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Amylin Acts in the Lateral Dorsal Tegmental Nucleus to Regulate Energy Balance Through Gamma-Aminobutyric Acid Signaling

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ABSTRACT

BACKGROUND: The pancreatic- and brain-derived hormone amylin promotes negative energy balance and is receiving increasing attention as a promising obesity therapeutic. However, the neurobiological substrates mediating amylin's effects are not fully characterized. We postulated that amylin acts in the lateral dorsal tegmental nucleus (LDTg), an understudied neural processing hub for reward and homeostatic feeding signals.

METHODS: We used immunohistochemical and quantitative polymerase chain reaction analyses to examine expression of the amylin receptor complex in rat LDTg tissue. Behavioral experiments were performed to examine the mechanisms underlying the hypophagic effects of amylin receptor activation in the LDTg.

RESULTS: Immunohistochemical and quantitative polymerase chain reaction analyses show expression of the amylin receptor complex in the LDTg. Activation of LDTg amylin receptors by the agonist salmon calcitonin dose-dependently reduces body weight, food intake, and motivated feeding behaviors. Acute pharmacological studies and longer-term adeno-associated viral knockdown experiments indicate that LDTg amylin receptor signaling is physiologically and potentially preclinically relevant for energy balance control. Finally, immunohistochemical data indicate that LDTg amylin receptors are expressed on gamma-aminobutyric acidergic neurons, and behavioral results suggest that local gamma-aminobutyric acid receptor signaling mediates the hypophagia after LDTg amylin receptor activation.

CONCLUSIONS: These findings identify the LDTg as a novel nucleus with therapeutic potential in mediating amylin's effects on energy balance through gamma-aminobutyric acid receptor signaling.

Keywords: Calcitonin, Food intake, IAPP, Motivated behavior, Obesity, Reward

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In the search for effective pharmacological treatments for obesity, much attention has focused on neuroanatomical targets in the central nervous system (CNS) (1), such as the hypothalamus and caudal brainstem, each historically linked with the homeostatic regulation of energy balance (2–7). While these studies have informed the field about cellular and molecular mechanisms mediating the metabolic effects of many gastrointestinal- and adipose tissue–derived hormones, the chronic hyperphagia underlying human obesity is not related to disproportionate homeostatic feeding, but rather is more likely based on excessive appetitive and motivational processes directed toward the consumption of highly palatable/rewarding food (8–11). Indeed, targeting nonhomeostatic/reward-based systems may provide a unique opportunity to treat obesity and metabolic diseases (12,13). Urgently needed, however, is a deeper understanding of the relevant CNS reward circuitry and how it responds to and integrates energy balance signals to control food intake and body weight.

The lateral dorsal tegmental nucleus (LDTg) (14) is a nucleus in the caudal midbrain that is uniquely positioned as a processing hub for the integration of reward-based and homeostatic energy balance signaling (14–16) yet has been understudied for its role in feeding and other motivational processes. Indeed, the LDTg has reciprocal projections with many feeding-relevant nuclei throughout the neuraxis, including but not limited to the nucleus tractus solitarius (NTS), the ventral tegmental area (VTA), the lateral hypothalamus, and the parabrachial nucleus. Given that the LDTg expresses receptors for a variety of feeding peptides (e.g., amylin, ghrelin, glucagon-like peptide-1, and peptide YY) (16–21), we hypothesized that energy balance–relevant neuroendocrine signals may act directly in the LDTg to modulate the neural processing of feeding-relevant information and affect motivational aspects of food reward.

Following initiation of a meal, a cascade of endocrine events occurs, including secretion of the peptide hormone amylin from the pancreatic β cells. Amylin activates its

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receptors within the CNS to suppress ongoing feeding during the meal and increase satiation (22,23). Historically, the contribution of central amylin signaling to food intake control has centered on its action in homeostatic feeding centers, primarily the area postrema of the caudal brainstem (24–31) and secondarily in hypothalamic subnuclei, including the arcuate nucleus and ventromedial hypothalamus (32–34). However, recent work has also established the VTA and nucleus accumbens as relevant nuclei for amylin's energy balance effects, particularly for reward-based feeding (35–37). While this growing body of literature highlights a more distributed CNS system mediating amylin's energy balance effects than originally thought, the action of amylin in these aforementioned nuclei cannot wholly explain the energy balance and food reward effects of amylin signaling (24,38,39). In fact, because the neural control of energy balance is distributed across the CNS (2,40) and in vitro radiography studies show that amylin binds to sites throughout the brain (20,21), the ability of amylin receptor signaling in other CNS nuclei to produce hypophagia requires more extensive evaluation.

Given that amylin is being considered as an antiobesity therapeutic, it is critical to more fully understand the neural substrates mediating amylin's effects on reward-based feeding in addition to its impact on homeostatic intake (22,41–44). That the LDTg binds amylin (21) and is widely connected with a variety of energy balance–relevant nuclei (14) collectively supports our hypothesis that amylin receptor signaling in the LDTg may control food intake, body weight, and motivated behaviors directed toward food reward. Thus, data presented here lend greater insight into amylin receptor signaling through the CNS by identifying the LDTg as a novel nucleus mediating the anorexigenic effects of amylin, while underscoring the LDTg–gamma-aminobutyric acidergic (GABAergic) system as a potential target for amylin-based therapies for the treatment of obesity.

METHODS AND MATERIALS

Details regarding all drugs used, stereotaxic surgery, quantitative polymerase chain reaction, immunohistochemical analyses, colchicine treatment, colocalization of calcitonin receptors (CTRs) and GABAergic markers, all behavioral experiments, and detailed statistical analyses are available in the Supplement.

Animals

Male Sprague Dawley rats (310–325 g upon arrival; Charles River, Wilmington, MA) individually housed in hanging wire cages (12-hour light/dark cycle) had ad libitum access to chow (Purina LabDiet 5001; Purina, St. Louis, MO) and water unless otherwise noted. For experiments labeling GABAergic neurons, male Sprague Dawley rats (250 g upon arrival; Envigo Labs, Indianapolis, IN) individually housed in hanging wire cages (12-hour light/dark cycle) had ad libitum access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee at University of Pennsylvania or University of Southern California and were performed according to the National Institutes of Health guidelines.

Behavioral Testing

Drug injections were made before the onset of the dark cycle unless otherwise specified. For experiments measuring ad libitum food intake, weights of food hoppers were recorded to the nearest 0.1 g, and food spillage was accounted for in cumulative food intake measurements. Food intake was recorded at 1, 3, 6, and 24 hours after injection, while body weight was measured at 0 and 24 hours after injection, except where noted. Injections were administered using a within-subjects counterbalanced design and were separated by at least 72 hours.

Statistical Analyses

All data are represented as mean ± SEM. The α level was set to $p \leq .050$ for all studies. Statistical analyses were performed using Statistica software version 13.0 (StatSoft, Tulsa, OK).

RESULTS

The Components of the Amylin Receptor Complex Are Expressed in the LDTg

Amylin receptors are formed by heteromeric interaction between one of two G_s/G_q-coupled calcitonin receptors (CTRa or CTRb) and one of three receptor activity–modifying proteins (RAMP1–3) (45,46). Although the LDTg binds amylin (21), no studies to date have examined expression of the amylin receptor complex within this nucleus. Therefore, we used quantitative real-time polymerase chain reaction to determine expression of the components of the amylin receptor (CTRa/b, RAMP1–3) in the LDTg, and found that both CTRs and all three RAMPs are indeed expressed in this nucleus ($n = 6$). CTRa gene expression is approximately fivefold greater than expression of CTRb, although this does not reach statistical significance ($F_{1,3} = 4.04$, $p = .14$; Figure 1A). Gene expression of RAMP1 is approximately twofold greater than the expression of RAMP2 ($F_{2,6} = 13.04$, $p < .01$; post hoc test, $p < .05$) and approximately 13-fold greater than the expression of RAMP3 (post hoc test, $p < .01$; Figure 1B). These findings are consistent with data from the area postrema and VTA, which also show higher expression of CTRa compared to CTRb and abundant RAMP1 expression (22,36).

Next, we performed immunohistochemical analyses to label cells that express CTR ($n = 6$). Data show labeling throughout the rostral-caudal axis of the LDTg, with particularly dense labeling in the caudal LDTg (8.6–9.1 mm posterior to bregma), providing evidence of amylin receptor expression at the protein level. Representative images from the caudal LDTg are shown (Figure 1C, D). Together, data in Figure 1 show that components of the amylin receptor complex are expressed in the LDTg at the gene and protein levels. Because of the dense CTR expression observed in the caudal LDTg, we targeted this subregion in our behavioral experiments.

LDTg Amylin Suppresses Cumulative Chow Intake and Body Weight

To test whether activation of amylin receptors in the LDTg by the native amylin peptide is sufficient to decrease food intake, rats ($n = 10$) were unilaterally injected in the LDTg with amylin

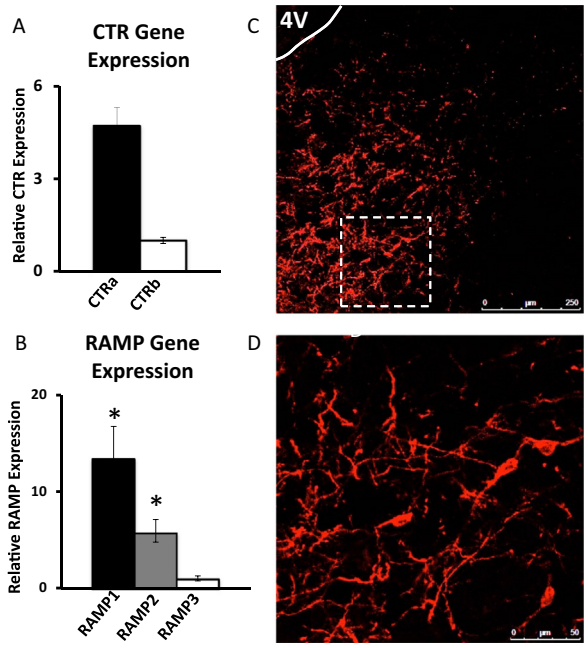

Figure 1. The components of the amylin receptor complex are expressed in the lateral dorsal tegmental nucleus. Micropunches of lateral dorsal tegmental nucleus–enriched tissue ($n = 6$) show that gene expression of calcitonin receptor a (CTRa) is approximately fivefold higher than calcitonin receptor b (CTRb) **(A)**, and gene expression of receptor activity–modifying protein 1 (RAMP1) is approximately twofold higher than RAMP2 and ~13-fold higher than RAMP3 **(B)**. Immunohistochemical data using CTR to label amylin receptor–expressing cells ($n = 6$) show dense labeling of cell bodies and projections in the caudal lateral dorsal tegmental nucleus **(C, D)**. The dashed box in **C** (×20) represents the field of view in **D** (×20 with a 2× optical zoom). *Significance by repeated measures analysis of variance ($p < .05$). 4V, fourth ventricle.

(0, 0.2, 0.4, and 0.8 μg; 100 nL artificial cerebrospinal fluid [aCSF]; see Figure 2A for representative injection placement) and subsequent chow intake and body weight change were recorded over a 24-hour period. Injection of amylin in the LDTg dose-dependently decreases food intake over 6 hours ($F_{3,27} \geq 3.00$, $p < .05$; Figure 2B) but not 24-hour food intake or body weight change ($F_{3,27} \leq 2.32$, $p > .05$; Figure 2C). Consistent with previous reports of amylin-induced hypophagia at early time points (47,48), all doses of amylin administered to the LDTg suppress chow intake at 1 hour ($p < .01$), but only the highest dose of amylin (0.8 μg) suppresses chow intake at 3 and 6 hours after injection ($p < .05$) compared to aCSF treatment.

LDTg Amylin Receptor Activation Suppresses Cumulative Chow Intake and Body Weight

To determine whether pharmacological LDTg amylin receptor activation with the long-acting amylin receptor agonist salmon calcitonin (sCT) produces more durable and more potent hypophagic effects, sCT (0, 0.01, 0.04, or 0.1 μg), was injected unilaterally into the LDTg and subsequent chow intake and body weight change were recorded over a 24-hour period ($n = 6$). Notably, the two lower doses of sCT, 0.01 and 0.04 μg, are subthreshold for prolonged effects on food intake and body weight when applied to the third ventricle (36). Results of this study show that intra-LDTg amylin receptor activation with sCT dose-dependently suppresses chow intake at 1, 3, 6, and 24 hours after injection ($F_{3,12} \geq 3.66$, $p < .05$; Figure 2D). Post hoc analyses reveal that all three doses of sCT produce a significant suppression of chow intake, compared to aCSF vehicle treatment, at 1 hour ($p < .01$) and 6 hours ($p < .05$) after injection. In addition, the two highest sCT doses (0.04 and 0.1 μg) decrease food intake at 24 hours after injection ($p < .05$). Body weight gain over the 24 hours after injection is also significantly reduced by intra-LDTg administration of 0.04 or 0.1 μg sCT ($F_{3,12} = 11.00$, $p < .01$; compared to aCSF, $p < .05$; Figure 2E). These data indicate that LDTg amylin receptor activation dose-dependently suppresses chow intake and body weight over 24 hours. Taken together and consistent with previous literature (49,50), LDTg amylin receptor activation with sCT results in more potent and longer-lasting hypophagic effects than LDTg administration of native peptide amylin.

LDTg Amylin Receptor Activation Suppresses Meal Size

To evaluate the behavioral mechanisms driving the hypophagia after LDTg amylin receptor activation, meal patterns were analyzed ($n = 5$). Unilateral injection of sCT in the LDTg at doses effective for reducing overall intake (0, 0.01, 0.04, or 0.1 μg) significantly suppresses meal size 24 hours after injection ($F_{3,15} = 5.18$, $p < .01$; Figure 3A). Post hoc analyses reveal that administration of the two highest doses of sCT, 0.04 and 0.1 μg, significantly decreases meal size compared to aCSF treatment ($p < .05$), consistent with the established role of amylin as a satiation signal (22,35). Along with this suppression in meal size, intra-LDTg administration of sCT also reduces meal duration at all doses tested ($F_{3,15} = 5.51$, $p < .01$; $p < .05$, compared to aCSF treatment; Figure 3B). LDTg amylin receptor activation increases latency to first meal ($F_{3,15} = 4.90$, $p < .05$; Figure 3C) at the highest dose ($p < .05$, compared to aCSF treatment), which indicates a decreased motivation to initiate feeding. Intra-LDTg administration of sCT decreases meal number over 24 hours after injection ($F_{3,15} \geq 3.77$, $p < .05$: Figure 3D), but only with the highest dose, 0.1 μg sCT ($p < .05$, compared to aCSF treatment). These data show that LDTg amylin receptor activation reduces food intake predominately via suppression of meal size rather than meal number. Importantly, this reduction in meal size is concomitant with a decrease in meal duration, which may be a consequence of reduced within-meal motivation to continue to feed and/or may reflect the normal physiological characteristics of amylin's effects on the behavioral satiation sequence.

LDTg Amylin Receptor Activation Attenuates Motivation for a Palatable Food

As the LDTg is a reward-relevant nucleus (15,16), we tested the hypothesis that LDTg amylin receptor activation attenuates motivated feeding as measured by sucrose self-administration on a progressive-ratio schedule of reinforcement ($n = 8$). Unilateral injection of either the native peptide amylin (0.4 μg) or amylin receptor agonist sCT (0.04 μg) into the LDTg significantly suppresses active lever responses for sucrose ($F_{2,14} = 11.52$, $p < .01$; Figure 4A), breakpoint ($F_{2,14} = 11.26$,

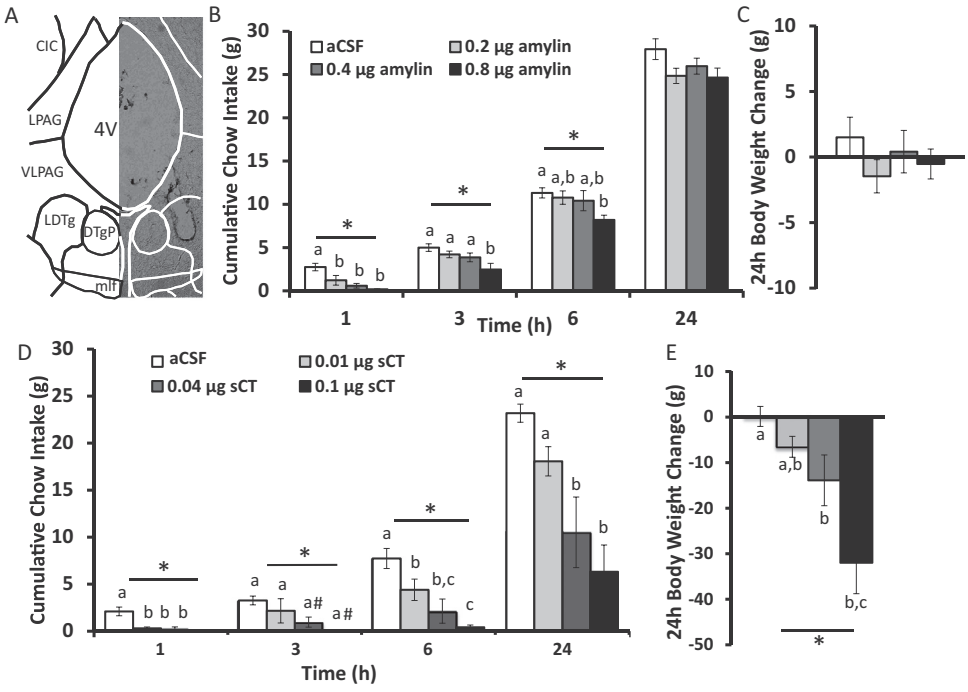

Figure 2. Intra–lateral dorsal tegmental nucleus (LDTg) amylin receptor activation dose-dependently suppresses chow intake and body weight. Amylin was unilaterally injected into the LDTg in a counterbalanced within-subjects design at the onset of the dark cycle using the following doses: 0 (artificial cerebrospinal fluid [aCSF]), 0.2, 0.4, and 0.8 µg (n = 10). A representative image of the LDTg injection site from a 35-µm-thick section is shown **(A)**. These doses of amylin dose-dependently decrease chow intake over 6 hours but have no effect on 24-hour chow intake **(B)** or body weight change **(C)**. The key in **(B)** also applies to **(C)**. In a separate cohort of rats, the amylin receptor agonist salmon calcitonin (sCT) was unilaterally injected into the LDTg in a counterbalanced within-subjects design at the onset of the dark cycle using the following doses: 0 (aCSF), 0.01, 0.04, and 0.1 µg (n = 6). These doses of sCT suppress chow intake over 24 hours **(D)** and decrease 24-hour body weight gain **(E)**. Different letters are significantly different from each other ($p < .05$) according to post hoc tests. The key in **(D)** also applies to **(E)**. Atlas image is –8.7 mm from bregma, based on Paxinos and Watson (69). *Significance by repeated measures analysis of variance ($p < .05$). #Indicates a trend for significance by post hoc Neuman-Keuls ($p < .1$). 4V, fourth ventricle; CIC, central nucleus inferior colliculus; DTgP, dorsal tegmental nucleus pericent; LPAG, lateral periaqueductal gray; mlf, medial longitudinal fasciculus; VLPAG, ventral lateral periaqueductal gray.

$p < .01$; Figure 4B), and sucrose pellets earned ($F_{2,14} = 7.72$, $p < .01$; Figure 4C) compared to aCSF treatment. Notably, there is no difference between treatments on inactive lever responding ($F_{2,14} = 1.13$, $p = .35$; Figure 4A). These data indicate that LDTg amylin receptor activation, both with the potent amylin receptor agonist sCT and with the native ligand amylin, reduces motivation to self-administer a palatable food.

LDTg Amylin Receptor Activation Does Not Produce Malaise

To determine if nausea/malaise contributes to the intake suppression after central amylin receptor activation, pica was measured after intra-LDTg sCT administration. Pica is the ingestion of nonnutritive substances, such as kaolin clay, and is a well-established model for nausea/malaise in non-vomiting species, such as the rat (51–55). The same doses of sCT used in the previous behavioral studies (0, 0.01, 0.04, or 0.1 µg) were injected unilaterally in the LDTg, and intakes of chow and kaolin clay were measured 24 hours after injection (n = 6). Intra-LDTg sCT does not increase kaolin intake at any dose ($F_{3,15} = 0.98$, $p = .45$; Figure 4D), but all three doses significantly suppress chow intake at 24 hours compared to aCSF treatment ($F_{3,15} = 7.93$, $p < .01$; post hoc test, $p < .05$; Figure 4E). These data suggest that the hypophagia and decreased motivation to feed following intra-LDTg amylin receptor activation are likely not caused by the induction of nausea/malaise.

LDTg Amylin Receptor Blockade Attenuates the Intake Suppressive Effects of Peripheral Amylin Receptor Activation

Given that pharmacological activation of amylin receptors directly in the LDTg suppresses food intake, the ability of peripherally administered amylin or amylin receptor agonists to access the CNS and act specifically within the LDTg is a key consideration in the development of amylin-based antiobesity pharmaceuticals and denotes potential preclinical relevance in animal models and clinical relevance in humans. Thus, to begin to address this critical question using a preclinical rodent model, we evaluated whether the intake- and body weight–suppressive effects of systemic sCT (5 µg/kg intraperitoneally) would be attenuated by acute LDTg amylin receptor blockade. We intentionally chose a dose of the amylin receptor antagonist AC187 that is subthreshold for an effect on feeding when delivered bilaterally within the LDTg (0.8 µg/hemisphere; n = 11) so as not to have competing orexigenic and anorexic behavioral responses. As expected, systemic administration of sCT significantly suppresses cumulative chow intake at 3, 6, and 24 hours after injection (main effects of sCT, $F_{1,10} \geq 11.31$, $p < .01$; planned comparisons of aCSF/sCT vs. aCSF/saline or AC187/saline at 3, 6, and 24 hours, $p < .05$; Figure 5A). A significant interaction between sCT and AC187 occurs at both 6 and 24 hours after injection ($F_{1,10} \geq 5.20$, $p < .05$); post hoc analyses reveal that pretreatment with intra-LDTg AC187 significantly attenuates the

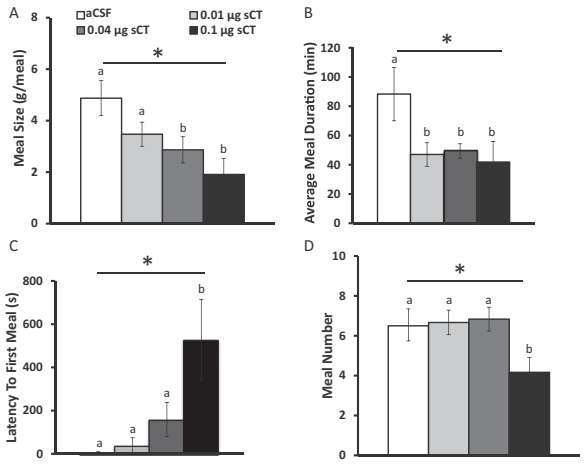

Figure 3. Intra–lateral dorsal tegmental nucleus amylin receptor activation predominately suppresses meal size rather than meal frequency. To determine the behavioral mechanism driving intake suppression, animals were housed in a custom-made automated feedometer to analyze meal patterns. The amylin receptor agonist salmon calcitonin (sCT) was unilaterally injected into the lateral dorsal tegmental nucleus in a counterbalanced within-subjects design at the onset of the dark cycle using the following doses: 0 (artificial cerebrospinal fluid [aCSF]), 0.01, 0.04, and 0.1 µg ($n = 5$). Intra–lateral dorsal tegmental nucleus sCT suppresses meal size over 24 hours at the two higher doses **(A)**, but all three doses suppress average meal duration over 24 hours **(B)**. Only the highest dose of sCT increases latency to first meal **(C)** and suppresses meal frequency over 24 hours **(D)**. *Significant by repeated-measures analysis of variance ($p < .05$); different letters are significantly different from each other according to post hoc tests ($p < .05$).

intake-suppressive effects of peripheral sCT at 24 hours ($p < .05$). Systemic administration of sCT also decreases 24-hour body weight gain ($F_{1,10} = 20.30$, $p < .01$, main effect of sCT; Figure 5B). Treatment with aCSF/sCT suppresses 24-hour body weight gain compared to aCSF/saline and AC187/saline conditions (planned comparisons, $p < .05$). Importantly, amylin receptor blockade alone (AC187/saline) does not significantly increase chow intake at any time point (no main effects of AC187, $F_{1,10} < 1.61$, $p > .2$) or body weight (no main effect of AC187, $F_{1,10} = 1.56$, $p > .2$). These data show that intra-LDTg amylin receptor blockade attenuates the intake-suppressive effects of a systemically delivered amylin receptor agonist, suggesting the potential preclinical relevance of LDTg amylin receptor signaling.

Knockdown of Calcitonin Receptors in the LDTg Increases Chow Intake and Body Weight

In order to determine if endogenous LDTg amylin receptor signaling is physiologically required for the normal day-to-day control of energy balance, an adeno-associated virus of serotype 1 (AAV1) that encodes a short hairpin RNA to knockdown CTR, the core component of the amylin receptor (AAV-CTR KD), or an empty vector control (AAV-Control) (35) was injected bilaterally into the LDTg (200 nL/hemisphere). Compared to AAV-Control animals, AAV-CTR KD decreases LDTg CTRa expression by approximately 67% ($F_{1,10} = 5.43$, $p < .05$; Figure 6A). Representative green fluorescent protein visualization of viral targeting and spread from a separate cohort of animals sacrificed 2 weeks after bilateral LDTg viral injection ($n = 3$/viral condition) is shown in Figure 6B.

Animals with LDTg amylin receptor knockdown show a sustained elevation in body weight compared to the AAV-Control rats (Figure 6C). Analyses of variance (ANOVAs) show that AAV-CTR KD animals weigh more than AAV-Control animals, either approaching ($F_{1,12} \geq 3.30$, $p < .1$) or reaching ($F_{1,12} \geq 4.75$, $p < .05$) statistical significance on any given experimental test day, beginning 3 days after viral injection. When analyzed as cumulative body weight gain from day 0 to day 31, AAV-CTR KD produces a significant increase in body weight gain compared to AAV-Control ($F_{1,12} = 4.73$, $p = .050$; Figure 6E).

AAV-CTR KD treatment causes small increases in 48-hour binned food intake (Figure 6D). ANOVAs show that AAV-CTR KD animals eat significantly more in 48-hour bins than AAV-Control animals on days 7 to 9 and 29 to 31 ($F_{1,12} \geq 5.78$, $p < .05$), with a trend for significance ($F_{1,12} \geq 3.50$, $p < .1$) on days 1 to 3, 9 to 11, and 19 to 21. When graphed cumulatively from day 0 to day 31, AAV-CTR KD rats have a trend for increased cumulative intake compared to AAV-Control rats ($F_{1,12} = 3.92$, $p < .1$; Figure 6F). Together, these data show that endogenous amylin accesses the LDTg and establish a physiological role for LDTg amylin receptor signaling in the normal control of food intake and body weight regulation.

LDTg Amylin Receptors Are Expressed on GABAergic Neurons

Next, we performed immunohistochemistry to determine the phenotype of amylin receptor–expressing cells in the LDTg. Sections were labeled for the amylin receptor (CTR), NeuN (a neuronal marker), and glial fibrillary acidic protein (a glia/astrocyte marker). CTR-expressing cells in the LDTg colocalize exclusively with NeuN (Figure 7A), suggesting that amylin receptor–expressing cells in the LDTg are primarily, if not exclusively, neuronal ($n = 3$).

To begin to evaluate the phenotype of LDTg CTR-expressing neurons, further immunohistochemical experiments tested if the CTR-positive neurons within the LDTg are cholinergic or GABAergic, as these represent classic LDTg neurotransmitter phenotypes (56). Results indicate that CTR in the LDTg does not colocalize with choline acetyltransferase, a marker for cholinergic neurons (Figure 7B; $n = 6$). After colchicine treatment (57), 13.7% of CTR neurons in the LDTg, specifically in the caudal LDTg (−8.6 mm to −9.1 mm from bregma) colocalize with the GABAergic neuronal marker Gad67 (Figure 7C; $n = 1$). These data suggest that at least a portion of amylin receptor–expressing cells in the LDTg are GABAergic neurons, although we cannot rule out the possibility that colchicine treatment did not result in labeling of all Gad67 cells.

Intra-LDTg $GABA_{A/B}$ Receptor Blockade Reverses the Intake-Suppressive Effects of LDTg Amylin Receptor Activation

As our data show that LDTg amylin receptor signaling regulates food intake and body weight, and that a portion of LDTg amylin receptor–expressing cells are GABAergic, we

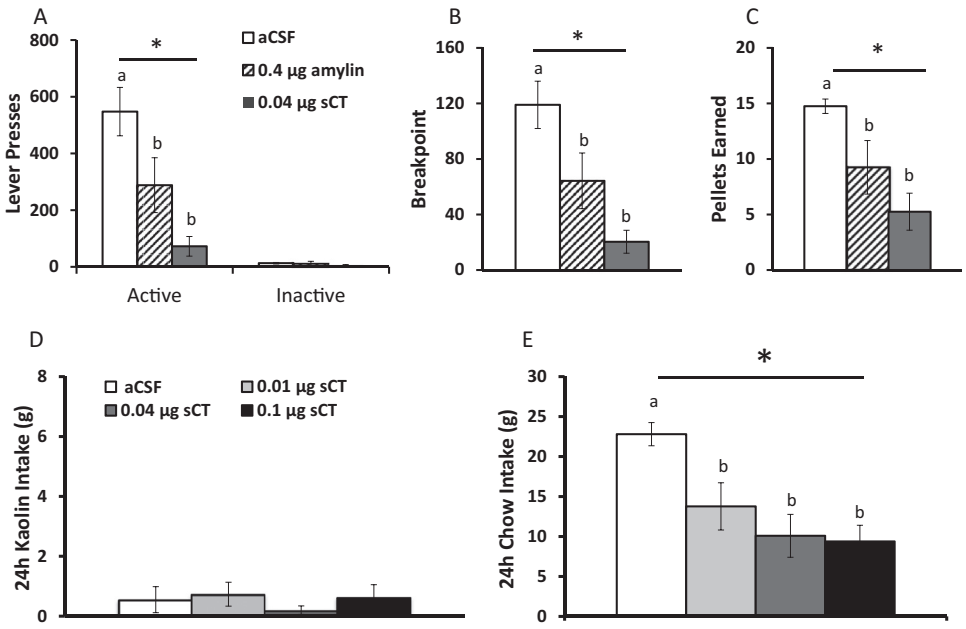

Figure 4. Intra–lateral dorsal tegmental nucleus (LDTg) amylin receptor activation suppresses motivated feeding but does not produce malaise. The ability of LDTg amylin receptor activation to reduce sucrose self-administration on a progressive-ratio schedule of reinforcement was assessed (n = 8). Intra-LDTg amylin receptor activation with amylin (0.4 µg) or salmon calcitonin (sCT; 0.04 µg) suppresses active lever presses **(A)**, breakpoint **(B)**, and pellets earned **(C)**. To determine if LDTg amylin receptor activation produces nausea/malaise, pica (ingestion of nonnutritive substances in response to a noxious stimulus) was measured. Animals received access to both chow and kaolin clay for 1 week before the beginning of the experiment. The amylin receptor agonist sCT was unilaterally injected into the LDTg using the following doses: 0 (artificial cerebrospinal fluid [aCSF]), 0.01, 0.04, and 0.1 µg (n = 6). Intra-LDTg amylin receptor activation does not increase kaolin clay intake **(D)** but suppresses chow intake at 24 hours **(E)**. Key in **(A)** applies to **(A-C)**; key in **(D)** applies to **(D, E)**. *Significant by repeated-measures analysis of variance ($p < .01$); different letters are significantly different from each other according to post hoc tests ($p < .05$).

next evaluated the hypothesis that LDTg GABA receptor signaling is downstream of LDTg amylin receptor activation and mediates LDTg amylin-induced hypophagia. To test this hypothesis, a cocktail composed of the $GABA_A$ receptor antagonist bicuculline (100 ng) and the $GABA_B$ receptor antagonist saclofen (500 ng) was unilaterally injected in the LDTg at doses subthrehold for an effect on feeding (100 nL, 50% dimethyl sulfoxide [DMSO] in aCSF) followed by a unilateral injection of sCT (0.04 µg; 100 nL, aCSF vehicle) in the ipsilateral LDTg; subsequent chow intake and body weight change were measured (n = 8).

For cumulative chow intake (Figure 8A), repeated-measures ANOVAs show a significant main effect of sCT at all time points ($F_{1,7} \geq 8.30$, $p < .05$) and a significant interaction between sCT and GABA receptor blockade at 24 hours after injection ($F_{1,7} = 7.47$, $p < .05$). Specifically, chow intake following 50% DMSO/sCT is significantly suppressed at 1 and 24 hours after injection, compared to all other conditions (planned comparisons, $p < .05$). Importantly, intra-LDTg $GABA_{A/B}$ receptor blockade does not affect feeding on its own ($F_{1,7} < 4.70$, $p > .1$) but reverses the intake-suppressive effects of intra-LDTg sCT at 24 hours after injection ($GABA_{A/B}$ receptor antagonists/sCT vs. 50% DMSO/sCT, $p < .05$; vs. 50% DMSO/aCSF, $p > .4$). Based on the feeding data, we analyzed the body weight change (Figure 8B) as a one-way repeated-measures ANOVA by treatment ($F_{3,21} = 7.77$,

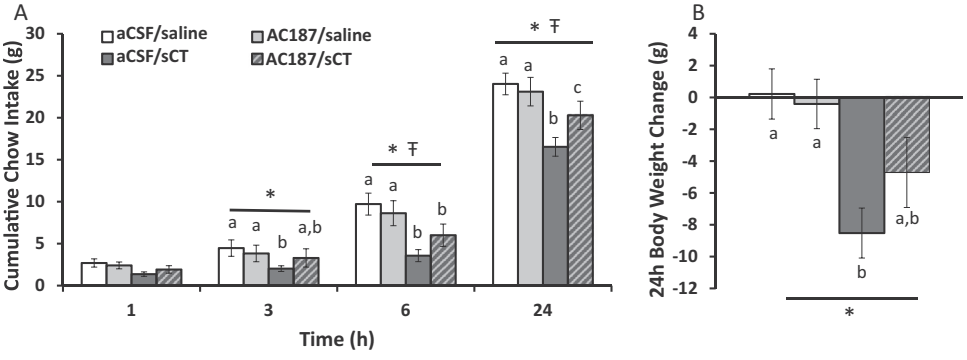

Figure 5. Lateral dorsal tegmental nucleus amylin receptor blockade attenuates the intake-suppressive effects of an amylin receptor agonist. To determine if lateral dorsal tegmental nucleus amylin receptor signaling is preclinically relevant, the amylin receptor antagonist AC187 was bilaterally injected in the lateral dorsal tegmental nucleus (0.8 µg/hemisphere), followed 45 minutes later by a systemic injection of salmon calcitonin (sCT) (5 µg/kg intraperitoneally) shortly before the onset of the dark cycle (n = 11). Pretreatment of AC187 alone has no significant effect on chow intake or body weight at any time point. Administration of sCT significantly suppresses intake at 3, 6, and 24 hours **(A)** as well as 24-hour body weight gain **(B)**. Pretreatment of AC187 with sCT significantly attenuates the intake-suppressive effects of systemically delivered sCT. The legend applies to both graphs. *Significant main effect of sCT by repeated-measures analysis of variance ($p < .01$). ŦSignificant interaction between sCT and AC187 by repeated-measures analysis of variance ($p < .05$); different letters are significantly different from each other according to post hoc planned comparisons ($p < .05$). aCSF, artificial cerebrospinal fluid.

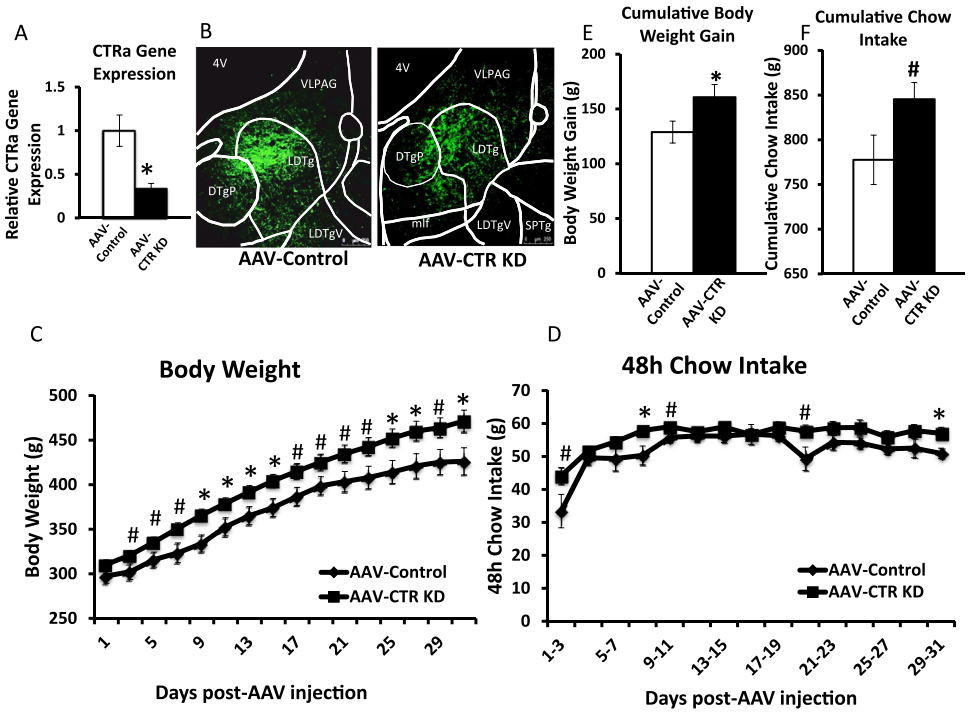

Figure 6. Calcitonin receptor knockdown in the lateral dorsal tegmental nucleus (LDTg) produces sustained increases in body weight and chow intake. To determine if LDTg amylin receptor signaling is physiologically relevant for the long-term control of food intake and body weight regulation, an adeno-associated virus (AAV) that knocks down the core component of the amylin receptor, the CTR (AAV-CTR KD), or an empty vector AAV (AAV-Control) was injected bilaterally in the LDTg (200 nL/hemisphere). Food intake and body weight was measured every 48 hours for 31 days following viral injection (n = 7/viral condition). **(A)** Compared to AAV-Control, the AAV-CTR KD produces a statistically significant 67% decrease of calcitonin receptor a (CTRa). A separate cohort of animals received either virus (n = 3/viral condition), were sacrificed 2 weeks later, and the brains were processed for green fluorescent protein visualization. **(B)** Representative images show green fluorescent protein labeling of viral expression in AAV-Control (left) and AAV-CTR KD (right). In behavioral studies, AAV-CTR KD produces an increase in body weight that was sustained over the behavioral test period **(C, E)**. Chow intake is transiently increased in AAV-CTR KD animals compared to AAV-Control animals when graphed in 48 bins **(D)**, and trending for significance when graphed cumulatively over the entire behavioral test period (**F**; $p < .1$). *Significance by analysis of variance ($p \leq .050$). #Trend for significance by analysis of variance ($p < .1$). 4V, fourth ventricle; DTgP, dorsal tegmental nucleus pericent; mlf, medial longitudinal fasciculus; SPTg, subpeducuncular tegmental nucleus; VLPAG, ventral lateral periaqueductal gray.

$p < .01$). Body weight gain after 50% DMSO/sCT treatment is significantly suppressed compared to all other treatments ($p < .01$). Importantly, GABA receptor blockade alone ($GABA_{A/B}$ receptor antagonists/aCSF) does not significantly alter body weight ($p > .9$) compared to 50% DMSO/aCSF treatment. These data show that intra-LDTg $GABA_{A/B}$ receptor blockade attenuates the anorexia produced by an intra-LDTg amylin receptor agonist.

DISCUSSION

The current obesity epidemic (1) highlights the urgent need to understand the neuroendocrine signals and neurobiological substrates that regulate energy balance, which in turn will inform the identification of novel opportunities for obesity pharmacotherapies. Recent attention has focused on targeting the amylin system for treating obesity, because the amylin analogue pramlintide has been approved by the U.S. Food and Drug Administration for the treatment of diabetes and also decreases food intake and body weight in obese patients (11,58). Although research on amylin's effects on energy balance has predominately focused on hindbrain and hypothalamic structures [see (22,42,59) for review], in vitro radiography data show that amylin binding sites are found throughout the brain (21), suggesting the likelihood of more distributed effects. The LDTg of the caudal midbrain represents one such amylin binding site; this nucleus receives information from and projects to several hindbrain, midbrain, and forebrain structures important for food intake, body weight regulation, and reward (14). Our experiments here show that the components of the amylin receptor complex are expressed in the LDTg and that amylin receptor signaling in the LDTg is important for the control of food intake and body weight regulation. In addition, our data identify a portion of LDTg amylin receptor–expressing cells as GABAergic neurons that we speculate may be interneurons. These findings highlight the LDTg as a potential energy balance hub and show that this nucleus is of potential preclinical relevance as a neural substrate that can be targeted for future amylin-based pharmacotherapies for obesity.

Despite the fact that the LDTg receives information from and projects to a number of feeding- and reward-relevant nuclei throughout the brain (14) and expresses receptors for a variety of feeding peptides (e.g., amylin, ghrelin, glucagon-like peptide-1, and peptide YY) (16–21), little attention has been paid to this nucleus for its role in energy balance control. The current data showing that LDTg amylin receptor activation suppresses food intake and body weight is highly novel and consistent with the satiating properties following systemic or intracerebroventricular administration of amylin [see (22,60) for review]. Importantly, the suppression in food intake by LDTg amylin receptor activation is not likely caused by nausea/malaise, because LDTg amylin receptor activation does not produce pica, suggesting the specificity of the energy balance effects. Two additional explanations underlying the body weight changes after LDTg amylin receptor activation are decreases in intestinal food weight and/or reductions in

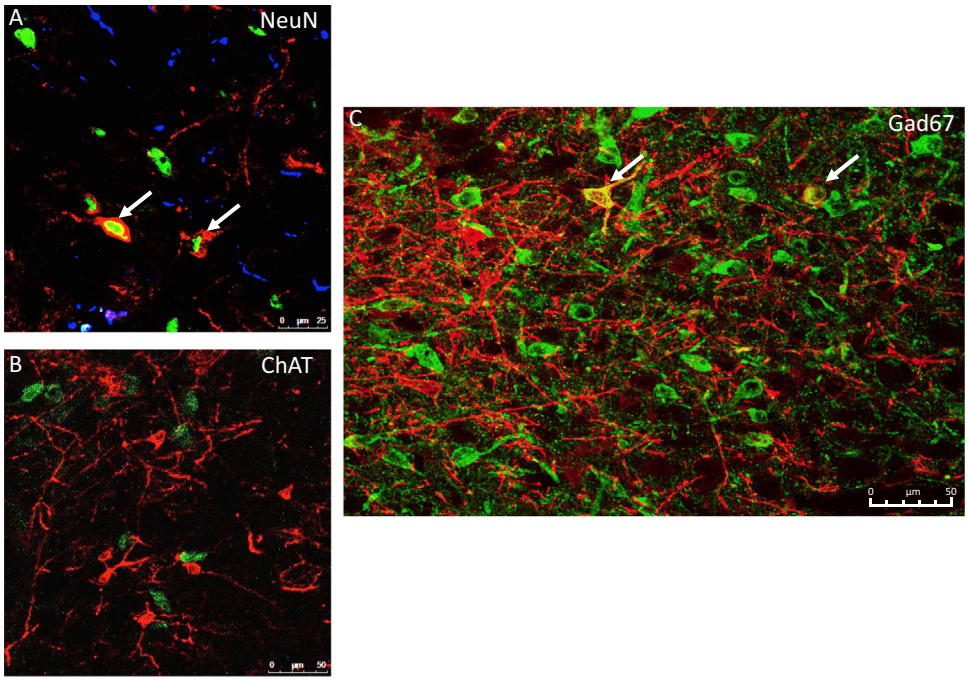

Figure 7. Calcitonin receptor (CTR)–expressing cells in the lateral dorsal tegmental nucleus are gamma-aminobutyric acidergic. Immunohistochemical analyses show that CTR-expressing cells in the lateral dorsal tegmental nucleus colocalize with the neuronal marker NeuN and not with the glial cell marker glial fibrillary acidic protein (**A**, ×20 with a 2× optical zoom; $n = 3$). CTR-expressing cells in the lateral dorsal tegmental nucleus do not colocalize with the cholinergic marker choline acetyltransferase (**B**, ×40; $n = 6$) but colocalize with the gamma-aminobutyric acidergic marker Gad67 (**C**; ×20; $n = 1$). Red cells are CTR-positive, blue cells are glial fibrillary acidic protein positive, and green cells show the cellular marker of interest: NeuN **(A)**, choline acetyltransferase **(B)**, and Gad67 **(C)**. White arrows indicate colocalization. ChAT, choline acetyltransferase.

prandial drinking. The aforementioned experiments use the amylin receptor agonist sCT, which binds irreversibly with high affinity to amylin receptors but also with low affinity to calcitonin receptors (49,61,62). In contrast, amylin itself binds with moderate affinity to amylin receptors and with very low affinity to calcitonin receptors (46,61). In addition, while we show evidence of gene expression of the amylin receptor complex in the LDTg, it is important to point out that the qualitative polymerase chain reaction micropunch data cannot establish whether both components of the amylin receptor are expressed in the same cell. However, given that intra-LDTg amylin administration suppresses food intake and body weight in a dose-dependent manner, the current collective data suggest that complete amylin receptors are likely expressed in the LDTg and that amylin receptor signaling is likely mediating the observed hypophagic response.

The within-meal intake inhibitory effects of LDTg amylin signaling may be explained by a reduction in the rewarding value of the ongoing meal. Indeed, LDTg amylin receptor activation not only suppresses the size of the meal, but also produces a concomitant decrease in meal duration, as well as a decrease in motivation to work for a palatable sucrose reward. The LDTg is reciprocally connected to both the NTS and VTA (14). Given the role of the NTS in meal size control [see (2,6) for review] and the VTA in reward processing [see (12,13) for review], the suppression of meal size observed after LDTg amylin receptor activation likely involves amylinergic modulation of NTS-LDTg-VTA neural processing. However,

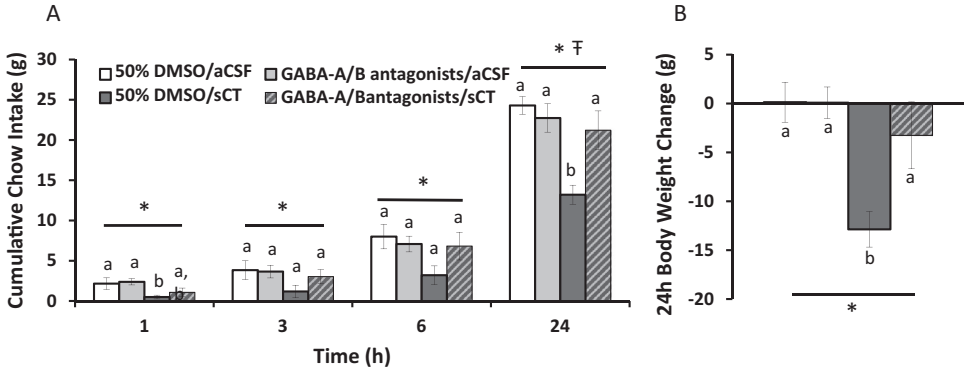

Figure 8. Intra–lateral dorsal tegmental nucleus (LDTg) gamma-aminobutyric acid (GABA) receptor blockade reverses the intake suppressive effects of intra-LDTg amylin receptor activation. To determine the role of GABA receptor signaling in the intake suppressive effects of LDTg amylin receptor activation, a cocktail of a $GABA_A$ receptor antagonist (bicuculline, 100 ng) and a $GABA_B$ receptor antagonist (saclofen, 500 ng) was administered unilaterally in the LDTg followed by salmon calcitonin (sCT) (0.04 μg; 100 nL; $n = 8$). GABA receptor blockade reverses the intake **(A)** and body weight–suppressive effects **(B)**. Key applies to both graphs. aCSF, artificial cerebrospinal fluid. *Significant main effect of sCT **(A)** or treatment **(B)** by repeated-measures analysis of variance ($p < .05$). ŦSignificant interaction between sCT and the GABA receptor antagonists by repeated-measures analysis of variance ($p < .05$); different letters are significantly different from each other according to post hoc planned comparisons ($p < .05$). DMSO, dimethyl sulfoxide.

future systematic neuroanatomical studies are needed to confirm that amylin receptor–expressing LDTg neurons impinge on this proposed NTS-LDTg-VTA circuitry through putative LDTg GABAergic inhibition of the NTS-LDTg-VTA polysynaptic communication. Alternatively, the decreased progressive-ratio responding may be a secondary response to LDTg amylin receptor signaling inducing satiation signaling more generally and potentially independent of reward signaling.

Previous studies established a role for the LDTg in reward processing for drugs of abuse and natural rewards (e.g., food and sex) through modulation of VTA dopaminergic cell firing (15,16,63–65). Our findings extend this literature on the role of the LDTg in modulating feeding behavior and energy balance, and provide novel evidence that LDTg signaling modulates the rewarding value of the ongoing meal. Given that activation of an LDTg–VTA pathway and its downstream targets can promote feeding and reward-associated behaviors, such as conditioned place preference and cocaine seeking (15,16, 66–68), amylin receptor activation of inhibitory GABA neurons in the LDTg may decrease VTA dopaminergic cell firing, ultimately leading to hypophagia and a reduction in motivated feeding. Our immunohistochemical and behavioral data provide converging evidence in support of this hypothesis. We speculate that LDTg amylin receptors may be expressed on putative GABAergic interneurons, suggesting that LDTg amylin receptor activation could result in local inhibition of a variety of output neural pathways, including those projecting to the VTA. Future studies should therefore examine whether LDTg amylin receptor activation suppresses VTA activity in response to a food reward, and whether this outcome is in fact LDTg-GABA mediated.

Arguably one of the most important findings from the current data set is that acute blockade of LDTg amylin receptors attenuates the intake-suppressive effects of a systemic amylin receptor agonist. Though a significant attenuation of the intake-suppressive effects is not observed until the 24-hour time point, these data are comparable to a similar experiment performed in the VTA (36) in which effects were also only observed at 24 hours. In contrast to the VTA and LDTg, previous reports have shown that systemically delivered amylin agonists are able to activate area postrema amylin receptors more rapidly (23,42). Thus, there appears to be a temporal difference in systemic amylin agonists' action in distributed nuclei throughout the neuraxis that requires further investigation. Nevertheless, the data suggest that amylin receptor agonists administered systemically can access the LDTg, and thus LDTg amylin receptors may represent a preclinically relevant CNS population that can be targeted by peripherally administered amylin receptor ligands for the treatment of obesity. Importantly, the dose of AC187 used here was selected to be subthreshold for an effect on food intake when administered in the LDTg. However, future experiments should conduct dose-dependent analyses of LDTg AC187 on food intake. Furthermore, the longer-term physiological role of LDTg amylin receptor signaling for energy balance control is supported by our study examining the effects of LDTg amylin receptor knockdown. Virogenetic knockdown of LDTg CTR increased body weight and food intake, suggesting that endogenous amylin can access the LDTg and that LDTg amylin receptors exert chronic control over energy balance. Interestingly, binned increases in food intake were modest compared to binned increases in body weight, suggesting an unexplored contribution of decreased energy expenditure following LDTg amylin receptor knockdown.

The novel findings here support the hypothesis that amylin receptor signaling in the LDTg is important for food intake and body weight regulation. These data highlight the importance of focusing further attention on this understudied nucleus in the field of obesity research. We have identified a subset of amylin receptor–expressing cells in the LDTg that are GABAergic neurons, which allows for future dissection of the downstream neurons and nuclei that are presumably inhibited by LDTg amylin receptor activation. As the LDTg also expresses receptors for other energy balance–relevant hormones (16–21), future studies should explore how amylin signaling in the LDTg potentially interacts with other feeding-related signals to exert integrated control of energy balance and food reward.

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