Cooperative interaction between leptin and amylin signaling in the ventral tegmental area for the control of food intake

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Mietlicki-Baase EG, Olivos DR, Jeffrey BA, Hayes MR. Cooperative interaction between leptin and amylin signaling in the ventral tegmental area for the control of food intake. Am J Physiol Endocrinol Metab 308: E1116–E1122, 2015. First published April 21, 2015; doi:10.1152/ajpendo.00087.2015.—Peripheral coadministration of amylin and leptin produces enhanced suppression of food intake and body weight, but the central nuclei mediating these effects remain unclear. Because each of these peptides controls feeding via actions at the ventral tegmental area (VTA), we tested the hypothesis that the VTA is a site of action for the cooperative effects of leptin and amylin on energy balance control. First, we show that intra-VTA injection of amylin and leptin at doses of each peptide that are effective in reducing food intake and body weight when administered separately produces an enhanced suppression of feeding when administered in combination. We also demonstrate that subthreshold doses of both amylin and leptin cause significant hypophagia and body weight loss when coadministered into the VTA. Additionally, we provide evidence that VTA amylin receptor blockade significantly attenuates the ability of intra-VTA leptin to reduce feeding and body weight gain. Together, these data provide the first evidence that the VTA mediates the interaction of amylin and leptin to cooperatively promote negative energy balance.

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METHODS

Subjects. Adult male Sprague-Dawley rats (346.5 ± 32.4 g at the start of experimental testing; Charles River Laboratories) were housed individually in hanging wire mesh cages in a temperature- and humidity-controlled environment (12:12-h light-dark cycle). Animals had ad libitum access to rodent chow (Laboratory Rodent Diet 5001; LabDiet) and water. All experimental procedures were approved by
the University of Pennsylvania Institutional Animal Care and Use Committee. All experiments were within subjects, with each rat receiving all treatments in a counterbalanced Latin squares design.

**Drugs.** Rat amylin (American Peptide) and the amylin receptor antagonist AC187 (Bachem) were dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus). Leptin (NHPP) was dissolved in artificial cerebrospinal fluid (aCSF) plus leptin (0.3 μg) or its vehicle (100 nl of 0.01 M sodium bicarbonate). The selected doses of drugs were based on the literature (17, 32, 33).

**Surgery.** Rats were anesthetized via intramuscular injection of a mixture containing ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg) and placed into a stereotaxic apparatus. Each rat was surgically implanted with a bilateral guide cannula aimed at the VTA (coordinates: ± 0.5 mm lateral to midline; 6.8 mm posterior to bregma, with internal cannula aimed 8.6 mm ventral to skull). Guide cannulae were affixed to the skull with jeweler’s screws and dental cement. Analgesic was provided for all surgeries (2 mg/kg meloxicam). VTA cannula placements were histologically verified postmortem by injection of 100 nl of pontamine sky blue ink. A representative image of unilateral VTA injection placement is shown in Fig. 1C.

**Intra-VTA coadministration of amylin and leptin.** Rats with chronically implanted VTA cannula were given a unilateral VTA injection of leptin or its vehicle (100 nl of 0.01 M sodium bicarbonate), followed by a second unilateral VTA injection of amylin or its vehicle (100 nl of aCSF) just before the onset of the dark phase. In separate cohorts of animals, different doses of leptin and amylin were used to explore the effects of different dose combinations (experiment 1: 0.3 μg of leptin and 0.4 μg of amylin; experiment 2: 0.1 μg of leptin and 0.04 μg of amylin) on food intake and body weight. Both injections were given into the same hemisphere of the VTA, and injection days were separated by a minimum of 48 h. For these studies, rats were housed in a custom-made automated feedometer system in which a small access hole in the hanging wire mesh cage leads to a food cup resting on an electronic scale. Food cup weights were automatically recorded by computer software (LabView) every 10 s for the 24-h test period, allowing for detailed food intake and meal pattern analyses. The criteria for defining a meal were 0.25 g or more of food intake and a minimum of 10 min between feeding bouts (31–33). Food intake, meal size, and meal number were calculated over the course of the 24-h test period. Spillage was accounted for in food intake measurements by assigning a percentage of total crumbs to each meal/time bin corresponding to the percentage of total intake for that meal/time bin, as reported previously (31–33). Twenty-four-hour body weight gain was assessed by subtracting each rat’s body weight at the beginning of the 24-h test period from its body weight at the end of the test (to the nearest 0.1 g).

**Intra-VTA coadministration of AC187 and leptin.** Approximately 1 h before lights-off, rats with indwelling VTA cannulae were each given a unilateral intra-VTA injection of the amylin receptor antagonist AC187 (0.1 μg) or vehicle (100 nl aCSF). One hour later, just before lights off, each rat received a second unilateral VTA injection into the same hemisphere of the VTA containing leptin (0.6 μg) or its vehicle (100 nl 0.01 M sodium bicarbonate). Food was unavailable between injections. After the second injection, preweighed food was returned to the rats, and 24-h food intake (accounting for spillage) and change in body weight were measured. A minimum of 48 h separated subsequent treatments.

**Statistical analyses.** All statistical analyses were run using Statistica (StatSoft). The α-level was set at P < 0.05 for all experiments. For each study, repeated-measures ANOVAs were conducted for each variable of interest (food intake, meal size/number, body weight). Each drug type (e.g., amylin, leptin, AC187) served as a within-subjects factor, with time as an additional within-subjects factor when multiple time points were assessed within a single experiment. Statistically significant main effects and interactions were probed using Student-Newman-Keuls post hoc analyses.

**RESULTS**

Intra-VTA doses of amylin and leptin that are independently effective to reduce food intake produce an enhanced suppression of food intake when combined. We evaluated whether amylin and leptin signaling interact in the VTA to control food intake and body weight. We first selected doses of each peptide that, when administered independently into the VTA, are suprathereshold for energy balance effects (0.4 μg of amylin and 0.3 μg of leptin; Figs. 1 and 2). We tested the ability of these intra-VTA doses of amylin and leptin, alone or in combination, to reduce feeding and body weight gain in chow-fed rats (n = 11). As expected, amylin alone and leptin alone each reduced food intake throughout the 24-h test period (statistically significant main effects of amylin and of leptin, ANOVAs F11,13 = 25.37, P < 0.001; main effect of time, F9,117 = 190.86, P < 0.0001; interactions between leptin/time and amylin/time, ANOVAs F9,117 = 2.58, P < 0.01; planned comparisons of vehicle/vehicle vs. leptin/vehicle, P < 0.05 from 6–24 h; vehicle/vehicle vs. vehicle/amylin, P < 0.05 from 1–24 h; Fig. 1A). However, the combination of these doses of amylin and leptin produced a reduction in chow intake that was greater than the effect of either hormone alone (planned comparisons between leptin/amylin and all other conditions, P < 0.05 from 4–24 h), suggesting that the VTA mediates the interactive effect of these peptides on feeding. We also measured 24-h body weight change in this study and found...
that a combination of leptin and amylin significantly suppressed body weight gain over the 24-h test (main effect of leptin, $F_{1,13} = 13.58, P < 0.01$; planned comparisons between vehicle/vehicle and leptin/amylin, $P < 0.05$; Fig. 1B).

To probe the behavioral mechanisms underlying the suppression of food intake, we conducted meal pattern analyses. We found that meal size (Fig. 2A) was durably and robustly reduced by the amylin plus leptin combination (statistically significant main effects of amylin and leptin, ANOVAs $F_{1,13} \geq 10.98, P < 0.01$; main effect of time, $F_{9,117} = 11.99, P < 0.0001$; interaction between amylin/time, $F_{9,117} = 4.19, P < 0.001$; planned comparisons between vehicle/vehicle and leptin/amylin, $P < 0.05$ at 0.5–3 and 12 h), whereas effects on meal number were observed at only one early time point and were relatively minor (statistically significant main effect of amylin, $F_{1,13} = 21.78, P < 0.001$; main effect of time, $F_{9,117} = 73.55, P < 0.0001$; interaction between amylin/time, $F_{9,117} = 1.96, P < 0.05$; planned comparisons between vehicle/vehicle and leptin/amylin, $P < 0.05$ at 2 h; Fig. 2B). This suggests that the primary mechanism by which VTA amylin plus leptin reduces feeding is likely through an enhancement of within-meal satiation signaling, with fewer effects on intermeal satiety processes.

**Combined intra-VTA injection of subthreshold doses of amylin and leptin significantly reduces chow intake and body weight gain.** The first study supported the hypothesis that the VTA is a site of action for the cooperative ability of amylin and leptin to promote hypophagia and suppress body weight gain. To further evaluate this effect, we conducted a similar experiment in a separate group of rats ($n = 19$) in which we delivered subthreshold doses of amylin (0.04 $\mu$g) and leptin (0.1 $\mu$g), alone or in combination, directly to the VTA. Although at these doses, neither amylin alone nor leptin alone reduced feeding or body weight gain over the 24-h postinjection, intra-VTA injection of the combination of the two peptides significantly suppressed both food intake (main effects of leptin ($F_{1,18} = 19.01, P < 0.001$) and amylin ($F_{1,18} = 12.00, P < 0.01$); interaction between amylin and leptin ($F_{1,18} = 6.90, P < 0.02$); planned comparisons of vehicle/vehicle vs. leptin/amylin, $P < 0.05$, vehicle/vehicle vs. leptin/vehicle or vehicle/amylin, $P > 0.05$; Fig. 3A) and body weight gain (main effect of leptin ($F_{1,18} = 15.23, P < 0.01$); planned comparisons of vehicle/vehicle vs. leptin/amylin, $P < 0.05$, vehicle/vehicle vs. leptin/vehicle or vehicle/amylin, $P > 0.05$; Fig. 3B). We again analyzed meal patterns and found results similar to the first amylin-leptin experiment in that the combination of intra-VTA leptin and amylin significantly reduced meal size (main effect of leptin ($F_{1,18} = 5.17, P < 0.04$); planned comparisons of vehicle/vehicle vs. leptin/amylin, $P < 0.05$, vehicle/vehicle vs. leptin/vehicle or vehicle/amylin, $P > 0.05$; Fig. 3C) but had no effect on meal number (no main effect of leptin or amylin and no significant interaction; all ANOVAs $F_{1,18} \leq 1.60, P > 0.05$; Fig. 3D). This further supports the notion that the VTA mediates the interactive effects of amylin and leptin on energy balance.

**Fig. 2. Meal patterns after intra-VTA administration of suprathreshold doses of amylin (0.4 $\mu$g) or its vehicle (100 nl of aCSF) plus leptin (0.3 $\mu$g) or its vehicle (100 nl of 0.01 M sodium bicarbonate). The hypophagia produced by VTA amylin combined with VTA leptin shown in Fig. 1 is driven primarily by reductions in meal size (A) rather than meal number (B). Within a time bin: #statistical significance ($P < 0.05$) compared with Veh/Veh; *significant difference ($P < 0.05$) from all other conditions. All data shown as means $\pm$ SE.**
Pharmacological blockade of VTA amylin receptors attenuates the intake- and body weight-suppressive effects of intra-VTA leptin. To begin to address the potential physiological relevance of the VTA for the cooperative energy balance effects of amylin and leptin, we tested whether endogenous amylin receptor activity is required for the ability of intra-VTA leptin to reduce feeding and body weight gain. Rats (n = 13) were given a direct VTA pretreatment injection of the amylin receptor antagonist AC187 (0.1 μg) or its vehicle (100 nl of aCSF), which was followed 1 h later by a second intra-VTA injection containing leptin (0.6 μg) or its vehicle (100 nl of 0.01 M sodium bicarbonate). This dose of intra-VTA-delivered AC187 was chosen for its ability to significantly attenuate suppression of food intake by systemic amylin agonist delivery, yet it is a subthreshold dose for independent effects when administered directly to the VTA (33). Chow intake and body weight gain were measured for 24 h posttreatment. The selected dose of intra-VTA leptin decreased both 24-h chow intake and body weight. However, VTA amylin receptor blockade with AC187 significantly attenuated both the hypophagia (main effect of leptin (F1,12 = 45.15, P < 0.0001); interaction between AC187 and leptin (F1,12 = 7.57, P < 0.02); post hoc comparisons, vehicle/vehicle vs. vehicle/leptin: P < 0.05, vehicle/leptin vs. AC187/leptin: P < 0.05; Fig. 4A) and suppression of body weight [main effect of leptin (F1,12 = 18.45, P < 0.01); main effect of AC187 (F1,12 = 13.07, P < 0.01); post hoc comparisons, vehicle/vehicle vs. vehicle/leptin: P < 0.05; vehicle/leptin vs. AC187/vehicle: P > 0.05; vehicle/leptin vs. AC187/leptin: P < 0.05; Fig. 4B] produced by VTA amylin receptor activation. This suggests that endogenous amylin is activating VTA amylin receptors to facilitate/enhance the anorectic and body weight-suppressive effects of VTA leptin signaling.

DISCUSSION

Several studies to date have established that peripheral administration of a combination of amylin and leptin produces effects on feeding and body weight that are additive or potentially synergistic in nature (48, 56, 57, 59). Both peptides act within the CNS to promote negative energy balance, with the actions of amylin on energy balance thought to be mediated exclusively by central nuclei (24). Therefore, it is critical to identify sites within the brain that support the interaction between these signals. The current findings demonstrate that the VTA is a novel central nucleus mediating the interaction of amylin and leptin to promote negative energy balance. Our data show that when amylin and leptin are coadministered directly into the VTA, the hypophagic and body weight-suppressive effects of the combination of the two peptides are greater than that of either peptide alone. This was observed with doses of amylin and leptin that, when administered separately, were either supra- or subthreshold for effects on food intake and body weight. Additionally, meal pattern analyses demonstrated that the reductions in feeding produced by the combination of intra-VTA leptin and amylin were due to suppression of meal size, consistent with the roles of both amylin and leptin acting as satiation signals (16, 18, 23, 25). In thinking about the reduction in food intake produced by VTA delivery of amylin and leptin, it is important to rule out alternative explanations for the observed hypophagia. This effect is unlikely to be due to impaired locomotion, as activation of VTA leptin receptors (17) or VTA amylin receptors (33) does not produce lasting reductions in locomotor activity. Furthermore, VTA amylin receptor activation does not cause pica (33) and does not produce conditioned taste avoidance (32), suggesting against the possibility that nausea/malaise causes the reductions in food intake observed with VTA amylin receptor activation. Together, these previous findings argue for a specific effect of VTA amylin plus leptin on feeding and body weight.

Leptin and amylin each can cross the blood-brain barrier (2–4), and within the VTA, both amylin receptor and leptin receptor signaling are physiologically relevant for energy balance control (17, 32, 33). Therefore, to begin to evaluate the potential physiological relevance of the VTA for the interaction between these peptides, we tested whether VTA amylin receptor blockade would attenuate the anorectic and body weight-suppressive effects of intra-VTA leptin. Indeed, our findings indicate that the reductions in chow intake and body weight gain produced by VTA delivery of leptin are blunted by intra-VTA pretreatment with the amylin receptor antagonist AC187. This hints at the possibility that activation of VTA amylin receptors by endogenous amylin may be required for the full expression of the food intake- and body weight-suppressive effects of VTA leptin receptor activation.

The present data describe a pharmacological and perhaps physiological role for the VTA in mediating the combined effects of amylin and leptin on food intake and body weight gain. The mechanism(s) underlying these effects is not yet known. Although our knowledge of the downstream effects of each peptide alone in the VTA is still somewhat limited, a few possible points of convergence are clear from the literature. One potential mechanism by which these two peptide signals could be integrated involves effects on dopaminergic signaling. Both amylin receptor and leptin receptor signaling within the VTA impact the activity of the mesolimbic dopamine system (14, 17, 32). Cooperative actions of VTA leptin and VTA amylin on dopaminergic signaling to key afferent targets such as the nucleus accumbens (14, 32) or central nucleus of the amygdala (21) could mediate the combined effects of these
hormones on energy balance. A critical empirical question that remains to be tested is whether amylin receptors and leptin receptors are coexpressed on dopamine neurons within the VTA. Within the VTA, amylin receptors are highly expressed on dopamine neurons (32), and VTA dopamine neurons also express the leptin receptor at the mRNA level (17). However, the lack of a widely validated antibody against the leptin receptor limits the ability to test for colocalization of these receptors at the protein level. Given that leptin can regulate the activity of VTA dopamine neurons via a presynaptic mechanism (55), it is also possible that leptin acts presynaptically within the VTA to regulate dopamine signaling, whereas amylin acts via a direct postsynaptic mechanism.

A related possibility is that amylin receptor and leptin receptor activation may impinge on common intracellular signaling pathways within VTA neurons. The intracellular signaling response to leptin within the VTA includes phosphorylation of STAT3 (17) and ERK (58). Although the intracellular signaling cascade engaged by VTA amylin receptor activation has not yet been established, amylin receptor activation in other areas of the brain stimulates phosphorylated (p)-ERK (45) and also increases p-STAT (59). Because intracellular signaling pathways are known to be important points of convergence for energy status signals, increased activation of one or more of these signals through the actions of both amylin and leptin could contribute to the enhanced energy balance effects produced by the combination of the peptides. In fact, several in vitro reports demonstrate the ability of amylin and leptin to produce cooperative effects on common intracellular signaling pathways (36–38). Given that leptin can induce p-STAT3 in VTA dopamine neurons (17), an interesting possibility is that coactivation of the same dopamine neuron by amylin and leptin may result in a potentiated response through the activation of common intracellular molecules within a single dopaminergic cell. Such analyses are certainly warranted for future studies.

Obese animals have reduced sensitivity to the effects of leptin, which is indicated by a reduction in leptin-induced p-STAT3 in several key feeding-relevant nuclei (28, 39). This has limited the utility of leptin-targeting monotherapies for the treatment of obesity (5). However, amylin remains effective at reducing food intake and body weight in the obese state (7). In fact, the amylin analog pramlintide is FDA approved for the treatment of diabetes but has the additional effect of reducing food intake and body weight in obese individuals (12, 52, 53). This finding, along with the ability of amylin receptor signaling to enhance the body weight-lowering effects of other neuroendocrine signals (22), has led to the notion that the amylin system is a strong candidate for future antiobesity drug development (30, 47, 51). Interestingly, the available data on the interactive effects of peripherally administered amylin and leptin suggest that amylin may improve leptin sensitivity (48, 56), highlighting the potential clinical utility of a combination of amylin- and leptin-targeting pharmacotherapies for more effective obesity treatment (9, 54). Given that a contributing factor to the development of obesity is the overconsumption of highly palatable, energy-dense foods (19), our finding that the VTA mediates the cooperative effects of leptin and amylin on energy balance control is particularly intriguing, as this nucleus plays an important role in the control of palatable food intake and food reward (29, 40, 60). Although the current studies examined the effects of the VTA amylin-leptin combination on chow intake, an exciting extension of our findings will be to evaluate whether VTA delivery of amylin and leptin can also attenuate the intake of palatable foods, such as those high in fat and/or carbohydrate, in both lean and diet-induced obese animals.

Neuroendocrine signals are often studied in isolation, but in reality these hormones and neuropeptides interact in complex ways to regulate behavior and physiology. Understanding the mechanisms by which feeding-related peptides interact to control energy balance is critically important not only for furthering our understanding of the in vivo actions of the signals but also potentially to develop more effective pharmacotherapies for obesity. Our data indicate that the VTA is a central site of action mediating the cooperative effects of amylin and leptin for the control of energy balance. This finding, along with recent reports identifying the importance of the hypothalamus for the effects of the combination of amylin and leptin (20, 59), highlights the importance of further investigation of the distributed neural substrates and mechanisms underlying this interaction.

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DISCLOSURES

The authors have no conflicts of interest to disclose, financial or otherwise.

AUTHOR CONTRIBUTIONS


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