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Ghrelin increases vagally-mediated gastric activity by central sites of action

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Abstract

Background—Vagally dependent gastric reflexes are mediated through vagal afferent fibers synapsing upon neurons of the nucleus tractus solitarius (NTS) which, in turn modulate the preganglionic parasympathetic dorsal motor nucleus of the vagus (DMV) neurons within the medullary dorsal vagal complex (DVC). The expression and transport of ghrelin receptors has been documented for the afferent vagus nerve, and functional studies have confirmed that vagal pathways are integral to ghrelin-induced stimulation of gastric motility. However, the central actions of ghrelin within the DVC have not been explored fully.

Methods—We assessed the responses to ghrelin in fasted rats using: 1) *in vivo* measurements of gastric tone and motility following IVth ventricle application or unilateral microinjection of ghrelin into the dorsal vagal complex (DVC); and, 2) whole cell recordings from gastric-projecting neurons of the dorsal motor nucleus of the vagus (DMV)

Results—1) IVth ventricle application or unilateral microinjection of ghrelin into the DVC elicited contractions of the gastric corpus via excitation of a vagal cholinergic efferent pathway; 2) Ghrelin facilitates excitatory, but not inhibitory, presynaptic transmission to DMV neurons.

Conclusions—Our data indicate that ghrelin acts centrally by activating excitatory synaptic inputs onto DMV neurons, resulting in increased cholinergic drive by way of vagal motor innervation to the stomach.

Keywords

vago-vagal reflexes; brainstem; gastrointestinal motility; gastrointestinal transit

Gastrointestinal peptide signaling occurs via neuronal or circulatory pathways, or through a combination of both pathways as we have proposed previously for cholecystokinin and GLP-1 [1,2]. The neuronal signaling pathway includes paracrine activation of luminal vagal afferent fibers or neurons that terminate in the nucleus tractus solitarius (NTS) within the brainstem dorsal vagal complex (DVC). The DVC is comprised of the NTS, the dorsal motor nucleus of the vagus (DMV) and the area postrema (AP) [3]. The medullary integration

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Disclosure
Competing Interests: the authors have no competing interests.

Author contribution
GMH designed the study and protocol, acquired and analyzed *in vivo* experimental data, and contributed to writing the manuscript. EMS acquired and analyzed *in vivo* experimental data, performed histological processing, and contributed to writing the manuscript. KNB contributed to the *in vitro* study design, data generation and analysis as well as the writing of the manuscript. AT contributed to all aspects of the study design and analysis and writing of the manuscript.

of gastric sensory and motor activity occurs within brainstem vagal circuits and vagal augmentation of gastric motility and intramural pressure is mediated ultimately by a subset of vagal preganglionic motor efferent fibers in the DMV that activate cholinergic enteric neurons (reviewed in [3]).

The neurohormone ghrelin, secreted from oxyntic cells within the gastric mucosa [4], is up-regulated during periods of negative energy balance, such as before meals, and is down-regulated after feeding [5]. Ghrelin secretion exerts profound stimulatory effects upon gastric motility and acid secretion as well as food intake and energy metabolism [6–10]. This stimulatory effect has been demonstrated in both fed and fasted states[11,12]. An inhibitory effect of ghrelin has been reported for fundic tone of fed animals [10]. This particular experimental design, however, included a potentially confounding manipulation of the stomach in order to squeeze out gastric chyme prior to implantation of a luminal-dwelling balloon for detecting pressure.

Ghrelin is considered to exert its gastroexcitatory effect via a vagally-mediated pathway which involves activation of growth hormone secretagogue receptors that originate in the vagal afferent neurons within the nodose ganglion and are transported to afferent terminals [13]. Furthermore, peripherally administered ghrelin diminishes vagal afferent activity while vagotomy, midbrain transection or perivagal capsaicin abolishes facilitation of feeding, growth hormone secretion, and activation of neuropeptide Y (NPY)- and growth hormone-releasing hormone (GHRH)-producing neurons [13,14]. Ghrelin receptor expression has also been reported within the medullary brainstem and brainstem application of ghrelin exerts behavioral [15], gastric [10] and cardiovascular [16] responses.

Studies investigating both ghrelin receptor expression and functional outcomes of ghrelin receptor activation clearly indicate that while peripheral vagal pathways are integral to ghrelin-induced stimulation of gastric motility, central sites of action must also be considered. However, our understanding of the central actions of ghrelin within the DVC remains incomplete and controversial. The aim of the present work was to test the hypothesis that the promotility effects of ghrelin involve activation of NTS neurons which, in turn, modulate the DMV preganglionic parasympathetic neuronal outflow projecting to the stomach.

Materials and Methods

All experiments were conducted on male Long-Evans rats (n = 40; Harlan Labs, Fredrick MD). Rats were ≥8 weeks of age upon entrance into the experiment and were double housed in a room maintained at 21–24°C on a 12:12-hours light-dark cycle and food and water *ad libitum*. All procedures were performed according to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the Penn State University College of Medicine.

In vivo studies

Following an overnight fast (water ad libitum), animals were anesthetized deeply with thiobutabarbitol (Inactin®; 100–150 mg kg^{-1} ip) and administered dexamethasone (1 mg kg^{-1} sc) to prevent cerebral edema. The trachea was intubated and a jugular catheter was inserted for intravenous delivery of drugs. The stomach was isolated and placed upon a moisturized gauze pad, an encapsulated miniature strain gage (RB Products, Minneapolis, MN) was aligned with the circular smooth muscle fibers and sutured to the gastric corpus before closing the abdominal incision. Animals were placed in a stereotaxic frame and maintained at 37±1 °C using a feedback-controlled warming pad. The head of the animal was oriented in order to surgically expose the fourth ventricle and the vagal trigone. After a

1 hour recovery period, drugs were applied to the surface of the fourth ventricle at the level of the calamus scriptorius (2 μl; *n* = 12). In one control group of rats (*n* = 5), prior to attaching the gastric strain gage, the posterior sub-diaphragmatic vagus was sectioned 5 mm caudal to the hiatus. A silk suture ligature was also placed gently around the left cervical vagus approximately 5–10 mm caudal to the nodose ganglion. The ligature was exteriorized though a length of PE-240 tubing for later sectioning of the vagus nerve. After the first application of ghrelin, and a 30 min observation period, the ligature was withdrawn, thus interrupting the remaining vagal outflow to the stomach. In a second control group of rats (*n* = 3) three applications of a subthreshold dose of ghrelin (3pmol) were made to the fourth ventricle to test for additive effects of ghrelin. This sub-threshold dose was selected on the hypothesis that subtle additive effects of ghrelin would be more clearly detected by increases against a background of low motility.

Baseline gastric motility and tone were monitored continuously on a polygraph (model 79, Grass, Quincy, MA) and the effects of each treatment, beginning immediately following application and for 10 min thereafter, were compared to the average of the 10 min preceding the microinjection. Due to the prolonged responses to higher doses of ghrelin, analysis of gastric motility was limited to the first 10 min of response in order to ensure that motility scores of the lowest doses were not biased by unequal recovery periods. A minimum of 30 min return to baseline levels occurred prior to subsequent administration of any experimental treatment.

Separate groups of rats were prepared similarly for ghrelin microinjection into the left dorsal vagal complex (DVC) through a glass micropipette (30–40 μm tip diameter) directed by a micromanipulator at the following co-ordinates: 0.1–0.3 mm lateral from midline, 0.1–0.3 mm rostral to calamus scriptorius and 0.5–0.7 mm dorsoventral to the surface. Sixty nanoliters of phosphate buffer saline (PBS) or ghrelin (100 pmol dissolved in PBS) were microinjected using a picospritzer (Toohey, Pressure System IIe, Fairfield NJ). One group (*n* = 5) was prepared for left cervical vagotomy as described above. Microinjections of ghrelin were administered utilizing the same time course as fourth ventricle application. An additional group of rats (n = 5) was implanted with a jugular catheter for intravenous delivery of the cholinergic muscarinic antagonist atropine methyl nitrate (50 $\mu g\ kg^{-1}$ bolus followed by continuous intravenous infusion 20 $\mu g\ kg^{-1}\ hr^{-1}$ for 20 min). Atropine methyl nitrate infusion began 30 min after the first microinjection of ghrelin. A second microinjection of ghrelin into the DVC occurred within 10–15 min of the onset of atropine methyl nitrate infusion. A final group of rats (*n* = 4) were vagotomized during instrumentation at the right cervical vagus in order that microinjections of ghrelin (100 pmol) directed at the right caudal NTS would activate a subset of NTS neurons that project to contralateral DMV neurons.

The strain gage amplifiers were calibrated by setting peak-to-peak sensitivity of individual gages to equal a 1g static load. Gastric motility was calculated using the following formula[17]:

$$\text{Motility Index} = (N_1 \times 1) + (N_2 \times 2) + (N_3 \times 4) + (N_4 \times 8)$$

Based upon this formula, N equals the total number of peaks in a particular milligram range. Therefore, presuming that a 0 mg signal is indicative of no gastric motility, the grouping of peak-to-peak sinusoidal signals were calculated as 25–50 mg, 60–100 mg, 110–200 mg and signals greater than 210 mg for N_1 through N_4, respectively. Unlike area under the curve measurements, the motility index is independent of baseline tone fluctuations that naturally occur across several seconds or minutes.

Histological Processing

At the conclusion of the experiment, rats were perfused transcardially with a bolus of lidocaine and heparin followed by cold PBS then PBS with 4% paraformaldehyde. The region of the brainstem containing the injection site was removed and refrigerated overnight in PBS containing 20% sucrose and 4% paraformaldehyde. Alternate coronal slices (40μm thick) were mounted on gelatin-coated slides, dried, stained with cresyl violet and coverslipped. Images of the microinjection site were captured using a digital camera attached to a Zeiss Axioskop Microscope and imported into Adobe Photoshop CS5 for analysis.

Electrophysiology

The methods of preparing the brainstem slices and the identification of DMV neurons have been described previously [18,19]. Briefly, Long-Evans rats (n=6) of either sex were anesthetized with isoflurane before being euthanized by a bilateral pneumothorax. After removal, 3–4 coronal slices (300μm-thick) of the brainstem were cut starting from the caudal area postrema and moving rostrally. The slices were incubated at 30±1°C in Krebs' solution (in mM: 126 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, and 11 dextrose, maintained at pH 7.4 by bubbling with 95% O_2-5% CO_2) for 60–90 minutes before use. A single slice was then transferred to a perfusion chamber on the stage of a Nikon E600FN microscope, kept in place with a nylon mesh and maintained at 35±1°C by perfusion with warmed Krebs' solution at a rate of 2.5–3.0 ml min^{-1}. DMV neurons were identified by their soma size and location adjacent to the smaller, dorsal NTS neurons and dorsal to the larger, more heavily myelinated neurons of the hypoglossal nucleus. Recordings were made from DMV neurons at levels spanning from the caudal tip of the area postrema to approximately 0.5 mm rostral to its anterior portion with patch pipettes (2–4MΩ resistance) filled with a potassium gluconate solution (in mM: K gluconate 128, KCl 10, $CaCl_2$ 0.3, $MgCl_2$ 1, Hepes 10, EGTA 1, ATP 2, GTP 0.25 adjusted to pH 7.35 with KOH) by using an Axopatch 1D amplifier (Molecular Devices, Sunnydale, CA). Liquid junction potential was compensated at the beginning of the experiment. Recordings were accepted only if the series resistance was <20 MΩ. Whole cell recordings of spontaneous and miniature glutamatergic excitatory postsynaptic currents (EPSC) were conducted on DMV neurons, voltage clamped at −60 mV, in the presence of 50 μM picrotoxin to isolate glutamatergic currents pharmacologically and 1μM tetrodotoxin (TTX) to isolate action potential independent synaptic transmission. We have shown previously that the currents recorded under these conditions comprise glutamatergic ionotropic currents only [18]. Whole cell recordings of spontaneous GABAergic inhibitory postsynaptic currents (IPSCs) were conducted on DMV neurons, voltage clamped at −50 mV, using patch pipettes (6–8MΩ) resistance filled with a potassium chloride solution (in mM:140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 EGTA, 2 ATP-Na, 0.25 GTP-Na, adjusted to pH 7.35 with HCl) in the presence of 1mM kynurenic acid to isolate GABAergic currents pharmacologically and 1 μM tetrodotoxin as above. We have shown previously that currents recorded under these conditions comprised GABAergic currents only [20].

Data were sampled at 10kHz and filtered at 2kHz, digitized via a Digidata 1320 interface, acquired with a PC utilizing pClamp9 software (Molecular Devices) and analyzed with Mini Analysis software (Jaejin Software, Leonia, NJ).

Drugs were applied to the bath *via* a series of manually operated valves. Cells were classified as responsive if perfusion with ghrelin (100nM) modified the frequency of s/mEPSC and mIPSC, respectively by a minimum of 50% from baseline (measured as the average frequency during 1min of recording in control conditions *vs.* 30s of recording

centered on peak drug response, identified as the maximal ghrelin-induced effect). A shift in holding current greater than 20pA was considered a postsynaptic effect.

Drugs and chemicals

Inactin® and all other salts were purchased from Sigma (St. Louis, MO). Ghrelin was purchased from Tocris (Ellsville, MO). All drugs were dissolved in sterile isotonic phosphate buffered saline (PBS; in mM: 147.6 NaCl, 83.3 NaH_2PO_4, 12.9 KH_2PO_4).

Statistical Analysis

Results are expressed as means ± S.E.M. with significance defined as $P < 0.05$. Between group results from *in vivo* studies, between pre- and post-treatment values, were compared by one-way ANOVA and Tukey *post hoc* analysis or paired *t*-test as appropriate (SPSS Inc, Chicago, IL). Results from *in vitro* studies were compared before and after drug administration, with each neuron serving as its own control (Student's paired *t*-test). Intergroup comparisons were conducted using the Student's grouped *t*-test or the χ^2 test.

Results

Fourth ventricle application of ghrelin increases gastric contractions

In fasted naïve rats ($n = 12$), application of PBS to the floor of the fourth ventricle did not induce any significant change in corpus tone or motility ($P > 0.05$; Fig. 1A&B). Fourth ventricle application of ghrelin (3–300 pmol) increased gastric corpus motility in a dose dependent manner ($P < 0.05$; Fig. 1B). Due to the variability of detectable responses following application of 3 pmol ghrelin, response duration was not calculated (Fig. 1C). For higher doses of ghrelin, the duration of the response was 17.1 ± 3.5 min for 10 pmol, 15.6 ± 2.1 min for 30 pmol, 20.1 ± 3.9 min for 100 pmol and 74.8 ± 16.9 min for 300 pmol. At the 300 pmol dose of ghrelin, 40% of animals (2/5) displayed a persistent increase in motility which did not return to baseline levels by the conclusion of the experiment. In a separate subset of animals, three sequential applications of 3 pmol ghrelin at intervals greater than 30 min induced comparable, non-significant, increases in corpus motility, suggesting that the effect of ghrelin was not additive in our preparation ($n = 3$; $P > 0.05$, 113±5%, 95±8% and 102±5%, respectively).

As with naïve rats, application of 100 pmol ghrelin to the fourth ventricle of rats which received a posterior subdiaphragmatic vagotomy (n = 4) induced a 233±35% increase in gastric corpus motility ($P < 0.05$; Fig. 1D) and the duration of the response was 16.3 ± 2.1 min. After 30 min stabilization and recovery, the bilateral vagotomy was completed by withdrawing the ligature around the left cervical vagus. This procedure reduced residual gastric contractions to 25±7% of pre-vagotomy levels ($P < 0.05$; data not shown). Following an additional 30-min stabilization and recovery, re-application of 100 pmol of ghrelin to the fourth ventricle no longer increased gastric corpus contractions ($P > 0.05$; Fig. 1D).

These data indicate that the increase in gastric motility following application of ghrelin to the fourth ventricle is mediated by activation of vagal neurocircuitry within the brainstem.

Microinjection of ghrelin into the DVC induced a vagally-mediated increase in corpus motility

To confirm that the effects of ghrelin were the result of localized activation of vagal neurocircuitry, dual-barrel microinjection pipettes were positioned within the left DVC of rats which received a posterior subdiaphragmatic vagotomy ($n = 4$) or rats with jugular intravenous catheters ($n = 5$). There was no significant difference in the baseline motility

index of either group ($P > 0.05$). Microinjection of PBS in the left DVC of either group of these rats did not induce any significant change in gastric motility ($P > 0.05$).

Microinjection of ghrelin (100 pmol) in the left DVC significantly increased corpus motility in the cohort of prevagotomized rats ($P < 0.05$; Fig. 2). Upon recovery of corpus motility to baseline levels, the vagal ligature was withdrawn to complete the vagotomy. Following an additional 30 min post-vagotomy stabilization period, 100 pmol of ghrelin was microinjected into the DVC of completely vagotomized animals and no longer increased corpus motility above baseline levels ($P > 0.05$; Fig. 2).

To verify that DVC microinjection of ghrelin increased corpus motility through the excitation of post-ganglionic cholinergic enteric neurons, peripheral muscarinic receptors were antagonized by the systemic administration of the cholinergic antagonist atropine methyl nitrate. Prior to administration of atropine, microinjection of 100 pmol of ghrelin in the DVC induced a 177±12% increase in corpus motility in the second cohort of rats ($P < 0.05$; Fig. 2). After 30 min recovery to return to baseline motility, atropine methyl nitrate administration (50 $\mu g\ kg^{-1}$ bolus followed by continuous iv infusion of 25$\mu g\ kg^{-1}\ hr^{-1}$) was initiated for 10–15 min prior to microinjection of ghrelin. Under these conditions, baseline motility was only reduced slightly in the presence of continuous systemic atropine (71±13% of baseline values pre-atropine; $P > 0.05$; data not shown). Following an additional 10 min of continuous atropine infusion, a second ghrelin microinjection into the DVC no longer increased corpus motility above baseline levels ($P > 0.05$; Fig. 2).

To demonstrate that the *in vivo* effects of ghrelin are through actions upon NTS neurons, microinjection of 100 pmol of ghrelin was directed at the ipsilateral DVC in rats that had the right cervical vagus transected. Microinjection of 100 pmol of ghrelin in the right DVC induced a 229±37 increase in gastric motility in this cohort of rats ($P < 0.05$).

Taken together, these data indicate that the ghrelin-mediated increase in corpus motility includes a central mechanism of action within the brainstem vagal pathways. In particular, the actions of ghrelin include a pro-motility effect likely mediated by NTS neurons. Furthermore, the increase in corpus motility includes vagal neurocircuitry that innervates the cholinergic enteric neurons of the stomach.

Ghrelin had no effect upon the membrane properties of DMV neurons

The action of ghrelin (100nM) to alter the membrane properties was assessed in 17 neurons under voltage clamp conditions. Of 11 neurons tested in control conditions, i.e. without TTX, ghrelin had no effect in 7 neurons (8.8±4.3pA; $P>0.05$) and induced an inward shift in holding current of −52.9±12.9pA in the remaining 4 neurons ($P<0.05$). In 6 further neurons, the effects of ghrelin were assessed in the presence of TTX (1μM) to block action potential dependent synaptic transmission; in these neurons ghrelin had no effect on holding current (−5.8±5.6pA; $P > 0.05$), suggesting the ghrelin-induced alterations in holding current observed in the absence of TTX were due to a synaptic-mediated action rather than a direct effect on the DMV neuronal membrane.

Ghrelin increased sEPSC frequency in DMV neurons

The effects of ghrelin to alter excitatory glutamatergic synaptic transmission was assessed in 6 DMV neurons. In 4 of these neurons, perfusion with ghrelin (100nM) increased the frequency of sEPSCs from 7.5±2.2 to 18.8±6.3 events s^{-1}, i.e. ghrelin increased sEPSC frequency to 308±90% of control ($P < 0.05$, Fig. 3). The frequency of sEPSCs returned to baseline levels after 20 minutes of wash-out (9.5±4.3 events s^{-1}). In these same neurons,

ghrelin had no effect upon the amplitude of sEPSCs (35.6±8.5pA in control *versus* 44.9±16.6pA in the presence of ghrelin; $P > 0.05$).

Ghrelin has no effect upon mEPSC frequency in DMV neurons

Since ghrelin increased the frequency but not amplitude of sEPSCs, this suggested a presynaptic site of action. To confirm that ghrelin was acting at pre- rather than post-synaptic sites, a series of experiments were conducted in the presence of tetrodotoxin (TTX; 1μM) to block action potential-dependent synaptic transmission, i.e., we assessed the effects of ghrelin perfusion on mEPSC amplitude and frequency. In the presence of TTX, mEPSC frequency decreased from 4.3±2.2 to 2.3±1.0 events s^{-1} ($P < 0.05$) while mEPSC amplitude was unchanged (33.6±3.4pA in control *versus* 27.9±0.8pA in the presence of TTX; $P > 0.05$).

Perfusion with ghrelin had no effect on mEPSC frequency or amplitude in 4 of the 5 neurons tested (2.6±1.2 events s^{-1} and 29.5±2.1pA, respectively, i.e. 93±5% and 108±10% of mEPSC frequency and amplitude in the absence of ghrelin; $P > 0.05$). In the remaining neuron, ghrelin increased mEPSC frequency but had no effect on mEPSC amplitude (206% and 104%, respectively). This suggests that, in the majority of neurons, ghrelin increases mEPSC frequency via an increase in action potential dependent synaptic transmission.

Ghrelin has no effect upon sIPSC frequency in DMV neurons

The actions of ghrelin to alter inhibitory GABAergic synaptic transmission was assessed in 7 DMV neurons. Superfusion with ghrelin (100nM) had no effect upon either sIPSC frequency or amplitude in any of the neurons tested, i.e., sIPSC frequency was 3.3±1.1 in control *versus* 3.4±1.2 events s^{-1} in the presence of ghrelin while sIPSC amplitude was 60.3±10.5pA in control *versus* 59.7±9.9pA in the presence of ghrelin ($P > 0.05$ for both).

Taken together, our electrophysiological data indicate the action potential-dependent effect of ghrelin which is selective for presynaptic glutamate terminals terminating on DMV neurons.

Discussion

In the present study, our experimental data indicate that 1) similar to IV^{th} ventricle application, ghrelin microinjected into the DVC elicits contractions of the gastric corpus; 2) ghrelin increases corpus contractions through the activation of a vagally-mediated cholinergic efferent pathway; 3) *in vitro* recordings suggest that ghrelin exerts its gastric prokinetic effects by facilitating excitatory synaptic transmission to DMV neurons; and 4) inhibitory synaptic transmission is not affected by ghrelin.

Previous studies report that intravenous or intracerebroventricular (ICV) administration of ghrelin facilitates contractility of the gastric corpus [6,9,12,21]. These studies did not fully test the *in vivo* central effects of ghrelin specifically within the DVC neurocircuitry. For example, ghrelin administration into the lateral ventricle stimulates gastric motility through a neuropeptide Y (NPY) mediated pathway, since immunoneutralization of NPY blocked the effects of i.c.v. ghrelin [12]. This specific action of ghrelin is probably mediated through one of many parallel descending systems originating in the hypothalamus and terminating in the DVC [19,22]. Furthermore, central effects of NPY upon *in vivo* gastric motility and *in vitro* DVC firing appear to be mixed and receptor specific [23–25]. The potential interactions between ghrelin and other peptidergic signaling pathways within the DVC are beyond the scope of our studies, but do highlight the complexity of brainstem-gastric neural circuits.

Previous studies have demonstrated that it is the acylated form of ghrelin that induces gastric motility in rats [26]. While the half life of acylated ghrelin in response to a single bolus glucagon challenge is relatively short (ca. 10 min) [27], fasting levels of total ghrelin remain elevated during a 24 hour period of negative energy balance [28] and are likely to provide sufficient serum titers to affect DVC control of gastric motility. The presence of detectable ghrelin-immunoreactive nerve terminals in the CNS are controversial (reviewed in [29]) and while it is often presumed that peptides do not cross the blood-brain barrier, there is evidence of blood-brain barrier permeability in regions of the NTS [30] and the presence of a saturable ghrelin transporter within the brainstem has been reported [31]. Therefore, we propose that the central actions of ghrelin may include the transport of circulating peptide into the DMV.

As with the previous studies which employed ICV administration of ghrelin, our observed increase in corpus motility following application of ghrelin to the fourth ventricle could be attributed to diffusion of ghrelin into the systemic circulation. Peripheral administration of ghrelin to animals that have been vagotomized has been shown to induce antral contractions similar to the fasted state, presumably occurring through activation of ghrelin receptors located in the gastrointestinal tract [12]. In the present study, the specificity of the effects of ghrelin on vagal brainstem neurocircuits were confirmed by the failure of repeated application of ghrelin, either to the fourth ventricle or microinjected into the DVC, to induce an increase in corpus motility following vagotomy. Furthermore, recent evidence supports a direct action of ghrelin on other neuronal systems [32] and reports of endogenous ghrelin receptor expression on the neurons of both the NTS and the DMV [16,33] supports a central site of action. We propose that in addition to peripheral or forebrain sites of action, ghrelin also acts directly within brainstem vagal neurocircuitry to increase gastric motility.

Our understanding of the gastric-directed motor output originating in the DMV reveals an increasingly complex level of organization [34]. With regard to the stomach, the majority of vagal afferent signaling is directed toward second order neurons within the NTS through a glutamatergic synapse. The neurochemical phenotype of these NTS neurons comprises glutamatergic, GABAergic and/or noradrenergic synapses that ultimately terminate onto DMV neurons. Evidence following application of GABAergic antagonists to the DVC, suggests strongly that it is the NTS GABAergic inputs onto DMV neurons which regulate the basal motor outflow to the stomach tonically [35–39]. Studies employing glutamatergic and noradrenergic antagonists within the DVC reveal little effect on basal response in the absence of ancillary reflex or pharmacological manipulation [35,40–42].

Ultimately, gastric motor reflexes are generated based upon the interplay between two separate postganglionic circuits. The parasympathetic preganglionic DMV neurons comprising the efferent vagal limb are cholinergic and activate postganglionic neurons via actions at a nicotinic receptor. Gastric projecting neurons within the DMV exhibit a basal rate of spontaneous action potentials [43–45] and it is this low frequency DMV firing which regulates an excitatory (cholinergic) circuit that is ultimately important to the antral milling of ingested solids and the delivery of reduced particles to the duodenum [46]. Gastric relaxation can occur as a consequence of inhibiting this tonically active excitatory pathway [47–51]. Conversely, activation of a non-adrenergic non-cholinergic (NANC) inhibitory vagal projection to the stomach potently elicits an inhibition of gastric motor functions. While this inhibition is often attributed to the release of nitric oxide (NO) [47,52–54], purinergic and vasoactive intestinal polypeptide mechanisms have also been identified (reviewed in [55]).

Peripheral ghrelin increases gastric motility and emptying via activation of the aforementioned cholinergic efferent vagal pathway [9]. In the present study, we confirmed the

role of post-ganglionic cholinergic neurons in the centrally-mediated effect of ghrelin on corpus motility by demonstrating that peripheral blockade of muscarinic receptors by atropine blocked the stimulatory effect of ghrelin.

In order to further identify which neuronal pool within the DVC is responsible for our *in vivo* observations following microinjection of ghrelin, we performed a right cervical vagotomy, followed by microinjection of ghrelin directed at the right (ipsilateral) DVC. The rationale for this approach was based upon the bilateral projection to the DMV by NTS neurons of the caudal medulla and the subsequent ipsilateral efferent output of the DMV [56,57]. Since the efferent outflow of ipsilateral DMV neurons was removed by the vagotomy, these injections targeted NTS neurons that also project contralaterally to intact DMV neural projections. Therefore, any preserved change in corpus motility would result from ghrelin acting upon NTS neurons that, in turn, would activate the intact contralateral DMV neurons in order for a change in corpus motility to occur.

Using an *in vitro* preparation in which a transgenic mouse model expressed enhanced green fluorescent protein under a tyrosine hydroxylase promoter (TH-EGFP), the effects of ghrelin upon identified neurons within the A2 region of the NTS has been investigated recently [58]. This study demonstrated that ghrelin acted presynaptically to inhibit glutamatergic transmission, presumably from vagal afferent terminals, to TH-positive NTS neurons. In contrast, ghrelin inhibited glutamatergic transmission to only a minority of either non-TH-positive or pro-opiomelanocortin-positive (POMC+) NTS neurons, suggesting that ghrelin acts in a pathway-specific manner to modulate afferent neurotransmission within the brainstem. In the present study, however, ghrelin *increased* glutamatergic transmission to rat DMV neurons. While these effects appeared to be mediated presynaptically, since ghrelin modulated the frequency but not the amplitude of spontaneous glutamatergic currents, they were abolished by tetrodotoxin, implying that ghrelin increases glutamatergic synaptic transmission via modulation of action potential dependent neurotransmission. Within the coronal brainstem slice preparation utilized in the present study, the majority of glutamatergic input to DMV neurons arises from the adjacent NTS. It is likely, therefore, that ghrelin is acting to increase activity of NTS neurons, either directly or indirectly, that project to the DMV, although the possibility of effects at other brainstem neurons cannot be excluded. It also must be borne in mind, however, that in the present study, recordings are made from DMV neurons that receive polysynaptic inputs. It is conceivable, therefore, that the increase in excitatory inputs to DMV neurons induced by ghrelin is due to a disinhibition of presynaptic glutamatergic inputs. If ghrelin, for example, decreased the activity of tonically active inhibitory GABAergic or catecholaminergic neurons, as in the study by Appleyard's group [58], the observed response in the recorded DMV neuron would be an increase in excitatory synaptic inputs. The precise site of action of ghrelin, and the phenotype of presynaptic neuron upon which it acts to regulate vagal efferent outflow, remains to be elucidated.

In conclusion, the present experiments describe the central actions of ghrelin upon gastric-projecting brainstem vagal neurocircuitry. Our observations demonstrate that ghrelin induces corpus motility through the indirect excitation of the preganglionic motor neurons within the DMV. These DMV neurons, in turn, activate the postganglionic cholinergic neurons which are responsible for gastric motility. Our data suggest that, in addition to peripheral activation of the afferent limb of vago-vagal reflex circuitry, circulating ghrelin may directly activate ghrelin receptors within the dorsal vagal complex. We have recently reported that alterations in the sensitivity of DVC neurons to central cholecystokinin in an animal model of spinal cord injury (SCI) [59]. We speculate that similar alterations in the sensitivity of vagal neurocircuitry to ghrelin may contribute to gastric dysmotility observed in pathophysiological conditions including SCI.

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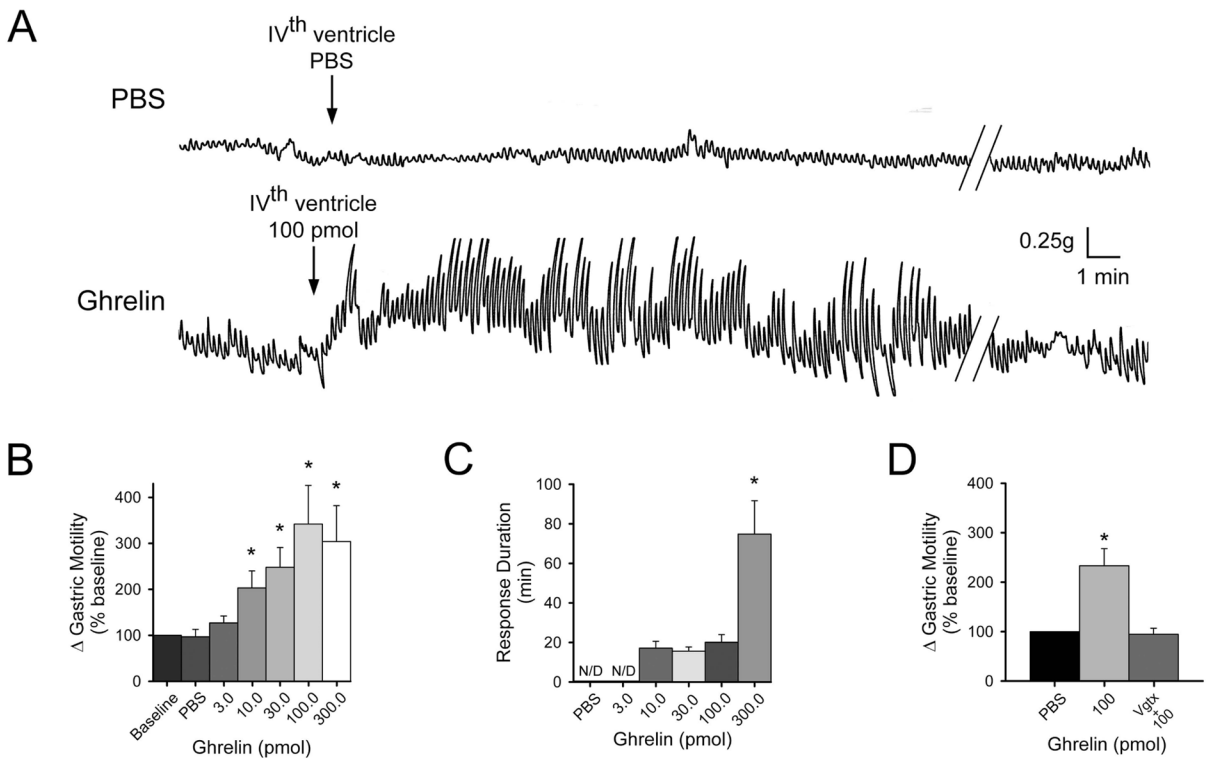

Figure 1. IVth ventricle application of ghrelin induces a vagally-mediated facilitation of corpus motility

A: representative original polygraph traces from fasted animals after **IVth** ventricle application of PBS or ghrelin demonstrating the dose-dependent increase in corpus motility of fasted animals.

B: graphic summary of the increase in corpus motility after **IVth** ventricle application of PBS or ghrelin (n=12; expressed as percent increase from baseline; * $P < 0.05$ vs. baseline).

C: graphic summary of the duration of the ghrelin response after **IVth** ventricle application of PBS or ghrelin (duration defined as contraction amplitude greater than 10 percent of baseline. N/D = no detectable increase in contraction amplitude or duration; * $P < 0.05$ vs. 10, 30 or 100 pmol).

D: graphic summary of the increase in corpus motility after **IVth** ventricle application of PBS or ghrelin in the cohort of animals that received posterior subdiaphragmatic vagotomy (n=4). Following completion of vagotomy (Vgtx) **IVth** ventricle ghrelin (100 pmol) no longer elevated corpus motility (* $P < 0.05$ vs. baseline).

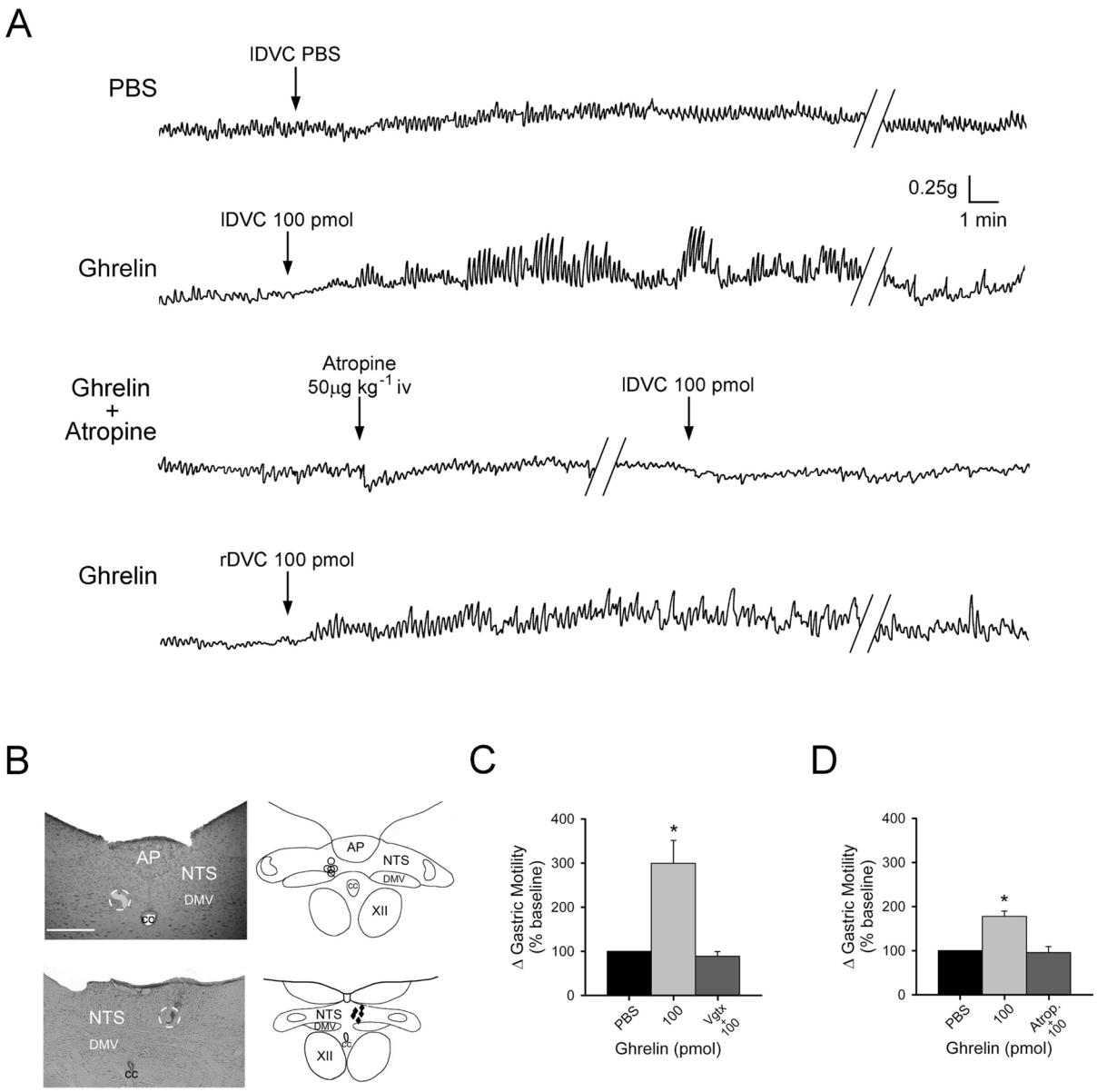

Figure 2. Microinjection of ghrelin in the left dorsal vagal complex (DVC) induces a vagally mediated increase in corpus motility

A: representative original polygraph records of anterior corpus motility from fasted animals after unilateral microinjection of PBS into the left DVC (lDVC, upper trace), 100 pmol of ghrelin into the lDVC (second trace), intravenous administration of atropine (50 μg kg^{-1} bolus followed by 20 μg kg^{-1} hr^{-1}) followed by a repeat lDVC injection of 100 pmol of ghrelin in the presence of systemic atropine (third trace), and unilateral microinjection of 100 pmol of ghrelin into the right DVC (rDVC, bottom trace) of an animal that had received a right cervical vagotomy prior to microinjection.

B: representative photomicrograph (*top left*) of left brain stem injection site with the injection region circled and schematic representation (*top right*) of effective injection areas (marked by ○). Representative injection site into the right brainstem (*bottom left*) with injection region circled and schematic representation (*bottom right*) of effective injection areas (marked by ◆). For clarity, only a few examples of the distribution of injection sites

are depicted. AP, area postrema; NTS, nucleus tractus solitarius; DMV, dorsal motor nucleus of the vagus; CC, central canal; XII, hypoglossus. Calibration 250 μm.

C: graphic summary of the increase in corpus motility induced by microinjection of PBS or ghrelin (100 pmol, expressed as percent increase from baseline) in the left DVC of the cohort of animals prepared for vagotomy (Vgtx; n=12). Following completion of vagotomy, microinjection of ghrelin (100 pmol) no longer elevated corpus motility (* $P < 0.05$ vs. baseline).

D: graphic summary of the increase in corpus motility induced by microinjection of PBS or ghrelin (100pmol, expressed as percent increase from baseline) in the left DVC of the cohort of animals administered atropine (Atrop; n=5). In the presence of systemic atropine, microinjection of ghrelin (100 pmol) no longer elevated corpus motility (* $P < 0.05$ vs. baseline).

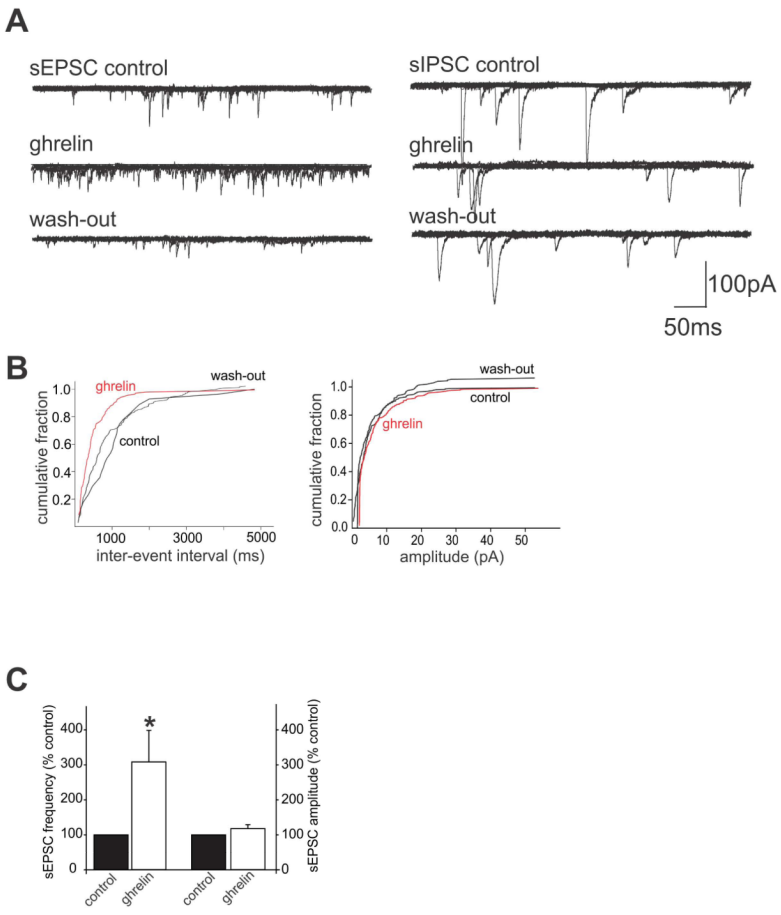

Figure 3. Ghrelin increases the frequency of sEPSCs but not sIPSCs

A: Six overlapping consecutive traces of sEPSCs (left) and sIPSCs (right) in DMV neurons voltage clamped at −60mV and −50mV, respectively. Ghrelin (100nM) was applied by superfusion for at least 3 minutes, or until the response reached plateau. Note that ghrelin increased the frequency of sEPSCs in a reversible manner, whereas ghrelin had no effect on sIPSC frequency.

B: computer generated graphics from the same neuron in A (left) showing the ghrelin-induced alteration in sEPSC frequency (left) but not amplitude (right).

C: Graphical summary of the effects of ghrelin to increase sEPSC frequency but not amplitude