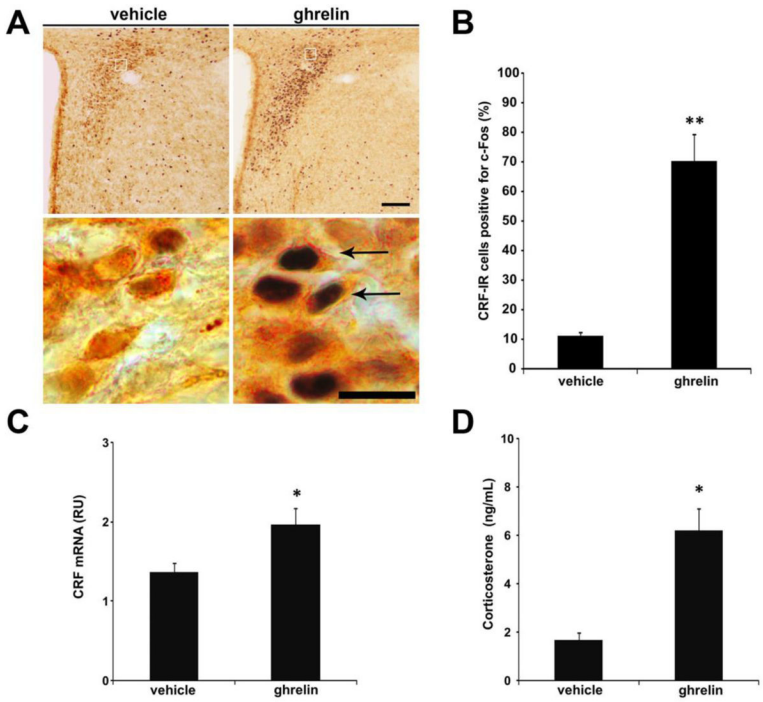

Figure 1. Ghrelin administration activates PVN CRF neurons and the HPA axis

Panel A shows representative photomicrographs of brains sections subjected to double immunohistochemistry using anti-CRF (brown staining) and anti-c-Fos (black staining) antibodies. Upper and bottom panels show low and high magnification images, respectively. Left and right images are from a vehicle- or a ghrelin-treated mouse, respectively. Arrows point to dual-labeled cells. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article. Scale bars, 100 μm (low magnification), 10 μm (high magnification). Panel B shows the percentage of CRF-IR cells positive for c-Fos in the PVN for each experimental group. Panel C shows comparative values of CRF mRNA, relative to the gene Cyclophilin A, in the PVN punches obtained from vehicle- or ghrelin-treated mice. Panel D shows comparative values of plasma corticosterone obtained from vehicle- or ghrelin-treated mice. Data represent the mean ±SEM. *, $p \leq 0.05$ vs. vehicle-treated mice. **, $p \leq 0.01$ vs. vehicle-treated mice.

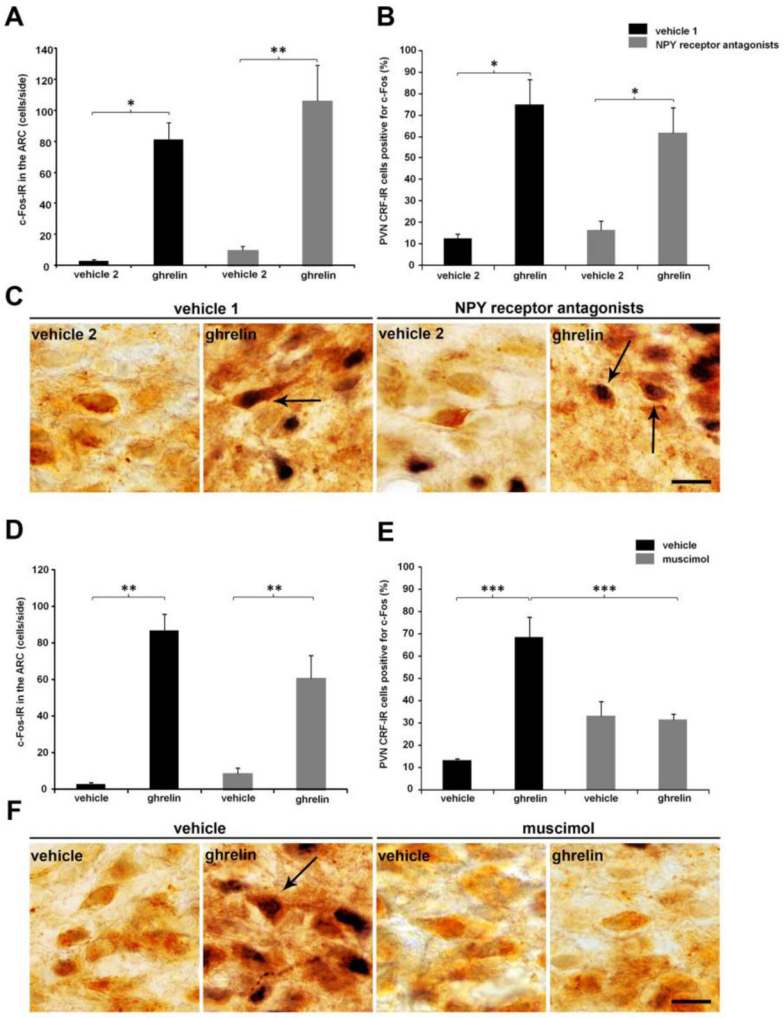

Figure 2. Ghrelin-induced activation of PVN CRF neurons does not require NPY signaling and depends on GABA signaling

Panels A, B and C display data of mice that were initially ICV-treated with vehicle containing or not a combination of selective Y1 and Y5 NPY receptor antagonists (BIBO3304 and CGP71683) and subsequently ICV-treated with vehicle or ghrelin. Panels D, E and F display data of mice that were initially intra PVN-treated with saline containing or not a GABA receptor agonist (muscimol) and subsequently ICV-treated vehicle or ghrelin. Panels A and D show bar graphs displaying the number of c-Fos-immunoreactive cells in the ARC in each experimental condition. Panels B and E show bar graphs displaying the percentage of CRF-IR cells positive for c-Fos in the PVN in each experimental condition. Panels C and F show representative high magnification images of the double immunostaining for CRF and c-Fos in the PVN of mice of each experimental group. Arrows point to dual-labeled cells. Scale bar: 10 μm. Data represent the mean±SEM. *, $p \leq 0.05$. **, $p \leq 0.01$. ***, $p \leq 0.001$.

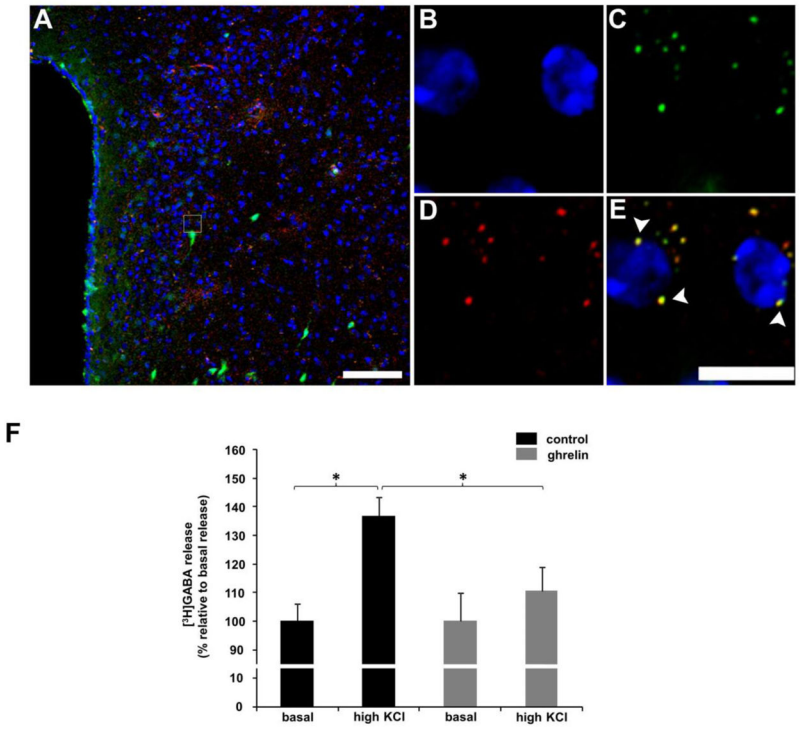

Figure 3. Ghrelin binds to GABAergic terminals within the PVN and inhibits *ex vivo* GABA release

Panels A–E show a representative confocal photomicrographs of the PVN of a mouse ICV-injected with F-ghrelin and then subjected to double fluorescent immunohistochemistry using anti-fluorescein (green) and anti-GAD67 (red) antibodies; sections were also counterstained using Hoechst (blue) to visualize cell nuclei. Panel A shows a low magnification merge image of the PVN. Panels B, C, D and E show high magnification images of the blue, green, red and merge channels, respectively. Arrowheads point to dual-labeled terminals. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article. Scale bars, 100 μm (low magnification), 10 μm (high magnification). Panel F shows basal and high KCL-induced *ex vivo* [^{3}H]-GABA secretion from PVN explants in the presence or absence of ghrelin. Data represent the mean±SEM. *, $p \leq 0.05$.

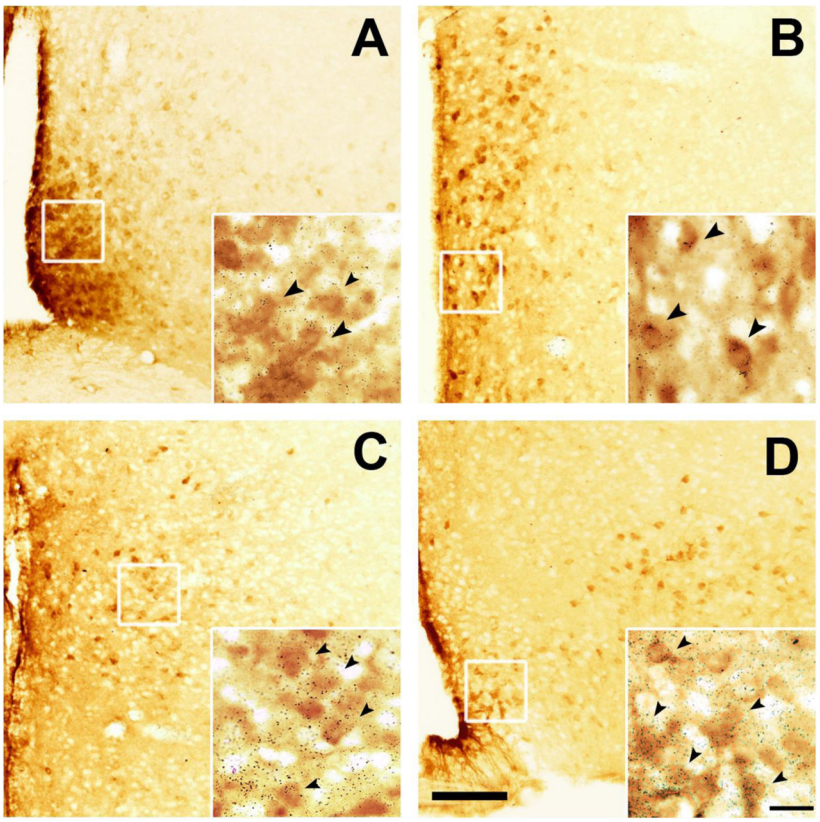

Figure 4. GABAergic neurons, located in hypothalamic nuclei known to innervate the PVN, bind ghrelin

Panels A, B, C and D show the mPOA, the Pe, the DMH and the ARC, respectively. Insets in each image show high magnification of areas marked in low magnification images. Dual-label histochemistry was performed on coronal brain sections of mice ICV-injected with F-ghrelin. F-ghrelin binding neurons were labeled by chromogenic immunohistochemistry (brown), and GAD67 mRNA-expressing neurons were labeled by ISHH (punctuate black sliver granules). Arrowheads point to dual-labeled cells. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article. Scale bars represent 100 and 10 μm for the low and high magnification images, respectively.

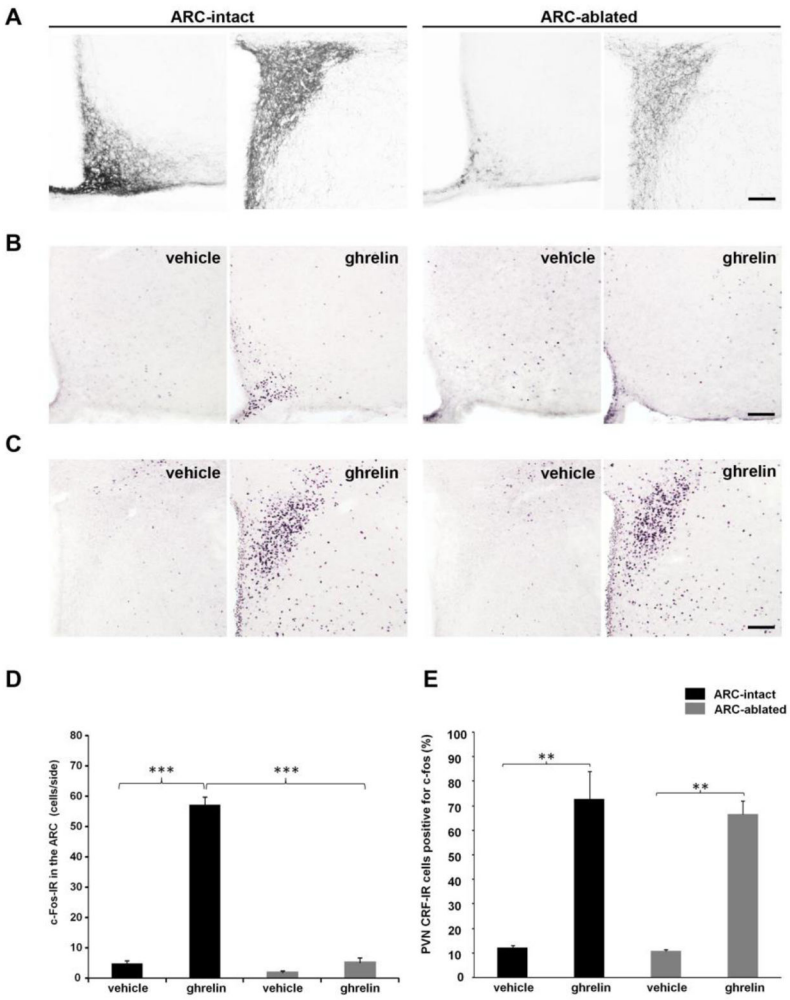

Figure 5. Peripherally-administered ghrelin activates PVN CRF neurons in ARC-ablated mice

Panel A shows representative photomicrographs of chromogenic NPY immunostaining in the ARC (left) and the PVN (right) of ARC-intact or ARC-ablated mice. Panels B and C show representative photomicrographs of c-Fos immunostaining (purple-black signal) in the ARC and the PVN, respectively, of ARC-intact or ARC-ablated mice treated with either saline or ghrelin. Panels D and E show a bar graph displaying the number of c-Fos-immunoreactive cells in the ARC and the percentage of CRF-IR cells positive for c-Fos in the PVN, respectively, in each experimental group. Scale bar: 100 μm **, $p \leq 0.01$. ***, $p \leq 0.001$.

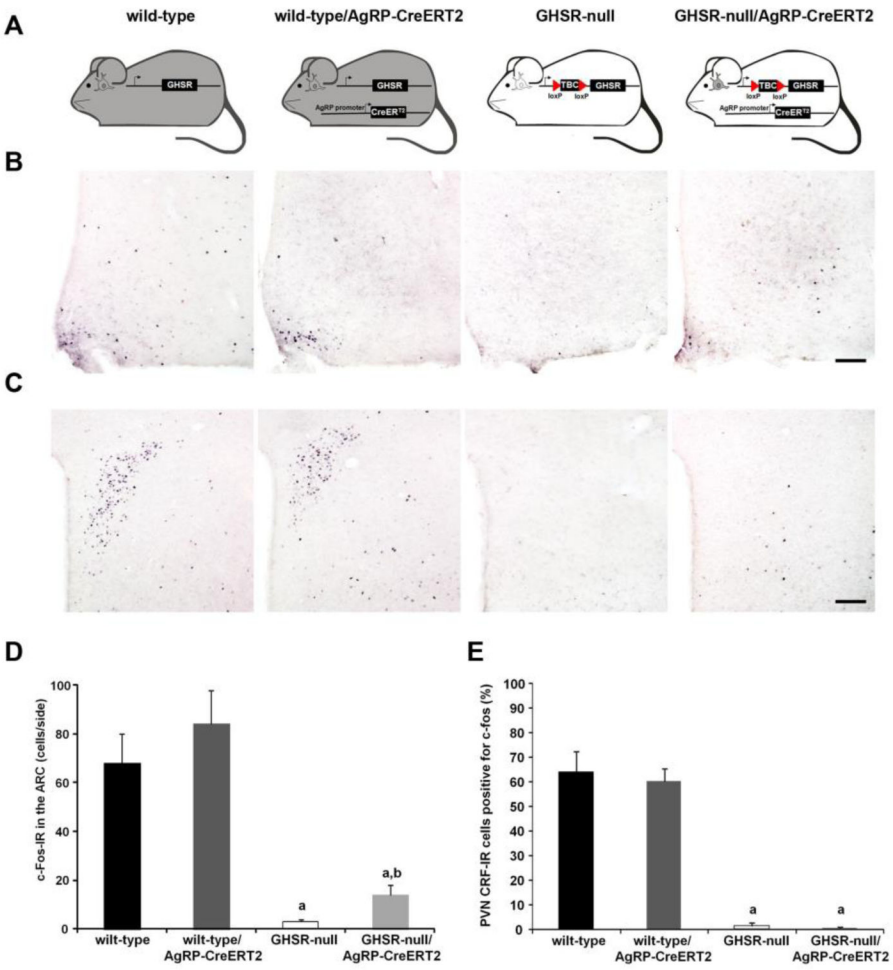

Figure 6. Peripherally-administered ghrelin fails to activate PVN CRF neurons in mice expressing GHSR exclusively in ARC AgRP neurons

Panel A displays a schematic diagram of the mouse genotypes used in this experiment. Each genotype is represented by a cartoon depicting a mouse, its AgPR neurons, and a diagram of the genes of interest contained in each mouse model (wild-type ghsr gene, loxP-flanked transcriptional blocking cassette (TBC) inserted into the ghsr gene or AgRP-CreER^{T2} transgene). Gray represents GHSR gene expression, and white represents lack of GHSR expression. Panels B and C show representative photomicrographs of c-Fos immunostaining (purple-black signal) in the ARC (B) or the PVN (C) of ghrelin-treated wild-type, wild-type/ AgRP-CreER^{T2}, GHSR-null, and GHSR-null/AgRP-CreER^{T2} mice. Panels D and E show a bar graph displaying the number of c-Fos-IR cells in the ARC and the percentage of CRF-IR cells positive for c-Fos in the PVN in each experimental group, respectively. Scale bar: 100 μm. a, $p \leq 0.05$ vs. ghrelin-treated wild-type mice. b, $p \leq 0.01$ vs. ghrelin-treated GHSR-null mice

Table 1

A summary of results

Experimental group	Control group	c-Fos in the ARC	c-Fos in the PVN CRF neurons	Food intake as assessed *de novo* here or previously (references)
Ghrelin (SC or ICV) in WT mice	Vehicle (SC or ICV, respectively) in WT mice	increased	increased	increased (Cabral et al. 2012)
Ghrelin (ICV) in Y1 and Y5 antagonist (ICV)-pretreated WT mice	Vehicle (ICV) in Y1 and Y5 antagonist (ICV)-pretreated WT mice	increased	increased	increased, but less than in ghrelin (ICV)-treated WT mice
Ghrelin (ICV) in GABAA agonist (intra-PVN)-pretreated WT mice	Vehicle (ICV) in GABAA agonist (intra-PVN)-pretreated WT mice	increased	not affected	not affected
Ghrelin (SC) in ARC-ablated mice	Vehicle (SC) in ARC-ablated mice	not affected	increased	not affected (Cabral et al. 2014)
Ghrelin (SC) in mice with GHSR in ARC AgRP neurons only	Ghrelin (SC) in GHSR-null mice	increased	not affected	increased, but less than in ghrelin (SC)-treated WT mice (Wang et al. 2013)

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