Hindbrain Catecholamine Neurons that Innervate Medial Hypothalamic Nuclei Modulate Growth Hormone Secretion but are not Required for the Orexigenic Response to Ghrelin

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Honors Thesis

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PASS WITH DISTINCTION
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As thesis advisor for Alan Emanuel,

I have read this paper and find it satisfactory.

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Précis

Ghrelin is a peptide produced and secreted into the bloodstream by specialized cells in the stomach. This peptide was initially shown to be capable of binding to receptor sites on cells in the brain and pituitary gland that secrete growth hormone (GH), a hormone required for normal growth. Binding of ghrelin to this receptor stimulates GH secretion. Subsequently it was discovered that ghrelin also stimulates food intake. The production of this peptide is enhanced during fasting, and ghrelin has thus been described as the "hunger hormone".

Currently, however, we do not have a clear concept as to the way in which ghrelin stimulates feeding behavior. The ghrelin receptor is predominately expressed at the base of the hypothalamus. This is a unique brain area with holes in the blood-brain barrier which allows for ghrelin and other molecules that nor~ly cannot cross the blood brain barrier to access the brain. One point of view is that the basal hypothalamus, which also contains feeding-stimulatory peptides, monitors the concentration of ghrelin in the blood. The alternative view is that ghrelin is monitored by the vagus nerve, which innervates the gut. The signal from the vagus is relayed through the hindbrain to the hypothalamus via neurons that synthesize and release catecholamine neurotransmitters.

The purpose of this thesis is to further evaluate the latter of these hypotheses. To do this, we injected an immunotoxin that is specific for catecholaminergic neurons into the hypothalamus. These neurons internalize the toxin at their terminals, and transport it back to the cell body where it acts on the nucleus to kill the cell. With this method, we were able to specifically eliminate the relay from the hindbrain to the hypothalamus. The feeding response to ghrelin was identical in lesioned and control animals, which were injected in the same location with a form of the toxin that could not be internalized. This result indicates that these neurons are not required for ghrelin-induced feeding.
While the catecholamine neurons targeted by the toxin are not required for ghrelin-induced feeding, it is clear that they are activated by ghrelin. We evaluated the activation of the hindbrain catecholamine neurons by microscopically looking for the expression of a protein, Fos, that has classically been used as a marker for neuronal activation. Fos expression was elevated in various groups of these neurons after ghrelin injections into the forebrain. This reveals that ghrelin is capable of increasing the activity of these neurons, but as we found, these neurons are not responsible for ghrelin's feeding effect. This suggests that activation of hindbrain catecholamine neurons by ghrelin mediates an effect other than stimulation of food intake.

To assess this possibility, we examined the role of hindbrain catecholamine neurons in another action of ghrelin, the GH response. We measured GH concentration in the blood at various time points after ghrelin injection in animals with and without the lesion to evaluate this hypothesis. The animals that lack the hindbrain catecholamine neurons had a prolonged growth hormone response to ghrelin. This prolongation of GH secretion suggests that the catecholamine neurons are part of a negative feedback loop for the control of GH.

This new hypothesis was evaluated by quantifying the activation, once again visualized with Fos expression, of the catecholamine neurons in response to GH. We found that GH activates the same populations of hindbrain catecholamine neurons as ghrelin, thus supporting the negative feedback hypothesis. This is a novel finding that contributes to our understanding of how GH secretion is controlled under different metabolic conditions as well as to our understanding of the functions served by hindbrain catecholamine neurons. Furthermore, our results do not support the previously proposed hypothesis that hindbrain catecholamine neurons are a required link in the pathways responsible for ghrelin-induced feeding. We conclude that ghrelin's effects on feeding most likely result from its direct actions on hypothalamic neurons.
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Introduction

Ghrelin is a peptide produced and secreted primarily by endocrine cells in the oxyntic gastric mucosa (Date et al., 2000b; Dornonville de la Cour et al., 2001). Ghrelin was originally discovered as an endogenous ligand for the growth hormone secretogogue receptor (GHSR) type 1a (Howard et al., 1996; Kojima et al., 1999; Date et al., 2000b; Date et al., 2000a). Ghrelin stimulates growth hormone (GH) release both by acting directly on GHSRs in the anterior pituitary and by increasing release of growth hormone releasing factor (GHRH) from hypothalamic parvocellular neurons (Cruz and Smith, 2008; Gahete et al., 2009). A second major metabolic effect of ghrelin is stimulation of food intake (Nagaya et al., 2001; Nakazato et al., 2001; Asakawa et al., 2003; Bagnasco et al., 2003; Geary, 2004; Kojima and Kangawa, 2005; Williams and Cummings, 2005). During fasting and instances of low glucose levels, ghrelin peptide content is decreased in the hypothalamus and stomach and ghrelin gene expression is increased in stomach, indicating that food deprivation is a stimulus for ghrelin production and secretion (Toshinai et al., 2001; Wren et al., 2001a; Wren et al., 2001b). Ghrelin is unique in its function as it is the only known endogenously produced molecule to generate feeding responses when injected peripherally, outside of the brain. Therefore, understanding the mechanism by which ghrelin stimulates feeding is important for enhancing knowledge about the physiological control of energy balance.

Ghrelin-induced stimulation of feeding appears to be mediated largely by the arcuate nucleus of the hypothalamus (Arc) (Bagnasco et al., 2003; Cowley et al., 2003). Ghrelin activates orexigenic (feeding stimulatory) neurons in the Arc that express neuropeptide Y (NPY) and Agouti-related peptide (AgRP). In addition, ghrelin inhibits anorexigenic neurons that express pro-opiomelanocortin (POMC), also localized to the Arc (Kamegai et al., 2001; Cowley
et al., 2003; Shrestha et al., 2004). Lesions of the Arc that destroy NPY/AGRP and POMC neurons impair or abolish ghrelin-induced stimulation of feeding (Bugarith et al., 2005; Luquet et al., 2007).

Although there is much evidence to support the view that ghrelin-induced GH secretion and feeding are dependent on ghrelin's actions within the Arc and possibly the adjacent pituitary, ghrelin and ghrelin receptors are also expressed in other brain sites (Ferrini et al., 2009) and on the vagus nerve (Date et al., 2002). In addition, ghrelin injections into hypothalamic sites outside the Arc and into nonhypothalamic sites stimulate feeding (Wren et al., 2001b; Faulconbridge et al., 2003; Olszewski et al., 2003; Naleid et al., 2005) and both ghrelin-induced feeding and GH secretion are reported to be blocked by vagotomy (Date et al., 2002). Therefore, ghrelin's actions at these various extrahypothalamic sites are potentially important for its major effects. Indeed, Date and colleagues recently reported that noradrenergic (NE) neurons in the A2 cell group within the nucleus of the solitary tract (NTS) are a required substrate for control of food intake by circulating ghrelin (Date et al., 2006). Specifically, Date et al. proposed that circulating ghrelin acts initially on vagal sensory neurons, which relay a signal from the periphery to activate A2 neurons that innervate the Arc. In support of their hypothesis, these investigators showed that ghrelin increases dopamine-beta-hydroxylase (DBH, an enzyme necessary for the synthesis of norepinephrine and epinephrine in noradrenergic and adrenergic neurons) mRNA expression in A2 cell bodies. In addition, they demonstrated that NE release in the Arc is enhanced, a result of increased activation of catecholamine neurons innervating the Arc. Conversely, Date and colleagues found that ghrelin-induced feeding is attenuated by intracerebroventricular (icv) α1 and β2 NE receptor antagonists or lesion of the catecholamine projection from the hindbrain to the Arc with two separate methods. The first was a knife cut
through the midbrain and the second was the injection of the retrogradely transported
catecholamine immunotoxin, anti-DBH-saporin (DSAP) into the Arc (Date et al., 2006). In a
separate report, this group also reported that both the feeding and GH responses to circulating
ghrelin were eliminated by vagotomy, suggesting that ghrelin does not act directly on the A2
neurons (Date et al., 2002) but peripherally either on the nodose ganglion, the collection of cell
bodies whose projections make up the vagus, or areas of peripheral vagal innervation.

Known functions of hindbrain catecholamine neurons are consistent with this proposed
role in mediating ghrelin’s actions. Some hindbrain catecholamine neurons, like ghrelin, exert
potent stimulatory effects on food intake (Leibowitz, 1988; Leibowitz et al., 1988; Taylor et al.,
2007), influence GH secretion (Chapman et al., 1993a, b; Willoughby et al., 1993) and their
activation increases expression of hypothalamic NPY and AGRP mRNA (Fraley and Ritter,
2003). However, while implicating A2 neurons in ghrelin-induced feeding, Date and colleagues’
previous work (Date et al., 2006) did not examine the potential contribution of hypothalamically-

Figure 1 – This diagram of a sagittal section of the rat brain just lateral to the midline represents the relative rostral-caudal
and dorsal-ventral positions of hindbrain catecholaminergic cell groups and medial hypothalamic nuclei discussed in this
study. Adapted from the Paxinos and Watson rat brain atlas. Abbreviations: Arc, arcuate nucleus of the hypothalamus; PVH,
paraventricular nucleus of the hypothalamus; 3v, third ventricle; 4v, fourth ventricle.
projecting catecholamine neurons other than those in A2 to ghrelin-induced feeding. NE and epinephrine (E) neurons from other hindbrain cell groups not only contribute to the innervation of the hypothalamus (Sawchenko and Swanson, 1982, 1983; Ritter et al., 2001), but a subpopulation of these non-A2 neurons is activated by glucose deficit (Ritter and Dinh, 1994; Ritter and Dinh, 1997; Ritter et al., 1998) and the activation of these neurons stimulates food intake (Ritter et al., 2001; Ritter et al., 2003; Li et al., 2009). Figure 1 demonstrates the relative anatomical locations of the hindbrain catecholamine cell groups and the medial hypothalamic areas that they innervate. Localized gene silencing or retrograde lesion of the hypothalamic NE/E projection with DSAP impairs this glucoregulatory feeding response (Ritter et al., 2001; Ritter et al., 2003; Li et al., 2009). Similarly, activation of hindbrain catecholamine neurons inhibits or stimulates GH secretion via hypothalamic α1 and α2 adrenergic receptors, respectively (Chapman et al., 1993a, b; Willoughby et al., 1993; Mounier et al., 1994), but the specific catecholamine cell population involved and whether ghrelin also activates this population are not known.

Therefore, the goal of the present study was to determine which hypothalamically-projecting catecholamine cell groups in the hindbrain are involved in these actions of ghrelin. We used double label immunofluorescence to examine the distribution of hindbrain catecholamine neurons expressing Fos protein, an indicator of neuronal activation (Cullinan et al., 1995), in response to ghrelin. Our results indicate that hindbrain catecholamine neurons that ghrelin activates are concentrated in the ventrolateral medulla (A1, A1/C1, C1 and A5), but are not present in A2, as suggested by Date, et al. (Date et al., 2006). We also examined GH secretion and food intake after targeted retrograde lesion of hypothalamically-projecting catecholamine neurons with DSAP. In contrast to Date et al. (Date et al., 2006), I found that
DSAP lesions that destroyed hypothalamically-projecting catecholamine neurons did not alter the feeding response to either central, within the brain, or systemic ghrelin administration. However, the GH secretory response to central ghrelin administration was enhanced by the DSAP lesion, suggesting a role for NE in feedback inhibition of ghrelin-induced GH secretion.

Hypotheses

1. Hindbrain catecholamine neurons relay the ghrelin-induced feeding signal to the arcuate nucleus and paraventricular nucleus of the hypothalamus. Lesion of these neurons will result in the ablation of ghrelin-induced feeding.

2. Catecholamine neurons that innervate the medial hypothalamus regulate an aspect of ghrelin-induced growth hormone secretion, the other major action of ghrelin. Specifically, the lesion of these neurons will result in prolongation of growth hormone secretion.

3. Ghrelin administration results in the activation (indicated by Fos protein expression) of hindbrain catecholamine neurons known to send projections to the medial hypothalamus.

4. The prolongation of the growth hormone response indicates that the catecholamine neurons might be part of a negative feedback response. Therefore, I hypothesize that the Fos expression observed after ghrelin is actually due to growth hormone or a signaling molecule that indicates instances of increased plasma growth hormone concentration. Peripheral growth hormone administration will result in increased Fos expression in hindbrain catecholamine neurons in a pattern similar to that observed after ghrelin administration.
Materials and Methods

Animals

Adult male Sprague Dawley rats from Simonsen laboratories (Gilroy, CA) were housed in suspended wire cages within a temperature controlled room illuminated between 0700 and 1900 h and used for all experiments. Rats had ad libitum access to water and standard pelleted rat chow (Harlan Teklad F6 Rodent Diet W, Madison, WI). The Washington State University Institutional Animal Care and Use Committee, which conforms to National Institute of Health rules and regulations, approved all experimental animal protocol.

Experiment 1: Effect of DSAP lesions on ghrelin-induced feeding and GH secretion.

In this experiment, DSAP or SAP was injected into the PVH, as described previously (Ritter et al., 2001; Ritter et al., 2003), to retrogradely lesion catecholamine neurons innervating medial hypothalamic nuclei. DSAP and SAP rats were subsequently tested for feeding and GH secretion responses to systemic and 4V administration of ghrelin. Feeding in response to 2-deoxy-D-glucose (2DG)-induced glucoprivation was used as a behavioral screening test to determine lesion effectiveness, since this response requires the catecholamine neurons targeted by the DSAP lesion (Ritter et al., 2001; Ritter et al., 2003). At the end of the experiment, the DSAP lesion was evaluated using immunohistochemical approaches.

DSAP and SAP Lesions: Rats were anesthetized with chloropent anesthesia (3 ml/kg ip), made by combining 21.25 g of choral hydrate, 10.6 g of magnesium sulfate, 4.43 g pentobarbital sodium, 75.26 ml ethyl alcohol and 169.00 ml propylene glycol, brought to 500 ml with sterile dd H2O and filtered. Saporin conjugated to a monoclonal antibody against dopamine-beta-hydroxylase (DSAP, 42ng/200naL, Advanced Targeting Systems, San Diego, CA) was stereotaxically microinjected over eight minutes into the paraventricular nucleus of the
hypothalamus (PVH; 1.8 mm caudal to bregma, 0.4 mm lateral to the midline on both sides, 7.35 mm ventral to the dura mater) with a glass micropipette (30 μm tip diameter) attached to a Picospritzer with polyethylene tubing to lesion DBH-expressing neurons projecting to the PVH. Unconjugated saporin (SAP, 42ng/200nL, Advanced Targeting Systems) was administered in the same manner to control animals. The progress of the injection solution through the micropipette was monitored microscopically. The animals were allowed to recover from surgery for at least two weeks before further experimentation.

*Fourth Ventricle (4V) Cannula Implantation:* Cannulas were implanted into the 4V of SAP and DSAP lesioned rats for administration of ghrelin and artificial cerebrospinal fluid (aCSF) control solution. Rats were anesthetized for cannula implantation with 1 ml/kg ketamine/xylazine/acepromazine cocktail [5 ml ketamine HCl, 100 mg/ml (Fort Dodge Animal Health, Fort Dodge, IA); 2.5 ml xylazine, 20 mg/ml (Ben Venue Laboratories, Bedford, Ohio); 1 ml acepromazine, 10 mg/ml (Vedco, Saint Joseph, MO); and 1.5 ml 0.9% saline solution]. Twenty six gauge cannulas, occluded with stainless steel obturators, were implanted stereotaxically at the midline, 2.0 mm rostral to the occipital suture and 6.5 mm ventral to the dura mater.

*2DG and Ghrelin-Induced Feeding Tests:* Feeding tests were conducted during the light phase, beginning at 1000 h in DSAP- (n = 14) and SAP- (n = 13) treated rats. Glucoprivic feeding was measured in a 4 h test immediately following injection of 2DG (200 mg/kg, sc, Sigma, St. Louis, MO) or saline (0.9%, 1 ml/kg) to assess the effectiveness of the DSAP lesion. Subsequently, feeding in response to systemic ghrelin (15ug/kg, ip, Peptide Institute, Osaka, Japan) or saline control injection (0.9%, 1 mL/kg ip or sc) was measured in a 2-h test in DSAP and SAP lesioned rats. Feeding responses were also measured in response to 4V ghrelin (2
μg/6μl, 150 pmol/1μl or 30 pmol/200nl) or artificial cerebrospinal fluid (aCSF; 6 μL, 1μl, or 200nl). Solutions were delivered into the 4V using 33 gauge injectors connected with polyethylene tubing to a Hamilton glass syringe.

Growth Hormone Response to LV ghrelin injection: Whole blood was collected for determination of GH concentrations in DSAP (n=7) and SAP (n=8) rats in response to LV ghrelin (2μg/6μl) or aCSF (6μl) injection. Samples (150 μl) were collected from the saphenous vein using glass capillary tubes at 4 time points: immediately before and 20, 40 and 60 minutes after ventricular injection. Samples were transferred to microcentrifuge tubes containing 4 μl 15% EDTA and promptly centrifuged for 10 minutes at 8,000 rpm. Plasma was transferred to new microcentrifuge tubes and stored at -20°C until analyzed. Rat GH concentration was measured in plasma using an ELISA kit (Millipore Corporation, Billerica, MA). The immunoassay has a sensitivity of 0.07ng/ml and produces a 5-parameter logistic standard curve. Plates were read using an uQuant microplate (Biotek) reader with Gen5 software (Biotek, Winooski, VT).

Immunohistochemical analysis of DSAP lesions: After the completion of behavioral testing, rats were euthanized by deep anesthesia induced by inhalation of isoflurane and transcardially perfused with a 0.1M phosphate buffer solution (PBS) followed by 4% formaldehyde in PBS. The brains were immediately extracted and post-fixed in 4% formaldehyde in PBS overnight and then submerged into 25% sucrose in PBS overnight. The hindbrain and hypothalamus were sectioned into four parallel sets of 30 μm slices using a cryostat and collected in 0.1M tris-sodium phosphate buffer (TPBS). The immunohistochemistry procedure was started immediately after sectioning. Free-floating hindbrain sections were processed for immunohistochemical detection of DBH. The sections
were rinsed three times for 5 min in TPBS and blocked overnight in 10% normal horse serum (NHS) made in TPBS. After removing the blocking solution, the sections were incubated in mouse anti-DBH (1:50,000; Millipore Corporation, Billerica, MA) antibody diluted in 10% NHS for 48 h. This solution was removed and the tissue was washed in TPBS (3x5 min) and then incubated in Alexa 555 donkey anti-rabbit secondary antibody for 3 h (Invitrogen, Carlsbad, CA). The tissue was washed again, mounted on VWR slides, and cover-slipped using Prolong Gold (Invitrogen). To quantify the effectiveness of the DSAP lesions, DBH-immunoreactive (-ir) cell bodies were counted in the A1, A2, and C1 catecholaminergic cell groups between the calamus scriptorius to the caudal border of the facial nucleus. DBH-ir terminals in the medial hypothalamus were evaluated. Data from any animal that did not have visibly decreased terminals innervating the hypothalamus were removed from analysis.

**Experiment 2: Effect of Ghrelin and GH on Fos Expression in Hindbrain Catecholamine Cell Groups.**

The purpose of this experiment was to determine whether ghrelin and GH activate hindbrain catecholaminergic neurons and, if so, to examine the distribution and phenotype of the activated neurons. We used double-label immunofluorescence to evaluate Fos expression in DBH-ir and phenylethanolamine N-methyltransferase (PNMT, the enzyme responsible for converting NE to epinephrine)-ir cells after ghrelin and GH treatment.

**Lateral Ventricle Cannula Implantation:** Cannulas were stereotaxically implanted into the lateral ventricle (LV), as described above for 4V cannula implantation, for administration of ghrelin or aCSF. Cannulas were implanted 1.0 mm caudal to bregma, 1.5 mm lateral to the midline and 3.9 mm ventral to the dura mater. The animals recovered to their pre-surgical body weight before further experimentation.
**Ghrelin injection and tissue Collection and Preparation:** Ghrelin (2μg/6μl; n=5 for DBH, n=3 for PNMT) or aCSF (6μl; n=2 for DBH, n=2 for PNMT) was injected into the LV, and human recombinant GH (75μg/100g body weight; n=4 for DBH, n=2 for PNMT; National Hormone and Peptide Program, A.F. Parlow, Torrance, CA) or 0.9% saline (1ml/kg; n=2 for DBH, n=1 for PNMT) was injected subcutaneously for induction of Fos signaling 90 minutes prior to euthanasia. Rats were killed by deep anesthesia induced by inhalation of isoflurane and transcardially perfused with a 0.1M phosphate buffer solution (PBS) followed by 4% formaldehyde in PBS. The brains were immediately extracted and post-fixed in 4% formaldehyde in PBS overnight and then submerged into 25% sucrose in PBS overnight. The hindbrain and hypothalamus were sectioned into four parallel sets of 30μm slices using a cryostat and collected in 0.1M tris-sodium phosphate buffer (TPBS). The immunohistochemistry procedure was started immediately after sectioning.

**Immunohistochemical Detection of Fos protein and DBH:** Free-floating hindbrain sections were processed for immunohistochemical detection of DBH or PNMT and Fos protein, using the protocol described for Experiment 1. Mouse anti-DBH (1:50,000; Millipore Corporation, Billerica, MA) and rabbit anti-Fos (1:10,000; EMD Biosciences Ab5, San Diego, CA) or rabbit anti-PNMT (1:10,000; Millipore Corporation) and goat anti-Fos (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies diluted in 10% NHS for 48 h were used as the primary antibodies. Secondary antibodies were Alexa 555 donkey anti-rabbit or donkey anti-goat and Alexa 488 donkey anti-mouse or donkey anti-rabbit (Invitrogen, Carlsbad, CA).

**Quantification of Fos Expression in DBH-ir neurons:** To determine the number and location of catecholamine cells expressing Fos in response to ghrelin or GH, cells co-expressing both DBH and Fos were counted in A1, A2, C1, C2, C3, C5, and area postrema cell groups in
one set of four parallel sections per animal. Cell group boundaries were defined according to Paxinos and Watson (Paxinos and Watson, 1997).

Statistical Analysis

Data for Experiments 1 and 2 are presented as mean ± SEM and were analyzed using three-way or two-way analysis of variance (ANOVA) for repeated measures or student's t test as appropriate. Differences with a P value ≤ 0.05 were considered to be significant. When a significant F value was revealed by ANOVA, the Holm-Sidak post hoc test was used to isolate significant differences.

Results

Experiment 1

Lesion verification. Rats with DSAP injections exhibited reduced total numbers of DBH-ir neurons in A1 (t9 = 7.245, P<0.001) and A1/C1 (t9 = 10.471, P<0.001) cell groups compared to SAP controls (Figure 2). Neurons in the A2 cell group form two morphologically distinct subtypes. The large multipolar cells predominately localized in the medial and commissural NTS were reduced by the DSAP lesion (t9 = 6.643, P < 0.001), as reported previously (Ritter et al., 2001) (Kalia et al., 1985). Conversely, the small round DBH-ir cells localized in the dorsal NTS were not reduced by the PVH DSAP injections (t9 = 0.968, P=0.358) (Kalia et al., 1985). DBH-ir terminals in medial hypothalamic nuclei (including ARC and PVH) of animals injected with DSAP were visibly reduced in comparison to those injected with SAP. An example of this reduction can be seen in Figure 2.
Figure 2 - Immunohistochemical and quantitative confirmation of DSAP lesion. Total hindbrain DBH-ir neurons were counted in A1, A2 and C1 cell groups for confirmation of reduced neuronal counts after DSAP lesion (A). DSAP rats (n=5) have significantly fewer A1 and A1/C1 neurons than the SAP controls (n=6). The reduction in A2 neurons was cell-type specific; the larger neurons in the medial NTS (A2-large) were reduced while the smaller neurons in the dorsal strip and dorsal NTS (A2-small) were not significantly reduced. The micrographs show typical DBH-ir in anatomically matched sections of a SAP control animal (B) and DSAP lesioned animal (C). There was a dramatic reduction of DBH immunoreactivity in both the arcuate and paraventricular hypothalamic nuclei with DSAP treatment. PVH, paraventricular nucleus of the hypothalamus. ARC, arcuate nucleus. 3V, third ventricle. *P < 0.05 in comparison to SAP control.

**Catecholamine neurons are essential for glucoprivic feeding but not ghrelin-induced feeding.** Rats were tested for an intact glucoprivic feeding response after DSAP lesion (n=14) or SAP (n=13) control injection. Analysis of the results with two-way repeated measures ANOVA indicates that there are significant main effects for 2DG compared to saline control ($F_{1,25}=79.00$, $P<0.001$) as well as the lesion ($F_{1,25}=41.290$, $P<0.001$). There was an interaction between the two factors ($F_{1,25}=41.555$, $P<0.001$) indicating that the response to the 2DG treatment depends on whether or not rats were subjected to the DSAP lesion. Post-hoc analysis revealed that 2DG significantly elevated feeding over baseline in SAP rats but not in DSAP rats (Figure 3A).
DSAP and SAP rats were tested for ghrelin-induced feeding and a two way repeated measures ANOVA was used to analyze the resulting data. There was a significant main effect of ip ghrelin or saline treatment (F_{1,25}=29.725, P<0.001) but no main effect due to the DSAP lesion (F_{1,25}=0.0000234, P=0.996). Post-hoc analysis revealed that both SAP and DSAP rats had significantly increased food intake 2 h after ip ghrelin treatment in comparison to the 2 h ip saline baseline (Figure 3B). There was a significant main effect of the 3 levels of icv ghrelin treatment versus the saline control (F_{2,22}=37.558, P<0.001) but there were no effects due to lesion status and no interaction between ghrelin treatment and lesion status. Increased food intake was also observed after fourth ventricular injection of ghrelin when compared to aCSF control (Figure 3C). There was a slight, but not significant, trend towards enhanced icv ghrelin-induced feeding in DSAP rats over ghrelin-induced feeding in SAP rats.

Figure 3 - Feeding in response to 2DG and ghrelin in 2- and 4- hr tests. SAP rats increased their food intake significantly in response to 200 mg/kg 2DG than after 1 ml/kg saline control injection, while DSAP rats did not increase their intake significantly in response to 2DG (A). Both SAP and DSAP rats responded to ghrelin with elevated 2 h feeding whether ghrelin was injected systemically (B; 15μg/kg ghrelin or 1 ml/kg saline) or into the 4V (C). There were no significant differences in ghrelin treatment groups in the SAP and DSAP animals. *P < 0.05 vs vehicle control.

GH secretion induced by fourth ventricular ghrelin administration was increased in DSAP lesioned rats. Figure 4 shows plasma GH concentration in DSAP and SAP rats immediately before (0) and 20, 40, and 60 minutes after aCSF or ghrelin treatment. A significant interaction among the three factors (2 x 2 x 4), ghrelin treatment, DSAP surgery, and time was
found with a three-way repeated measures ANOVA ($F_{3,104}=2.975$, $P=0.035$). The data at each timepoint was analyzed with two-way repeated measures ANOVA. There were no main effects due to treatment or lesion status at the 0 or 60 minute time points. At 20 minutes after injection, there was a main effect for ghrelin treatment of the animal ($F_{1,13}=37.80$, $P<0.001$). At 40 minutes after treatment, there were significant main effects for both lesion status ($F_{1,13}=5.531$, $P=0.035$) and ghrelin treatment ($F_{1,13}=5.332$, $P=0.038$). Post-hoc analysis indicated that ghrelin treatment resulted in elevated plasma GH levels over GH levels after saline treatment in DSAP rats at 20 and 40 minutes after treatment and at 20 minutes after treatment in SAP rats. Treatment of DSAP and SAP rats with aCSF did not result in any significant differences between sampling times or between groups.

![Figure 4](image)

**Figure 4** - Concentration of rat GH after icv ghrelin or control aCSF stimuli in SAP and DSAP rats. GH concentration in both SAP and DSAP rats peaked 20 minutes after treatment. The GH concentration rats in SAP animals returned to baseline levels at 40 minutes while that of DSAP animals remained elevated at near peak concentration at that time point. *$P < 0.05$ DSAP ghrelin in comparison to DSAP aCSF. †$P < 0.05$ for SAP ghrelin vs SAP aCSF.

**Experiment 2**

_Ghrelin and GH increase Fos expression in hindbrain catecholamine neurons._

Physiological saline and aCSF treated animals were pooled into a vehicle group because they
were not different. Higher levels of Fos expression in DBH-ir cells were observed in rats treated
with ghrelin or GH than those treated with vehicle (Figure 5). One way ANOVA revealed
significant differences between treatment groups in the percentage of cells co-expressing Fos and
DBH A1 (F2,10=12.95, P=0.002), A1/C1 (F2,10=54.79, P<0.001), C1 (F2,10=8.22, P=0.008) and
A5 (F2,10=53.95, P<0.001) cell groups. Holm-Sidak post-hoc analysis revealed that ghrelin
significantly elevated Fos expression in DBH positive neurons in A1, A1/C1, C1 and A5 cell
groups and that sc GH significantly increased Fos and DBH co-expression in A1, A1/C1, and A5
cell groups, compared to vehicle control levels. There were no significant differences in Fos
expression in A2, C2, C3, or area postrema catecholamine neurons in ghrelin-treated animals or
in A2, C1, C2, C3 or area postrema catecholamine neurons in GH-treated animals. Typical
immunofluorescent labeling can be seen in the A1/C1 and A2 cell groups in Figure 7.

![Figure 5 - Co-expression of Fos and DBH after icv ghrelin (n=5), sc GH (n=4), or vehicle (n=4) injections, shown as the percentage of DBH cell bodies in each cell group that were positive for Fos expression. *P<0.05 vs vehicle control. †P<0.05 vs ghrelin.]

Fos expression in PNMT-ir (epinephrine) neurons was similar to that observed in the C1, C2 and C3 DBH-ir cell groups defined anatomically. The sample size in this experiment is too
small to do a powerful enough statistical analysis but the trend towards increased Fos expression in C1 neurons after ghrelin treatment is still apparent (Figure 6).

Discussion

Immunohistochemical results presented here reveal that ghrelin administration increased Fos expression in DBH-ir cell bodies in cell groups A1, in the area of A1/C1 overlap, to a limited extent in C1, and in A5. The largest percentage of activated cells was in cell group A1. Thus, NE neurons were the predominant catecholamine phenotype activated by ghrelin. We also found that ghrelin increased Fos expression in the NTS, as others have found (Li et al., 2006), but Fos was not expressed in A2 neurons. The absence of ghrelin-induced Fos expression in A2 neurons is consistent with other results (Date et al., 2005; Faulconbridge et al., 2008). Failure of A2 neurons to express Fos in response to ghrelin does not eliminate the possibility that ghrelin’s effects are mediated by inhibition of these neurons. However, absence of Fos expression in A2 neurons was surprising because Date et al. (Date et al., 2006) reported that ghrelin increased DBH mRNA in A2 neurons, suggesting that they are activated, not inhibited, by ghrelin.
Cell groups A1 and caudal C1, where DBH- and Fos-ir were co-expressed after ghrelin administration, provide dense innervation of the medial hypothalamus, including the paraventricular nucleus and Arc (Sawchenko and Swanson, 1982, 1983; Ritter et al., 2001; Ritter et al., 2003). Stimulation of feeding in response to glucose deficit is an important function of
neurons in these cell groups (Ritter et al., 2001; Ritter et al., 2003; Li et al., 2009). Injections of DSAP into either the Arc or paraventricular hypothalamic nucleus virtually eliminate DBH terminals throughout the medial hypothalamus, reduce cell numbers in catecholamine cell groups (mainly A1, A2 and caudal C1), and significantly reduce or eliminate glucoprivic feeding (Ritter et al., 2001; Fraley and Ritter, 2003; Ritter et al., 2003). In the present study, we used the glucoprivic feeding response as an independent behavioral test of the DSAP-induced lesion of the hypothalamic NE/E projection. We found that DSAP lesions eliminated the glucoprivic feeding response, as expected. Immunohistochemical analysis of the lesion site showed the typical loss of DBH terminals in Arc, paraventricular nucleus and other medial hypothalamic sites, as well as reduction in the number of hindbrain cell bodies in the cell groups known to contribute to the hypothalamic innervation. Thus, judged by these two criteria, the DSAP lesion was effective. However, this lesion did not impair the feeding response to either central or systemic ghrelin administration. Together, these findings fail to support the hypothesis, proposed previously by Date et al. (Date et al., 2006), that ghrelin-induced feeding requires NE neurons that innervate the Arc.

The discrepancy between our DSAP data and that reported by Date et al. (Date et al., 2006) might be attributable to the difference in the DSAP injection sites in the two experiments. In Date’s experiment, DSAP was injected into the Arc, while in ours DSAP was injected into the PVH. Differences in the pattern of DBH terminal loss are not likely to have caused the differences in experimental outcomes because injection of DSAP into either site causes a loss of DBH terminals in the Arc and medial hypothalamus. However, with injections into the Arc, nonspecific damage to Arc ghrelin-sensitive neurons, rather than the specific loss of NE cells and terminals, could conceivably have accounted for the failure of the DSAP-injected rats to respond
Published findings using a variety of approaches have shown that Arc lesions abolish
the feeding responses to central and peripheral ghrelin administration (Bugarith et al., 2005;
Luquet et al., 2007). Date et al. did not provide evidence to rule out this possibility (Date et al.,
2006).

Date and colleagues also supported their hypothesis that hindbrain NE neurons mediate
ghrelin-induced feeding by demonstrating that this response was reduced after midbrain
transection of the ascending NE fiber bundle. These data are also inconclusive, however, since
midbrain transection eliminates both afferent and efferent fiber pathways between the forebrain
and hindbrain, such that the reduced feeding could be due to the elimination of efferent fibers
essential for appetitive behavior and overall motor function. Without data showing that the
midbrain transected animals were capable of maintaining their body weight and of responding to
other appetitive stimuli, it is not possible to interpret the loss of the feeding response to ghrelin as
being a specific consequence of NE fiber damage.

It has been suggested previously (Date et al., 2005) that ghrelin enhances feeding by
antagonizing the effects of peripheral satiety peptides, such as cholecystokinin (CCK), on vagal
sensory neurons. The signal generated by this interaction is subsequently transmitted to the
NTS, where vagal sensory fibers terminate, and then to the hypothalamus by A2 neurons.
However, our data show that DSAP lesions, which eliminate A2 neurons that project to the
hypothalamus, neither change the magnitude of ghrelin-induced feeding (this experiment) nor
impair the response to CCK (Ritter et al., 2001). Thus, if ghrelin stimulates feeding by
antagonizing vagally-mediated satiety signals, central transmission of this effect does not require
NE neurons with direct projections to the hypothalamus, although A2 neurons projecting to other
sites could still be involved. It is important to note, however, that the importance of the vagal
sensory neurons in ghrelin-induced feeding and GH secretion is still unclear due to conflicting bodies of evidence both in rats and in humans (Date et al., 2002; Takeno et al., 2004; le Roux et al., 2005; Arnold et al., 2006).

Ghrelin is a GH secretagogue capable of acting directly on GH secreting cells in the pituitary and on parvocellular GHRH cells in the Arc. Release of NE at the level of the hypothalamus is capable of producing either excitatory or inhibitory effects on GH secretion, depending on the receptor type activated, but the predominant effect of NE appears to be the inhibitory effect mediated by alpha-1 adrenoreceptor activation (Willoughby et al., 1993). A novel and important result of our study was the finding that the DSAP lesion caused a prolongation of the GH secretory response to ghrelin, indicating that catecholamine neurons exert a predominantly inhibitory effect on ghrelin-induced GH secretion and may normally exert negative feedback control of this response. This modulatory effect could arise either from the direct action of ghrelin on catecholamine neurons or it could reflect the action of GH on catecholamine neurons. Our Fos studies indicate that GH activates catecholamine neurons in many of the same hindbrain groups that are activated after ghrelin administration, suggesting that catecholamine neuron activation after ghrelin may be largely a response to elevated GH levels, rather than to ghrelin itself. If so, our findings support a broader involvement of NE neurons in modulation of GH secretion and would lead to the prediction that GH secretion in response to other GH activators would also be enhanced by the DSAP lesion, and that pharmacologically blocking the GH binding to its receptor would result in decreased activation of hindbrain catecholamine neurons due to ghrelin or GH treatment.

The similarities and differences of expression of Fos in DBH-ir neurons after GH and ghrelin treatment are interesting. As mentioned, we observed elevation of Fos expression over
control animals in both groups, but the magnitude of the elevation clearly differs in the more rostral cell groups. In fact, there are significant differences between the ghrelin- and GH-induced Fos expression in both DBH-ir A1/C1 and C1 groups, demonstrating that ghrelin has a somewhat wider anatomical range of action than GH. The duplication of the increase in C1-induced Fos expression in PNMT-ir neurons in ghrelin- but not in growth hormone-treated animals similarly indicates a difference. The function of the difference between the activation distributions of ghrelin and growth hormone is not known but there are various other actions of ghrelin not investigated in this study. Ghrelin has been implicated in sleep (Szentirmai et al., 2009), alcohol-seeking (Addolorato et al., 2009), reward learning (Jerlhag, 2008), and many other physiological and behavioral activities. The differences in activation of catecholamine neurons in the hindbrain between ghrelin and growth hormone could be due to ghrelin's other physiological actions and much more investigation is needed to distinguish the involvement of hindbrain catecholamine neurons in these actions.

Conclusion

In summary, the present data indicate that a population of catecholamine neurons with projections to the hypothalamus serves as a negative feedback mechanism to modulate ghrelin-induced GH release, and possibly to control GH secretion induced by other stimuli as well. However, our data clearly do not indicate a role for hypothalamically projecting catecholamine neurons in ghrelin-induced feeding, and certainly do not support the proposal that they are required for this response. Since injection of ghrelin directly into the Arc stimulates feeding (Bagnasco et al., 2003), ghrelin receptors are present in the Arc and accessible to circulating ghrelin (Howard et al., 1996) and Arc lesions impair feeding induced by both systemic administration of ghrelin agonist (Luquet et al., 2007) and icv ghrelin (Bugarith et al., 2005), it
seems most likely from present data that circulating ghrelin, or arguably ghrelin produced in the hypothalamus (Sato et al., 2005; Ferrini et al., 2009), induces feeding primarily through its direct actions in the Arc.

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References


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