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Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility

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Abstract

Ghrelin is an octanoylated peptide hormone that potently and rapidly increases food intake. The orexigenic action of ghrelin involves the hypothalamic arcuate nucleus (ARC), which is accessible to plasma ghrelin and expresses high levels of the ghrelin receptor. Local administration of ghrelin in a variety of other brain nuclei also increases food intake. It is currently unclear, however, if these non-ARC ghrelin brain targets are impacted by physiological increases of plasma ghrelin. Thus, the current study was designed to clarify which ghrelin brain targets participate in the short-term orexigenic actions of ghrelin. First, c-Fos induction into mouse brains centrally or peripherally treated with ghrelin was analyzed. It was confirmed that peripherally administered ghrelin dose dependently increases food intake and mainly activates c-Fos in ARC neurons. In contrast, centrally administered ghrelin activates c-Fos in a larger number of brain nuclei. To determine which nuclei are directly accessible to ghrelin, mice were centrally or peripherally injected with a fluorescent ghrelin tracer. It was found that peripherally injected tracer mainly accesses the ARC while centrally injected tracer reaches most brain areas known to express ghrelin receptors. Following that, ghrelin effects in ARC-ablated mice were tested and it was found that these mice failed to increase food intake in response to peripherally administered ghrelin but fully responded to centrally administered ghrelin. ARC-ablated mice showed similar patterns of ghrelin-induced c-Fos expression as seen in control mice with the exception of the ARC, where no c-Fos was found. Thus, peripheral ghrelin mainly accesses the ARC, which is required for the orexigenic effects of the hormone. Central ghrelin accesses a variety of nuclei, which can mediate the orexigenic effects of the hormone even in the absence of an intact ARC.

Keywords

arcuate nucleus; monosodium glutamate; dorsal vagal complex

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INTRODUCTION

Ghrelin is a 28-amino acid octanoylated peptide synthesized mainly by endocrine cells of the stomach (1). Ghrelin is the only known orexigenic peptide hormone, and many studies suggest that it plays an essential role in body weight regulation (2). In order to increase food intake, ghrelin acts via its specific receptor named the growth hormone secretagogue receptor (GHSR), which is highly expressed within several food intake regulatory brain centers (3, 4). Increases of circulating ghrelin concentration stimulate feeding almost instantly (2). Thus, the orexigenic action of ghrelin presumably depends on the quick accessibility of the circulating hormone to its brain targets. One key site of action for ghrelin is the hypothalamic arcuate nucleus (ARC), which expresses high levels of GHSR and is located in close apposition to the median eminence (ME), a circumventricular organ with fenestrated capillaries that allow the rapid access of circulating ghrelin (5). In line with this possibility, peripheral administration of ghrelin strongly increases c-Fos expression in the ARC, and ghrelin fails to increase food intake in ARC-ablated rats (6–9). However, other reports suggest that orexigenic actions of peripheral ghrelin may also involve the area postrema (AP), another circumventricular organ located in the caudal brainstem (10). The AP together with the nucleus of the solitary tract (NTS) and the dorsal motor nucleus (DMX) form the dorsal vagal complex (DVC), which also expresses high levels of GHSR and increases c-Fos expression in response to peripheral administration of ghrelin (3, 11, 12). In addition to the ARC and the DVC complex, GHSR is also expressed in other food intake-related brain areas without obvious direct access to ghrelin circulating in the bloodstream. Among others, these areas include the lateral hypothalamus (LHA), the anterior hypothalamic area (AHA), the dorsomedial nucleus (DMH) and the ventromedial nucleus (VMN) within the hypothalamus and also the ventral tegmental area (VTA) in the midbrain. Direct micro-injections of ghrelin into these brain areas increases food intake (13–16), and they are accessible to ghrelin present in the cerebrospinal fluid (CSF) (17). However, the relative physiological relevance of ghrelin's distributed brain targets and its integration in order to modulate the orexigenic actions of the hormone are currently unclear. Here, food intake and c-Fos induction in the brains of control and ARC-ablated mice subjected to either peripheral or central administration of ghrelin was systematically studied. Additionally, a recently developed ghrelin fluorescent tracer to examine the accessibility of CSF and circulating hormone to these food intake-related brain areas was used (17). It was found that peripheral ghrelin shows a limited accessibility to the brain and that peripheral and central ghrelin regulate food intake through different neuronal circuitries.

MATERIAL AND METHODS

Animals

C57BL6/J mice were generated in the animal facility of the IMBICE. In order to generate the ARC-ablated mice, 4-day old pups were injected subcutaneously with either 2 mg/g body weight (BW) of monosodium glutamate (Sigma Aldrich) or saline. Experiments were performed with adult (7–9 week old) male mice, which were housed in a 12-h light/dark cycle with regular chow and water available *ad libitum.* This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory

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Animals of the National Research Council, USA, and all efforts were made to minimize suffering. All experimentation received approval from the Institutional Animal Care and Use Committee of the IMBICE.

Treatments and stereotaxic surgeries

Experimental mice were peripherally or centrally injected with acyl-ghrelin (Global Peptide, cat# PI-G-03). For peripheral ghrelin treatment, individually housed mice were subcutaneously injected with saline or ghrelin (0.06 or 0.6 nmol/g BW) between 8:00 and 10:00 a.m. Here, a total of 37 mice were used and grouped as follows: vehicle (n= 13), low (n= 12) and high (n= 12) dose of ghrelin. For experiment with ARC-ablated mice, a total of 22 mice were used and grouped as follows: vehicle (n= 5), low (n= 10) and high (n= 7) dose of ghrelin. For central infusion of ghrelin, mice were first stereotaxically implanted with a single indwelling sterile guide cannula (4 mm long, 22 gauge, Plastics One) into the lateral ventricle (intra-cerebro-ventricular, ICV). The placement coordinates for the lateral ventricle were: antero-posterior:−0.34 mm, lateral:+1 mm and ventral:−2.3 mm. After surgery, animals were individually housed and allowed to recover for at least 5 days. On the morning of the experimental day, animals were ICV-injected, through a 30 gauge needle, with 4 μL of saline with or without acyl-ghrelin (0.6 nmol/mouse). Here, a total of 18 mice were used and grouped as follows: vehicle (n= 5) and ghrelin (n= 13). For experiment with ARC-ablated mice, a total of 12 mice were used and grouped as follows: vehicle (n= 3) or ghrelin (n= 9). Two hours after treatment, mice were anesthetized and perfused with formalin. Food intake was calculated by subtracting the remaining food weight from the initial food weight at the end of the experiment.

Assessment of c-Fos localization by immunohistochemistry (IHC)

Brains of perfused mice were removed, post-fixed, immersed in 20% sucrose and cut coronally at 25 μm into three equal series on a sliding cryostat as previously described (18). C-Fos IHC was performed as described (18). Briefly, sections were pretreated with 0.5% H_2O_2, treated with blocking solution (3% normal donkey serum and 0.25% TritonX in PBS) and incubated with anti-c-Fos antibody (Calbiochem/Oncogene, cat# PC38, 1:15,000) for two days at 4°C. Then, sections were treated with biotinylated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:1,000) for 1 h and with Vectastain Elite ABC kit (Vector Laboratories, cat # PK6200) for 1 h, according to manufacturer's protocols. A visible signal was developed with 3-3′-diaminobenzidine (DAB)/Nickel solution, giving a black/purple precipitate. Sections were sequentially mounted on glass slides and coverslipped with mounting media.

Visualization of sites accessible to ghrelin

The procedure for analysis of sites accessible to ghrelin was adapted from a recently described protocol (17). Fluorescein-ghrelin[1–18] (hereafter referred to as F-ghrelin) is an 18 amino acid analog of the hormone with an optical dye attached through a Lys19 at its C-terminus that behaves similarly to the endogenous ghrelin in terms of GHSR affinity (19). Mice were treated with vehicle containing, or not, the F-ghrelin using procedures and doses, as described above. Fifteen minutes after treatment, mice were anesthetized and perfused with formalin. Brains were processed as described above in order to generate coronal brain

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sections, which were then used for IHC. Brain sections were pretreated with 0.5% H_2O_2 and treated with blocking solution. Next, sections were incubated with goat anti-fluorescein antibody (Molecular Probes, cat# A-11095, 1:1,500) 48 h at room temperature. Then, sections were treated with biotinylated donkey anti-goat antibody (Vector Laboratories, 1:1000), and then with Vectastain Elite ABC kit, according to manufacturer's protocols. Finally, a visible signal was developed with DAB/Nickel solution, giving a black/purple precipitate. Negative controls were also performed using the same procedure but omitting the primary antibody or secondary antibody. Sections were sequentially mounted on glass slides, and coverslipped with mounting media. Importantly, we have recently validated the specificity and accuracy of this experimental strategy (17). Briefly, we have shown that: a) F-ghrelin is fully bioactive *in vivo*; b) the pattern of F-ghrelin labeling is in general agreement with previous studies using in situ hybridization immunohistochemistry for visualization of GHSR mRNA in the mouse brain; c) F-ghrelin signal is significantly reduced by an excess of unlabeled ghrelin; and d) F-ghrelin fails to label brain nuclei of GHSR-null mice.

Validation of ARC-ablated mouse model

The ARC lesion was confirmed by ARC cell nuclei counting after thionin staining and by estimations of the number of specific neuronal populations known to be located within this nucleus including NPY, dopamine or POMC-producing neurons that were identified after immunostaining against NPY, tyrosine hydroxylase (TH) or adrenocorticotropic hormone (ACTH), respectively. In order to visualize cell nuclei, brain sections were stained with thionin (Sigma, cat# T7029), dehydrated in an ascending alcohol series, cleared in xylene and coverslipped. In order to perform IHC, brain sections were pretreated with 0.5% H_2O_2, treated with blocking solution and incubated with the primary antibody (anti-NPY Abcam, cat# ab30914, 1:10,000; anti-TH, Santa Cruz (H-196), cat# sc-14007, 1:20,000; and, anti-ACTH, generated in rabbit by our laboratory, 1:3,000) overnight at room temperature. Then, sections were treated with biotinylated anti-rabbit antibody, with Vectastain Elite ABC kit and DAB/Nickel solution, as described above. Sections were sequentially mounted and cover slipped with mounting media.

Quantitative Analysis

Bright-field images were acquired with a Nikon Eclipse 50i and a DS-Ri1 Nikon digital camera. Total c-Fos-immunoreactive (IR) cells and fluorescein-IR signal in each brain region were bilaterally estimated in sections between −1.58 and −1.94 mm for the ARC and ME and between bregma −7.32 and −7.56 mm for the NTS and AP. Total c-Fos-IR cells and fluorescein-IR signal for the AHA and LHA were estimated between bregma −0.70 and −0.94 mm; in this case, signal medially or laterally located to a dorso-ventral line drawn through the column of the fornix was considered AHA or LHA, respectively. In the DMH and VTA, estimations were performed between bregma −1.34 and −1.94 mm, and between bregma −3.08 and −3.80 mm, respectively. Anatomical limits of each brain region were identified using a mouse brain atlas (20). Cells containing distinct nuclear black/purple precipitate were quantified in one out of three complete series of coronal sections through the whole nuclei. Blind quantitative analysis was performed independently by two observers and expressed as c-Fos-IR cells per coronal section per side. Fluorescein-IR signal was

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estimated in images taken in comparable areas under the same optical and light conditions. Then, microphotographs were converted to grey scale images of 8 bits, inverted and the mean optical density (OD) for each region was measured by using the Image J software. Notably, fluorescein-IR signal in the ME was quantified in the region that comprises both its internal and external layers. The fluorescein-IR signal is expressed as OD, which is the mean grey of the pixels with a 256 grey scale as a reference. Total thionin-stained cell nuclei were estimated in the ARC and DVC of control and ARC-ablated mice. In the ARC-ablated and control mice's ARC, total TH-IR, ACTH-IR, NPY-IR signals were estimated as described for fluorescein-IR signal and expressed as a percentage relative to the control group.

Determination of plasma ghrelin levels

To determine plasma ghrelin levels induced in each experimental group, a separate cohort of mice was treated with acyl-ghrelin as described above. Blood samples were collected, 0.5 and 2 h after ghrelin administration, from mouse tails into tubes containing EDTA on ice (21). The protease inhibitor p-hydroxymercuribenzoic acid (Sigma-Aldrich, cat# 12425) was added to each sample to achieve a final concentration of 1 mM. The samples were centrifuged and plasma was immediately treated with one-tenth volume of 1 N HCl, and then stored at −80°C until use. Acyl-ghrelin levels were determined using ghrelin EIA kits according to the manufacturer's instructions (Cayman Chemical Company).

Statistical analyses

Data is expressed as the mean±SEM. No significant differences ($p>0.09$) were observed between mice subcutaneously treated with vehicle and mice ICV-treated with vehicle in any measures taken and thus their data were pooled and named vehicle-treated group. One-way ANOVA followed by the Newman Keuls test was used to compare food intake or quantitative analysis of neuroanatomical data from different groups. In order to compare data from control and ARC-ablated mice, t-test was performed. Analyses were performed using GraphPad Prism 5.0. Significant differences were considered when $P<0.05$.

RESULTS

The ARC shows an increased responsiveness to ghrelin as compared to other brain areas

To assess the physiological implications of our study, plasma ghrelin levels achieved in each experimental condition were determined. Plasma ghrelin concentrations were 250±32 and 268±26 pg/mL in vehicle-treated group after 0.5 and 2 h post-treatment, respectively. In mice peripherally treated with the low dose of ghrelin, plasma ghrelin levels were elevated to 584±76 pg/mL 0.5 h post-treatment ($P<0.05$, as compared to vehicle-treated group) and returned to 301±23 pg/mL 2 h after treatment. In mice peripherally treated with the high dose of ghrelin, plasma ghrelin levels were elevated to 4207±583 and 1030±154 pg/mL after 0.5 and 2 h post-treatment, respectively ($P<0.01$, as compared to vehicle-treated group). In mice ICV-treated with ghrelin, plasma ghrelin concentrations were 317±39 and 240±32 pg/mL after 0.5 and 2 h post-treatment, respectively. In terms of food intake, all groups of ghrelin-treated mice showed a significant increase of their food intake. Subcutaneous administration of ghrelin stimulated food intake dose-dependently (3.2±0.7, 13.3±1.0 and

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20.2±1.7 mg/g BW for vehicle, low and high dose of ghrelin, respectively, P<0.05), and ICV administration of ghrelin stimulated food intake in the same magnitude as the high dose of peripherally administered ghrelin (18.1±1.5 mg/g BW, Figure 1).

To determine which brain nuclei were responsive to ghrelin in each experimental condition, IHC for c-Fos was performed. Brain areas analyzed included those which are known to be involved in feeding behavior and to express GHSR. In mice peripherally treated with the low dose of ghrelin, c-Fos-IR cells were exclusively observed in the ARC. In mice peripherally treated with the high dose of ghrelin, c-Fos-IR cells were observed in the ARC as well as in other brain areas, including the NTS and hypothalamic paraventricular nucleus. In mice ICV injected with ghrelin, c-Fos-IR cells were detected not only in the above mentioned nuclei but also in other brain areas such as the AHA, LHA, DMH and VTA. Since the ARC and NTS are known to modulate acute food intake, and they showed the strongest ghrelin-induced increase of c-Fos expression, a detailed quantitative analysis was performed in these brain areas (Figure 2). In the ARC, mice peripherally treated with ghrelin showed a dose-dependent increase in the number of c-Fos-IR cells compared with vehicle-treated group (5±1, 37±7 and 57±3 cells/section for vehicle, low and high dose of ghrelin, respectively; P<0.05). Mice ICV treated with ghrelin showed the highest number of c-Fos-IR cells in the ARC (87±9 cells/section, P<0.05, as compared with the other experimental groups). In the NTS, mice injected with vehicle or the low dose of ghrelin showed similar number of c-Fos-IR cells (18±2 and 21±4 cells/section, respectively; P=NS). In contrast, mice injected with ghrelin either peripherally at the high dose or ICV showed a significant increase of the number of c-Fos-IR cells in the NTS, as compared with the vehicle-treated group (60±9 and 75±12 cells/section, respectively; P<0.05 vs. vehicle-treated group). The ghrelin-induced increase in the number of c-Fos-IR cells in the NTS was not statistically different between these two experimental groups. In the AHA, LHA, DMH and VTA, the amount of c-Fos-IR cells was significantly higher exclusively in mice ICV injected with ghrelin (Figure 3).

The ARC shows increased accessibility to circulating ghrelin in comparison to other brain areas

In order to assess which brain areas are accessible to exogenously administered ghrelin in each experimental condition, a recently developed ghrelin fluorescent tracer was used (Figure 4A). In mice peripherally treated with the low dose of F-ghrelin, fluorescein-IR signal was exclusively found in the ARC and tanycyte-like cells of the ME. In mice peripherally treated with the high dose of F-ghrelin, fluorescein-IR signal was found not only in the ARC and tanycyte-like cells of the ME but also in the AP. In mice ICV-injected with F-ghrelin, fluorescein-IR signal was found in most of the brain areas expressing GHSR as well as in the ependymal cells of the ventricular system, as recently reported (17). The pattern of fluorescein-IR signal showed a diffuse profile within the ARC of mice peripherally treated with F-ghrelin at the low dose while it mainly presented a cell body-shape in the other two groups of mice treated with F-ghrelin (see insert of Figure 4A). Quantitative analysis indicated that mice peripherally treated with F-ghrelin showed a dose-dependent increase of fluorescein-IR signal intensity in the ARC (18.5±2.8, 32.0±4.4 and 46.9±1.4 OD for the ARC of vehicle, low and high dose of F-ghrelin, respectively, P<0.05).

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Mice ICV-treated with F-ghrelin showed the highest levels of fluorescein-IR signal intensity in the ARC (151.8±14.6 OD, P<0.05, as compared to the other experimental groups, Figure 4B). In the ME, quantitative analysis indicated that mice peripherally treated with F-ghrelin showed a significant increase of fluorescein-IR signal intensity compared with vehicle-treated group (24.7±3.1, 60.9±12.9 and 74.7±5.0 OD for the ME of vehicle, low dose and high dose of ghrelin, respectively, P<0.05). As found in the ARC, mice ICV-treated with F-ghrelin showed the highest levels of fluorescein-IR signal intensity in the ME (146.5±28.5 OD, P<0.05, compared to the other experimental groups, Figure 4C). Mice injected with vehicle, low or high doses of F-ghrelin showed similar levels of fluorescein-IR signal intensity in the NTS (25.0±4.8, 26.1±1.1 and 26.4±1.0 OD, respectively) while mice ICV-treated with F-ghrelin showed a significant increase of fluorescein-IR signal intensity (75.1±12.3 OD, P<0.05, compared to the other experimental groups, Figure 4D). In the AP, mice injected with vehicle or the low dose of F-ghrelin showed similar levels of fluorescein-IR signal intensity (26.6±3.4 and 28.2±2.1 OD, respectively; P=NS). In contrast, mice peripherally treated with a high dose of F-ghrelin or ICV-treated with F-ghrelin showed a significant increase of fluorescein-IR signal intensity in the AP (40.0±0.9 and 105.9±2.0 OD, respectively, P<0.05 as compared to vehicle-treated group). Of note, mice ICV treated with F-ghrelin showed significantly higher levels of fluorescein-IR signal intensity in the AP, compared to levels found in mice peripherally treated with a high dose of F-ghrelin (Figure 4E). In the AHA, LHA, DMH and VTA, fluorescein-IR signal was exclusively detected in mice ICV injected with F-ghrelin (Figure 5).

The ARC is required for orexigenic actions of circulating ghrelin

To assess the requirement of the ARC for ghrelin-induced food intake in the different experimental conditions, ARC-ablated mice were generated. These studies were performed in 7–9 week old ARC-ablated mice before the development of energy balance abnormalities (22, 23). Food intake and BW did not differ between control and ARC-ablated mice from weaning until the experimental day, when overnight food intake and BW were 136±3 mg/g BW and 23.9±0.5 g or 154±14 mg/g BW and 24.9±0.6 g for ARC-ablated or control mice, respectively. Monosodium glutamate-induced neurotoxic lesions were selective in the ARC, as Nissl staining showed that ARC-ablated mice presented intact morphology of the DVC complex (Figure 6) and other brain regions (not shown). Quantitative analysis of Nissl staining indicated that ARC-ablated mice had a significant decrease in the number of thionin-stained cells in the ARC (77±14 and 244±12 cells/section, P<0.05 as compared to control mice), while the number of cells in the AP and NTS was not affected (164±28 vs. 206±24 cells/section and 168±9 vs. 169±17 cells/section for the AP and the NTS of ARC-ablated and control mice, respectively, P=NS). In the ARC, ARC-ablated mice had a 17.1±7.3, 24.7±8.1 and 42.3±7.4 % of NPY-IR, TH-IR and ACTH-IR signal intensities, respectively, compared to control mice, which had 100.0±7.1, 100±10.4 and 100±8.8 %, respectively (P<0.05).

ARC-ablated mice failed to increase food intake in response to peripheral injections of ghrelin (2.1±1.9, 2.2±0.5 and 4.4±0.9 mg/g BW for vehicle, low and high dose of ghrelin, respectively, P=NS). In contrast, they fully responded to the orexigenic effects of the hormone when it was ICV administered (13.2±2.2 mg/g BW, P<0.05 compared to control

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group, Figure 7A). In order to discard the possibility that food intake divergence was due to the variability in the lesion, the above mentioned results were confirmed in an independent cross-over study in which food intake following sc or ICV ghrelin administration was evaluated in the same ARC-ablated animals (n=5, data not shown). As expected, ARC-ablated mice showed no ghrelin-induced increase of the number of c-Fos-IR cells in the ARC in any experimental protocol (Figure 7B). In the NTS, ARC-ablated mice injected with vehicle or the low dose of ghrelin showed similar numbers of c-Fos-IR cells (8±3 and 17±2 cells/section, respectively; P=NS), but showed a significant increase in the number of c-Fos-IR cells when ghrelin was administered either peripherally at the high dose or ICV (28±2 and 68±8 cells/section, respectively; $P<0.05$, Figure 7C). Ghrelin-induced increase of c-Fos in the NTS of ARC-ablated mice peripherally treated with the high dose of the hormone was statistically lower than the increase observed in control mice. In contrast, ARC-ablated mice ICV-treated with ghrelin showed an increase of c-Fos in the NTS similar to the increase observed in control mice ICV-treated with ghrelin. ARC-ablated mice ICV-injected with ghrelin also showed a significant increase of the number of c-Fos-IR cells in the AHA, LHA, DMH and VTA (not shown).

Discussion

This study helps to clarify which ghrelin brain targets are involved in the short-term orexigenic actions of the hormone. Current data indicate that small and acute increments of circulating ghrelin, sufficient to increase food intake, mainly impact at the ARC level as indicated by c-Fos induction and accessibility of F-ghrelin. In contrast, higher and acute increments of circulating ghrelin increase c-Fos expression and access not only the ARC but also the AP. Central ghrelin is able to reach and increase c-Fos expression in a wider variety of brain areas. Interestingly, ARC-ablated mice fail to increase food intake in response to peripherally administered ghrelin but remain fully responsive to the orexigenic effects of the centrally administered hormone.

Plasma ghrelin levels rise in response to negative energy balance conditions or stress, and these variations likely play a role in food intake regulation (4). Current experimental protocols of peripheral hormone administration mimic physiological increases of plasma ghrelin. In particular, the low dose of ghrelin used in this study induced a ~2-fold increase of plasma ghrelin levels, and such a level of increase is observed in rodents upon a 12- or 24-h fast, just prior to food availability in a 4-h calorie restriction experimental paradigm or after exposure to stressful stimuli, such as a tail pinch, water immersion or chronic social defeat (21, 24–27). In human beings, plasma ghrelin levels rise ~2-fold shortly before meals and after overnight fasting (2). The high dose of ghrelin used in this study induced a ~17-fold increase of plasma ghrelin levels, which are exceptionally high for most physiological conditions. However, a recent study has reported that mice subjected to a chronic calorie deprivation (intake of 40% less of normal calories over 8 days) develop a progressive elevation in plasma ghrelin levels, which end up reaching concentrations of around 12- to 18-fold higher than observed in *ad lib*-fed mice (28). Thus, the lower dose of ghrelin used in the current study appears to mimic many physiological conditions where plasma ghrelin increases while the higher dose of ghrelin resembles a severe and specific pathophysiological condition. Importantly, ghrelin increases under our experimental

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conditions were transient, while fasting or caloric restriction induces a more sustained increase of plasma ghrelin levels. CSF ghrelin levels are difficult to quantify in mice given the large amounts of sample required for the available assays. To our knowledge, neither basal nor levels reached after central administration of ghrelin have been previously determined in the mouse CSF. In other species, CSF ghrelin levels are ~1000-fold lower than plasma ghrelin levels suggesting that CSF ghrelin levels reached in the current study are likely supraphysiological (29). Still, data obtained from mice ICV-treated with ghrelin is useful to better dissect the potential neuronal circuits by which ghrelin can affect food intake.

Centrally administered ghrelin is able to reach and increase c-Fos expression in most of the brain areas where GHSR is present (3, 17). The current profile of c-Fos induction in response to ICV ghrelin confirms previous observations made by other studies (12, 30, 31). It is important to stress that ICV ghrelin likely increases c-Fos expression not only in GHSR-expressing neurons but also in some of the early targets of the GHSR-expressing neurons. In addition, the absence of c-Fos expression is not proof of the absence of ghrelin's action on a given neuronal population. For instance, VTA neurons of the mesolimbic pathway have been shown to be a target of circulating ghrelin, as indicated by behavioral and nucleus accumbens dopamine release studies in mice (26, 32). However, few c-Fos-IR cells in this midbrain region were detected, even in animals ICV-treated with ghrelin. It is possible that ghrelin actions on these neurons mainly involve effects on the neuronal firing or neurotransmitter release, independently of c-Fos gene transcription (33). Another explanation is that the effect of ghrelin in some brain areas may be delayed and become evident after a prolonged period of time. The current study was focused on the acute effect of ghrelin, as the orexigenic effects of ghrelin is very rapid, starting within 5–10 minutes after its administration (1). Indeed, the c-Fos distribution 2-h after treatment likely reflects early events after ghrelin's action as the presence of nuclear c-Fos protein requires not only gene expression, but also protein biosynthesis and mobilization from cytoplasm to the cell nucleus (33). In addition, ghrelin accessibility to the brain was studied 15 minutes after F-ghrelin treatment. Thus, current data do not invalidate that ghrelin may act on or access other brain areas over prolonged time periods, after a sustained increases of the hormone or even under particular conditions such as fasting, when the accessibility to the brain of other hormones has been shown to increase (34).

Interestingly, ARC-ablated mice were fully responsive to the orexigenic effects of centrally administered ghrelin. These orexigenic effects of ghrelin may involve direct actions of the hormone on different hypothalamic (i.e. LHA, DMH) and/or extra-hypothalamic (i.e. NTS, VTA) nuclei, which increased the number of c-Fos-IR cells in ARC-ablated mice ICV-treated with ghrelin and are known targets of the orexigenic actions of ghrelin (13–16). Thus, it seems that CSF ghrelin is able to increase food intake via anatomically distributed brain sites, even in the absence of the ARC. Given the limited accessibility of peripheral ghrelin to the brain, the physiological relevance of these ghrelin-responsive circuits is unclear. One possibility is that these neuronal circuits are engaged by centrally-produced ghrelin; however, the existence of authentic brain-derived ghrelin in mice is a matter of debate (35). In particular, results regarding the distributions of ghrelin-IR neurons described by different authors vary, and physiological significance of endogenous central ghrelin

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production in rodents has been inconsistent (35), Another possibility includes that GHSR could modulate appetite-regulating neuronal circuits in a ghrelin-independent manner since this receptor shows a very strong constitutive activity that makes it capable to signal in the absence of its ligand (36). Additionally, GHSR heterodimerization with other G protein-coupled receptors could serve as an allosteric mechanism, independent of ghrelin binding, to modulate either GHSR or the other receptors signaling and, as a consequence, food intake (37). Future studies are required to elucidate the physiological relevance of these circuits activated in response to central infusions of ghrelin.

Our current data support the notion that the ARC is the main target of the orexigenic effects of peripheral ghrelin in mice since the low dose of ghrelin used in this study mainly accessed and increased c-Fos in ARC neurons. In particular, the NPY/Agouti related protein (AgRP)-producing neurons of the ARC have been established as the main mediators of the orexigenic effects of peripheral ghrelin (30, 38). A recent study, however, has shown that re-expression of GHSR in AgRP neurons only partially restores the orexigenic response to peripherally administered ghrelin (39). Interestingly, larger increases of plasma ghrelin accessed not only the ARC but also the AP and increased c-Fos expression in the NTS. Despite increased NTS c-Fos expression in response to the high dose of ghrelin, ARC-ablated mice failed to increase food intake in this experimental condition. Thus, the ARC is required for increases of food intake induced by peripheral ghrelin, even though high concentrations of plasma ghrelin can also access the AP. Current observations may help to reconcile previous disparate findings in terms of brain areas responsive to ghrelin. In particular, some previous studies have shown that peripheral ghrelin administration in rodents induces c-Fos expression exclusively in the ARC and not in other brain areas (6, 7). In contrast, ours and other studies have found that peripheral administration of ghrelin also increases the number of c-Fos-IR neurons in the DVC (11, 12, 40, 41). Current results suggest that these discrepancies are due to differences in the dose of ghrelin administered. However, the possibility that the disagreements in expression pattern of c-Fos after different ghrelin treatments may be related to technical variability between different laboratories cannot be completely ruled out.

The role of the DVC as a mediator of ghrelin-induced food intake has been a matter of debate. Ghrelin could act on the DVC either directly or indirectly via activation of the vagal afferent neurons of the nodose ganglion, which express GHSR, has access to peripheral ghrelin and innervate the NTS. Initial studies reported that total subdiaphragmatic vagotomy reduces the orexigenic effect of peripherally administered ghrelin in rodents and humans (24). However, further studies using more selective methods to disconnect abdominal vagal afferents showed that these afferents are not necessary for ghrelin-induced food intake (42). Ghrelin can act directly on the DVC as suggested by the finding that administration of ghrelin to the caudal brainstem, via either nuclei microinjection or injection into the fourth ventricle, increases food intake and c-Fos in the NTS (43). It has been proposed that the neural circuit by which the DVC mediates the orexigenic effects of ghrelin involves activation of noradrenergic neurons of the NTS projecting to the ARC (44). However, other studies have failed to support such hypothesis (43, 45, 46). Current data indicates that peripheral ghrelin cannot access the NTS, even when it reaches very high concentrations in plasma. Thus, c-Fos increases in the NTS induced by peripheral ghrelin may be, in part,

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secondary to ghrelin action in the AP. In support of this possibility, it has been shown that AP ablation blocks the increase of c-Fos in DMX and NTS induced by peripheral administration of ghrelin (40). Notably, c-Fos increase in the NTS induced by high levels of plasma ghrelin was significantly reduced in ARC-ablated mice suggesting that ARC outputs may also play a role in DVC activation. CSF ghrelin was able to access the NTS, but the physiological relevance of this effect is unclear. Direct action of ghrelin in the NTS may be one of the pathways mediating orexigenic effect of the centrally administered hormone in ARC-ablated mice. Additionally, ghrelin action in the DVC may mediate other effects of the hormone, including regulation of blood glucose (41), relaxation of the proximal stomach (47) or pancreatic protein secretion (40). It is currently unknown if these effects of ghrelin are conserved in ARC-ablated mice.

Previous studies have suggested that peripheral or central ghrelin engages different neuronal circuitries. In particular, bilateral midbrain transections specifically blocked orexigenic effects of peripherally administered ghrelin, but failed to affect feeding induced by centrally administered ghrelin (44). In addition, centrally, but not peripherally, administered ghrelin induced wakefulness in mice (48). Previous evidence has shown that CSF ghrelin is able to reach most of the brain areas where GHSR is expressed (17). In contrast, it seems that circulating ghrelin mainly gains access to the ARC, at least in acute experimental conditions. It has been shown that ghrelin is mainly transported from the brain to the blood via a saturable transport system in mice while ghrelin transport into the brain is very limited and no such system has been identified (49). The ME, located in close apposition to the ARC, is a circumventricular organ where plasma ghrelin easily diffuses to reach neuronal GHSR (5). Thus, the increased accessibility of ghrelin in the ARC, in comparison with other brain areas, is likely an important reason why peripheral or central ghrelin engage different neuronal circuitries.

It has been previously reported that centrally administered ghrelin fails to increase food intake in ARC-ablated rats (9). The discrepancy between previously reported and current observations might be attributable to some differences of the experimental design, including the use of different species or different protocols of monosodium glutamate administration. The protocol used to ablate the ARC by Tamura et al. required five neonatal injections of monosodium glutamate while the protocol used in the current study required only one administration of the drug. Both protocols of monosodium glutamate administration markedly diminished all neuronal population of the ARC; however, the simplicity of the current protocol presumably reduces the variability of the degree of the lesions. Indeed, the experimental variability in the responses of ARC-ablated mice was similar to the observed in the control group. Another major difference between previous and the current study is the age of the animals. Here, young animals (7–9-weeks old), which had BW and food intake similar to those observed in control mice, were used. It is well established that ARC-ablated rodents develop a series of abnormalities as they age, including hypophagia, decreased BW and excessive accumulation of intra-abdominal fat (22, 23). Tamura et al study was performed with 14-week old rats, which were leaner and showed altered food consumption patterns as compared to control animals (9). Therefore, these energy balance alterations raise concerns if the loss of the feeding response to ICV-administered ghrelin is a specific consequence of ARC ablation. Moreover, it has been shown that obese rodents are resistant

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to ghrelin-induced food intake (50). Thus, one may wonder whether Tamura et al. observations have been affected by the obese phenotype of the ARC-ablated rats. Further investigations using different rodent models, such as GHSR-conditional knockout mouse in which GHSR is eliminated exclusively from ARC neurons, will be needed to better understand neural circuits mediating the orexigenic effects of ghrelin.

Overall, our current findings complement previous studies and highlight the notion that ghrelin can act via multiple circuits to regulate food intake. Figure 8 shows schematic models of the brain nuclei that may be involved in feeding responses to central or peripherally administered ghrelin. Hopefully, these models will be useful for the comprehensive understanding of orexigenic effects of ghrelin.

Acknowledgments

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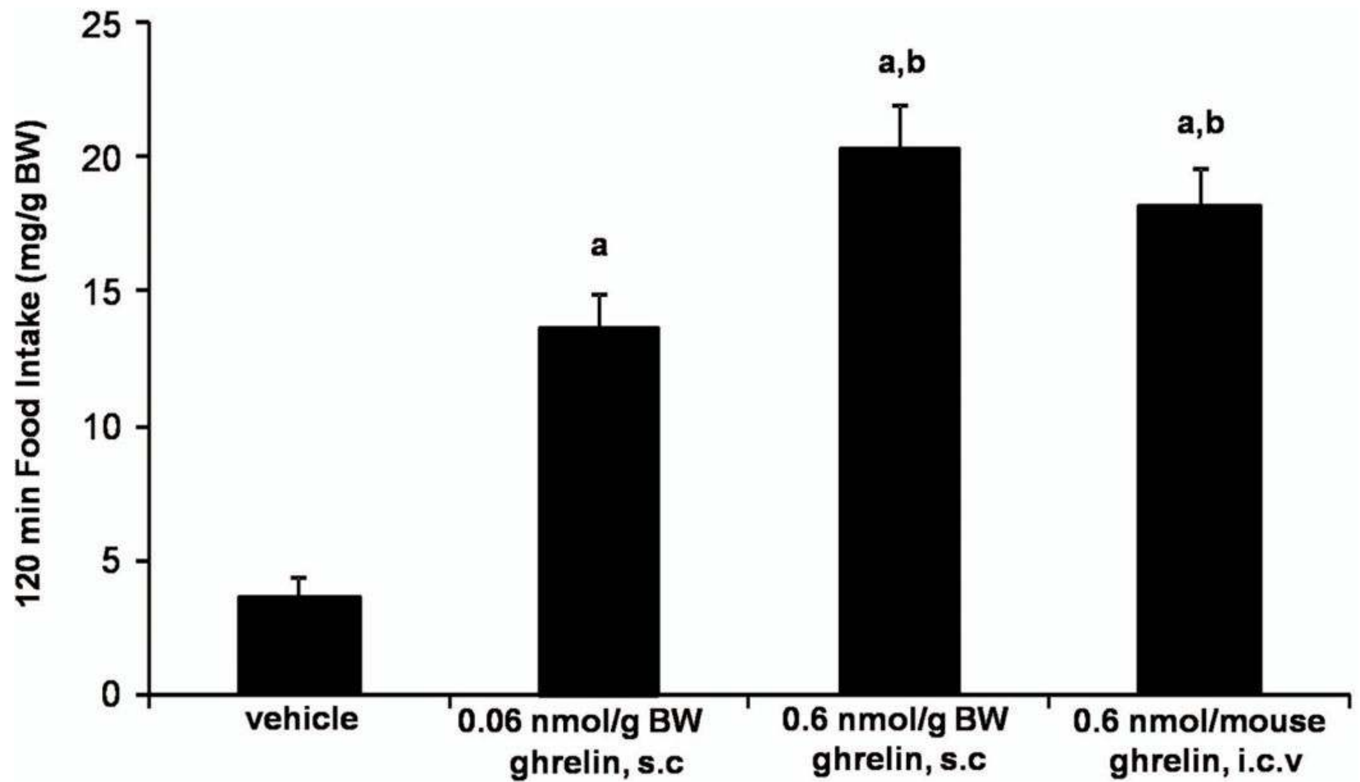

Figure 1.

Ghrelin-induced food intake in the different experimental groups. Figure shows 120 min food intake in mice subcutaneously or centrally injected with vehicle alone or containing ghrelin. Data represent the mean±SEM.

a, $p<0.05$ vs. control group.

b, $p<0.05$ vs. low dose of ghrelin s.c group

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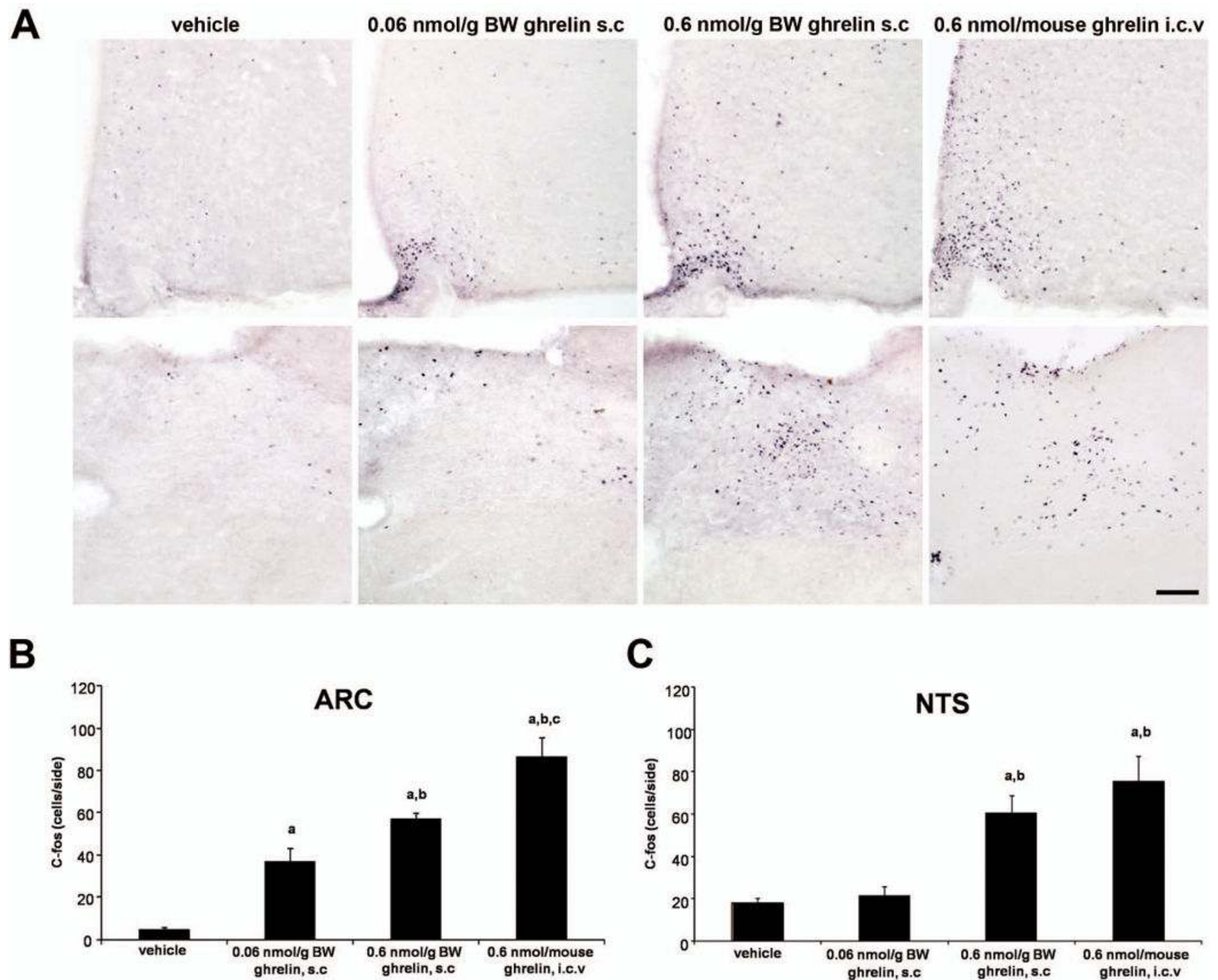

Figure 2.

Ghrelin-induced c-Fos in the ARC and the DVC of the different experimental groups. Panel A shows representative low (×20) magnification images of c-Fos staining in the ARC (upper line) and the DVC (bottom line) of vehicle- and ghrelin-treated mice. Scale bar: 100 μm. Bar graphs show quantitative analysis of the number of c-Fos-IR cells in the ARC (panel B) and the NTS (panel C) of vehicle- and ghrelin-treated mice. Data represent the mean±SEM.

a, $p<0.05$ vs. control group.

b, $p<0.05$ vs. low dose of ghrelin s.c group.

c, $p<0.05$ vs. high dose of ghrelin s.c group.

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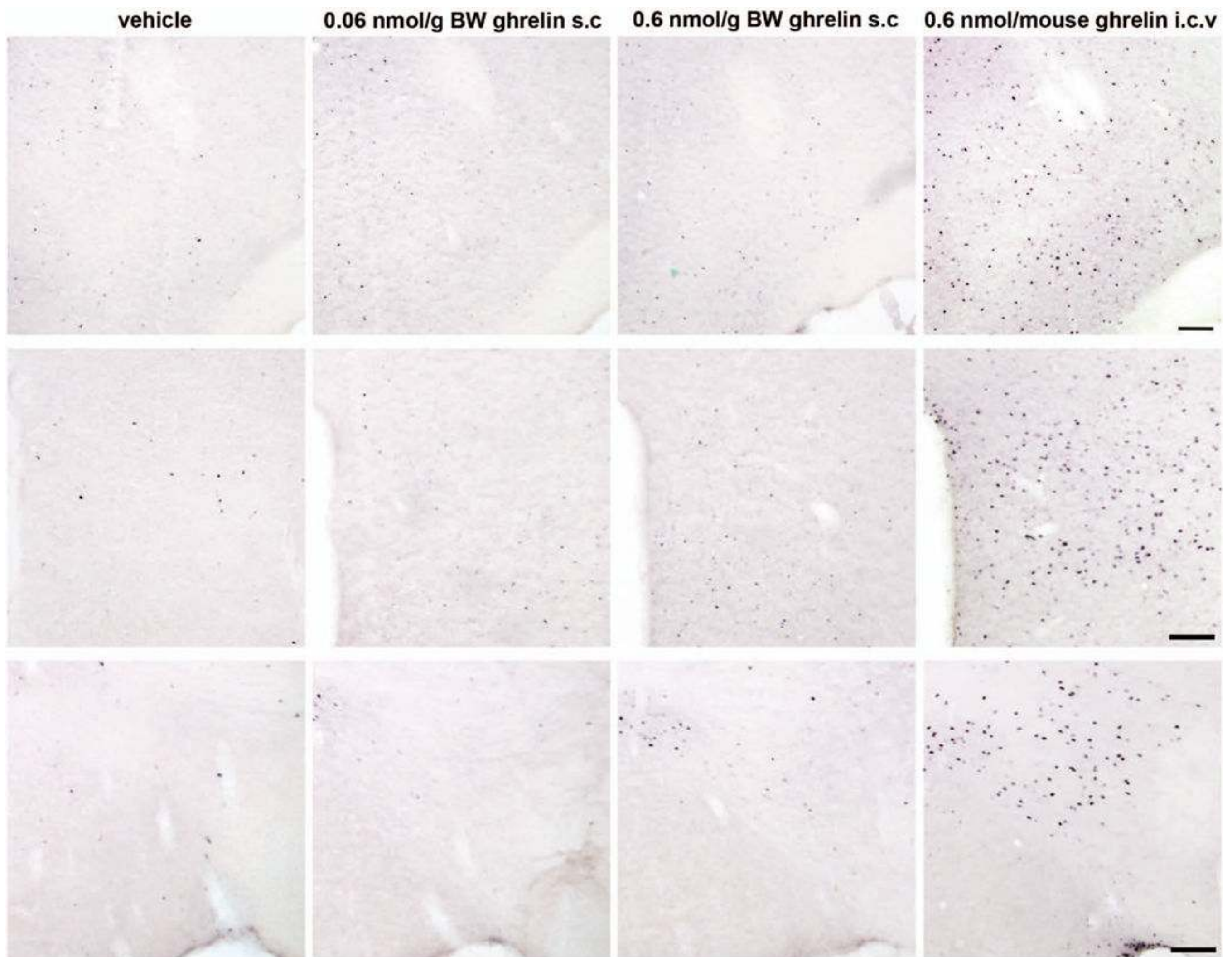

Figure 3.

Ghrelin-induced c-Fos in the AHA, the LHA, the DMH and the VTA of the different experimental groups. Panels show representative low (×20) magnification images of c-Fos staining in the AHA and LHA (upper line), the DMH (middle line) and the VTA (bottom line) of vehicle- and ghrelin-treated mice. Scale bar: 100 μm.

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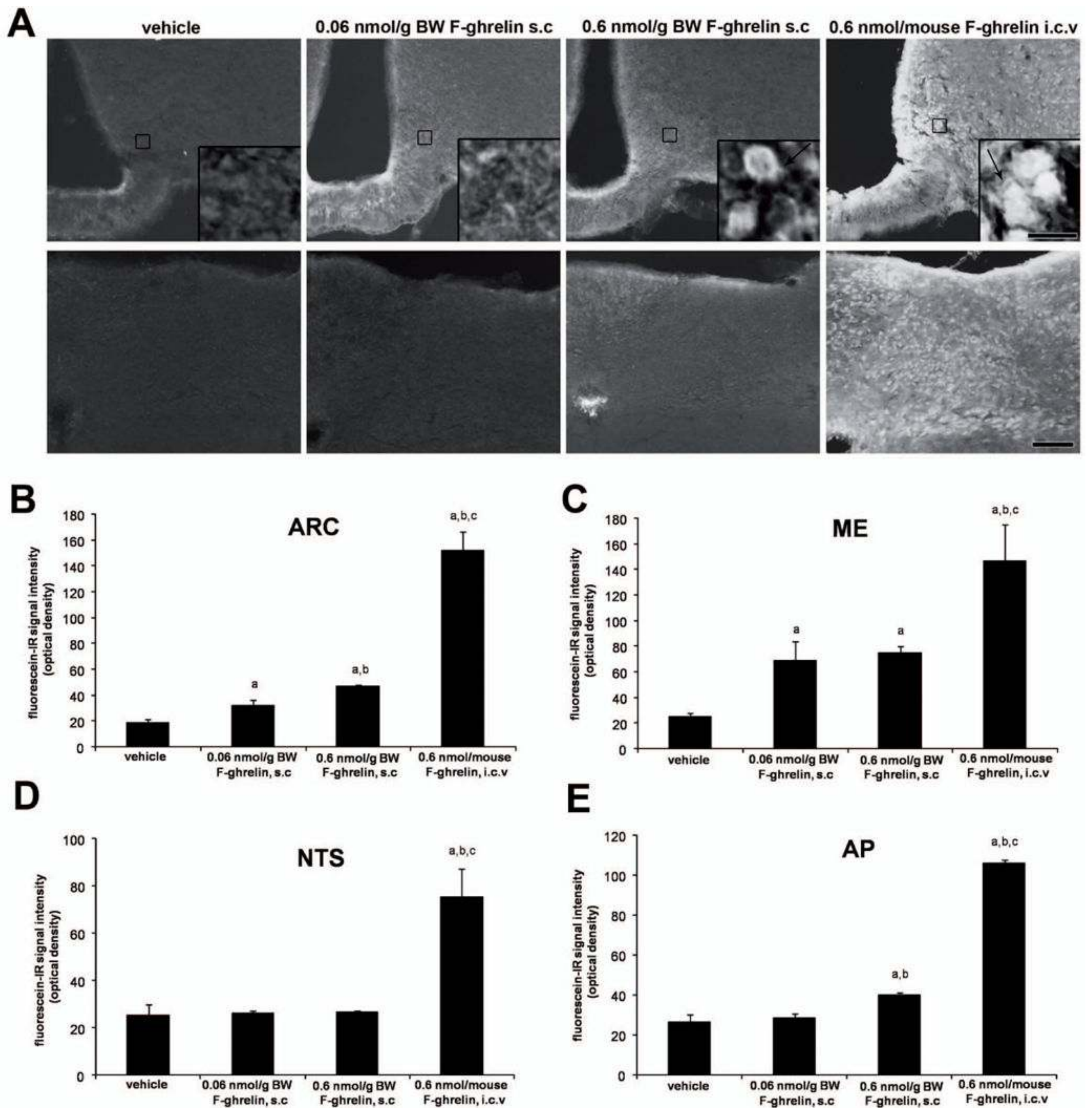

Figure 4.

Fluorescein-IR in the ARC and the DVC of the different experimental groups. Panel A shows inverted series of representative low (×20) magnification images of cromogenic IHC for fluorescein in ARC and ME (upper line) and DVC (bottom line) of vehicle- and F-ghrelin-treated mice. Scale bar: 100 μm. Bar graphs show quantitative analysis of fluorescein-IR intensity in the ARC (panel B), the ME (panel C), the NTS (panel D) and the AP (panel E) of vehicle- and F-ghrelin-treated mice. Data represent the mean±SEM. a, $p<0.05$ vs. control group.

b, $p<0.05$ vs. low dose of ghrelin s.c group.

c, $p<0.05$ vs. high dose of ghrelin s.c group.

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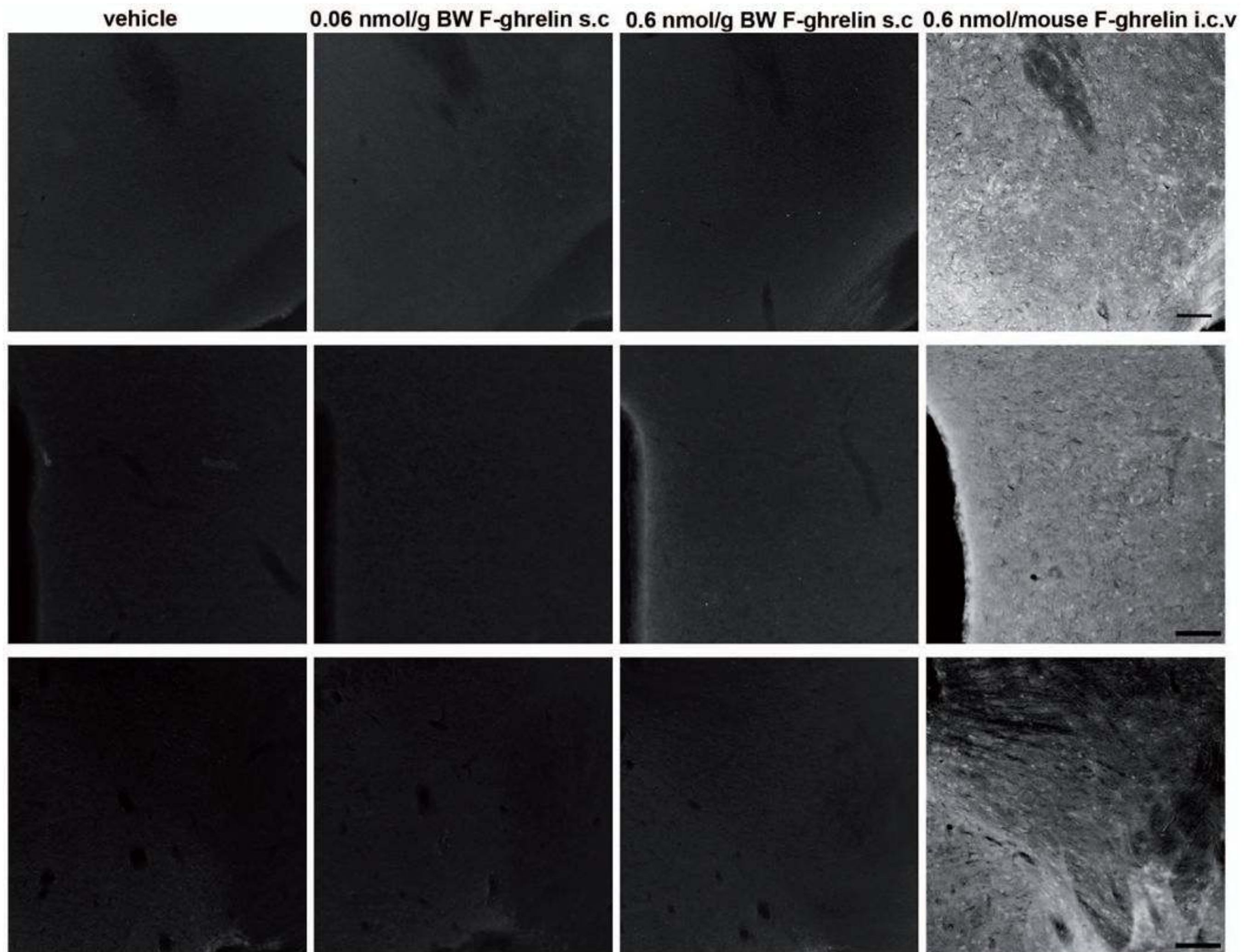

Figure 5.

Fluorescein-IR in the AHA, the LHA, the DMH and the VTA of the different experimental groups. Panel A shows inverted series of representative low (×20) magnification images of cromogenic IHC for fluorescein in the AHA and LHA (upper line), the DMH (middle line) and the VTA (bottom line) of vehicle- and ghrelin-treated mice. Scale bar: 100 μm.

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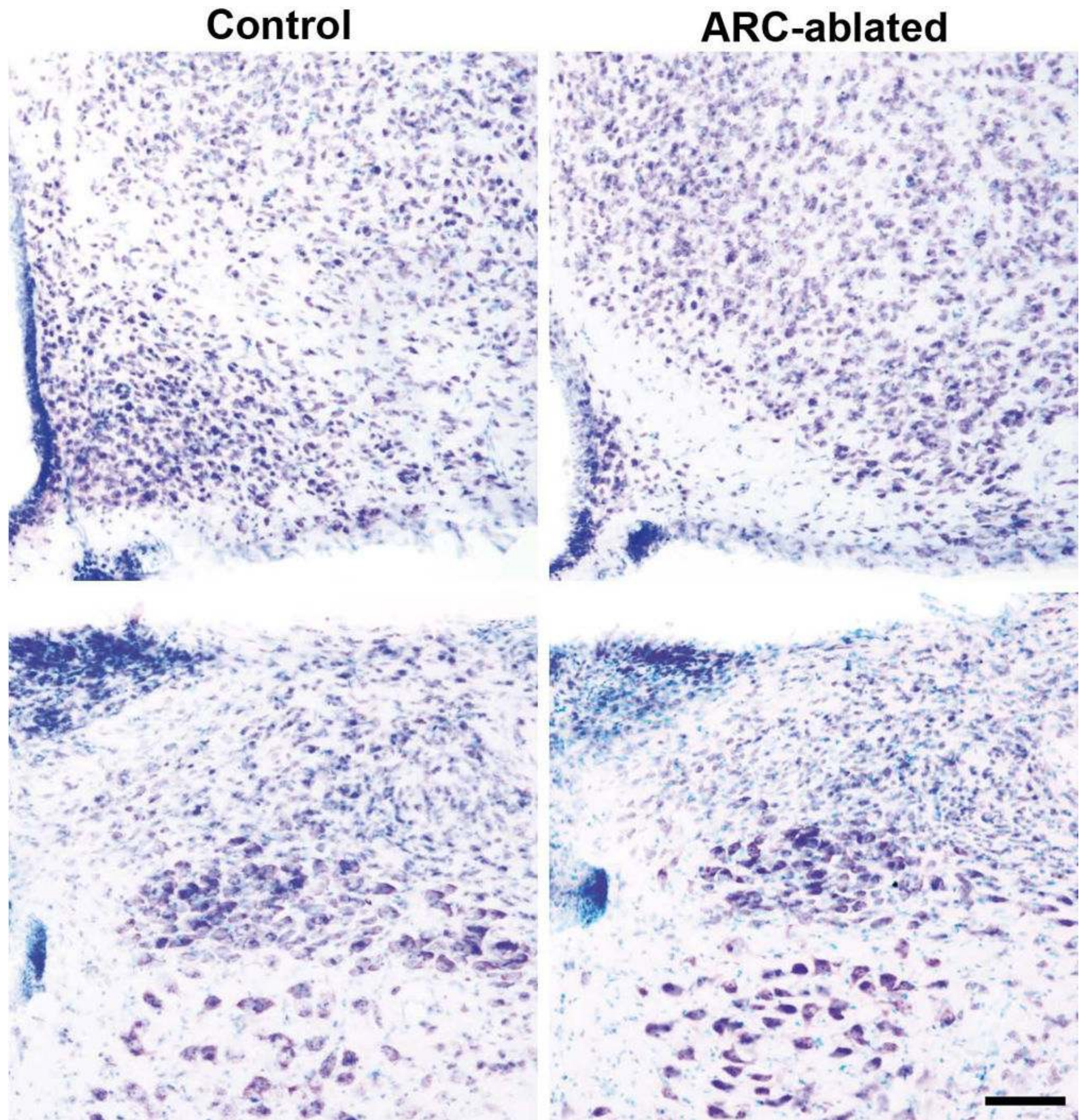

Figure 6.

Validation of ARC-ablated mouse model. Panels show representative images of cresyl violet staining of the ARC (upper line) and DVC (bottom line) of control and ARC-ablated animals. Scale bar: 100 μm.

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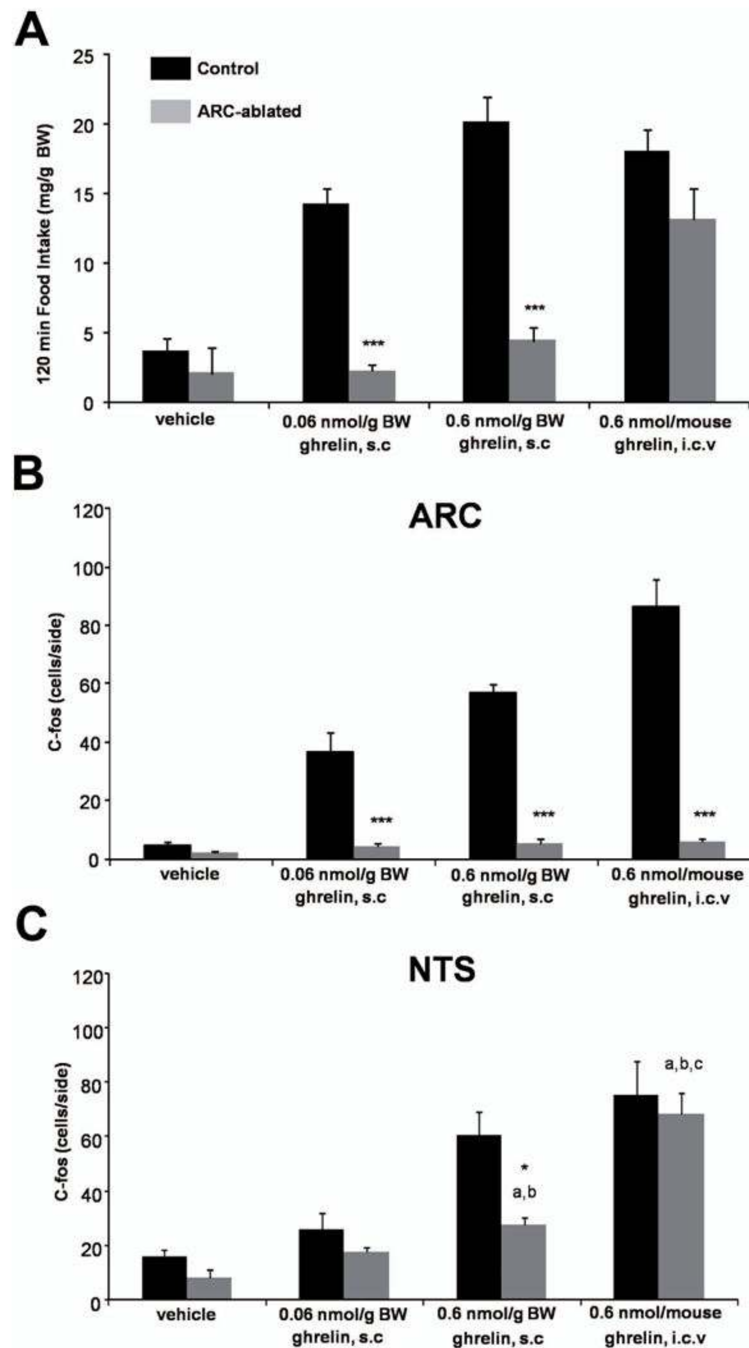

Figure 7.

Ghrelin-induced food intake and ghrelin-induced c-Fos in the ARC and the DVC of control and ARC-ablated mice subject to the different experimental protocols. Panel A shows 120 min food intake in control and ARC-ablated mice peripherally or centrally treated with vehicle or ghrelin. Panels B and C show quantitative analysis of the number of c-Fos-IR cells in the ARC and the NTS, respectively, of control and ARC-ablated mice peripherally or centrally treated with vehicle or ghrelin. Data represent the mean±SEM.

***, $p<0.001$ vs. control group with same treatment.

*, $p<0.05$ vs. control group with same treatment.

a, $p<0.05$ vs. ARC-ablated control group.

b, $p<0.05$ vs. ARC-ablated low dose of ghrelin s.c group.

c, $p<0.05$ vs. ARC-ablated high dose of ghrelin s.c group

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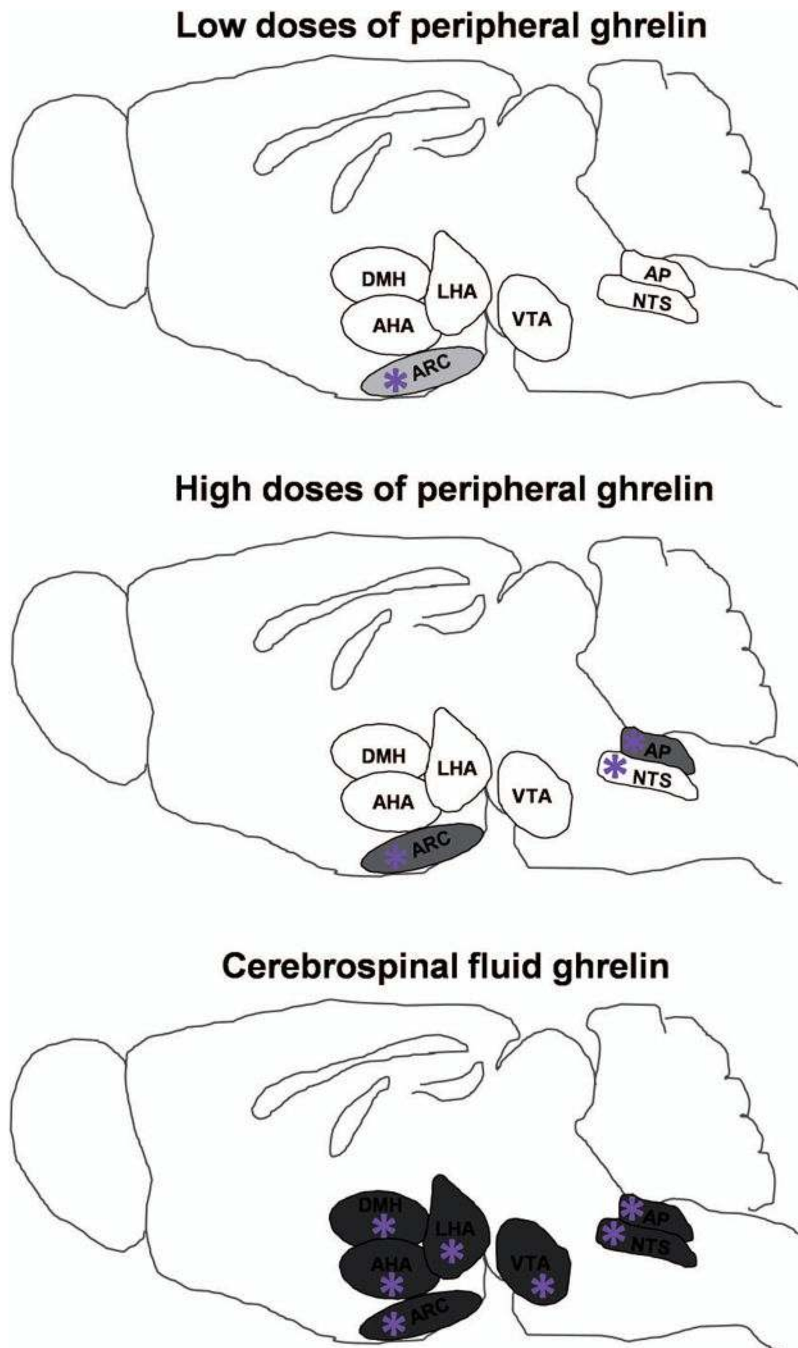

Figure 8.

Model of potential neuronal nuclei mediating orexigenic effects of peripheral and central ghrelin. Images depict a sagittal view of the rodent brain in which regions implicated in ghrelin's regulation of food intake are highlighted. Asterisks within brain nuclei represent that ghrelin-induced c-Fos was found, and lighter-to-darker grey tone represents that F-ghrelin accessibility was detected. Abbreviations are as follows: AHA, anterior hypothalamic area AP: area postrema, ARC: arcuate nucleus, DMH: dorsomedial nucleus,

LHA: lateral hypothalamic area, NTS: nucleus of the solitary tract and VTA: ventro tegmental area.

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