Distinct Forebrain and Caudal Brainstem Contributions to the Neuropeptide Y Mediation of Ghrelin Hyperphagia

Lucy F. Faulconbridge, Harvey J. Grill, and Joel M. Kaplan

Neuropeptide Y (NPY) has been implicated in the downstream mediation of ghrelin hyperphagia, with the site of action for both peptides considered to be intrinsic to the hypothalamus. Here, however, we observed robust hyperphagia with caudal brainstem (CBS) (fourth intracerebroventricular) ghrelin delivery and, moreover, that this response was reversed with coadministration of either of two NPY receptor antagonists (1229U91 and D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$) with contrasting NPY receptor subtype–binding properties. The same results were obtained after forebrain (third intracerebroventricular) administration, but the sites for both ghrelin and antagonist action were open to question, given the caudal flow of cerebrospinal fluid (CSF) through the ventricular system. To control for this, we occluded the cerebral aqueduct to restrict CSF flow between the forebrain and CBS ventricles and tested all combinations (same and cross ventricle) of ghrelin (150 pmol/1 μl) and NPY receptor antagonist delivery. With fourth intracerebroventricular ghrelin delivery after aqueduct occlusion, preadministration of either of the two antagonists through the same cannula reversed the hyperphagic response but neither was effective when delivered to the third ventricle. With third intracerebroventricular ghrelin administration, however, 1229U91 reversed the ingestive response only when delivered to the fourth ventricle, whereas D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ was effective only when delivered to the forebrain. These results demonstrate distinct mediating pathways (due to location and subtypes of relevant NPY receptor) for the hyperphagic response driven separately by forebrain and CBS ghrelin administration. ***Diabetes*** **54:1985–1993, 2005**

From the Department of Psychology, University of Pennsylvania, Philadelphia, Pennsylvania.

Address correspondence and reprint requests to Lucy F. Faulconbridge, Department of Psychology, University of Pennsylvania, 3720 Walnut St., Philadelphia, PA 19104. E-mail: lucyhf@sas.upenn.edu.

Received for publication 17 December 2004 and accepted in revised form 7 April 2005.

ARC, arcuate nucleus of the hypothalamus; CBS, caudal brainstem; CSF, cerebrospinal fluid; NPY, neuropeptide Y; PVN, paraventricular nucleus.

© 2005 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Ghrelin, the endogenous ligand for the growth hormone secretagogue (GHS) receptor (1), induces a hyperphagic response when delivered centrally (2–6). GHS receptors are located on neuropeptide Y (NPY)/agouti-related protein neurons in the arcuate nucleus of the hypothalamus (ARC) (7), and it is generally held that projections from these neurons to those in the paraventricular nucleus (PVN) and perhaps in other hypothalamic regions are an essential link in the downstream mediation of the orexigenic actions of ghrelin (8,9). Consistent with this view is evidence showing that ghrelin induces c-*fos* expression (5,10–12) and NPY mRNA expression in ARC (10,13,14) and stimulates electrophysiological activity in NPY/agouti-related protein neurons (8,15). Central administration of either ghrelin or NPY also activates c-*fos* expression in the PVN (16–18), an area with strong expression of NPY-Y1 and Y5 receptors (19). Functional support for the mediation of ghrelin hyperphagia by NPY systems comes from pharmacological studies in which NPY antibodies (14) or receptor antagonists (3,5,20,21) were shown to abolish ghrelin-induced feeding. The results of these studies, in which ligands were delivered to forebrain ventricles, are consistent with the view that the relevant GHS and NPY receptors are contained within the hypothalamus.

The anatomical extent of the substrates supporting the ghrelin-NPY interaction relevant to feeding control, however, remains open to question. Attribution of a hypothalamic site of action for the pharmacological studies is tenuous, given that the caudal flow of cerebrospinal fluid (CSF) through the ventricular system carries ligands to behaviorally potent receptor populations in the caudal brainstem (CBS). Thus, with ghrelin and the NPY receptor antagonist both delivered to the third or lateral ventricles (20,21), it is possible that CBS receptors and circuits contribute to either or both the primary ghrelin effect and its reversal. GHS receptors are clearly represented in the CBS (22,23), as are the NPY receptor subtypes most commonly implicated in the control of ingestive behavior (24–27). Targeted stimulation of brainstem GHS receptors (6) or NPY receptors (28,29), moreover, yields robust hyperphagic responses at low doses. Furthermore, as shown below, delivery of NPY antagonists to the fourth (hindbrain) ventricle completely reverses the hyperphagic response observed when ghrelin is administered through the same cannula. The parity of the results derived from

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

CBS and forebrain drug administration points to a number of possible sites for ghrelin-NPY interactions, within and between different brain regions, any or all of which might be necessary for the expression of ghrelin hyperphagia.

The substrates underlying the ghrelin-NPY interactions are parsed here via the "cerebral aqueduct occlusion" method, in which a grease plug restricts the flow of injected ligands through the ventricular system. Aqueduct occlusion causes no overt effects on the animal and no changes in body weight or food intake and has provided decisive evidence about CBS versus forebrain sites of action for other drug treatments. Examples include the hypophagic effects of the cocaine- and amphetamine-related transcript (30) and the hyperphagic and glycemic effects of 5-thio-D-glucose (31), which are observed upon either forebrain or brainstem ventricle administration. In both cases, however, when the flow of CSF was blocked via aqueduct occlusion, forebrain treatment was no longer effective, indicating that the effects observed reflected stimulation of receptor populations specifically within the CBS (see also 32). By contrast, a forebrain site of action for the dipsogenic response to angiotensin II is affirmed by the persistence of the third intracerebroventricular elicited response when the aqueduct is occluded and by the ineffectiveness of fourth intracerebroventricular treatment (33).

Here, the aqueduct occlusion method is applied in a series of experiments in which ghrelin is delivered to either the third or fourth ventricles. Possible within-level mechanisms for the NPY mediation of ghrelin hyperphagia are explored in "same-ventricle" studies, in which ghrelin and NPY antagonists are delivered together to the fourth or to the third ventricle. "Cross-ventricle" studies (in which ghrelin and antagonists are delivered to opposite sides of the occluded aqueduct) explore the possibility that long projection systems are (also) required for the NPY mediation of ghrelin hyperphagia.

A full set of same-ventricle and cross-ventricle studies are performed with each of two NPY receptor antagonists. The first, 1229U91, is a well-characterized and relatively selective NPY-Y1 receptor antagonist (34) with some agonist activity at the NPY-Y4 receptor (35). The second, D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ (36), is a COOH-terminal NPY fragment with contrasting subtype selectivity; available evidence suggests that it is unlikely to bind to the Y1 receptor (37). The two antagonists were similarly effective at reversing ghrelin hyperphagia in "open-ventricle" experiments but differentially potent in aqueduct occlusion experiments, depending on site of administration. Overall, the results presented below reveal the limitations of the intrinsic hypothalamic model for the downstream mediation of ghrelin hyperphagia and compel a broader anatomical perspective for ghrelin-NPY interactions in the control of ingestive behavior.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 295–330 g at surgery were housed in hanging stainless steel cages under a 12-h light/12-h dark cycle (lights on at 9:00 A.M.). Pelleted Chow (Ralston Purina, St. Louis, MO) and water were available ad libitum. The experimental protocols used conform 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.). Rats received a 22-gauge guide cannula (Plastics One, Roanoke, VA) implanted 2 mm above an injection site in the third and/or fourth ventricle (experiments 1 and 4), and for experiments 2, 3, 5, and 6, an additional 19-gauge guide cannula was implanted into the cerebral aqueduct. The third-ventricle cannula was positioned on the midline, 2.0 mm posterior to bregma and 7.7 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 aqueduct cannulas were angled 11° in the lateral-midline direction and guided to a position on the midline, 7 mm posterior to bregma and 5 mm below the dura. The cannulas were attached to the skull with jeweler's screws and dental acrylic and closed with obturators.

Verification of cannula positions. Ventricular cannula placements were evaluated functionally after at least 7 days of recovery from surgery through measurement of the sympathetically mediated hyperglycemic response to 210 μg of 5-thio-D-glucose in 2 μl of artificial CSF (31). Only rats that showed at least a twofold increase of plasma glucose level in response to this treatment were used in the experiments.

Drug preparation and injection. Rat ghrelin (Phoenix Pharmaceuticals, Belmont, CA) and the NPY receptor antagonists 1229U91 (Neosystems) and D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ (Bachem, King of Prussia, PA) were dissolved in artificial CSF and stored at −80°C. The 150-pmol ghrelin dose is known to be a moderate, supra-threshold dose for the feeding response upon forebrain ventricular delivery (5). The 5-nmol dose of 1229U91 had been shown to reverse melanin-concentrating hormone-induced hyperphagia by 86% (38); a lower dose (2 nmol) reduced NPY-induced feeding by 48% (39). The 10-μg dose of D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ was shown to reverse ghrelin-induced hyperphagia when delivered to the lateral ventricle (21).

Drugs and vehicle were delivered via a microsyringe (Hamilton, Reno, NV) with a 28-gauge injector that extended beyond the guide cannula and into the ventricle. All treatment conditions consisted of two injections: ghrelin + vehicle, NPY receptor antagonist + vehicle, vehicle + vehicle, or NPY antagonist + ghrelin, 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. All drug injections were delivered in the 2nd h of the light phase. On at least two occasions before experimental testing, rats were given vehicle injections to habituate them to the injection procedure. In all experiments, food intake (dish weight difference minus spillage) was measured 1.5, 3, and 24 h after injection.

Aqueduct occlusion: delivery and verification. For experiments in which cerebral aqueduct occlusion was employed (experiments 2, 3, 5, and 6), 5 μl of a 50:50 ratio of High Vacuum grease (Dow Corning) and Dialectic Connector grease (VersaChem) mixed with 0.1 μg of fast green dye (for visualization) was injected via a 100-μl Hamilton syringe into the cerebral aqueduct 1 h before drug injections on the aqueduct occlusion test day.

Aqueduct occlusion was verified functionally by 5-thio-D-glucose injection after testing. Rats with third intracerebroventricular cannulas were included in the analysis only when the injection failed to increase plasma glucose. For histological verification, black India Ink (2 μl) was injected through the cannula just before the rats were killed. Brains were removed and postfixed in a 10% sucrose-formalin solution. Mid-sagittal sections were cut and then examined under a dissection microscope for verification of aqueduct occlusion. Only data for animals showing no ink in the opposite ventricle were included in the analysis.

Experimental design. The study consisted of six experiments in two series of three experiments each: the first three (experiments 1, 2, and 3) used 1229U91, and the second three (experiments 4, 5, and 6) used D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$. The initial experiment in each series (experiments 1 and 4) consisted of two groups of rats, one with third intracerebroventricular cannulas (experiment 1a, $n = 10$; experiment 4a, $n = 7$) and one with fourth intracerebroventricular cannulas (experiment 1b, $n = 13$; experiment 4b, $n = 7$). All rats in each group received each of four treatment combinations (ghrelin + vehicle, NPY antagonist + vehicle, vehicle + vehicle, or NPY antagonist + ghrelin) delivered in counterbalanced order with 2 days between test sessions.

The second in each series (experiments 2 and 5) consisted of two groups of rats, one with third ventricle and cerebral aqueduct cannulas (experiment 2a, $n = 12$; experiment 5a, $n = 14$) and one with fourth ventricle and aqueduct cannulas (experiment 2b, $n = 16$; experiment 5b, $n = 9$). All rats received vehicle + vehicle as a baseline condition before insertion of the aqueduct occlusion. For the postocclusion treatment conditions, rats were divided into two subgroups receiving either ghrelin + vehicle or ghrelin + NPY antagonist.

The third in each series (experiments 3 and 6) consisted of two groups of rats, with all rats receiving three cannulas positioned, respectively, above the third and fourth ventricles and into the cerebral aqueduct. In experiment 3a, rats received ghrelin into the third ventricle and 1229U91 into the fourth

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

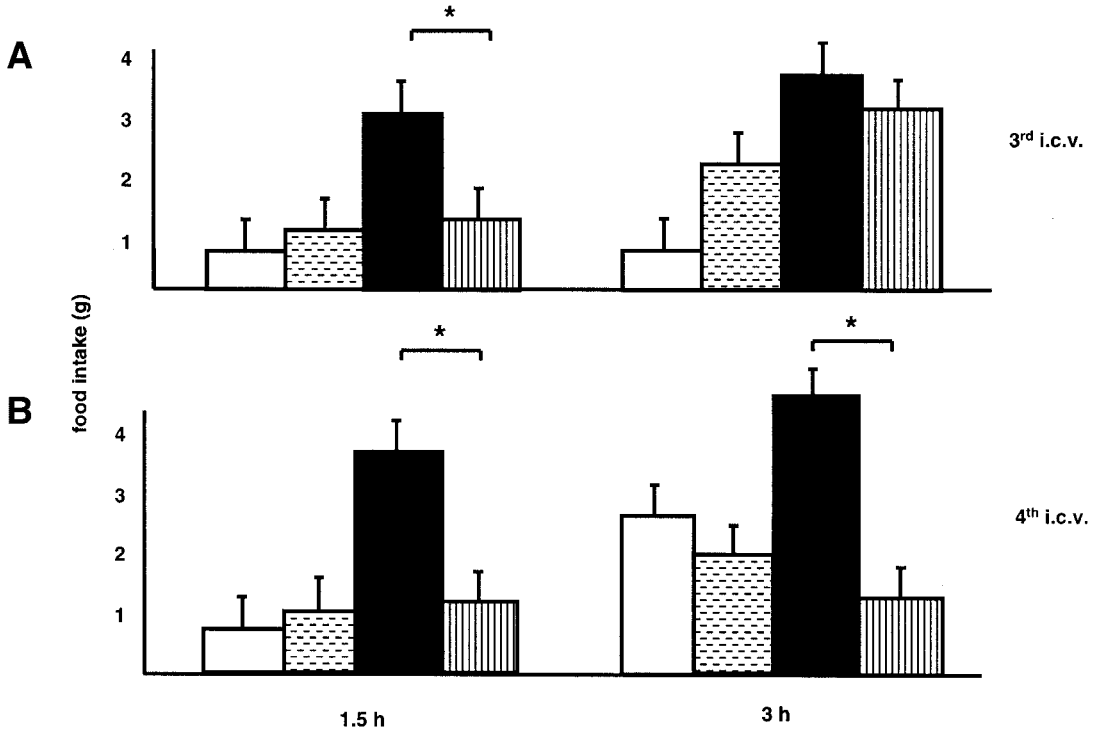

FIG. 1. The effects of 1229U91 on ghrelin hyperphagia when both ligands are delivered intracerebroventricularly (i.c.v.) to either the third (*A*) or fourth (*B*) ventricle: open-ventricle study. Values shown are means ± SE cumulative intakes 1.5 and 3 h after treatment for the four injections conditions: vehicle + vehicle (□), 1229U91 + vehicle (▨), ghrelin + vehicle (■), and ghrelin + 1229U91 (▩). In all cases, intake was greater after ghrelin treatment than under vehicle baseline conditions ($P < 0.01$). *Significant reversal of ghrelin hyperphagia with antagonist coadministration ($P < 0.05$). See text for further details.

ventricle ($n = 6$) or ghrelin into the third ventricle and vehicle into the fourth ventricle ($n = 6$) after aqueduct occlusion. In experiment 3b, rats received ghrelin into the fourth ventricle and 1229U91 into the third ventricle ($n = 8$) or ghrelin into the fourth ventricle and vehicle into the third ventricle ($n = 8$) after aqueduct occlusion. In experiment 6a, animals received ghrelin into the third ventricle and D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ into the fourth ventricle ($n = 5$) or ghrelin into the third ventricle and vehicle into the fourth ventricle ($n = 4$). In experiment 6b, animals received ghrelin into the fourth ventricle and

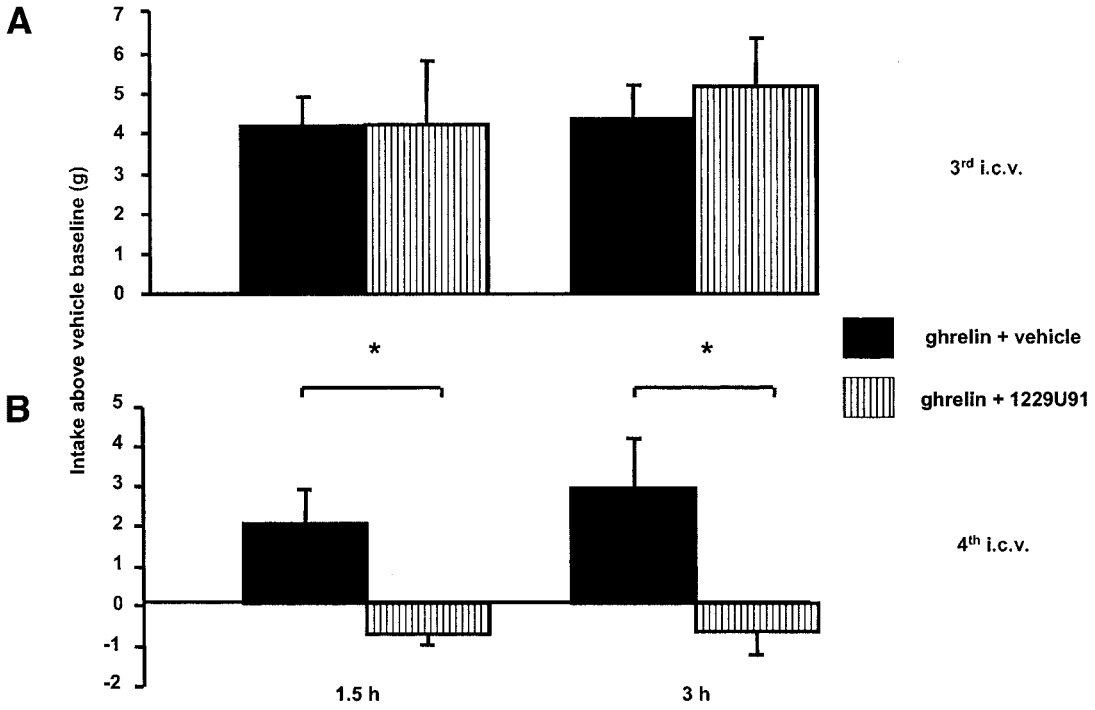

FIG. 2. The effects of 1229U91 on ghrelin hyperphagia at 1.5 and 3 h when both ligands are delivered, after aqueduct occlusion, to the third (*A*) or the fourth (*B*) ventricle. Values shown represent the means ± SE differences in intake between drug (ghrelin + vehicle or ghrelin + Y receptor antagonist) and vehicle + vehicle baseline conditions for the respective groups. *Significant reversal of ghrelin hyperphagia ($P < 0.02$). See text for further details. i.c.v., intracerebroventricular.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

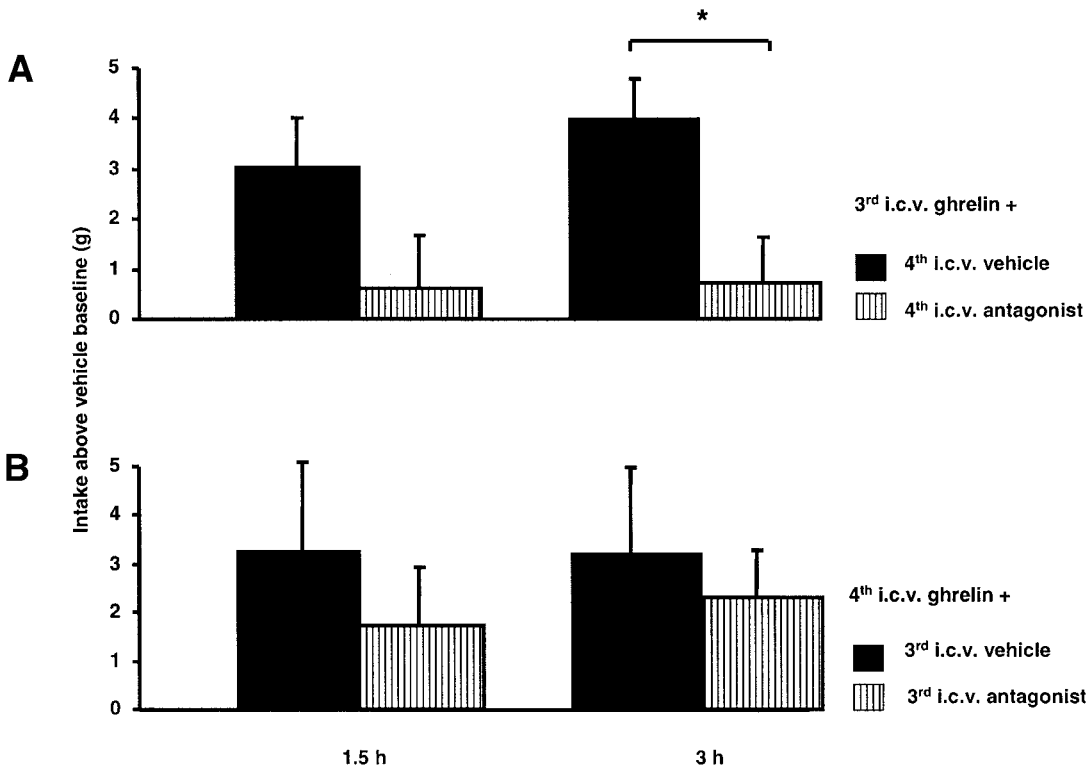

FIG. 3. The effects of 1229U91 on ghrelin hyperphagia 1.5 and 3 h after delivery of ghrelin and 1229U91 to different ventricles after aqueduct occlusion. *A*: Means ± SE differences in intake between the vehicle + vehicle baseline condition and ghrelin delivery to the third ventricle with either vehicle (■) or 1229U91 (▥) to the fourth ventricle. *B*: Difference in intake between baseline conditions and ghrelin delivery to the fourth ventricle with either vehicle (■) or 1229U91 (▥) to the third ventricle. *Significant reversal of ghrelin hyperphagia ($P < 0.02$). See text for further details. i.c.v., intracerebroventricular.

D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ into the third ventricle ($n = 6$) or ghrelin into the fourth ventricle and vehicle ($n = 7$) into the third ventricle.

Data analysis. For experiments 1 and 4, two-way (ventricle × drug condition) repeated-measures ANOVAs were used to evaluate overall treatment effects, and Tukey's honestly significant difference post hoc tests were used to evaluate pairwise intake differences among the four conditions. Separate analyses were conducted for intake measured 1.5 and 3 h after treatment. For experiments 2, 3, 5, and 6, reversal of ghrelin hyperphagia was assessed by a two-tailed independent-samples t test comparing the size of the ghrelin effect in rats receiving ghrelin alone after aqueduct occlusion (ghrelin + vehicle minus vehicle + vehicle) with the intake difference between the ghrelin + Y receptor antagonist condition and vehicle + vehicle baseline.

RESULTS

Experiment 1. Ghrelin and 1229U91: open-ventricle studies. Figure 1 shows the values for the four treatment conditions (vehicle + vehicle, ghrelin + vehicle, 1229U91 + vehicle, and ghrelin + 1229U91) delivered to the third (experiment 1a) and fourth (experiment 1b) ventricles. The respective ANOVAs (for each ventricle placement and each of the two time points) revealed a significant main effect of injection condition in all cases ($P < 0.01$). A hyperphagic response to ghrelin was obtained in each case (vehicle + ghrelin versus vehicle + vehicle; $P < 0.01$). At the 1.5-h time point for both third and fourth ventricle groups, the antagonist reversed the effect of ghrelin (i.e., ghrelin + 1229U91 < ghrelin + vehicle [$P < 0.015$], and there were no significant differences between ghrelin + 1229U91 and vehicle + vehicle conditions). For the fourth intracerebroventricular experiment, this reversal was sustained at the 3-h time point, whereas for the third intracerebroventricular conditions, there was no longer a statistically significant reversal.

Experiment 2. Ghrelin and 1229U91: same-ventricle studies with aqueduct occlusion

Experiment 2a: third ventricle injections. The results with aqueduct occlusion (Fig. 2*A*) were in marked contrast to those of the open-ventricle study (experiment 1a). The ghrelin effect was apparent in both cases, but with occlusion, the antagonist failed to reverse the hyperphagic response; that is, the comparisons between the difference scores (ghrelin + vehicle minus vehicle + vehicle versus ghrelin + 1229U91 minus vehicle + vehicle) were not significant (1.5 h: $t(10) = -0.04$, NS; 3 h: $t(10) = -0.60$, NS). This result suggests that the Y1 receptors critical for the reversal of the third intracerebroventricular ghrelin response in the open-ventricle case reside in the CBS, an inference supported by the results of experiment 3a.

Experiment 2b: fourth ventricle injections. The pattern of results was comparable with that obtained from the corresponding open-ventricle study (experiment 1b). The reversal of ghrelin hyperphagia in this experiment (Fig. 2*B*) is shown by the significant difference between the size of the ghrelin effect and the intake difference between ghrelin + 1229U91 and its vehicle control values (1.5 h: $t(14) = 3.38$, $P < 0.005$; 3 h: $t(14) = 2.95$, $P < 0.02$).

Experiment 3. Ghrelin and 1229U91: cross-ventricle studies with aqueduct occlusion

Experiment 3a: ghrelin into third ventricle; 1229U91 into fourth ventricle. Figure 3*A* (*right*) shows the reversal of the hyperphagic response to third intracerebroventricular ghrelin delivery at the 3-h time point with Y1 receptor antagonist delivery to the fourth ventricle ($t(7) =$

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

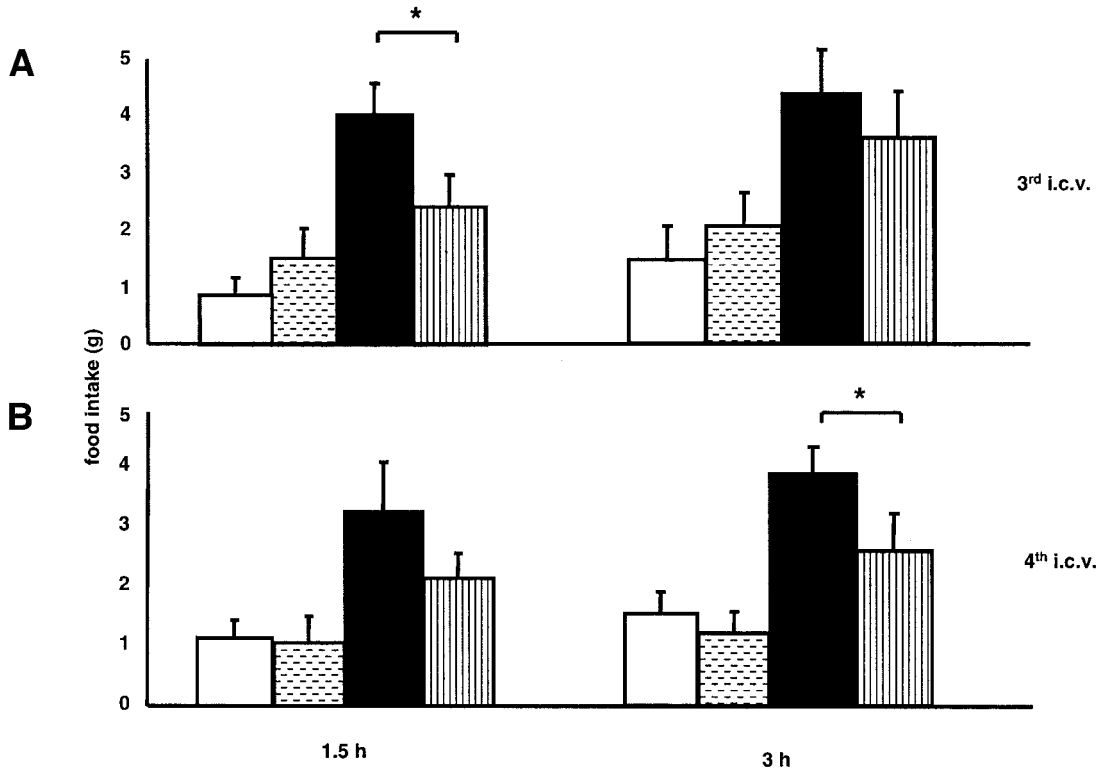

FIG. 4. The effects of D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ on ghrelin hyperphagia when both ligands are delivered to either the third (*A*) or fourth (*B*) ventricle: open-ventricle study. Values shown are means ± SE cumulative intakes 1.5 and 3 h after treatment for the four injections conditions: vehicle + vehicle (□), 1229U91 + vehicle (▨), ghrelin + vehicle (■), and ghrelin + D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ (▥). In all cases, intake was greater after ghrelin treatment than under vehicle baseline conditions ($P < 0.01$). *Significant reversal of ghrelin hyperphagia with antagonist coadministration ($P < 0.03$). See text for further details. i.c.v., intracerebroventricular.

−3.04, $P < 0.02$). For the 1.5-h time point, the difference was not statistically significant ($P = 0.09$).

Experiment 3b: ghrelin into fourth ventricle; 1229U91 into third ventricle. The lack of a significant difference between values for the ghrelin + vehicle group and the ghrelin + Y1 receptor antagonist group (1.5 h: $t(8) = -0.75$, ns; 3 h: $t(8) = -0.47$, NS) (see Fig. 3*B*) indicates that forebrain Y1 receptor activation is not required for the hyperphagia obtained with CBS ghrelin delivery.

Experiment 4. Ghrelin and D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$: open-ventricle studies. Figure 4 shows the results for the four treatment conditions delivered to the third (experiment 4a) and fourth (experiment 4b) ventricles. The results with the present antagonist were generally consistent with those described for 1229U91 (experiment 1). The ANOVAs (for each ventricle placement and each of the two time points) indicated a significant main effect of injection condition in all cases ($P < 0.001$). A hyperphagic response to ghrelin was obtained in each case (vehicle + ghrelin versus vehicle + vehicle; $P < 0.01$). For third intracerebroventricular delivery, the antagonist reversed the hyperphagic response to ghrelin at the 1.5-h time point ($P < 0.03$), but at 3 h, there was no longer a significant difference between the ghrelin + D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ and ghrelin + vehicle conditions ($P = 0.66$). For the fourth ventricle (Fig. 4*B*), the trend toward reversal approached but did not reach statistical significance ($P = 0.07$) 1.5 h after treatment. By the 3-h time point, a highly significant reversal was obtained ($P = 0.008$).

Experiment 5. Ghrelin and D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$: same-ventricle studies with aqueduct occlusion. As shown in Fig. 5, the antagonist coadministered with ghrelin significantly reversed the hyperphagic response for both third intracerebroventricular (experiment 5a: 1.5 h, $t(12) = -3.70$, $P < 0.002$; 3 h, $t(12) = -3.82$, $P < 0.003$) and fourth intracerebroventricular (experiment 5b: 1.5 h, $t(6) = -2.81$, $P < 0.04$; 3 h, $t(6) = -2.29$, $P = 0.06$) placements. The third intracerebroventricular results for D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ contrast with those described above for 122U91 (experiment 2a), in which that antagonist failed to reverse ghrelin hyperphagia when the aqueduct was occluded.

Experiment 6. Ghrelin and D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$: cross-ventricle studies with aqueduct occlusion. For all cross-ventricle combinations, D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ failed to reverse the hyperphagic response when the antagonist was delivered to the fourth ventricle and ghrelin to the third ventricle (experiment 6a) and when delivered to the third ventricle along with fourth intracerebroventricular ghrelin administration (experiment 6b) (Fig. 6). An anomalous finding was obtained for the 3-h intake measurement in experiment 6b, where the hyperphagic response was apparently enhanced by antagonist treatment ($P < 0.03$).

DISCUSSION

The present results confirm an important role for NPY systems in the downstream mediation of ghrelin hyperpha-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

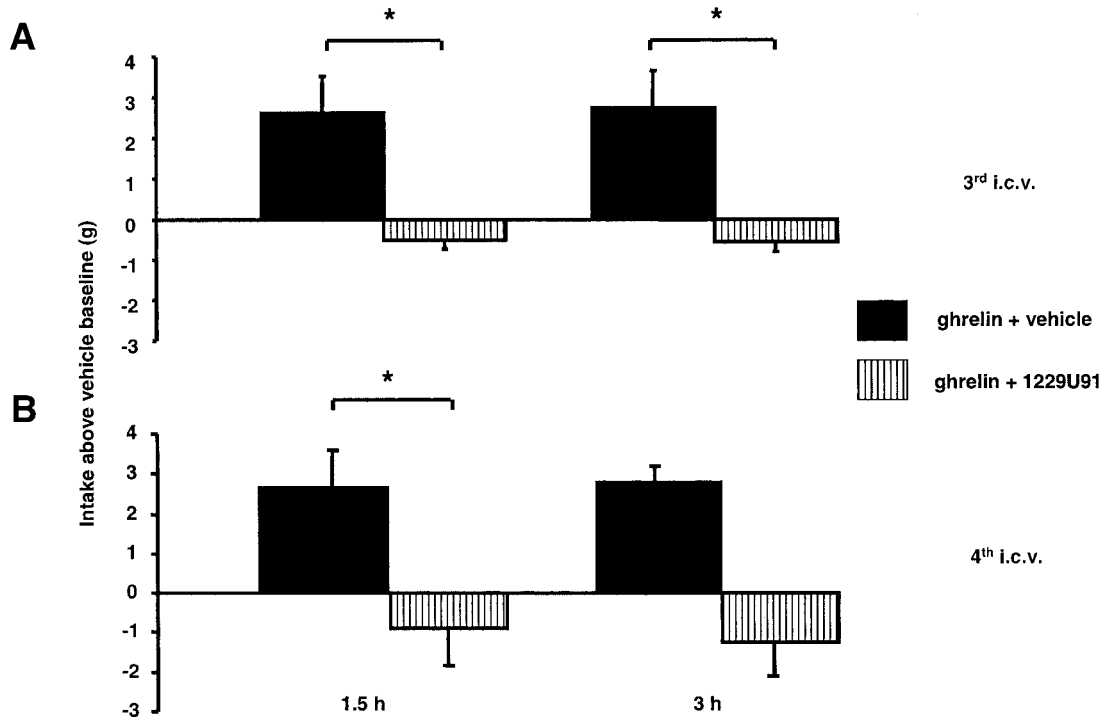

FIG. 5. The effects of D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ on ghrelin hyperphagia at 1.5 and 3 h when both ligands are delivered, after aqueduct occlusion, to the third (*A*) or the fourth (*B*) ventricle: same-ventricle study. Values shown represent the means ± SE differences in intake between drug (ghrelin + vehicle or ghrelin + Y receptor antagonist) and vehicle + vehicle baseline conditions for the respective groups. *Significant reversal of ghrelin hyperphagia ($P < 0.05$). i.c.v., intracerebroventricular.

gia but strongly qualify the often-posited view of an intrinsic hypothalamic circuit sufficient to explain the ghrelin-NPY interactions of relevance to food intake control. A broader neuroanatomical perspective is compelled by the existence of separate GHS receptor "triggers" for the hyperphagic response in the CBS as well as in the hypothalamus (see also 6), and the clear indication that NPY receptor stimulation within the CBS is required for the expression of the hyperphagic response to central ghrelin administration.

Perhaps the most salient counterexamples to the intrinsic hypothalamic model were provided by studies with the NPY-Y1 receptor antagonist 1229U91. A reversal of the hyperphagic response to ghrelin by 1229U91 delivery to the third ventricle was obtained, but only when the caudal flow of CSF was not obstructed (experiment 1a). Ghrelin hyperphagia persisted with aqueduct occlusion (experiment 2a), but the response was no longer affected by administration of the antagonist. It appears, then, that activation of hindbrain Y1 receptors is critical for the expression of the response to forebrain GHS receptor stimulation. This inference was further supported by the cross-ventricle study (experiment 3a) in which the hyperphagia triggered by third intracerebroventricular ghrelin was reversed by 1229U91 delivery to the fourth ventricle. Activation of CBS Y1 receptors (19) also appears to be necessary for the ingestive response stimulated by CBS ghrelin administration. This was indicated by complete reversal of the hyperphagic response when ghrelin and 1229U91 were coadministered to the fourth ventricle in experiments both with (experiment 2b) and without (experiment 1b) aqueduct occlusion. These results, taken together, indicate that endogenous activation of Y1 receptor within the CBS is necessary for the hyperphagic response triggered by either forebrain or hindbrain ghrelin treatment. Activation of forebrain Y1 receptors is not required for ingestive response to ghrelin delivery at either placement.

Although ghrelin-NPY interactions within the hypothalamus are not sufficient to fully account for the forebrain-elicited ghrelin response, work with the second receptor antagonist D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ indicates that there is a within-hypothalamus interaction that is necessary for this response. In contrast to the result obtained with 1229U91, D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ coadministered with ghrelin to the third ventricle with aqueduct occlusion (experiment 5a) reversed the hyperphagic response. Thus, the response arising from GHS receptor stimulation within the forebrain, interestingly, appears to require the simultaneous activation of NPY receptors in both forebrain and CBS but with different subtypes of relevance in the two regions: Y1 receptors within the CBS and a different NPY receptor subtype (36), perhaps the Y5 receptor, within the forebrain (25,40).

A different set of conclusions is reached for the NPY mediation of ghrelin hyperphagia elicited from the CBS placement. First, there appears to be no requirement for activation of forebrain NPY receptors; results of the cross-ventricle studies (experiments 3b and 6b) were negative. Within the CBS, activity at both sets of NPY receptors bound by the respective antagonists is necessary for the hyperphagic response. The fourth intracerebroventricular–elicited ghrelin response was reversed by Y1 receptor antagonist delivery to the CBS. This, in fact, is the common feature for response reversal for both intracerebroventricular placements, although it is not clear whether activation

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

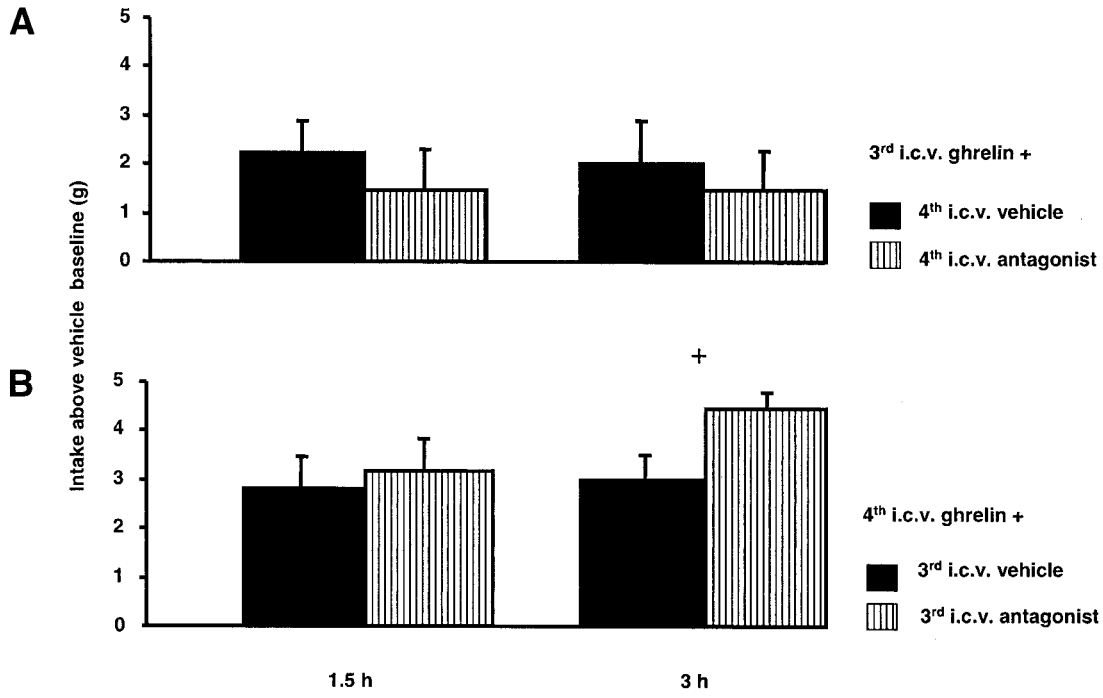

FIG. 6. The effects of D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ on ghrelin hyperphagia at 1.5 and 3 h after delivery of ghrelin and D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ to different ventricles, after aqueduct occlusion: cross ventricle study. *A*: Means ± SE differences in intake between the vehicle + vehicle baseline condition and ghrelin delivery to the third ventricle with either vehicle (■) or D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ (▥) to the fourth ventricle. *B*: difference in intake between baseline conditions and ghrelin delivery to the fourth ventricle with either vehicle (■) or D-Tyr^{27,36}, Thr^{32} NPY$_{27–36}$ (▥) to the third ventricle. i.c.v., intracerebroventricular.

of the same or different subsets of CBS Y1 receptors is relevant to the responses driven by forebrain and CBS GHS receptor stimulation. For receptors bound by D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$, those relevant for the CBS-elicited response reside within the CBS proper. This was the clearest contrast between the respective responses to CBS and forebrain ghrelin delivery. This antagonist was effective when coadministered with ghrelin through the same intracerebroventricular cannula (fourth or third intracerebroventricular), but not effective when the ligands were delivered to different sides of the occluded aqueduct (experiments 6a and 6b).

Our analysis of the NPY receptors underlying ghrelin hyperphagia raises a set of questions concerning the location of the NPY neurons driven, respectively, by forebrain and CBS GHS receptor stimulation. It is possible that ARC neurons are required for all centrally elicited responses, regardless of the trigger site. The account would be straightforward for the third intracerebroventricular–elicited ghrelin response. Thus, ARC projections to neurons expressing receptors bound by D-Tyr^{27,36}, D-Thr^{32} NPY$_{27–36}$ (in, for example, PVN or lateral hypothalamus) could anchor an intrinsically hypothalamic component of the mediating circuit. Descending projections from ARC, although considered weak and not far reaching (41), may be sufficient to provide the requisite stimulation of Y1 receptors expressing neurons in the CBS. An ARC-based account of the fourth intracerebroventricular–elicited response, however, would appear less parsimonious. It would require "a long-loop arrangement," given that the GHS and all relevant NPY receptors reside within the CBS. Here, ascending projections of unknown phenotype must engage ARC NPY neurons that would then, via descending projections, provide the presynaptic drive for both classes of relevant NPY Y receptor subtypes within the CBS.

Another limitation for the ARC NPY model for the mediation of ghrelin hyperphagia is the exclusion of a potential contribution from NPY neurons within the CBS (42,43), some of which, particularly those in the nucleus of the solitary tract, are of demonstrated relevance to the control of ingestive behavior (44,45). It is possible that the fourth intracerebroventricular ghrelin response is mediated by a circuit contained entirely within the CBS and also that hindbrain NPY neurons, by virtue of their local and ascending projection systems (46), contribute in some measure to the hyperphagic response to forebrain GHS receptor stimulation. A number of questions must be addressed before the mediating circuitry for centrally elicited ghrelin hyperphagia can be elucidated. Further attention should be directed toward the NPY neurons in the hindbrain, including investigation of possible GHS receptor coexpression, and of the location and neurochemical properties of behaviorally relevant projection sites elsewhere within the CBS. Functional-anatomical studies may prove particularly useful if coordinated with strategies allowing stimulation of restricted subpopulations of GHS receptors. For example, it should be possible in rats with cerebral aqueduct occlusion to ascertain whether NPY neurons in either or both CBS and ARC are activated after delivery of ghrelin to the third or fourth ventricle and to characterize the neuraxial location and phenotypes of NPY receptor–expressing neurons that are activated by these treatments.

In summary, the present results introduce a considerable degree of complexity for the NPY mediation of

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

centrally elicited ghrelin hyperphagia, with the subtype and location of NPY receptors critical for the behavioral response differing, depending on whether the response was elicited by forebrain or hindbrain GHS receptor stimulation. The area of overlap for hyperphagia triggered in the separate locations was a requirement for activation of NPY-Y1 receptors in the CBS, whereas critical receptors bound by D-Tyr^{27,36}, D-Thr^{32} $NPY_{27–36}$ (possibly the Y5 receptor) were always local to (or at modest distances from) the site of ghrelin delivery. The cells of origin for the endogenous ligand mediating CBS- and forebrain-elicited ghrelin hyperphagia remain to be determined, with likely candidates including NPY neurons in the ARC and those in CBS nuclei such as the nucleus of the solitary tract. Also open is the question of which NPY neurons and receptor subpopulation(s) are most relevant under physiological conditions where both forebrain and CBS receptors are likely to be stimulated simultaneously. Some of the considerations raised in the present study are likely to be relevant for analysis of various neurochemical systems implicated in food intake and body weight control, particularly when a given hormone (e.g., leptin [47]) or peptide mediator (e.g., melanocortins [48,49]) yields comparable effects when delivered selectively to different regions of the brain.

ACKNOWLEDGMENTS

H.J.G. has received support from National Institutes of Health Grant R01-DK-21397. J.M.K. has received support from National Institutes of Health Grant DK-42284.

We thank Rebecca Stack, Gina Gubitosa, and Joanna Wapner for their technical contribution to the studies.

NOTE ADDED IN PROOF

While the current report was in process, we completed a full set of experiments with a third NPY receptor antagonist, L-152-804, a selective antagonist at the Y5 receptor subtype (Kantani et al., *Biochem Biophys Res Comm* 272:169–173, 2000). The results were comparable with those reported for D-Tyr^{27,36}, D-Thr^{32} $NPY_{27–36}$ in experiments 4–6 (i.e., reversal of ghrelin hyperphagia in same-ventricle studies and lack of effect with cross-ventricle administration). The results are consistent with the hypothesis that in addition to the Y1 receptor requirement, endogenous activation of the Y5 receptor (at the anatomical level of the pharmacologically stimulated GHS receptor) is critical for the expression of ghrelin hyperphagia.

REFERENCES

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656–660, 1999
2. Tschop M, Smiley DL, Heiman ML: Ghrelin induces adiposity in rodents. *Nature* 407:908–913, 2000
3. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M: Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 120:337–345, 2001
4. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR: The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 141:4325–4328, 2000
5. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S: A role for ghrelin in the central regulation of feeding. *Nature* 409:194–198, 2001
6. Faulconbridge LF, Cummings DE, Kaplan JM, Grill HJ: Hyperphagic effects of brainstem ghrelin administration. *Diabetes* 52:2260–2265, 2003
7. Willesen MG, Kristensen P, Romer J: Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology* 70:306–316, 1999
8. Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL: The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* 37:649–661, 2003
9. Chen HY, Trumbauer ME, Chen AS, Weingarth DT, Adams JR, Frazier EG, Shen Z, Marsh DJ, Feighner SD, Guan XM, Ye Z, Nargund RP, Smith RG, Van Der Ploeg LH, Howard AD, MacNeil DJ, Qian S: Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* 145:2607–2612, 2004
10. Seoane LM, Lopez M, Tovar S, Casanueva FF, Senaris R, Dieguez C: Agouti-related peptide, neuropeptide Y, and somatostatin-producing neurons are targets for ghrelin actions in the rat hypothalamus. *Endocrinology* 144:544–551, 2003
11. Wang L, Saint-Pierre DH, Tache Y: Peripheral ghrelin selectively increases Fos expression in neuropeptide Y-synthesizing neurons in mouse hypothalamic arcuate nucleus. *Neurosci Lett* 325:47–51, 2002
12. Hewson AK, Dickson SL: Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J Neuroendocrinol* 12:1047–1049, 2000
13. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I: Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. *Diabetes* 50:2438–2443, 2001
14. Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K: Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* 50:227–232, 2001
15. Kumarnsit E, Johnstone LE, Leng G: Actions of neuropeptide Y and growth hormone secretagogues in the arcuate nucleus and ventromedial hypothalamic nucleus. *Eur J Neurosci* 17:937–944, 2003
16. Olszewski PK, Grace MK, Billington CJ, Levine AS: Hypothalamic paraventricular injections of ghrelin: effect on feeding and c-*fos* immunoreactivity. *Peptides* 24:919–923, 2003
17. Li BH, Xu B, Rowland NE, Kalra SP: c-*fos* expression in the rat brain following central administration of neuropeptide Y and effects of food consumption. *Brain Res* 665:277–284, 1994
18. Lambert PD, Phillips PJ, Wilding JP, Bloom SR, Herbert J: c-*fos* expression in the paraventricular nucleus of the hypothalamus following intracerebroventricular infusions of neuropeptide Y. *Brain Res* 670:59–65, 1995
19. Wolak ML, DeJoseph MR, Cator AD, Mokashi AS, Brownfield MS, Urban JH: Comparative distribution of neuropeptide Y, Y1 and Y5 receptors in the rat brain by using immunohistochemistry. *J Comp Neurol* 464:285–311, 2003
20. Lawrence CB, Snape AC, Baudoin FM, Luckman SM: Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology* 143:155–162, 2002
21. Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D, Muller EE: Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. *Regul Pept* 111:161–167, 2003
22. Lin Y, Matsumura K, Fukuhara M, Kagiyama S, Fujii K, Iida M: Ghrelin acts at the nucleus of the solitary tract to decrease arterial pressure in rats. *Hypertension* 43:977–982, 2004
23. Zhang W, Lin TR, Hu Y, Fan Y, Zhao L, Stuenkel EL, Mulholland MW: Ghrelin stimulates neurogenesis in the dorsal motor nucleus of the vagus. *J Physiol* 559:729–737, 2004
24. Glass MJ, Chan J, Pickel VM: Ultrastructural localization of neuropeptide Y Y1 receptors in the rat medial nucleus tractus solitarius: relationships with neuropeptide Y or catecholamine neurons. *J Neurosci Res* 67:753–765, 2002
25. Dumont Y, Fournier A, Quirion R: Expression and characterization of the neuropeptide Y Y5 receptor subtype in the rat brain. *J Neurosci* 18:5565–5574, 1998
26. Parker RM, Herzog H: Regional distribution of Y-receptor subtype mRNAs in rat brain. *Eur J Neurosci* 11:1431–1448, 1999
27. Kopp J, Xu ZQ, Zhang X, Pedrazzini T, Herzog H, Kresse A, Wong H, Walsh JH, Hokfelt T: Expression of the neuropeptide Y Y1 receptor in the CNS of rat and of wild-type and Y1 receptor knock-out mice: focus on immunohistochemical localization. *Neuroscience* 111:443–532, 2002
28. Corp ES, Melville LD, Greenberg D, Gibbs J, Smith GP: Effect of fourth

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023

ventricular neuropeptide Y and peptide YY on ingestive and other behaviors. *Am J Physiol* 259:R317–R323, 1990
29. Corpa ES, McQuade J, Krasnicki S, Conze DB: Feeding after fourth ventricular administration of neuropeptide Y receptor agonists in rats. *Peptides* 22:493–499, 2001
30. Aja S, Sahandy S, Ladenheim EE, Schwartz GJ, Moran TH: Intracerebroventricular CART peptide reduces food intake and alters motor behavior at a hindbrain site. *Am J Physiol Regul Integr Comp Physiol* 281:R1862–R1867, 2001
31. Ritter R, Slusser PG, Stone S: Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science* 213:451–452, 1981
32. Grill HJ, Carmody JS, Sadacca LA, Williams DL, Kaplan JM: Attenuation of lipopolysaccharide anorexia by antagonism of caudal brainstem but not forebrain GLP-1-R. *Am J Physiol Regul Integr Comp Physiol* 287:R1190–R1193, 2004
33. Buggy J, Fisher AE: Anteroventral third ventricle site of action for angiotensin induced thirst. *Pharmacol Biochem Behav* 4:651–660, 1976
34. Kanatani A, Ishihara A, Asahi S, Tanaka T, Ozaki S, Ihara M: Potent neuropeptide Y Y1 receptor antagonist, 1229U91: blockade of neuropeptide Y-induced and physiological food intake. *Endocrinology* 137:3177–3182, 1996
35. Matthews JE, Jansen M, Lyerly D, Cox R, Chen WJ, Koller KJ, Daniels AJ: Pharmacological characterization and selectivity of the NPY antagonist GR231118 (1229U91) for different NPY receptors. *Regul Pept* 72:113–119, 1997
36. Myers RD, Wooten MH, Ames CD, Nyce JW: Anorexic action of a new potential neuropeptide Y antagonist [D-Tyr^{27,36}, D-Thr^{32}]-NPY (27–36) infused into the hypothalamus of the rat. *Brain Res Bull* 37:237–245, 1995
37. Roscoe AK, Leach SM, Nyce JW, Wooles WR: Hypotensive effects of [D-Tyr^{27,36}, D-Thr^{32}]neuropeptide Y (27–36). *Peptides* 16:1411–1415, 1995
38. Chaffer CL, Morris MJ: The feeding response to melanin-concentrating hormone is attenuated by antagonism of the NPY Y(1)-receptor in the rat. *Endocrinology* 143:191–197, 2002
39. Yokosuka M, Kalra PS, Kalra SP: Inhibition of neuropeptide Y (NPY)-induced feeding and c-*fos* response in magnocellular paraventricular nucleus by a NPY receptor antagonist: a site of NPY action. *Endocrinology* 140:4494–4500, 1999
40. Gerald C, Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, Smith KE, Vaysse P, Durkin MM, Laz TM, Linemeyer DL, Schaffhauser AO, Whitebread S, Hofbauer KG, Taber RI, Branchek TA, Weinshank RL: A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* 382:168–171, 1996
41. Broberger C, Johansen J, Johansson C, Schalling M, Hokfelt T: The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. *Proc Natl Acad Sci U S A* 95:15043–15048, 1998
42. Hokfelt T, Lundberg JM, Lagercrantz H, Tatemoto K, Mutt V, Lindberg J, Terenius L, Everitt BJ, Fuxe K, Agnati L, Goldstein M: Occurrence of neuropeptide Y (NPY)-like immunoreactivity in catecholamine neurons in the human medulla oblongata. *Neurosci Lett* 36:217–222, 1983
43. Chronwall BM, DiMaggio DA, Massari VJ, Pickel VM, Ruggiero DA, O'Donohue TL: The anatomy of neuropeptide-Y-containing neurons in rat brain. *Neuroscience* 15:1159–1181, 1985
44. Sahu A, Kalra SP, Crowley WR, Kalra PS: Evidence that NPY-containing neurons in the brainstem project into selected hypothalamic nuclei: implication in feeding behavior. *Brain Res* 457:376–378, 1988
45. Li AJ, Ritter S: Glucoprivation increases expression of neuropeptide Y mRNA in hindbrain neurons that innervate the hypothalamus. *Eur J Neurosci* 19:2147–2154, 2004
46. Sawchenko PE, Swanson LW, Grzanna R, Howe PR, Bloom SR, Polak JM: Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. *J Comp Neurol* 241:138–153, 1985
47. Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, Baskin DG: Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 143:239–246, 2002
48. Grill HJ, Ginsberg AB, Seeley RJ, Kaplan JM: Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. *J Neurosci* 18:10128–10135, 1998
49. Brown KS, Gentry RM, Rowland NE: Central injection in rats of alpha-melanocyte-stimulating hormone analog: effects on food intake and brain Fos. *Regul Pept* 78:89–94, 1998

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/54/7/1985/381239/zdb00705001985.pdf by guest on 04 October 2023