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Fourth ventricle injection of ghrelin decreases angiotensin II-induced fluid intake and neuronal activation in the paraventricular nucleus of the hypothalamus

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Abstract

Ghrelin acts in the CNS to decrease fluid intake under a variety of dipsogenic and natriorexigenic conditions. Previous studies on this topic, however, focused on the forebrain as a site of action for this effect of ghrelin. Because the hindbrain contains neural substrates that are capable of mediating the well-established orexigenic effects of ghrelin, the current study tested the hypothesis that ghrelin applied to the hindbrain also would affect fluid intake. To this end, water and saline intakes were stimulated by central injection of angiotensin II (AngII) in rats that also received injections of ghrelin (0.5 µg/µl) into either the lateral or fourth ventricle. Ghrelin injected into either ventricle reduced both water and 1.8% NaCl intake that was stimulated by AngII. The nature of the intake effect revealed some differences between the injection sites. For example, forebrain application of ghrelin reduced saline intake by a reduction in both the number of licking bursts and the size of each licking burst, but hindbrain application of ghrelin had a more selective effect on burst number. In an attempt to elucidate a brain structure in which hindbrain-administered ghrelin and forebrain-administered AngII interact to cause the ingestive response, we used Fos-immunohistochemistry in rats given the treatments used in the behavioral experiments. Although several brain areas were found to respond to either ghrelin or AngII, of the sites examined, only the paraventricular nucleus of the hypothalamus (PVN) emerged as a potential site of interaction. Specifically, AngII treatment caused expression of Fos in the PVN that was attenuated by concomitant treatment with ghrelin. These experiments provide the novel finding that the hindbrain contains elements that can respond to ghrelin and cause decreases in AngII-induced fluid intake, and that direct actions by ghrelin on forebrain structures is not necessary. Moreover, these studies suggest that the PVN is an important site of interaction between these two peptides.

Keywords

ghrelin; angiotensin II; water intake; saline intake; paraventricular nucleus; hindbrain

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Introduction

Ghrelin is a 28 amino acid peptide produced in the gut [1] and brain [2] that is well known for its orexigenic properties. More recent studies have demonstrated a role for ghrelin in fluid intake, but its effects are in the opposite direction of that for food intake. For instance, both peripheral [3] and central [3–7] injections of ghrelin reduce water intake in laboratory rats. This anti-dipsogenic effect of ghrelin is not restricted to water intake, but also decreases saline intake [8]. Importantly, ghrelin only affects the intake of these fluids when it is stimulated by certain dipsogenic [7] or natriorexigenic [8] conditions, including central administration of angiotensin II (AngII). As such, the opposite direction of the effects of ghrelin provide an opportunity to understand separate, but perhaps overlapping, circuits that are involved in the control of drinking behaviors.

The neural substrates that mediate the effects of ghrelin on fluid intake have not been identified. Further clouding the issue, previous studies testing the CNS effects of ghrelin on drinking had a focus on the forebrain. This is potentially limiting, especially in light of studies looking at other effects of ghrelin. For instance, studies have shown clear hindbrain-mediated effects of ghrelin on food intake [9, 10] and on the maintenance of fasting blood glucose levels [11]. Accordingly, we used injections of ghrelin into either the lateral ventricle (LV) or fourth ventricle (4V) to test the hypothesis that ghrelin acting in the hindbrain decreases fluid intake. We used AngII as the stimulus for drinking because it increases consumption of both water and saline, allowing us to test for effects that may be selective for one fluid type or the other, and because ghrelin has been shown to decrease intake of both fluids when drinking is stimulated by AngII [7, 8]. To help elucidate a neural substrate that is involved in the integration of AngII and ghrelin, we used Fos-immunohistochemistry to test for sites of convergence in the brain between structures responding to these two peptides.

Methods

Animals

Adult, male Sprague-Dawley rats (Envigo Research Models and Services, Indianapolis, IN) weighing 325–349g were housed individually in hanging wire-mesh cages in a temperature- and humidity-controlled room with standard chow and water provided *ad libitum*, except when stated. Rats were maintained on a 12:12-h light:dark cycle with lights on at 8:00 AM. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at State University of New York at Buffalo.

Surgery

Rats were surgically implanted with either one chronic indwelling cannula aimed at the LV or two chronic indwelling cannulae; one aimed at the LV and the one aimed at the 4V. To accomplish this, rats were anesthetized (70 mg/kg ketamine and 5 mg/kg xylazine, I.M.) and placed in a stereotaxic instrument. For LV placements, a 26-gauge guide cannula (Plastics One, Roanoke, VA) was implanted (coordinates: 0.9mm posterior to bregma, 1.4mm lateral to mid-line, 1.8mm ventral to dura) and secured to the skull using bone screws and dental

cement. For 4V placements, the guide cannula was implanted (coordinates: 2.5mm anterior to the occipital structure, 4.8mm ventral to skull) and secured to the skull using bone screws and dental cement.

Peptides

Angiotensin II (AngII; Bachem Bioscience Inc., King of Prussia, PA) and ghrelin (Pi Proteomics, Huntsville, AL) were used in each experiment. AngII was dissolved in sterile trisbuffered saline (TBS; 0.9%) and ghrelin was dissolved in artificial cerebral spinal fluid.

Verification of cannula placement

Rats were given a minimum of five days to recover from surgery before cannula placement was verified. LV placement was considered accurate if a drinking response (≥ 6 ml in 30 min) occurred after an injection of AngII (10ng in 1 ul). Accurate 4V placement was verified by testing for at least a doubling of blood glucose after an injection of 5-thio-d-glucose (210ug in 1 ul; MP Biomedicals, Santa Ana, CA, USA). Animals without accurate cannula placement were excluded from the experiments.

Intake measurements

Fluid intake tests in Experiments 1 and 2 (see below) were each 2h. Fluid volume consumed was determined by weighing the bottles before and after each intake test. The pattern of intake throughout the test was measured using a contact lickometer (designed and constructed by the Psychology Electronics Shop, University of Pennsylvania, Philadelphia, PA) that recorded individual licks throughout each intake test. The lickometer interfaced with a computer using an integrated USB digital I/O device (National Instruments, Austin, TX). Home cages were affixed with an electrically isolated metal plate with a 3.175-mm-wide opening, through which the rat needed to lick to reach the drinking spout, minimizing the possibility of non-tongue contact with the spout. Food intake was quantified by comparing the weight before and after the food intake test, which occurred for two hours after, but not during, the fluid intake test. Spilled crumbs were collected using plastic transparencies placed under each cage during the food intake test. Drinking microstructure was analyzed to determine the number of bursts and the average number of licks per burst during each drinking test. A bout of licking was considered a burst when at least 2 licks occurred with a maximum inter-lick interval of 1 s.

Immunohistochemistry

Brain sections from rats in Experiments 3 and 4 (see below) were washed in TBS for 90 minutes during which time the TBS was replaced 9 times. Next, the sections were placed in a 3% hydrogen peroxide-TBS solution for 15min. After 3 5-min rinses with TBS, the sections were incubated in a rabbit anti-c-Fos antibody (1:5000; Ab-5; Calbiochem, USA) for one hour on a rocker at room temperature, then overnight at 4°C. The sections then were washed for 1 h with TBS and then incubated in biotin-SP-conjugated affinipure donkey anti-rabbit IgG (1:1000; Jackson Laboratories, USA) for 2 h at room temperature. After a 30min wash with TBS, the sections were incubated in avidin-biotin complex (1:111; Vectastain Elite ABC Kit, Vector Laboratories, California, USA) for one hour at room temperature. The

sections were washed with TBS three times and then visualized with 3,3'-diaminobenzidine tetrahydrochloride hydrate (1:50; DAB; Sigma Aldrich, MO, USA). This protocol was followed for both experiments, but the immunohistochemistry was done at different times. Thus, direct comparisons between experiments 3 and 4 were not made in any analysis.

Quantitative analysis of Fos-immunoreactivity

The number of Fos-immunoreactive (Fos-ir) cells was quantified using Nikon software (NIS-Elements BR 3.2 64-bit, Melville, NY, USA). This software indicates the number of cells, in a designated area of a brain section, that meet a criterion threshold for intensity, circularity, and size. Criteria were set to be consistent within brain regions and batches of staining. Criterion selection and cell counts were performed without knowledge of treatment condition. Analyses of brain regions used Paxinos and Watson [12] as a guide, and regions for quantification of Fos were outlined and segregated from the rest of the section using NIS-Elements (Nikon Instruments, Inc).

Statistical analysis

Statistica (version 9; StatSoft Inc., Tulsa, OK, USA) was used to analyze data with α -level set at $p < 0.05$. The fluid intake tests and Fos-immunoreactivity studies were analyzed using a two-way ANOVA to test the between-subjects effect of drug treatment (AngII or ghrelin). One rat in the AngII+ghrelin group from experiment 4 was excluded from this analysis because it was found to be an outlier based on being more than 2 standard deviations from the mean. For the analysis of drinking microstructure, burst number and burst size, t-tests were conducted to compare the AngII-treated groups. Newman-Keuls *post hoc* tests were used to further probe statistically significant main effects or interactions.

Experiment 1: Effect of LV ghrelin on water and 1.8% NaCl intakes

Rats were habituated to 2-bottle cages with access to tap water and 1.8% NaCl, as well as standard chow for at least 5 days before testing. On the day of testing, each rat was given two consecutive 1ul injections into the LV. The first injection containing either AngII (10ng in 1ul TBS) or vehicle (1ul TBS), and the second injection contained either ghrelin (0.5ug in 1 ul) or vehicle (1ul aCSF). The doses of AngII and ghrelin were chosen based on previous studies in our laboratory [7, 8]. After the second injection, rats were placed back into their cages with pre-weighed water and 1.8% NaCl, but no food, for 2 h. Fluids were measured again at the end of the 2-h test and then returned to the cages along with pre-weighed food. Remaining food and food spillage were measured at the end of the 2-h food test. A contact lickometer recorded licks for water and 1.8% NaCl during the 2-h fluid intake test.

Experiment 2: Effect of 4V ghrelin on water and 1.8% NaCl intake

Experiment 2 used the same approach as experiment 1 except that rats received the second injection (ghrelin or vehicle) into the 4V instead of the LV.

Experiment 3: Neuronal activation by angiotensin II and lateral ventricle administered ghrelin

Rats were given the same drug treatments as described in Experiment 1, but after the second injection, rats were returned to their cages with no access to food or fluids. Ninety minutes after the second injection, rats were anesthetized and transcardially perfused with 150 ml isotonic saline followed by 450 ml of 4% paraformaldehyde. Brains were extracted and postfixed in 4% paraformaldehyde overnight and then switched to 20% sucrose for 2 days before being cut into 40- μ m coronal sections using a freezing microtome (Spencer 820, American Optical Company, Buffalo, NY, USA). Sections were stored in cryoprotectant at -20°C between cutting and immunohistochemistry.

Experiment 4: Neuronal activation by angiotensin II and fourth ventricle administered ghrelin

Experiment 4 used the same methods as experiment 3 except that rats received the second injection (ghrelin or vehicle) into the 4V instead of the LV.

Results

Experiment 1: Effect of LV-administered ghrelin on water intake and 1.8% NaCl intakes

In an initial experiment, we gave rats two consecutive injections into the LV: the first containing AngII (10 ng in 1 μ l) or vehicle (TBS), and a second containing ghrelin (0.5 μ g in 1 μ l) or vehicle. Total licks for water or 1.8% NaCl in the 2 h after the injections are shown in Figure 1A and 1B. The interaction between the two peptides (AngII and ghrelin) was statistically significant for water intake (Fig 1A; $F = 4.3903$, $P < 0.05$) and for 1.8% NaCl (Fig 1B; $F = 8.9620$, $P < 0.01$). *Post hoc* tests revealed that both water and 1.8% NaCl intakes were increased by AngII and that treatment with ghrelin attenuated the increase in water intake and completely blocked the increase in 1.8% NaCl.

To further probe the effect of ghrelin on AngII-induced intake, we analyzed licking burst patterns of AngII and AngII+ghrelin-treated rats. The results of the burst pattern analyses are found in Figures 2 and 3. This analysis found that the decreased water intake observed after ghrelin injection into the LV was not produced by a statistically significant decrease in either burst number (Fig 2A; $T = 1.1031$; $P = 0.2854$) or burst size (Fig 3A; $T = 0.6159$; $P = 0.5461$). In contrast, the more robust decrease in saline intake observed after ghrelin injection into the LV was due to decreases in both burst number (Fig 2B; $T = 4.5489$; $P < 0.001$) and burst size (Fig 3B; $T = 2.1841$; $P < 0.05$).

Experiment 2: Effect of 4V-administered ghrelin on water and 1.8% NaCl intakes

To examine the effect of hindbrain-administered ghrelin on AngII-stimulated fluid intake, we measured water and 1.8% NaCl after injection of AngII into the LV (10ng in 1 μ l) and ghrelin (0.5 μ g/ μ l) into the 4V of rats and compared the subsequent intake with rats given appropriate control injections. Total licks for water and 1.8% NaCl during the 2-h drinking test are shown in Figures 1C and 1D. The interaction between the two drugs (AngII and ghrelin) was statistically significant for water (Fig 1C; $F = 9.4639$, $P < 0.01$) and for 1.8% NaCl (Fig 1D; $F = 4.6637$, $P < 0.05$). *Post hoc* tests revealed that AngII increased intake of

both water and 1.8% NaCl and the AngII-induced increases in intake were attenuated by ghrelin.

Analysis of licking burst patterns in rats given ghrelin injections into the 4V revealed a more selective effect on burst number, but a less selective effect of fluid type. Specifically, the analysis of licking burst patterns in 4V-injected rats found that ghrelin treatment was associated with a decrease in burst number for either fluid type (water: Fig 2C, $T = 2.2563$, $P < 0.05$; saline: Fig 2D, $T = 2.1093$, $P < 0.05$). Although the effect was observed for both fluids, in contrast to the more selective effect on saline observed in Experiment 1, injections of ghrelin into the 4V appeared to cause no changes in burst size (water: Fig 3C, $T = 0.4025$, $P = 0.6907$; 1.8% NaCl: Fig 3D, $T = 0.7900$, $P = 0.4367$).

Experiment 3: Neuronal activation by angiotensin II and lateral ventricle administered ghrelin

To elucidate potential sites in the brain at which ghrelin and AngII interact, we tested for Fos-immunoreactivity in the brains of rats given two consecutive injections into the LV: one of AngII (10ng) or vehicle and one of ghrelin (0.5 μ g) or vehicle. No results indicated a potential site of interaction between AngII and ghrelin when both peptides were given to the LV. Either a main effect of AngII (Table 1) or ghrelin (Table 2) was found in each site examined, but we did not find any statistically significant interactions of the treatments in any brain area examined.

Experiment 4: Neuronal activation by angiotensin II and fourth ventricle administered ghrelin

In contrast to the results of Experiment 3, when ghrelin was given to the 4V of rats that were also given AngII into the LV, the paraventricular hypothalamic nucleus (PVN), emerged as a potential site of interaction between these two peptides. Specifically, we found a significant interaction between the two drug treatments (AngII vs ghrelin; Fig 4A; $F = 6.1759$; $P < 0.05$) in the number of Fos-positive cells. *Post hoc* tests revealed that the number of Fos-positive cells was increased after treatment with AngII + vehicle, and that this increase was not found in rats also given ghrelin into the 4V. Representative photomicrographs of Fos-immunoreactivity in the PVN from rats in each treatment group are shown in Figure 4B–E. Either a main effect of AngII (Table 1) or ghrelin (Table 2) was found in the other areas examined. Representative photomicrographs showing high levels of Fos-immunoreactivity in each of these structures are shown in Figure 5A–H to illustrate the regions of interest for the analyses.

Discussion

The present study shows for the first time that elements in the rat hindbrain can respond to ghrelin and cause a suppression of fluid intake. Our findings replicate and extend previous studies [4, 7, 8] by showing that forebrain ventricle injections of ghrelin reduced AngII-induced fluid intake and that this effect also occurred when the injections of ghrelin were made into the hindbrain ventricle. The finding that injections into the hindbrain were effective raises the intriguing possibility that injections into the forebrain were effective only

because the ghrelin was able to follow the rostral-caudal flow of CSF, thereby allowing it to act on hindbrain structures. This possibility seems unlikely, however, because of some notable differences in the responses, at both the behavioral and protein levels, observed after the different routes of injection. Specifically, the analysis of drinking microstructure conducted on licks recorded during experiments 1 and 2 revealed that ghrelin injected into the forebrain produced a decrease in burst number and burst size for saline with no change in either of those parameters for water, whereas ghrelin injected into the hindbrain resulted in a decrease in burst number for both water and saline with no effect on burst size. Injecting ghrelin into different sites also resulted in different patterns of neuronal activity. In particular, ghrelin injected into the hindbrain, but not the forebrain, blocked an AngII-induced increase in neuronal activity in the PVN. If ghrelin was acting only in the hindbrain to affect AngII-stimulated drinking, even when injected into the forebrain, the same changes to lick patterns and neuronal activity would be expected after forebrain and hindbrain injected ghrelin, but that was not found in the present study. Accordingly, we believe that the most favorable conclusion to be drawn from these studies is that both forebrain and hindbrain sites are able to respond to ghrelin and cause the observed changes in behavior.

The results of the present experiments draw attention to the previously unrecognized ability of the hindbrain to mediate these responses on AngII-induced drinking and neuronal activity in the PVN. Based on the ability of ghrelin injected into the hindbrain to reduce AngII-stimulated drinking, we conclude that direct contact between ghrelin and receptors in forebrain structures is not required in order for ghrelin to suppress AngII-induced fluid intake. This is in line with studies that have examined the hindbrain contribution of some of ghrelin's other effects. Studies by Faulconbridge et al. [9, 10], for example, demonstrated that hindbrain injection of ghrelin is sufficient to mediate its hyperphagic effects. Similarly, Scott et al. [11] showed that fasting blood glucose levels could be rescued in ghrelin receptor deficient mice by selectively restoring the ghrelin receptor in the hindbrain. Thus, it appears that a number of ghrelin's effects, including the effect on AngII-stimulated fluid intake from the present study, can be mediated by hindbrain structures and do not require direct contact between ghrelin and forebrain structures. Taken together with the observation that AngII-induced Fos expression in the PVN was attenuated by 4V injections of ghrelin, the results suggest the existence of an ascending pathway from ghrelin-responsive structures in the hindbrain to forebrain substrates that respond to AngII. An alternate possibility is that the ghrelin injected into the hindbrain was able to diffuse against the flow of CSF, but this is unlikely based on several previous studies, including experiments from our laboratory using AngII-induced drinking as a model [13]. Further studies are needed to confirm the existence of these putative ascending pathways and identify their source.

In addition to providing steps toward elucidating a ghrelin-responsive network that plays a role in the control of fluid intake, our studies provide the first investigation of licking patterns relevant to the fluid intake suppression caused by ghrelin. These data are potentially informative because early work by Davis and colleagues [14–16] suggests that observable changes in lick patterns are indicative of the nature of the change. More specifically, a change in the number of licking bursts (burst number) is associated with a difference in post-ingestive feedback, whereas changes in the number of licks per burst (burst size) is associated with changes in orosensory properties of the tastant. With respect to the present

study, the changes in burst size and burst number observed after the various treatments and routes of administration of ghrelin suggests that ghrelin injected into the LV reduced saline intake through effects on both post-ingestive and orosensory changes, but that ghrelin injected into the 4V had a more selective effect on postingestive feedback. As discussed above, these data support our conclusion that forebrain and hindbrain elements effect changes through separable, although likely overlapping, neural substrates.

The neuroanatomical approach used in the present experiments also provided new information about the anatomical specificity of the interaction between ghrelin and AngII. The experiments using Fos immunohistochemistry allowed us to probe the CNS for areas showing differences in rats injected with each peptide alone or the combination of the two, and also allowed us to compare the profile of activation when the injection of ghrelin was made into either the LV or 4V. Our analysis focused on a limited set of brain regions based on previous studies of Fos expression after ghrelin [17–22] or AngII [23–26] administration. Perhaps the most notable finding in the present studies was that rats given ghrelin into the 4V did not show elevated Fos in the PVN that was observed in rats given AngII alone. An earlier study [27] used Fos expression to test the hypothesis that ghrelin administered to the 4V affected forebrain substrates. Consistent with the present results, these studies failed to find activation in the PVN after 4V injection of ghrelin, and therefore concluded that ascending projections to at least some forebrain substrates were not required for intake effects of ghrelin. The present studies do not necessarily invalidate that conclusion, but do provide evidence for ghrelin-responsive ascending circuits that play a role in the control of ingestive behavior. The present findings also provide another reason that caution is urged when drawing conclusions based on the absence of Fos after a given treatment. Using the present studies as an example, in the absence of AngII, the lack of ghrelin-induced Fos in the PVN could be reasonably interpreted as evidence that the PVN is not part of a ghrelin-responsive circuit. The lower level of Fos observed in the PVN after ghrelin administration to the 4V was only observable when levels of Fos were elevated by AngII, suggesting that previous studies and the present study showing a lack of Fos after ghrelin was given alone was limited by a floor effect, but that the PVN is, actually, part of a ghrelin responsive circuit that can be engaged by ghrelin acting in the hindbrain. In the context of the present focus on fluid intake, these data suggest that the PVN is an important brain region for the subject of future studies on the fluid intake suppression caused by ghrelin. Because we did not attempt to isolate particular portions of the PVN (magnocellular vs parvocellular, peptide expression, etc), future studies are needed to determine if the observed interaction is specific to any sub-regions or cell types. The present studies are an important step toward that end.

It is fitting that the PVN would play a role in integrating the effects of these two peptides on drinking because it has long been recognized as an integrative structure in the control of ingestive behaviors. For instance, the PVN receives input from structures including the SFO [28–31] and OVL [31, 32] that are involved in detecting signals of thirst, such as AngII [33, 34], and integrates this to aid in producing the proper behavioral and physiological responses. Similarly, efferents from structures involved in controlling food intake converge on the PVN. In particular, the PVN has been shown to detect and integrate feeding relevant signals from the arcuate nucleus including AgRP, NPY, and melanocortins [35] as well as feeding relevant signals from the hindbrain [36, 37]. Given that the PVN is clearly involved

in integrating signals relevant to ingestive behaviors, it is not surprising to see it emerge as a site of interaction between ghrelin and AngII in the current study.

Although the present experiments demonstrated that the hindbrain is sufficient to mediate the anti-dipsogenic effect of ghrelin, it does not identify which structures in the hindbrain are involved in this effect. The results of our Fos experiments identified ghrelin responsive neurons in the PBN and the NTS at the level of area postrema and obex. This is consistent with others that have shown neural activation in these structures after ghrelin treatment [17, 20, 27, 38]. These are also attractive targets because several studies have demonstrated that the fluid intake responses to AngII are enhanced after manipulations that inhibit cells of, or produce lesions in these hindbrain sites [39–41]. Thus, they appear to provide an inhibitory influence on AngII-induced fluid intake under normal conditions. Collectively, these findings point to these hindbrain structures as potentially being involved in mediating the effect of hindbrain administered ghrelin on AngII-stimulated drinking. Ongoing follow-up experiments in our laboratory will help test this open question.

The present studies are not without limitations. There are at least two key issues that we believe deserve consideration. First, the findings of the current study may not generalize to drinking in response to other dipsogenic or natriorexigenic conditions. These studies used a single means to induce fluid intake, and it is quite possible that the hindbrain is not sufficient to mediate the suppression of drinking by ghrelin under other conditions. Indeed, our previous studies found that ghrelin affected drinking only under some conditions [7, 8]. Our working hypothesis is that this was related to the strength of the stimulus to drink, but it may reflect differences in the pathways affected by the different stimuli, and the ability of ghrelin to engage these pathways. Similarly, the PVN may not be a site of interaction between ghrelin and other treatments that induce drinking and may be specific to AngII-induced fluid intake, and perhaps even more specific to AngII that is delivered to the lateral ventricle. The present data, nevertheless, provide additional information that could lead to a better understanding of how the system can respond to endogenous AngII and ghrelin. Second, the neuroanatomical studies are limited by the targeted analysis used here. Specifically, we focused on a limited set of brain areas, supported by previous research, but future examination of other brain regions could reveal additional and unexpected sites of interaction between ghrelin and AngII. Follow-up experiments are needed to answer these questions.

The present experiments replicate and extend what is known about how ghrelin affects AngII-induced drinking. First, we demonstrate a previously unappreciated capability of the hindbrain to mediate the anti-dipsogenic effects of ghrelin on AngII-stimulated water and saline intake. This is a novel departure from previous studies that had focused on the forebrain as a site of action for ghrelin's anti-dipsogenic effects. Second, these experiments provide new information about where in the brain these two peptide systems are interacting, a question that had not previously been addressed. Also of importance, the current study suggests that the PVN can serve as an important starting point for future studies that can help reveal specific hindbrain structures that may be involved in mediating the anti-dipsogenic effect of hindbrain-administered ghrelin, thereby furthering our understanding of the means by which thirst and salt appetite are controlled.

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Highlights

- 4V injection of ghrelin decreased AngII-induced water and saline intake.
- Ghrelin affected lick patterns differently depending on injection site (LV vs 4V).
- 4V injection of ghrelin reduced AngII-induced neural activity in the PVN.

Figure 1.

Total licks for water (A, C) or 1.8% NaCl (B, D). Rats ($n=8-14$ per group) were injected with AngII ($10\text{ng}/\mu\text{l}$) or vehicle ($1\mu\text{l}$ TBS) into the LV plus ghrelin ($0.5\mu\text{g}/\mu\text{l}$) or vehicle ($1\mu\text{l}$ aCSF) into either the LV (A, B) or the 4V (C, D). Licks for water and 1.8% NaCl were recorded using a contact lickometer for 2h after the injections during which time food was not available. AngII increased licks for water and 1.8% NaCl, but ghrelin injected into the LV attenuated the increase in licks for water and blocked the AngII-induced licks for 1.8% NaCl. Similarly, ghrelin injected into the fourth ventricle caused an attenuation of AngII-

stimulated licks for water (C) and 1.8% NaCl (D). Bars with different letters are significantly different from each other ($P < 0.05$).

Figure 2.

Number of bursts for water (A, C) and 1.8% NaCl (B, D). An analysis of drinking microstructure was performed on the total licks for water and 1.8% NaCl that were recorded during Experiments 1 (Figure 1A and 1B) and 2 (Figure 1C and 1D) from AngII treated rats (AngII+vehicle vs AngII+ghrelin). Ghrelin injected into the LV caused a decrease in the number of bursts for 1.8% NaCl intake, but did not affect the number of bursts for water intake compared to AngII+vehicle treated rats. Ghrelin injected into the 4V reduced the

number of bursts for both water and 1.8% NaCl intake compared to AngII+vehicle treated rats. Significant differences denoted by * ($P<0.05$).

Figure 3.

Burst size (average licks per burst) for water (A, C) and 1.8% NaCl (B, D). An analysis of drinking microstructure was performed on the total licks for water and 1.8% NaCl that were recorded during Experiments 1 (Figure 1A and 1B) and 2 (Figure 1C and 1D) from AngII treated rats (AngII+vehicle vs AngII+ghrelin). Ghrelin injected into the LV caused a decrease in the burst size for 1.8% NaCl intake, but did not affect the number of bursts for water intake compared to AngII+vehicle treated rats. Ghrelin injected into the 4V did not

affect the burst size for either water or 1.8% NaCl intake compared to AngII+vehicle treated rats. Significant differences denoted by * ($P<0.05$).

Figure 4.

Number of Fos-immunoreactive cells in the PVN. Rats ($n=5-7$ per group) were given an injection of AngII ($10\text{ng}/\mu\text{l}$) or vehicle ($1\mu\text{l}$ TBS) into the LV plus an injection of ghrelin ($0.5\mu\text{g}/\mu\text{l}$) or vehicle ($1\mu\text{l}$ aCSF) into the 4V. Food and fluids were removed after the injections and rats were transcardially perfused 90 minutes later. AngII caused an increase in the number of Fos-immunoreactive cells in the PVN, but this increase was blocked by an injection of ghrelin into the 4V (A). Representative photomicrographs of fos-immunoreactivity in the PVN after treatment with vehicle/vehicle (B), vehicle/ghrelin (C), AngII/vehicle (D), or AngII/ghrelin (E). Significant differences denoted by * ($P<0.05$).

Figure 5.

Representative photomicrographs showing high levels of fos-immunoreactivity in each structure examined: AV3V (5A), dorsal portion of cMnPO (5B), ventral portion of cMnPO (5C), SFO (5D), SON (5E), ARC (5F), PBN (5G), NTS-AP (5H), and NTS-OBX (5I).

Hemi-sections are shown for structures not on midline, but both sides were included in the analysis.

Structures in which a main effect of AngII on Fos-immunoreactivity was found ($p<0.05$), without a main effect of ghrelin or an interaction between AngII and ghrelin. Injection site refers to the location of the injection of ghrelin or its vehicle, all AngII was injected into the LV.

Table 1

Structure	Injection site	VEH (TBS)/ VEH (aCSF)	VEH (TBS)/ GHR (0.5µg)	ANG (10ng)/ VEH (aCSF)	ANG(10ng)/ GHR (0.5µg)
AV3V	LV	14.8 ± 3.08	30.95 ± 11.13	95.20 ± 9.00	75.58 ± 8.82
	4V	20.61 ± 4.35	40.39 ± 13.19	87.05 ± 16.86	67.94 ± 25.98
cMnPO	LV	34.45 ± 5.32	86.67 ± 14.95	127.40 ± 16.56	137.67 ± 31.66
	4V	23.13 ± 8.22	35.70 ± 5.30	62.86 ± 10.84	67.52 ± 16.82
PVN	LV	20.98 ± 5.07	36.50 ± 13.07	96.05 ± 17.45	100.90 ± 29.92
SFO	LV	16.23 ± 5.44	23.77 ± 13.27	56.46 ± 14.46	78.71 ± 10.15
	4V	3.74 ± 0.92	4.81 ± 1.91	46.93 ± 10.62	45.72 ± 10.61
SON	LV	4.12 ± 1.44	20.50 ± 4.10	107.45 ± 20.95	69.82 ± 13.32
	4V	3.77 ± 2.11	18.48 ± 9.35	33.86 ± 9.44	34.40 ± 11.37

The mean number of Fos-immunoreactive cells ± SEM is given for each of the four treatments. Sections (2–4 per animal) from each region were used to compute the mean for each brain and the mean for each region and each treatment below is based on 5–11 brains.

Abbreviations: AV3V = anteroventral third ventricle region; cMnPO = caudal Median Preoptic area; PVN = paraventricular nucleus; SFO = subfornical organ; SON = supraoptic nucleus.

Structures in which a main effect of ghrelin on Fos-immunoreactivity was found ($p<0.05$), without a main effect of AngII or an interaction between AngII and ghrelin. Injection site refers to the location of the injection of ghrelin or its vehicle, all AngII was injected into the LV.

Table 2

Structure	Injection site	VEH (TBS)/ VEH (aCSF)	VEH (TBS)/ GHR (0.5ug)	ANG (10ng)/ VEH (aCSF)	ANG(10ng)/ GHR (0.5ug)
ARC	LV	47.97 ± 9.06	95.50 ± 9.25	48.69 ± 12.18	94.78 ± 9.14
	4V	24.83 ± 4.74	34.64 ± 4.84	16.10 ± 2.85	29.75 ± 2.15
PBN	LV	14.60 ± 1.45	121.28 ± 18.00	22.80 ± 8.84	114.79 ± 17.90
	4V	14.54 ± 4.67	108.46 ± 24.41	34.55 ± 5.77	115.15 ± 23.47
NTS-AP	LV	42.31 ± 4.66	177.16 ± 23.23	56.68 ± 7.60	131.03 ± 21.84
	4V	10.93 ± 2.68	34.49 ± 8.36	18.97 ± 4.70	63.26 ± 17.35
NTS-OBX	LV	26.88 ± 8.18	69.53 ± 12.31	24.54 ± 9.95	73.38 ± 13.36
	4V	10.17 ± 2.21	28.15 ± 7.80	16.84 ± 3.45	27.12 ± 10.09

The mean number of Fos-immunoreactive cells ± SEM is given for each of the four treatments. Sections (2–4 per animal) from each region were used to compute the mean for each brain and the mean for each region for each treatment below included 5–11 brains.

Abbreviations: ARC = arcuate nucleus; PBN = parabrachial nucleus; NTS-AP = nucleus of the solitary tract at the level of area postrema; NTS-OBX = nucleus of the solitary tract at the level of obex.