Hyperphagic Effects of Brainstem Ghrelin Administration

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The role of ghrelin in feeding control has been addressed from a largely hypothalamic perspective, with little attention directed at ingestive consequences of stimulation of the peptide's receptor, the growth hormone secretagogue receptor (GHS-R), in the caudal brainstem. Here, we demonstrate a hyperphagic response to stimulation of GHS-R in the caudal brainstem. Ghrelin (150 pmol) delivered to the third and fourth ventricles significantly and comparably increased cumulative food intake, with maximal response ~3 h after injection. The meal patterning effects underlying this hyperphagia were also similar for the two placements (i.e., significant reduction in the time between injection and first-meal onset, an increase in the number of meals taken shortly after the injection, and a trend toward an increase in the average size of the first meals that approached but did not achieve statistical significance). In a separate experiment, ghrelin microinjected unilaterally into the dorsal vagal complex (DVC) significantly increased food intake measured 1.5 and 3 h after treatment. The response was obtained with a 10-pmol dose, establishing the DVC as a site of action with at least comparable sensitivity to that reported for the arcuate nucleus. Taken together, the results affirm a caudal brainstem site of action and recommend further investigation into multisite interactions underlying the modulation of ingestive behavior by ghrelin. *Diabetes* 52: 2260–2265, 2003

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (1), is secreted primarily from the gastrointestinal tract (2) and induces a hyperphagic response when delivered centrally via lateral or third intracerebroventricular injection (3–6). The arcuate nucleus of the hypothalamus (ARC) has been taken as the principal central site of ghrelin action because of *1*) the abundance of GHS-R in this structure (7), *2*) the expression of GHS-R in almost all ARC neuropeptide Y/agouti-related protein neurons (8), which are considered important in the downstream mediation of various peripherally and centrally stimulated effects on feeding and energy balance (9), *3*) a variety of evidence indicating the functional significance of ghrelin action on neuropeptide Y/agouti-related protein neurons (4,6,10,11), and *4*) the hyperphagic response obtained upon injection of doses as low as 30 pmol to this structure (12). At the same time, however, it is important to note that central nervous system GHS-R expression is not limited to the ARC (7) and that other structures relevant to feeding control, some of which are outside the hypothalamus, may also contribute to pharmacological and physiological actions of ghrelin.

GHS-Rs are clearly represented in the caudal brainstem (7,13), although the anatomical extent of the distribution has not yet been fully described. Least studied in this regard are medullary sites, such as the dorsal vagal complex (DVC), which are clearly relevant to the neural control of feeding behavior. A recent study by J.M. Zigman and J.K. Elmquist (personal communication) using in situ hybridization, demonstrates the presence of GHS-R in all three divisions of the DVC. The DVC also contains receptors for other blood-borne correlates of the animal's nutritive status (14–18), and low doses of signaling molecules, such as leptin, yield significant feeding effects when microinjected here (14). The presence of GHS-R in this structure suggests that this is a site at which a variety of important blood-borne signals, including ghrelin, are integrated and brought to bear on energy control.

In the present study, we explored the functional significance of brainstem GHS-R by delivering ghrelin to the fourth (brainstem) ventricle at a dose shown to be effective for forebrain intracerebroventricular injection (6) and via unilateral injection into the DVC of doses below the reported threshold (12) for hyperphagic response to ARC microinjection. In addition, the feeding responses to equal doses of ghrelin delivered to the third and fourth ventricles were compared. For this comparison, cumulative intake response was evaluated, as was drug action on the size and timing of meals initiated in the hours after treatment. The meal-pattern analysis, more generally, addresses basic questions about the behavioral mechanisms underlying ghrelin-induced hyperphagia. An increase in meal frequency, expressed as a decreased latency to feed and/or a decreased intermeal interval, would suggest that ghrelin contributes to processes that govern meal initiation. Increases in meal size would be consistent with a modulatory action of ghrelin on the impact of signals arising from the gut that underlie satiation and meal termination. A hyperphagic response with comparable meal patterning underpinnings obtained with forebrain and brainstem ven-

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AP, area postrema; ARC, arcuate nucleus of the hypothalamus; DVC, dorsal vagal complex; GHS-R, growth hormone secretagogue receptor; NTS, nucleus tractus solitarius.

tricular ghrelin administration would suggest that a common behavioral control mechanism can be engaged by stimulation of anatomically distinct populations of GHS-R.

RESEARCH DESIGN AND METHODS

Animals. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 295–330 g at surgery, were housed in hanging stainless steel cages in a room under a 12/12-h light/dark cycle (lights on 9:00 A.M.). Powdered chow (Ralston Purina, St. Louis, MO) and water were available ad libitum. The experimental protocols conformed to institutional standards of animal care and the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

Surgery. Rats were anesthetized with a mixture of ketamine (9 mg/kg) and xylazine (1.5 mg/kg i.m.). Each animal received a 22G guide cannula (Plastics One, Roanoke, VA) that was implanted 2 mm above an injection site in the third or fourth ventricle (experiment 1) or the DVC (experiment 2). The third ventricle cannula was positioned on the midline, 2.0 mm posterior to bregma and 7.6 mm below the skull surface. Fourth ventricle coordinates were on the midline, 2.5 mm anterior to the occipital suture and 5.2 mm below the skull surface. The DVC cannulas were angled 15° in the anterior-posterior direction and positioned 0.4 mm lateral to midline, 2.2 mm anterior to the occipital suture, and 6.8 mm below the dura. The cannulas were cemented to four jeweler's screws, attached to the skull, and closed with obturators.

Verification of cannula positions. Ventricular cannula placements were evaluated functionally after at least 7 days recovery from surgery through measurement of the sympathetically mediated hyperglycemic response to 210 μg of 5-thio-D-glucose in 2 μl saline (19). Only rats that showed at least a twofold increase in plasma glucose level in response to this treatment were used in the experiments. The parenchymal placement was verified histologically. Fast green dye or india ink (0.2 μl) was injected through the cannula just before transcardial perfusion with saline followed by 10% formalin. Brains were removed and postfixed in a 10% sucrose-formalin solution. Sections (50 μm thick) were cut in the coronal plane and then stained with cresyl violet. Only data for animals whose injection site was located within the DVC were included in the behavioral analysis.

Drug preparation and injection. Rat ghrelin (Phoenix Pharmaceuticals, Belmont, CA) was dissolved in sterile saline (experiment 1) or artificial cerebrospinal fluid (experiment 2) and stored at −4°C for no more than 6 weeks before use. Drug or vehicle was delivered with a 28-G injector via a Hamilton microsyringe (Reno, NV). For experiment 1, ghrelin (150 pmol) or vehicle was delivered in two 1-μl bolus injections, separated by a 30-s pause. The injector was kept in place for an additional 30 s after the last bolus. For experiment 2, 0.2 μl ghrelin (5 or 10 pmol) or vehicle was delivered unilaterally to the DVC over a 2-min period via microsyringe pump (Harvard Apparatus, Holliston, MA). All injections were delivered in the first hour of the light phase. On at least two occasions before experimental testing, rats were given vehicle injections to habituate them to the injection procedure.

Experimental procedures

Experiment 1: Cumulative food intake and meal-pattern effects of third or fourth intracerebroventricular ghrelin administration. Two respective groups of rats were prepared with third ($n = 14$) or fourth ($n = 11$) intracerebroventricular cannulas. Each received injections of 150 pmol ghrelin and vehicle, delivered in counterbalanced order on different occasions separated by 3–4 days. The 150-pmol dose is known to be a moderate supra-threshold dose for the feeding response with forebrain ventricular delivery (6). A given level of hyperphagic response, expected for the third intracerebroventricular treatment, would provide a reference against which to evaluate any response to ghrelin delivered to the brainstem ventricle.

Apparatus. Cumulative food intake and meal patterns were measured with a specialized device (DiaLog Instrument, Tallahassee, FL) that recorded intake in 30-s intervals throughout a 23-h period. The apparatus consists of stainless steel hanging cages, each with an opening to a food cup seated on a load cell circuit that reports to an interface and a computer with customized software for data storage, display, and analysis. The system was initiated each day ~1 h after the onset of the light phase. During the first hour, animals were weighed and food cup weights were obtained to confirm system calibration. On the experimental schedule, animals received intracerebroventricular injections over a 20-min period just before system initiation and presentation of the food cup.

Statistical analyses. Cumulative intake records with 30-min resolution were generated for purposes of display. Drug and ventricle effects were analyzed via two-way ANOVA on cumulative intakes at 1.5, 3, 12, and 24 h. Given the pattern of results obtained, the analysis of meal pattern parameters was limited to the first 3 h after treatment. Meals were defined as a bout of feeding in which a minimum of 0.25 g was ingested, with a meal termination criterion as the beginning of a pause in ingestive behavior no less than 10 min in duration. (Of all the meals evaluated, only four bouts of feeding failed to meet the intake criteria. The parameters evaluated via two-way ANOVA included latency to the first meal, interval between the first and second meal, size of the first meal, two-meal average meal size, and the number of meals taken in 3 h.

Experiment 2: Cumulative food intake effects of DVC ghrelin microinjection. Rats ($n = 13$) received ghrelin or vehicle into the DVC, in counterbalanced order separated by 3 days. All rats were tested with a 10-pmol dose. Among these, three had been previously tested in a pilot experiment with 30 pmol ghrelin (and counterbalanced vehicle condition). The remaining animals subsequently received a 5-pmol dose (with counterbalanced vehicle condition). As in experiment 1, injections were delivered in the first hour of the light phase. Food intake was measured at 1.5, 3, and 24 h after injection by weighing the food dish. Results for the 10- and 5-pmol doses were analyzed separately against the respective vehicle values via two-tailed paired t tests.

RESULTS

Experiment 1: Cumulative food intake and meal pattern effects of third or fourth intracerebroventricular ghrelin administration.

Ventricle injection site comparisons. For every parameter evaluated, the two-way ANOVA (injection site × drug) showed no main effect of injection site ($P > 0.2$). In addition, with one arguably minor exception noted below, drug treatment effects for third and fourth intracerebroventricular injection sites were of comparable magnitude, as indicated by the lack of significant two-factor interactions ($P > 0.5$). Because there were no differences in ghrelin action with third and fourth intracerebroventricular delivery, the statistical results are presented below only for the drug factor.

Cumulative food intake. The hyperphagic response to 150 pmol ghrelin is depicted in Fig. 1*A* and *B*, which show the cumulative intake records for third and fourth intracerebroventricular conditions, respectively. The ANOVA for the four time points evaluated revealed a significant main effect of drug in each case (1.5 h: $F_{1,23} = 19.1$, $P < 0.001$; 3 h: $F_{1,23} = 26.6$, $P < 0.001$; 12 h: $F_{1,23} = 7.79$, $P < 0.01$; 24 h: $F_{1,23} = 4.59$, $P < 0.05$). It is clear from inspection of the cumulative intake records for the entire day (Fig. 1*A* and *B* insets) that the maximal size of the ghrelin effect was achieved by 3 h after injection. Because there was no additional hyperphagic effect of ghrelin beyond this point, we have limited the time frame of the meal-pattern analysis to the early hours after treatment.

Meal patterning. Ghrelin treatment significantly increased meal frequency, which is reflected in the significant increase in the number of meals taken during the 3 h after injection ($F_{1,23} = 16.35$, $P < 0.001$). Values (mean ± SE) for this parameter were 0.73 ± 0.19 and 2.18 ± 0.4 meals for vehicle and ghrelin delivered to the fourth ventricle, respectively, and 0.86 ± 0.31 and 2.00 ± 0.33 for these same injections into the third ventricle. This ghrelin-related increase in meal frequency was accounted for by a substantial decrease in the interval between injection and the onset of the first meal ($F_{1,12} = 14.63$, $P < 0.001$) (Fig. 2). There was no main effect of ghrelin treatment on the interval between the first and second meals ($F_{1,23} = 3.98$, $P < 0.06$), although the interaction between drug and intracerebroventricular placement factors was significant ($F_{1,23} = 5.01$, $P < 0.05$), reflecting a decrease in intermeal interval observed with the fourth intracerebroventricular group.

As shown in Fig. 3, there was a sizeable trend toward an

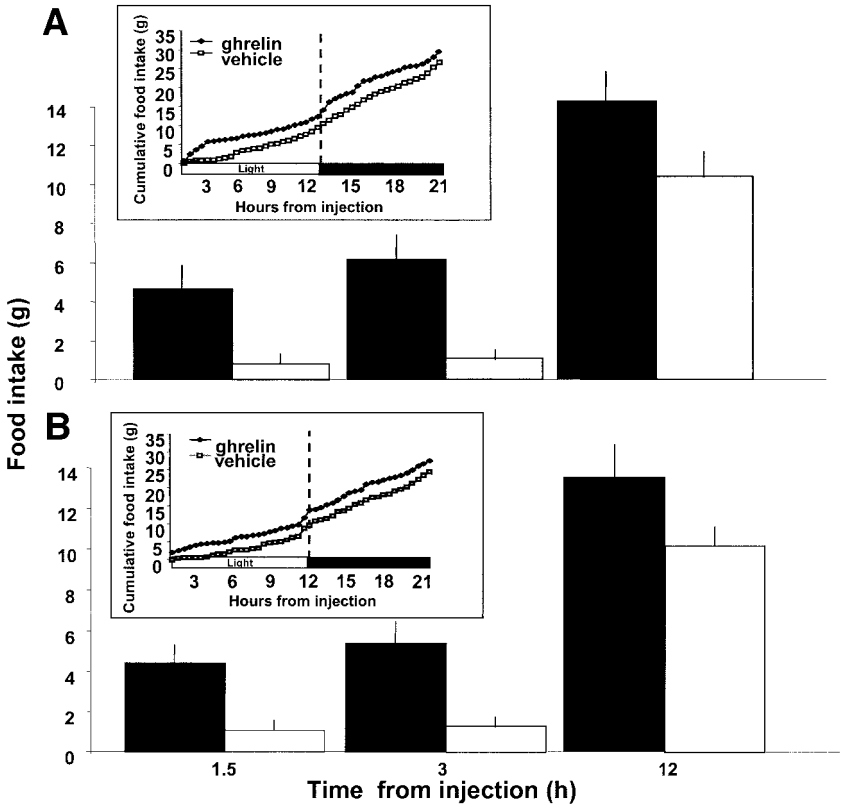

FIG. 1. Cumulative food intake (mean ± SE) at 1.5, 3, and 12 h following injection of 150 pmol ghrelin (■) or vehicle (□) into third (*A*) and fourth (*B*) ventricles in experiment 1. Insets show, with 30-min resolution, group mean cumulative intake records for drug and vehicle conditions. See text for statistical analysis.

increase in first meal size after ghrelin treatment. The ANOVA on the raw data yielded a significant main effect of drug treatment ($F_{1,23} = 4.98$, $P < 0.04$). However, inspection of Fig. 3, in which individual subject values are represented, reveals that high-value outliers contributed heavily to the size of the trend. Indeed, when these outliers are removed from the analysis (one from the third and two from the fourth intracerebroventricular group), or when their impact is reduced through a log transform of the raw data, the overall effect of ghrelin treatment on first-meal size is no longer statistically significant. A similar pattern of results was obtained for the average size of the first two meals taken after treatment, where the significant treatment effect ($F_{1,23} = 5.31$, $P < 0.04$) in the analysis of the raw data is no longer reliable with outliers removed or with log-transformed data.

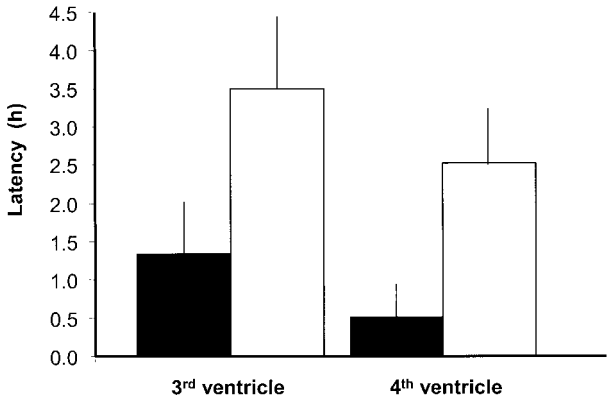

FIG. 2. Time (mean ± SE) taken for animals to initiate a meal following injection of 150 pmol ghrelin (■) or vehicle (□) in experiment 1. See text for statistical analysis.

Experiment 2: Cumulative food intake effects of ghrelin microinjection into the DVC. Of the 13 cannula placements judged to lie within the DVC, 7 were at the level of the area postrema (AP). The other six were clustered in a position within 1 mm anterior to the AP (Fig. 4). Ghrelin at the 10-pmol dose unilaterally delivered to the DVC significantly increased intake at the 1.5-h [$t(12) = 2.99$, $P < 0.01$] and 3-h [$t(12) = 4.08$, $P < 0.004$] measurement points (Fig. 5). At the 5-pmol dose, values after ghrelin injection were, on average, higher than after vehicle, but the difference was not statistically reliable at either time [90 min: $t(9) = 0.68$, $P = 0.51$; 3 h: $t(9) = 0.66$, $P = 0.52$].

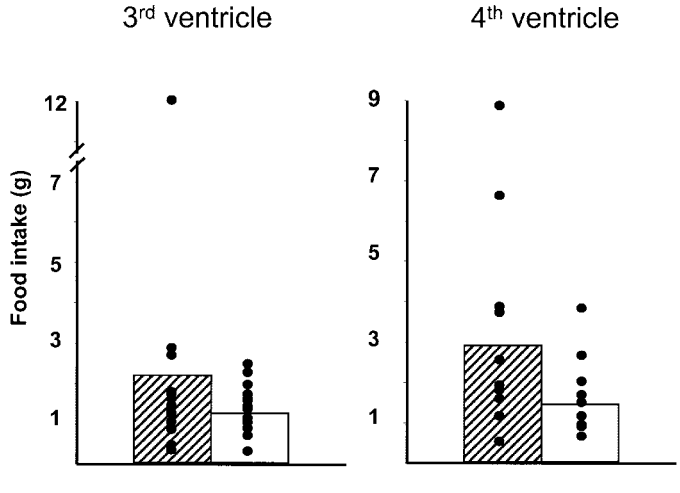

FIG. 3. Size of the first meal (mean ± SE) following injection of ghrelin (▨) or vehicle (□) in experiment 1. Data points shown represent meal sizes of individual animals in each condition. See text for statistical analysis.

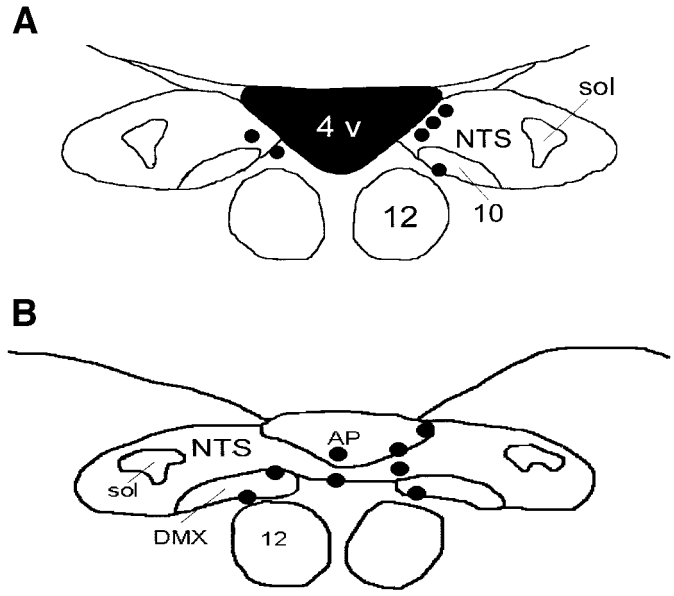

FIG. 4. Reconstruction of dorsal medullary injection sites. The centers of injections are represented by black dots; all were deemed to lie within the dorsal vagal complex. Placements shown were between −13.24 (*A*) and −13.68 mm (*B*) from bregma (25). Of the 13 cannula placements, 6 were clustered in a position within 1 mm anterior to the AP (*A*). The other seven injector sites were at the level of the AP (*B*). 12, hypoglossal nucleus; 10, dorsal motor nuclear of the vagus nerve; sol, solitary tract; four v, fourth ventricle; NTS, nucleus of the solitary tract.

DISCUSSION

The present results indicate that delivery of ghrelin to the brainstem induces a hyperphagic response similar to that obtained by us and by others with the same dose of ghrelin delivered to the forebrain. This conclusion is supported by complementary results of the two experiments in which ghrelin was delivered to the fourth ventricle and to the brainstem parenchyma at low doses. The magnitude of the orexigenic response to fourth intracerebroventricular ghrelin administration was comparable to the effects of third intracerebroventricular delivery. The 150-pmol dose elevated cumulative intake at 1.5 and 3 h to approximately the same extent with both placements. Given the essentially flat character of the dose-response curve for intracerebroventricular ghrelin (4,6,11,12) over two or more orders of magnitude in the supra-threshold range, the present results are not sufficient for a judgement of equivalent sensitivity to the peptide at the two placements. The results do qualitatively establish that a hyperphagic response is obtained after brainstem intracerebroventricular delivery and, moreover, that the form of the behavioral response was similar for the two injection sites. In each case the cumulative intake effect was primarily attributable to a reduced interval between the first two meals and/or a reduced latency to the first meal initiated after injection. In both cases, there were group-average increases in meal size, which accounted for a minority share of the cumulative intake effects of ghrelin. In neither case, however, was the meal-size effect statistically significant (see below). Thus, very similar response profiles were obtained from the third intracerebroventricular placements, providing optimal stimulation of hypothalamic structures, including ARC, and from the fourth intracerebroventricular placement, bearing more directly on feeding-relevant structures in the caudal brainstem.

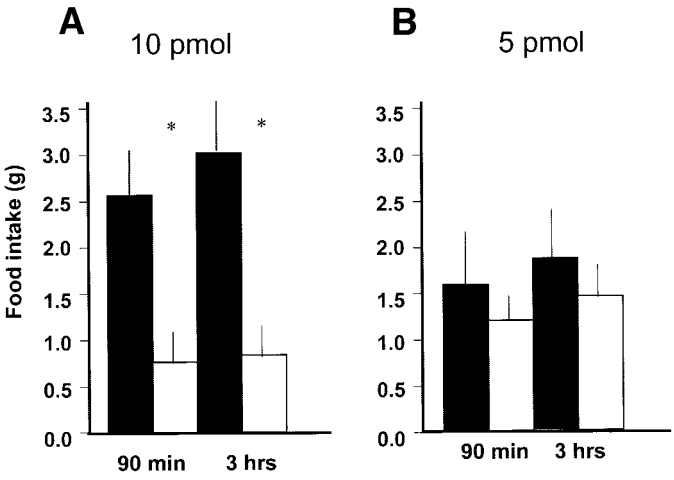

FIG. 5. Cumulative food intake (mean ± SE) at 1.5 and 3 h following injection of 10 (*A*) or 5 pmol (*B*) ghrelin (■) or vehicle (□) into DVC parenchyma in experiment 2. **P* < 0.01, drug vs. vehicle.

A brainstem trigger for the behavioral action of ghrelin is strongly reinforced by the hyperphagic response observed on unilateral microinjection of the peptide into the DVC. Wren et al. (12) showed that the most sensitive hypothalamic site for ghrelin-induced hyperphagia was ARC, at which 30 pmol was the lowest effective dose. Here, a similarly robust and significant response was obtained from the DVC with a lower (10-pmol) dose, establishing the DVC as a parenchymal placement of at least comparable sensitivity. Viewed along with the hypothalamic results, our findings suggest that ingestive responses to ghrelin can be elicited from more than one location; effective placements include the ARC and at least one site within the caudal brainstem.

The DVC is particularly interesting as a putative site of ghrelin action. It is already clear that this substrate is an important site for the convergence and integration of various peripheral and central signals relevant to energy homeostasis. Two of its component structures, the nucleus tractus solitarius (NTS) and AP, receive visceral afferent inputs that drive a number of autonomic reflexes and relay visceral sensory information to other components of the broader central nervous system for the control of energy balance (20–22). The DVC also contains receptors responsive to blood-borne signals correlated with the animal's metabolic status. These include long-form leptin receptors (14,15) and mechanisms for the detection of changes in circulating glucose level (16–18). We noted earlier that GHS-R has recently been localized throughout the DVC (J. M. Zigman and J. K. Elmquist, personal communication). Our functional results complement this anatomical evidence and suggest that the hyperphagic effects of ghrelin delivered to the fourth ventricle are triggered, at least in part, by ghrelin acting on GHS-R in the DVC. The demonstration of ghrelin sensitivity in the DVC expands the range of peripherally generated signals relevant to energy balance that are received and potentially integrated within this region of the brainstem and draws attention to a functional parallel between the DVC and the hypothalamic arcuate nucleus.

The meal patterning observations enhance the comparison between the effects of third and fourth intracerebroventricular ghrelin treatment and also speak more generally to ghrelin's behavioral mode of action. Significant reductions in latency to the first meal (and in the case of fourth intracerebroventricular delivery, in the first intermeal interval) are consistent with the view that ghrelin acts as a meal-initiating signal (23,24). It should be noted, however, that the interval between ghrelin treatment and first-meal onset, albeit one shorter than after-vehicle injection, was appreciable. An explanation for the somewhat delayed effect under these pharmacological conditions cannot be provided at this time. Possibilities include a minimum temporal interval over which the effects of receptor stimulation must be integrated, a genomically mediated response, or a cascade of events within the neural network underlying meal initiation. Any neurological or pharmacological interpretation of this result, of course, must be considered alongside structural aspects of the experiment, including the particular configuration of the testing environment, and the relatively low level of arousal and exploratory behavior during the light phase of the circadian cycle. Such considerations notwithstanding, it is clear that the predominant effect underlying the hyperphagia that attends central ghrelin administration is an increase in the probability of meal initiation.

In contrast to the robust findings for latency and meal frequency, a mixed picture was obtained from the analysis of meal-size results. On average, a sizeable increase in the amount consumed during the first meal (and in the average of the first two meals) was observed, which represented a salient fraction of the treatment-related effects on cumulative intake. The trends, however, were carried largely by excessively high meal sizes for one or two rats, with the remaining values for drug and vehicle conditions similarly distributed over the same intake range (Fig. 3). A prominent trend was also obtained for the average size of the first two meals, which approached but did not achieve statistical significance. Given the variability in this parameter under the present conditions, it may be fair to view the negative results on meal size with some reservation. Indeed, prospects for capturing a reliable effect of ghrelin on meal size may be improved with other testing paradigms; for example, one in which the drug is delivered before a scheduled access to food, where the probability of feeding is high and the feeding latency effect is discounted. In any event, the present meal-size results do not provide a clear indication that ghrelin acts on feedback inhibitory mechanisms mediating satiation and the termination of individual meals.

Two conclusions may be drawn from the present work; they concern the form of the behavioral response and the central sites from which hyperphagic responses to ghrelin can be obtained. The most reliable behavioral effect of ghrelin was a reduction in the latency to feed and an increase in the number of meals taken during the first few hours after treatment. These results are consistent with a role for central ghrelin signaling in meal initiation. The meal patterning results for third and fourth intracerebroventricular administration were comparable, as was the size of the cumulative intake response, suggesting a role for both forebrain and brainstem GHS-R in short-term food-intake control. The response to low-dose microinjection of ghrelin into the DVC strongly supports a brainstem site of action and recommends further investigation into multisite interactions underlying the modulation of ingestive behavior by this hormone.

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