Leptin influences the excitability of area postrema neurons

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Leptin influences the excitability of area postrema neurons. Am J Physiol Regul Integr Comp Physiol 310: R440–R448, 2016. First published December 30, 2015; doi:10.1152/ajpregu.00326.2015.—The area postrema (AP) is a circumventricular organ with important roles in central autonomic regulation. This medullary structure has been shown to express the leptin receptor and has been suggested to have a role in modulating peripheral signals, indicating energy status. Using RT-PCR, we have confirmed the presence of mRNA for the leptin receptor, ObRb, in AP, and whole cell current-clamp recordings from dissociated AP neurons demonstrated that leptin influenced the excitability of 51% (42/82) of AP neurons. The majority of responsive neurons (62%) exhibited a depolarization (5.3 ± 0.7 mV), while the remaining affected cells (16/42) demonstrated hyperpolarizing effects (−5.96 ± 0.95 mV). Amylin was found to influence the same population of AP neurons. To elucidate the mechanism(s) of leptin and amylin actions in the AP, we used fluorescence resonance energy transfer (FRET) to determine the effect of these peptides on cAMP levels in single AP neurons. Leptin and amylin were found to elevate cAMP levels in the same dissociated AP neurons (leptin: % total FRET response 25.3 ± 4.9, n = 14; amylin: % total FRET response 21.7 ± 3.1, n = 13). When leptin and amylin were coapplied, % total FRET response rose to 53.0 ± 8.3 (n = 6). The demonstration that leptin and amylin influence a subpopulation of AP neurons and that these two signaling molecules have additive effects on single AP neurons to increase cAMP, supports a role for the AP as a central nervous system location at which these circulating signals may act through common intracellular signaling pathways to influence central control of energy balance.

circumventricular organ; caudal brain stem; energy balance; obesity; amylin; cAMP

LEPTIN IS AN ADIPOCYTE-DERIVED peptide hormone encoded by the ob gene that circulates at concentrations proportional to adipose tissue mass and communicates levels of fat stores to the central nervous system (CNS), thus playing an important role in long-term regulation of energy balance. Circulating leptin exerts its effects on feeding and energy expenditure by binding to leptin receptors in the CNS (11, 29). Although five leptin receptor isoforms have been identified (70), only the long form of the receptor, ObRb, possesses the cytoplasmic domains required for signal transduction (3, 8, 39), and this form is essential for the weight-reducing effect of leptin (6–9, 19).

The arcuate nucleus of the basal hypothalamus has been a principle focus of research directed toward elucidating leptin’s actions in the CNS (63) (for review, see Ref. 15). However, more recently, the role of other hypothalamic, forebrain, and brain stem regions have been shown to be critical in mediating the central effects of leptin (20, 28, 33, 36, 37, 50, 54).

Whether at the arcuate nucleus or in these other CNS regions where leptin receptors are found, central actions of leptin require that this adipokine cross the blood-brain barrier (BBB). The BBB, a regulatory interface between the brain and the periphery, restricts the central access of circulating molecules (4) and leads to the obvious question as to how leptin gains access to central sites to influence body weight regulation. Although a saturable leptin transport system (5) and transendothelial signaling (51) represent potential mechanisms through which peripheral signals may reach neurons protected by the BBB, recent evidence (68) suggests that the sensory circumventricular organs (CVOs), a group of specialized CNS structures that lack the normal BBB, may play a role.

The area postrema (AP), located on the wall of the fourth ventricle, is a sensory CVO well known for its role in the emetic reflex (46). The AP also has well-documented roles in immune function, cardiovascular regulation, and energy homeostasis (see Refs. 17 and 53 for a review). The AP sends extensive efferent projections to hypothalamic and medullary autonomic nuclei (31, 65) (for review, see Ref. 23) and has receptors for numerous peripheral signals involved in feeding and metabolism (34) (for review, see Ref. 53). Thus, the AP provides a potential route through which circulating leptin may act to influence downstream autonomic nuclei that control feeding behavior.

Although few studies have examined the responsiveness of the AP to leptin, the demonstration of ObR-like immunoreactivity (45) and mRNA expression (30) in the AP suggests potential actions for leptin in this sensory CVO. In addition, gene array studies from our own laboratory have revealed the presence of leptin receptor mRNA (LepR) in the AP (34). The specific leptin receptor subtypes were not, however, determined in any of the above studies. Increases in activated phosphorylated-signal transducer and activator of transcription 3 (pSTAT3) immunoreactivity, a known direct downstream marker of leptin receptor activation, has also been observed in the AP in response to leptin (21, 37). At the physiological level, leptin injection into the fourth ventricle has been shown to suppress appetite (67), while knockdown of LepR and LepRb in the median nucleus tractus solitarius (mNTS) and AP causes hyperphagia, as well as increased body weight and adiposity (33, 38), providing further evidence for a role of the AP in mediating the central effects of leptin.

The present study was undertaken to determine whether the signaling form of the leptin receptor (ObRb) is found in AP and to examine the functional effects of activation of this receptor on AP neurons.
METHODS

All rats (Charles River) were maintained on a 12:12-h light-dark cycle (lights on 0700) and were provided with food (LabDiet 5001, composition 13.5% kcal fat, 58% kcal carbohydrate, and 28.5% kcal protein) and water ad libitum before decapitation (which occurred between 0900 and 1200). All animal protocols conformed to the Canadian Council for Animal Care standards and were approved by the Queen’s University Animal Care Committee.

Leptin receptor localization using RT-PCR. Male Sprague-Dawley rats (100–150 g) were decapitated, and their brains were quickly removed and placed into oxygenated (95% O₂-5% CO₂), ice-cold (1–4°C) artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 2 KCl, 1.25 KH₂PO₄, 2.0 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, and 20 glucose. Brain slices (300 μm) were cut through the medulla using a vibratome (Leica, Nussloch Germany) and placed in Hibernate medium (BrainBits, Springfield, IL) supplemented with 1 × B27 (Invitrogen, Burlington, ON, Canada). Under a dissecting microscope, the AP was identified on the basis of its distinct anatomical boundaries and its location on the border of the fourth ventricle, and it was carefully microdissected to ensure no surrounding tissue was included.

Total RNA was extracted from AP from three rats using an Ambion RNAqueous kit and was then DNase-treated (Fermentas) by adding 1 μl 10X buffer with MgCl₂, and 1 μl deoxyribonuclease to the total RNA, followed by incubation at 37°C for 30 min. After incubation, 1 μl of 25 mM EDTA was added to the solution and incubated at 65°C for 10 min to stop the DNase reaction. Reverse transcription of the RNA to cDNA was immediately undertaken by adding 26 mM solution consisting of (in mM): 87 NaCl, 2.5 KCl, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 1.25 NaH₂PO₄, 25 glucose, and 75 sucrose. A mediallular tissue block containing the AP was isolated, and 300-μm coronal slices were obtained using a vibratome (Leica, Nussloch Germany). Slices were then incubated at 32°C for at least 1 h in oxygenated aCSF (external recording solution) composed of (in mM): 126 NaCl, 2.5 KCl, 26 NaHCO₃, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, and 10 glucose, pH 7.2 with NaOH. Slices were transferred to a recording chamber and continuously perfused at a rate of 1–2 ml/min with 32°C aCSF. Neurons were visualized using an infrared differential interference contrast system on an upright microscope (Nikon, Japan).

Electrophysiology. Whole cell current-clamp recordings from AP neurons were obtained using an Axopatch 700B (dissociated cell recording) or Multiclamp 700B (slice recordings) patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Stimulation and recording parameters were controlled by Spike2 (version 6) and Signal (version 3) software (Cambridge Electronics Design, Cambridge, UK). For dissociated cell recordings, data were acquired at 8 kHz, filtered at 2 kHz, and digitized using a Micro 1401 interface (Cambridge Electronics Design), while for slice recordings, data were acquired at 10 kHz and filtered at 2.4 kHz. Capacitive transients and series resistance.

Table 1. Primer sets used to detect leptin mRNA from the area postrema

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
<th>Product Size, bp</th>
<th>Ascension Number</th>
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<td>570</td>
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</table>
errors were minimized before recording. For dissociated cell recordings, the external recording solution contained the following (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, and glucose (10), at pH 7.2 with NaOH. Patch electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL) on a Flaming Brown micropipette puller (model P97; Sutter Instrument, Novato, CA). Electrodes used for dissociated cell recordings were then fire polished and had resistances of 2.5–5 MΩ when filled with internal recording solution that contained (in mM) 130 K-glucuron, 10 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 EGTA, and 2 NaATP. For slice recording, pipettes were filled with an intracellular solution made of (in mM): 125 potassium gluconate, 10 KCl, 2 MgCl₂, 0.1 CaCl₂, 5.5 EGTA, 10 HEPES, 2 NaATP, at pH 7.2 with KOH and had a resistance of 3–5 MΩ. All chemicals were purchased from Sigma (Oakville, ON, Canada).

Once whole cell configuration was achieved, cells were perfused via a gravity-fed perfusion system with external recording solution at a rate of 1–2 ml/min. Cells were defined as neurons by the presence of ≥50 mV action potentials. Following a minimum of 5-min stable baseline recording period (control), 100 pM or 10 nM leptin (Phoenix Pharmaceuticals, Belmont, CA; reconstituted in external recording solution) was bath applied for 100–200 s followed by a wash with external recording solution. Leptin concentrations were chosen on the basis of the low and high boundaries of leptin concentrations normally measured in rat circulation (40, 41).

We also examined the effects of leptin in amylin-sensitive AP neurons, by determining the effect of bath administration of amylin (10 nM) and leptin (10 nM) on the same dissociated AP neurons. Leptin was applied before amylin in some recordings, and in other recordings, the order of peptide application was reversed. The second peptide was not applied until the neuron fully recovered and stabilized at the original baseline potential, or the neuron partially recovered and stabilized at a new membrane potential.

**Analysis of electrophysiological data.** Changes in membrane potential were calculated from the maximal difference between the average membrane potential in 100-s segments immediately before and for 100-s epochs after peptide application. AP neurons were considered responsive when this difference was ≥2 SDs of the mean baseline membrane potential, and the cell showed recovery toward baseline. To be considered significant, a response must have initiated during the period of leptin application. The response magnitude was measured at its maximum value (mean over 100-s epoch) before returning to baseline. Changes in action potential frequency (in cells that displayed spontaneous action potentials) were assessed by comparison of the difference between the mean action potential frequency for 100 s immediately before leptin application and that after leptin application.

**Measurements of cAMP levels in dissociated AP neurons by fluorescence resonance energy transfer.** To elucidate possible mechanisms of leptin and amylin actions in single AP neurons, since amylin was known to act through cAMP signaling, we measured changes in levels of cAMP in AP neurons exposed to amylin and or leptin using a previously described fluorescence resonance energy transfer (FRET)-based cAMP sensor. This unimolecular sensor contained the cAMP-binding domain of exchange protein activated by cAMP-1 (or EPAC1), flanked by enhanced cyan and yellow fluorescent protein (eCFP, eYFP), respectively (55, 74) and was introduced into freshly dissociated AP neurons in neurobasal-A medium (Invitrogen) supplemented with B27 containing 0.5 mM l-glutamine (Invitrogen) by adenoviral infection. FRET-based measurements of cAMP were carried out for 6 h postinfection in a HBSS, containing (in mM) 137 NaCl, 5 KCl, 1 Na₂HPO₄, 5.6 dextrose, 20 HEPES, at pH 7.4 and maintained at room temperature. Prior to the addition of any agents, at least three randomly preselected regions of interest (ROI) per cell were chosen in which FRET changes would be measured. Changes in FRET in these ROIs were obtained by capturing eCFP (470 nm) and eYFP (530 nm) emissions in images every 5 s using a Zeiss Axio Observer Z1 inverted microscope at a magnification of ×40 and calculating the ratio of these emissions (CFP/YFP) using Image J-based analysis of the captured fluorescent images (National Institutes of Health, Bethesda, MD).

After establishment of a 5-min period of stable basal FRET, the impact of bath-applied leptin (25 nM) or amylin (100 nM) on AP neuron cAMP levels were measured sequentially, without washout. In these experiments, the second peptide was introduced after the change in FRET caused by the first peptide had stabilized. To obviate the effects caused by the sequence of peptide addition, leptin or amylin was randomly selected as the first peptide tested. After the effect of the second peptide had stabilized, maximal cAMP levels that could be measured by the sensor expressed in each individual AP neuron analyzed were established by the addition of a maximal stimulus, a combination of a nonselective phosphodiesterase inhibitor [3-isobutyl-1-methylxanthine (IBMX), 100 μM], and an adenylyl cyclase activator [ forskolin (FSK), 1 μM] for 5 min. In light of recent studies demonstrating synergistic effects of leptin and amyl in food intake and body weight (60, 71, 73), a second series of experiments was performed to determine the effect of simultaneous addition of both leptin and amyl in on AP neuron cAMP levels.

Data are presented either as representative tracings from individual cells or as the mean of averaged values in multiple identical experiments. Mean changes in cAMP were obtained by calculating the peak FRET change in individual AP neurons from baseline and normalizing these FRET changes to the maximal possible increase, which could be measured (IBMX + Forskolin), taken as 100%. Routinely, nonresponders, AP neurons in which peptides caused a change in FRET of less than 1%, were excluded from our analysis.

**Statistics.** Mean change (means ± SE) in membrane potential and spike frequency (for cells that were spontaneously active) before (control) and during leptin peptide administration (leptin) were calculated, and differences were tested using a paired Student’s t-test. A Fisher’s exact test was used to determine whether the proportion of depolarizing and hyperpolarizing responses were different at different concentrations of leptin (100 pM and 10 nM). Means of changes in FRET values within 300-s of individual treatments were derived from values obtained for individual cells in individual experiments. A one-way ANOVA (followed by Tukey post hoc analysis, where appropriate), was used to determine whether changes in FRET emission (and thus, cAMP levels) differed in respect to the peptide applied (alone or in combination). All statistical analyses were performed using GraphPad Prism (version 5.0; San Diego, CA).

**RESULTS.**

**ObRb mRNA is expressed in the area postrema.** RT-PCR analysis of cDNA obtained from mRNA isolated from acutely microdissected AP using primer sets directed toward an extracellular domain common to all leptin receptor isoforms confirmed the presence of leptin receptor (LepR) mRNA in AP (see Fig. 1). To determine whether the signaling form of the leptin receptor (ObRb) was present in AP, primer sets directed toward two different unique intracellular signaling domains on the ObRb receptor were used and revealed the presence of ObRb mRNA in AP (see Fig. 1). ObRa mRNA was also present in AP, as illustrated in Fig. 1. LepR, ObRb1, ObRb2, and ObRa mRNA were also localized to the hypothalamus, an area of the brain previously shown to express ObRb (22), which, thus, served as a positive control (see Fig. 1). GAPDH served as a positive control for the PCR reactions. PCR products for GAPDH and all leptin receptors (ObRb1, ObRb2, ObRa, and LepR) were not observed in the "no template control" lane, in which the template was omitted from the cDNA synthesis reaction, and no labeling was found in the cDNA synthesis reaction, and no labeling was found in the
negative controls (data not shown). The validity of all PCR products was confirmed by sequencing.

**Leptin influences the excitability of area postrema neurons.** Whole cell current-clamp techniques were used to evaluate the direct effects of leptin receptor activation on neuronal excitability of dissociated AP neurons Bath application of leptin (10 nM) influenced the excitability of 51% (42/82) of AP neurons tested. The majority of responsive neurons (26/42 or 62%) exhibited a depolarization (mean change in membrane potential $5.3 \pm 0.7$ mV, $n = 26$) in response to leptin administration (see Fig. 2). All depolarizing responses began within 100 s of leptin application. The depolarizing responses seen in response to leptin administration were of a long duration, often lasting several minutes upon termination of leptin application, and

![Fig. 1. ObRb receptor mRNA is expressed in the area postrema (AP). Agarose gels showing RT-PCR analysis of AP cDNA for leptin receptor expression (left). ObRb receptor (ObRb1, ObRb2) mRNA, as well as ObRa receptor (ObRa) mRNA, and leptin receptor (LepR) mRNA (expression common to all leptin receptor isoforms) were also expressed in the AP. The hypothalamus (right) served as a positive control tissue and shows ObRb (ObRb1, ObRb2), ObRa, and LepR receptor expression. GAPDH served as a positive control in both AP and hypothalamic tissue. Product size (base pairs) is shown in the leftmost lane of each gel.](Image)

![Fig. 2. Leptin influences the excitability of AP neurons. Left (Dissociated): current-clamp recordings from two different dissociated AP neurons showing that bath application of 10 nM leptin caused a depolarization (top) and an accompanying increase in action potential firing frequency, while a different neuron (bottom) exhibited a hyperpolarization and an accompanying decrease in action potential firing frequency in response to leptin administration. Right (Slice): current-clamp recordings from two different AP neurons obtained from slice preparations. The upper trace shows a depolarizing response to bath administration of 10 nM leptin, while the neuron shown in the lower trace was hyperpolarized by leptin application. Time and duration of leptin administration are indicated by the gray bar above each current-clamp recording.](Image)
they were typically accompanied by an expected increase in action potential firing frequency [control mean spike frequency 0.6 ± 0.4 s/s (n = 9) and leptin mean spike frequency 1.13 ± 0.5 s/s (n = 9), P < 0.01]. The remaining affected cells (16/42) demonstrated hyperpolarizing effects (mean change in membrane potential −5.96 ± 0.95 mV, n = 16), in response to leptin administration (see Fig. 2). Similar to the depolarizing responses, these effects began within 100 s of peptide administration, lasted several minutes and were often accompanied with a decrease in action potential firing frequency [control mean spike frequency prior to leptin 2.8 ± 0.9 s/s (n = 6), leptin mean spike frequency 0.9 ± 0.5 s/s (n = 6), P < 0.05].

A 100-fold lower concentration of leptin (100 pM) was bath-applied to an additional 21 dissociated neurons to determine whether leptin responses were concentration dependent. There was no difference in the number of AP neurons affected, the distribution of depolarizing and hyperpolarizing responses, or the magnitude of the response. Of the 21 neurons tested, 52% (11/21) were influenced by bath administration of leptin with eight neurons showing a depolarizing response (mean change in membrane potential 4.3 ± 2.7 mV), three neurons exhibiting a hyperpolarization (mean change in membrane potential was −3.7 ± 1.1 mV), and the remaining cells (n = 10) being unaffected by leptin administration. Although the magnitude of the effects of 100 pM leptin on membrane potential were numerically smaller than observed after administration of 10 nM, these changes were not statistically significant (depolarization: P = 0.6; hyperpolarization: P = 0.32), nor were the proportion of cells demonstrating depolarizing and hyperpolarizing responses significantly changed (P = 0.72, using Fisher’s exact test).

To ensure that the effects on the neuronal excitability of dissociated AP neurons in response to leptin administration were not the result of changes in these cells as a consequence of the dissociation procedure, the effect of leptin administration on AP neurons in acute AP slice preparations was also evaluated. Whole cell current-clamp recordings from 10 AP neurons in such medullary slices showed similar responsiveness to bath application of 10 nM leptin with 30% of cells exhibiting depolarizations (mean change in membrane potential 6.95 ± 1.2 mV, n = 3; see Fig. 2), while four cells hyperpolarized (−4.5 ± 0.95 mV, see Fig. 2). The remaining cells tested (n = 3) were unaffected by leptin application.

**Leptin influences amylin-sensitive area postrema neurons.** We next examined whether leptin influenced amylin-sensitive AP neurons, using whole cell current-clamp recordings from 14 dissociated AP neurons, which were tested for responsiveness to both leptin (10 nM) and amylin (10 nM). Of the seven cells that depolarized in response to bath administration of leptin, five also depolarized in response to similar bath perfusion of amylin (see Fig. 3). The mean depolarization of these sensitive neurons (n = 5) to leptin was 5.5 ± 0.7 mV and 6.6 ± 0.8 mV to amylin. None of the AP neurons that hyperpolarized in response to leptin were influenced by amylin (n = 2). The remaining five cells were unaffected by application of either peptide.

**Leptin and amylin elevate cAMP levels in the same dissociated AP neurons.** The effects of amylin on intracellular cAMP levels are well documented (2, 14, 26, 32, 66). In addition, mRNA for a variety of phosphodiesterases (PDEs) (PDE4A, PDE4B, PDE4D, and PDE7B) are present in AP (48, 56, 69), and 8-bromo-cAMP has been shown to excite AP neurons (13). As such, we sought to examine potential interactions between leptin and amylin in cAMP-mediated signaling using a FRET-based cAMP sensor in AP neurons. Consistent with the fact that the cells expressing the cAMP sensor were healthy, 17 of the 19 cells used in our analysis of changes in cAMP in response to either leptin or amylin, responded to a saturating cAMP stimulus (IBMX+FSK). In these cells, amylin elicited clear increases in cAMP in 13 of these 17 cells (amylin induced an increase in cAMP = 21.7% ± 3.1% of maximum, see Fig. 4). The impact of leptin on cAMP levels in these AP neurons was also tested and, intriguingly, 14 of the 17 cells also responded to this peptide with an increase in cAMP (le!ptin-induced increase in cAMP = 25.3% ± 4.9% of maximum, see Fig. 4). In these experiments, 12 of the 14 cells that responded to amylin with an increase in cAMP also responded similarly to leptin. We next examined effects of coadministration of amylin and leptin and found that in six of the seven cells, the addition of both of these peptides additively increased cAMP, with amylin and leptin coadministration increasing cAMP by 53.0% ± 8.3% of the maximal possible response.

![Fig. 3. Leptin depolarizes amylin-sensitive AP neurons. Current-clamp recording obtained from the same AP neuron illustrating depolarizing responses to 10 nM amylin (top) and 10 nM leptin (bottom). Time and duration of amylin (top, black bar) and leptin (bottom, gray bar) administration are indicated by the bar above each current-clamp recording.](http://ajpregu.physiology.org/)
The increase in cAMP caused by the simultaneous addition of both leptin and amylin was significantly greater than the effects observed when either of these peptides was used alone ($P < 0.0016$, see Fig. 4).

**DISCUSSION**

In this study, we have confirmed the presence of the signaling form of the leptin receptor, ObRb, using RT-PCR in acutely dissected AP, and have also shown direct effects of the adipocyte-derived hormone, leptin, on the excitability of AP neurons. Although the hypothalamic arcuate nucleus is perceived as the principal mediator of leptin signaling, there is a growing body of evidence suggesting that leptin also acts in the caudal brain stem (27, 50). LepR immunoreactivity (common to all receptor isoforms) (12, 35) has been demonstrated in AP, as has LepR mRNA (12, 35). More recently, ObRb immunoreactivity has been shown to be expressed in the caudal brain stem, with strong expression being observed in the NTS and dorsal motor nucleus of the vagus (12, 35) and weaker expression seen in the AP (12, 35), while fluorescent in situ hybridization and immunohistochemistry showed strong ObRb expression in AP, NTS, and the dorsal motor nucleus of the vagus (30). Early studies suggested ObRb mRNA to be present in the AP and NTS, showing “inconsistent hybridization” in these regions (22). Using RT-PCR, we have confirmed the presence of the LepR in the AP and have confirmed that the signaling form of the leptin receptor, ObRb, is present in this medullary CVO, suggesting a role for the AP in mediating the central effects of leptin. A functional role for ObRb in AP is supported by the fact that pSTAT3 activation is observed in the AP (12, 35, 37). Further support for a functional role of ObRb receptor activation in AP is shown by the results of our electrophysiological recordings in the present study. Using current-clamp techniques to record from AP neurons in both dissociated cell and medullary slice preparations, we showed that more than half of the AP neurons were influenced by leptin, with either depolarization or hyperpolarization observed in response to this adipokine. Although it is possible that the dissociation procedure itself or the 1–5-day culture period influenced receptor expres-
Leptin and amylin, two circulating factors, affect food intake, energy balance, and body weight. Leptin is a pancreatic peptide that is cosecreted with insulin in response to food intake, and its levels are elevated by food restriction, reducing food intake and increasing blood glucose. Leptin signaling in these other brain regions is mediated by actions in the parabrachial nucleus, both of which are major sites of sensory-autonomic integration. The NTS is of particular interest due to its role in the sympathetic nervous system, and the presence of proopiomelanocortin (POMC) neurons, which are activated by both leptin and amylin, in the AP. Previous work has shown that the excitatory responses elicited by leptin in AP neurons are mediated by cAMP, and our observation that leptin-induced depolarization of neurons was abolished in AP-lesioned animals suggests that the AP is a point of integration for these circulating factors.

The presence of mRNA for a variety of PDEs in AP neurons suggests that the AP may be a point of integration for these circulating factors related to circulating factors at the AP. Given that leptin and amylin both inhibit food intake in response to positive energy balance, albeit that they function over different time periods (leptin is a long-term adiposity signal, whereas amylin is a short-term satiation signal), it is plausible that the subpopulation of AP neurons may mediate the anorexie effects of both of these circulating factors, and, thus, exhibit excitatory responses to both peptides. This premise is also supported by the observation that leptin and amylin receptors have been colocalized on AP neurons (Liberini CG and Lutz TA, in press). Our observation that neurons depolarized by leptin were also depolarized by amylin, while neurons that were hyperpolarized by leptin were insensitive to amylin is in accordance with such a hypothesis. Our observation that leptin and amylin depolarized the same AP neurons and that cAMP is elevated by leptin and amylin in the same AP neurons suggests that the excitatory responses elicited by leptin in AP neurons may be mediated by cAMP. Previous work showing that amylin elevates cGMP in AP neurons (57, 77) and the majority of these AP neurons express the amylin receptor (CTR) suggests that cGMP may also mediate responses to amylin in the AP (77). Possible effects of leptin on cGMP in AP neurons and possible interaction of leptin and amylin on cGMP levels warrant investigation.

Recently, synergistic effects of leptin and amylin have been demonstrated on food intake and body weight in lean and DIO rats and obese humans (60, 71, 73). Numerous studies have suggested that the AP is not the site of action for these synergistic effects but rather downstream autonomic nuclei that control feeding behavior, energy expenditure, and fat utilization, such as the NTS, hypothalamus (arcuate nucleus, lateral hypothalamic, ventromedial and dorsomedial hypothalamus), or the mesolimbic reward system (nucleus accumbens or ventral tegmental area) (42, 73) (see Refs. 52 and 72 for a review) underlie these effects. However, the results of the current study demonstrate that leptin and amylin influence the same AP neurons and that leptin and amylin act in an additive manner to increase cAMP in the AP. These results suggest that the AP might be an important site of integration of these two circulating signals, which then act to influence downstream autonomic nuclei that control feeding behavior.

Many recent studies have evaluated the effect of leptin and amylin coadministration on a variety of intracellular signaling pathways; however, the mechanism(s) of leptin/amylin interaction remain to be fully elucidated. It has been demonstrated that, in human peripheral tissues (adipose tissue, primary adipocytes, blood mononuclear cells), leptin and amylin have additive effects on STAT3 phosphorylation, extracellular signal-related kinase (ERK) activation, as well as PKB (Akt) and AMPK activation (47). In vivo, coadministration of leptin and amylin has been shown to stimulate central histaminergic signaling (64).

The presence of mRNA for a variety of PDEs in AP neurons (48, 56, 69) and the demonstration that 8-bromo-cAMP excite AP neurons (13) suggest a role for cAMP in AP signaling. As the effects of amylin on intracellular cAMP levels are well documented (2, 14, 26, 32, 66), our finding that amylin increased cAMP levels in AP neurons was not unexpected. However, our finding that leptin administration elevates cAMP levels in AP neurons was unexpected in light of recent work suggesting that leptin promoted increased cAMP hydrolysis through an effect at a cAMP-PDE and that this effect was important for normal leptin signaling in these other brain regions (62, 76, 75, 25) (see Refs. 1 and 61 for review). Our further demonstration that leptin and amylin elevate cAMP levels in the same AP neurons and that coadministration causes additive effects on cAMP levels in single AP neurons, not only supports the notion that the AP may be a point of integration for these circulating substances, but also suggest that complex alterations in single-cell cAMP-mediated signaling may mediate this interaction.

Leptin-amylin agonism has implications for pharmacological obesity interventions, as preclinical trials in rats and humans have shown synergistic weight loss with leptin-amylin coadministration, exceeding the predicted additive effect of therapy with either peptide alone (60, 71). The physiological effects of amylin on food intake have been shown to be almost exclusively mediated by actions in the AP (59). Our finding that amylin and leptin activate the same population of AP neurons possibly via cAMP may provide insight into mechanisms of leptin-amylin synergistic effects on weight loss.
In summary, the present study demonstrates the direct actions of leptin on the excitability of AP neurons and suggests that leptin action in the AP may contribute to its modulation of long-term energy balance. The AP, an ideal target for leptin due to its ability to monitor the contents of the circulation and transmit this information via well-established projections to autonomic nuclei in the brain stem and hypothalamus, provides a route by which the circulating adiposity signal, leptin, which does not freely diffuse across the BBB, can act to control energy expenditure and feeding behavior. Furthermore, the effects of leptin and amylin on the same subpopulation of neurons, possibly mediated via cAMP, in the AP may be relevant to the development of obesity interventions based on the synergistic effects of leptin and amylin on weight loss.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
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