Published in final edited form as:

Neuropharmacology. 2017 September 01; 123: 67–79. doi:10.1016/j.neuropharm.2017.05.024.

Amylin receptor activation in the ventral tegmental area reduces motivated ingestive behavior

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Abstract

Amylin is produced in the pancreas and the brain, and acts centrally to reduce feeding and body weight. Recent data show that amylin can act in the ventral tegmental area (VTA) to reduce palatable food intake and promote negative energy balance, but the behavioral mechanisms by which these effects occur are not fully understood. The ability of VTA amylin signaling to reduce intake of specific palatable macronutrients (fat or carbohydrate) was tested in rats in several paradigms, including one-bottle acceptance tests, two-bottle choice tests, and a free-choice diet. Data show that VTA amylin receptor activation with the amylin receptor agonist salmon calcitonin (sCT) preferentially and potently reduces intake of fat, with more variable suppression of sucrose intake. Intake of a non-nutritive sweetener is also decreased by intra-VTA administration of sCT. As several feeding-related signals that act in the mesolimbic system also impact motivated behaviors besides feeding, we tested the hypothesis that the suppressive effects of amylin signaling in the VTA extend to other motivationally relevant stimuli. Results show that intra-VTA sCT reduces water intake in response to central administration of the dipsogenic peptide angiotensin II, but has no effect on *ad libitum* water intake in the absence of food. Importantly, open field and social interaction studies show that VTA amylin signaling does not produce anxiety-like behaviors.

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Declaration of Interest: MRH and EGM-B receive funding from Zealand Pharma that was not used in support of these studies. MRH receives funding from Novo Nordisk that was not used in support of these studies. The authors declare no other competing financial interests.

Collectively, these findings reveal a novel ability of VTA amylin receptor activation to alter palatable macronutrient intake, and also demonstrate a broader role of VTA amylin signaling for the control of motivated ingestive behaviors beyond feeding.

Keywords

obesity; IAPP; reward; mesolimbic; macronutrient; diet

1. Introduction

Amylin is a pancreatic- and brain-derived peptide that reduces food intake and body weight by promoting satiation (Dobolyi, 2009; Li et al., 2015; Lutz, 2005; Lutz et al., 1995b). These energy balance effects of amylin are mediated through actions in the central nervous system (CNS) (Lutz et al., 1995a). Research on amylin's feeding effects has largely focused on the hindbrain (Lutz et al., 1998; Mollet et al., 2004; Potes et al., 2010) and hypothalamus (Chance et al., 1991; Dunn-Meynell et al., 2016; Turek et al., 2010), but recent findings support the ventral tegmental area (VTA) of the mesolimbic reward system as a novel and important nucleus that mediates the energy balance effects of amylin. Amylin receptor activation in the VTA is both physiologically and pharmacologically relevant for the control of food intake and body weight gain (Mietlicki-Baase et al., 2015a; Mietlicki-Baase et al., 2015b; Mietlicki-Baase et al., 2013), but the behavioral mechanisms underlying these effects are unresolved. As amylin-based pharmacotherapies are thought to be promising for treating obesity (Jorsal et al., 2016; Sadry and Drucker, 2013), a comprehensive understanding of the mechanisms by which VTA amylin signaling suppresses feeding is critical.

Human obesity is driven in part by the overconsumption of energy-dense, highly palatable foods (Meye and Adan, 2014; Rolls, 2007). Interestingly, VTA amylin signaling has particularly potent suppressive effects on the intake of palatable foods. For example, acute administration of an amylin receptor agonist directly into the VTA suppresses intake of a high-fat diet (HFD) at lower doses than those required for suppression of chow intake (Mietlicki-Baase et al., 2015b; Mietlicki-Baase et al., 2013). Furthermore, rats with AAV-mediated knockdown of VTA amylin receptors exhibit chronic hyperphagia and weight gain when maintained on HFD, with minimal to no effect on food intake or body weight in chow-fed rats (Mietlicki-Baase et al., 2015b). As HFD is a mixed-macronutrient diet that is elevated in both fat and refined carbohydrate (sucrose), it remains unclear whether amylin may affect intake of HFD based on reduced incentive salience for one or both of these macronutrients. Indeed, previous work suggests that systemic amylin can impact intakes of isolated macronutrients (Michel et al., 2007). However, this possibility has not been empirically tested.

Here, the effect of VTA amylin receptor activation on macronutrient intake is investigated in a series of one-bottle acceptance and two-bottle choice tests in which rats have access to carbohydrate and/or fat solutions. A free-choice diet model (la Fleur et al., 2010) is also used to evaluate the impact of VTA amylin signaling on food selection when animals have a simultaneous choice of pure fat, pure carbohydrate, and chow. To test the possibility that

VTA amylin receptor activation suppresses other motivated ingestive behaviors, water intake as well as intake of a non-nutritive sweetener are evaluated after VTA injection of an amylin receptor agonist. Finally, an important consideration is that some feeding-related peptides can induce anxiety-like behaviors in rodents (Anderberg et al., 2016; Gulec et al., 2010; To and Bagdy, 1999), and hypophagia can be induced by anxiety-like responses (Blasio et al., 2014; Strongman, 1965). Therefore, to rule out the possibility that the suppression of feeding/motivated behaviors by VTA amylin is due to anxiogenesis, the effect of VTA amylin receptor activation on anxiety-like behaviors is tested in two separate paradigms. Collectively, the results of these studies suggest that amylin signaling in the VTA has more potent suppressive effects on fat intake versus carbohydrate intake, and that motivated fluid intake is reduced by VTA amylin receptor activation. These suppressive effects on motivated ingestive behavior following VTA amylin receptor activation occur without producing anxiety-like behaviors, collectively revealing a more expansive role for mesolimbic amylin receptor signaling in modulating motivated behaviors beyond feeding.

2. Material and Methods

2.1. Animals

Adult male Sprague-Dawley rats (Charles River) were individually housed in hanging wire cages in a temperature- and humidity-controlled environment, under a 12h–12h light cycle. Except where noted, rodent chow (Purina 5001) and water were available *ad libitum*. Procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Experiments were conducted using a counterbalanced within-subjects design, except where noted. Treatments were separated by at least 48h.

2.2. Drugs

Salmon calcitonin (sCT; Bachem) was dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus) for central injections and in sterile 0.9% saline for peripheral injections. Angiotensin II (AngII; Bachem) and AC187 (Tocris) were dissolved in aCSF for central injections. Behaviorally relevant doses were selected from the literature (Daniels et al., 2005; Mietlicki et al., 2009; Mietlicki-Baase et al., 2015b; Mietlicki-Baase et al., 2013).

2.3. Surgeries

After one week of acclimatization, rats were anesthetized via IM injection of a cocktail of ketamine (90mg/kg), xylazine (2.7mg/kg), and acepromazine (0.64mg/kg) and placed into a stereotaxic apparatus. Rats were surgically implanted with a bilateral guide cannula (Plastics One; 26-ga) aimed at the VTA (guide cannula coordinates: ± 0.5 mm lateral to midline, 6.8mm posterior to bregma, 6.6mm ventral to skull; internal cannula aimed 8.6mm ventral to skull). For AngII experiments, rats also were implanted with a single guide cannula aimed at the lateral ventricle (LV; guide cannula coordinates: 0.9mm posterior to bregma, 1.6mm lateral to midline, 2.8mm ventral to skull; internal cannula extended 1.5–2.5mm beyond guide cannula, based on functional verification). Analgesia was provided for all surgical procedures (2mg/kg meloxicam, SC).

2.4. Verification of cannula placements

LV cannula placement was verified prior to behavioral testing by injecting a dipsogenic dose of AngII (10ng in 1µl aCSF) and measuring subsequent water intake. Animals passed this functional verification if they drank at least 5ml of water in the 30min post-injection period (Mietlicki et al., 2009). VTA cannula placements were verified post-mortem via injection of 100nl pontamine sky blue ink. A representative image of VTA cannula placement is shown in Figure 1A, based on coordinates of Paxinos and Watson (1998). Only data from animals with correct cannula placements were included in analyses.

2.5. Intake of fat and carbohydrate solutions

Rats were trained to consume macronutrient solution(s) for at least 4 days prior to initial testing. During training and testing, rats were deprived of food and water for 1.5h prior to a 60min access bout to macronutrient solution(s). No other food or water were available during this access bout. Buret access began 30min after lights on. On test days, rats received a unilateral intra-VTA injection of sCT (0.04µg) or vehicle (100nl aCSF) just before macronutrient access, and intakes of the solution(s) were measured every 10min for the 60min access period. The dose of sCT was selected from our previous work and is subthreshold for prolonged effects on feeding when delivered directly into the cerebroventricular system (Mietlicki-Baase et al., 2013). Fat solution consisted of Intralipid® (Baxter; soybean oil-based emulsion), while sucrose was used for the carbohydrate solution. Sucrose was made with deionized water (DIW), and Intralipid® was diluted with DIW as needed. Due to the within-subjects design of the experiments, it was ensured that at least one training (e.g., non-injection) day always preceded the next test day. Separate groups of rats were used for each of these fat/carbohydrate intake studies except where noted.

First, rats (n=7) had simultaneous access to two burets, one of which contained 20% Intralipid® (2 kcal/ml) and the other, 10% sucrose (0.4 kcal/ml). On test days, intakes of Intralipid® and sucrose were measured to the nearest 0.1 ml. To evaluate the effect on each macronutrient when no other solution was available, separate one-bottle acceptance tests were conducted in which rats (n=6–9 per macronutrient) had access to either 20% Intralipid® or 10% sucrose. Again on test days, intake was measured to the nearest 0.1ml.

Next, to eliminate the variable of differences in caloric density between solutions, one-bottle acceptance tests using isocaloric solutions of Intralipid® (10%, 1kcal/ml) or sucrose (25%, 1kcal/ml) were conducted. Rats (n=7) were initially trained for 4 days to consume one of these solutions (half assigned to each macronutrient), and then were tested in a within-subjects design. After 4 days of no macronutrient access, the same rats were switched to the opposite macronutrient solution, retrained for 3 days, and tested again in a within-subjects design. Intakes were measured to the nearest 0.1ml.

Finally, to control for volume of intake of each solution in a two-bottle test, rats (n=11) were given simultaneous access to solutions of Intralipid® (10%, 1 kcal/ml) and sucrose (10%, 0.4 kcal/ml) that were equally consumed under vehicle conditions. In this study, intake to the nearest 1ml was analyzed.

2.6. Saccharin intake

A subset of rats previously used for Intralipid®/sucrose two-bottle experiments were trained for at least 3 days to consume 0.05% saccharin. During training and subsequent test sessions, rats were deprived of food and water for 1.5h prior to access to a single buret of 0.05% saccharin. Buret access began 30min after lights-on and lasted for 60 min, during which food and water remained unavailable. Because of the high individual variability in saccharin intake in rats (Lu et al., 2005; Nachman, 1959), only animals that consumed at least 5ml of saccharin during the access period on the final two consecutive training days were tested (n=8). On test days, food and water were removed and saccharin was made available according to the same schedule as training. Shortly before buret access, each rat received a unilateral VTA injection of sCT (0.04µg) or vehicle (100nl aCSF). Saccharin intake was recorded to the nearest 0.1 ml every 10 min during the 60 min access period.

2.7. AngII-stimulated water intake

During the light phase, rats (n=7) had food removed from their cages and received a unilateral VTA injection of sCT (0.04µg) or its vehicle (100nl aCSF), followed by LV injection of either AngII (10ng) or its vehicle (1µl aCSF). Graduated burets containing tap water were made available immediately after LV injections; food remained unavailable. Water intake was measured to the nearest 1ml at 15, 30, 60, and 120 min.

2.8. Ad libitum water intake in the presence or absence of food

To test the effects of VTA amylin receptor activation on water intake when food is available, rats (n=10) were given a unilateral intra-VTA injection of sCT (0.04µg) or vehicle (100nl aCSF) just before lights off. Pre-weighed chow and a graduated buret containing tap water were returned to the cage. Food and water intakes were measured at 0.5, 1, 2, 4, and 24h, with water intake measured to the nearest 1ml. Crumb spillage was collected and was accounted for in food intake measurements.

A similar experiment in which food was unavailable was subsequently conducted. Rats (n=10) received unilateral VTA administration of sCT (0.04µg) or vehicle (100nl aCSF) just before lights off, but food was removed from the cage and only a graduated buret containing tap water was made available. Water intake was measured to the nearest 1ml at 0.5, 1, 2, 4, and 24h.

2.9. Open field testing

An open field test was used to evaluate whether VTA amylin receptor activation produces anxiety-like behavior (Crawley, 1985; Sestakova et al., 2013). Rats were habituated to open field boxes (76.2cm × 76.2cm) for 15min a day for 5 total days before experimental testing, including 3 consecutive habituation days leading up to the test day. These rats had previously been used for saccharin and/or macronutrient fluid intake testing. Habituation and testing were conducted under dim white light during the light phase, with a thin layer of beta chip bedding in the box to reduce glare. On test day, rats were deprived of food beginning 1h before lights on, and food remained unavailable until testing was complete. Beginning just after lights on, each rat received a unilateral VTA injection of either sCT (0.04µg) or vehicle (100nl aCSF) in a between-subjects design (n=5–7 per drug condition). One hour after each

rat was injected, it was placed in the open field box for 15min. Time spent in the inner zone [middle 25cm × 25cm square; (Bueno et al., 2007; Salim et al., 2010)] and number of entries to the inner zone were tracked and scored automatically using ANY-Maze video tracking software (Stoelting Co., version 4.99).

2.10. Social interaction testing

To evaluate potential anxiogenic effects of VTA amylin receptor activation in another paradigm, anxiety-like behavior in a social interaction task was monitored (File and Seth, 2003). The apparatus consisted of an open field box (76.2cm × 76.2cm) with a wire mesh enclosure (22.9cm long, 17.8cm wide, 16.5–17.8cm tall) inverted on one side of the box (long end 10.2cm from wall, short end 26.7cm from either side). An additional hanging wire cage was placed on top of the enclosure with a glass bottle inside, providing weight on top of the enclosure to prevent the stimulus rat from escaping. Test rats were allowed to explore to this setup during 10min habituation sessions, during which the enclosure was empty. On test day, the enclosure contained a novel male rat (stimulus animal; habituated to being in enclosure prior to test days).

Test rats were deprived of food beginning 1h before lights on, and food remained unavailable until testing was complete. All injections and testing were completed during the first half of the animals' light phase. Rats were given unilateral VTA injection of sCT (0.04µg) or vehicle (100nl aCSF) in a between-subjects experimental design (n=5–8 per drug condition). One hour after each rat was injected, it was placed in the social interaction apparatus for 10min and allowed to roam freely. The use of a wire mesh enclosure allowed the test rat to be exposed to various sensory cues from the stimulus rat, while not being able to directly access the animal. Activity during this time was video recorded using ANY-Maze software.

After testing was complete, videos were scored by two independent scorers. Social interaction was quantified by assessing the amount of time that the test rat spent in contact with the stimulus rat's enclosure; any part of the test rat's body in contact with the cage, except for tail-only contact, was counted. This information was processed for total percentage of time spent in social interaction, total number of interaction bouts, and average bout duration. For each rat, the scorers' values were averaged to obtain a single value for each dependent variable.

2.11. Free-choice diet

To test the impact of VTA amylin receptor activation on food selection, rats were maintained on a free-choice diet similar to that developed by la Fleur and colleagues (la Fleur et al., 2010). Animals had *ad libitum* access to water as well as three sources of energy: vegetable shortening (Crisco®, J.M. Smucker Company) as a pure fat; 30% sucrose (Sigma) as a pure carbohydrate; and standard rodent chow, for at least 3 weeks prior to and throughout experimental testing. Baseline 24h intakes of each source of energy, along with 24h body weight gain, were measured prior to experimental testing. To evaluate whether VTA amylin receptor signaling is required for the intake-suppressive effects of peripheral sCT in animals on this free-choice diet, rats were pretreated with unilateral VTA injection of either the

amylin receptor antagonist AC187 (0.1µg) or vehicle (100nl aCSF) approximately 1h before lights off, followed just before lights off by an IP injection of either sCT (5µg/kg) or vehicle (1ml/kg 0.9% NaCl). The dose of AC187 used was intentionally selected to be subthreshold for an effect on feeding when administered to the VTA (Mietlicki-Baase et al., 2015a; Mietlicki-Baase et al., 2013). Data were evaluated from rats exhibiting a consistent food preference, defined as those who chose to eat the most calories from the same energy source (e.g., either from shortening or from sucrose) at both their final complete pre-testing baseline reading as well as under vehicle/vehicle conditions during testing. Sixteen rats fully completed testing and had verified VTA cannula placement. Data from 12 of these rats (n=6 fat-preferring, n=6 sucrose-preferring) were included in our final analyses. Of the remaining 4 animals, 3 had inconsistent food preferences, and 1 rat consumed the most calories from chow.

2.12. Statistical analyses

Data were analyzed using Statistica (Statsoft), with the α level set at $p < 0.05$. For fluid and food intake studies, data were analyzed using a repeated measures ANOVA accounting for the within-subjects experimental design, with drug and time as factors. When multiple macronutrient solutions (e.g., Intralipid® and sucrose) or multiple energy sources (e.g., shortening, sucrose, and chow in free-choice diet experiments) were available simultaneously within a single experiment, the ANOVA also accounted for food/fluid type. For free-choice diet studies, separate ANOVAs were run for fat-preferring and for sucrose-preferring animals for both food intake and body weight gain. To evaluate baseline differences between fat-preferring and sucrose-preferring rats, separate ANOVAs for the percent of daily kcal from each food were run for each preference group, with food type as a within-subjects factor. Baseline total energy intake and body weight gain for these rats were evaluated by ANOVA with preference group as a between-subjects factor. Finally, data from the open field and social interaction experiments were analyzed by one-way ANOVA, with drug treatment as a between-subjects factor. Significant results from ANOVAs were probed using Student-Newman-Keuls post-hoc analyses. All data are expressed as mean $\pm$ SEM.

3. Results

3.1. VTA amylin receptor activation suppresses intake of palatable macronutrient solutions, with more consistent intake-suppressive effects on fat than carbohydrate

Previous studies demonstrate that VTA amylin receptor activation suppresses intake of a palatable HFD (Mietlicki-Baase et al., 2015b). However, HFD is a mixed-macronutrient diet containing 60% of kilocalories from fat, 20% from carbohydrate, and 20% from protein. It is possible that VTA amylin receptor activation may have more potent suppressive effects on the intake of a particular palatable macronutrient (either fat or carbohydrate). To address this possibility, the effects of VTA amylin receptor activation on intake of fat and carbohydrate solutions were evaluated. Specifically, fat (Intralipid®, a soybean oil-based emulsion) and/or carbohydrate (sucrose) solutions were made available either alone (one-bottle acceptance testing) or simultaneously (two-bottle choice testing).

First, rats were presented with 20% Intralipid® (2 kcal/ml) and 10% sucrose (0.4 kcal/ml) in a 1 hour, two-bottle choice test. These concentrations were selected such that the percentage of energy consumed from the solutions under vehicle conditions (fat: $69.0 \pm 6.1\%$, sucrose: $31.0 \pm 6.1\%$) roughly approximated the proportions of kcal derived from fat and carbohydrate in HFD (60% fat, 20% carbohydrate). Intakes of the solutions were tested after VTA injection of the amylin receptor agonist sCT (0.04µg) or vehicle (100nl aCSF, see Figure 1A). When energy intake (kcal) of each solution was evaluated, repeated measures ANOVA revealed a significant drug $\times$ macronutrient $\times$ time interaction ($F_{5,30}=3.53$, $p=0.01$; Figure 1B). Post hoc analyses showed that cumulative fat intake was reduced by sCT from 20–60min, whereas sucrose intake was decreased only at 50–60min (all $p<0.05$), suggesting that in this paradigm, fat intake is more rapidly suppressed by VTA amylin receptor activation than is sucrose intake.

Next, to test the impact of VTA amylin receptor activation on intake when only a single macronutrient solution is available, one-bottle acceptance tests using these same concentrations of 20% Intralipid® and 10% sucrose were conducted in separate groups of rats. The results here were similar to those obtained in the two-bottle choice test. As shown in Figure 1C, in rats with access to a single bottle containing 20% Intralipid®, VTA sCT (0.04µg) reduced intake of the solution (drug $\times$ time interaction, $F_{5,40}=5.04$; $p=0.001$; vehicle versus sCT, $p<0.05$ at all times). However, intra-VTA sCT had no significant effect on intake in rats given access to a bottle of 10% sucrose (Figure 1D; main effect of drug, $F_{1,5}=0.06$, $p=0.81$; drug $\times$ time interaction, $F_{5,25}=1.22$, $p=0.33$).

To rule out the possibility that differences in caloric density between solutions might explain the stronger reduction in fat intake after VTA amylin receptor activation, the effect of intra-VTA sCT on intake of isocaloric solutions of Intralipid® (10%, 1 kcal/ml) or sucrose (25%, 1 kcal/ml) was analyzed. In rats with access to 10% Intralipid®, VTA sCT (0.04µg) significantly reduced energy intake of the fat solution at all times tested (Figure 2A; main effect of sCT, $F_{1,6}=26.75$, $p=0.002$; drug $\times$ time interaction, $F_{5,30}=8.76$, $p=0.00003$; post hoc for vehicle versus sCT, $p<0.05$ at all times). In contrast, although a main effect of sCT was observed for rats consuming 25% sucrose (Figure 2B; $F_{1,6}=11.05$, $p=0.02$), the drug $\times$ time interaction was not significant ($F_{5,30}=2.34$, $p=0.07$). To make a more direct comparison between macronutrients, the percent suppression of intake for each macronutrient by VTA sCT compared to intake under vehicle conditions was calculated. Intralipid® intake was suppressed by $37.45 \pm 6.60\%$, compared to $19.42 \pm 5.16\%$ suppression of sucrose intake; however, this difference did not reach statistical significance by paired t-test (Figure 2C).

These studies addressed the intake of fat and carbohydrate solutions with regards to energy intake (kcal), but the concentrations of each solution used do not account for possible differences in the relative palatability of the solutions, or the animal's preference for one solution over the other. To address this issue, concentrations of Intralipid® (10%) and sucrose (10%) solutions were determined such that, under vehicle conditions, rats would consume *similar volumes* of each solution when presented simultaneously. As shown in Figure 2D, when rats given concurrent access to 10% Intralipid® and 10% sucrose received an intra-VTA injection of sCT (0.04µg) just prior to the access period, the volume of Intralipid® intake was suppressed, but sucrose intake was unaffected (drug $\times$ macronutrient

interaction, $F_{1,10}=13.80$, $p=0.004$; vehicle/Intralipid® versus sCT/Intralipid®, $p<0.05$; vehicle/sucrose versus sCT/sucrose, $p>0.05$). As expected, no differences in volume of macronutrient intakes were observed under vehicle conditions (vehicle/Intralipid® versus vehicle/sucrose, $p>0.05$). This provides evidence that when rats are given concentrations of Intralipid® and sucrose that are similarly consumed by volume, VTA amylin receptor activation selectively reduces fat intake.

3.2. Amylin receptor activation in the VTA reduces intake of a palatable, non-nutritive sweetener

All of the present macronutrient studies use caloric solutions, making it impossible to disentangle the hypophagic effects of VTA amylin receptor activation from motivational effects less tied to the nutritive value of the food or solution. To test whether the intake-suppressive effects of VTA amylin receptor activation extend to non-nutritive but motivationally relevant stimuli, the ability of intra-VTA sCT to suppress intake of saccharin (0.05%), a non-nutritive but palatable sweetener, was examined. When rats received a direct VTA injection of sCT (0.04µg) just prior to a 60min saccharin access period, intake of saccharin was decreased (Figure 3A; drug $\times$ time interaction, $F_{5,35}=3.09$, $p=0.02$; vehicle versus sCT, $p<0.05$ at all times). This provides the first evidence that VTA amylin receptor activation can reduce intake of palatable but non-nutritive substances.

3.3. Stimulated water intake is decreased by VTA amylin receptor activation

The finding that saccharin intake was suppressed by VTA sCT raises the question of whether this effect extends to intake of other non-caloric but motivationally relevant stimuli. To test whether VTA amylin receptor activation suppresses stimulated water intake, rats were given an intra-VTA pretreatment of sCT (0.04µg) or vehicle (100nl aCSF), followed by LV injection of the dipsogenic hormone AngII (10ng) or vehicle (1µl aCSF). Subsequent water intake was measured for 2h, during which time food was unavailable. As shown in Figure 3B, a significant sCT $\times$ AngII interaction was observed ($F_{1,6}=10.62$, $p=0.02$). Post hoc analyses revealed that, as anticipated, the water intake of rats treated with AngII was greater than rats who did not receive AngII (vehicle/AngII or sCT/AngII versus vehicle/vehicle or sCT/vehicle, all $p<0.05$). Furthermore, drinking was reduced in AngII-treated rats pretreated with VTA sCT (vehicle/AngII versus sCT/AngII, $p<0.05$), suggesting that VTA amylin receptor activation suppresses motivated water intake.

3.4. Ad libitum water intake is only suppressed by VTA amylin signaling in the presence, but not the absence, of food

The experiments described thus far all tested fluid intake, bringing up the possibility that VTA amylin receptor activation may generally suppress intake of any fluid. Although the fact that intakes of different types of macronutrient solutions are differentially impacted by VTA sCT argues against this possibility, to directly test the effect of VTA amylin receptor activation on unstimulated water intake, water intake was measured over the course of a 24h test period in VTA sCT-treated rats with or without simultaneous food access.

When both food and water were available, intra-VTA sCT (0.04µg) significantly suppressed water intake (Figure 3C; drug $\times$ time interaction, $F_{4,36}=11.99$, $p=0.000003$; vehicle versus

sCT, $p < 0.05$ from 1–24h). Importantly, and consistent with prior studies (Mietlicki-Baase et al., 2013), feeding was also suppressed by VTA amylin receptor activation (Figure 3D; drug $\times$ time interaction, $F_{4,36} = 6.29$, $p = 0.0006$) at the same times that reduced water intake was observed (vehicle versus sCT, $p < 0.05$ from 1–24h), suggesting that the decreases in water intake could be secondary to sCT-induced hypophagia. Indeed, when the ratios of water (ml)/food (g) intake over the 24h test period are calculated, there is no significant difference between drug conditions (Figure 3E; $F_{1,9} = 2.23$, $p = 0.17$). Therefore, these findings suggest that the observed decreases in water intake are likely tied to the hypophagic effect of sCT, reflecting reduced prandial drinking.

In a separate experiment, water intake was measured after VTA sCT (0.04 μ g) when food was unavailable. These data are depicted in Figure 3F. In contrast to the suppression of water intake that occurs when food is present, VTA sCT had no effect on water intake in the absence of food. No significant main effect of drug condition ($F_{1,9} = 0.13$, $p = 0.73$) or drug $\times$ time interaction ($F_{4,36} = 0.21$, $p = 0.93$) were observed. Thus, the collective body of data provides evidence that VTA sCT does not ubiquitously suppress all fluid intake.

3.5. VTA amylin receptor activation does not increase anxiety-like behaviors

To rule out the possibility that the reductions in motivated ingestive behavior observed after VTA sCT are due to anxiogenic effects, anxiety-like behaviors after VTA amylin receptor activation were assessed in two separate paradigms: open field and social interaction. In the open field test, rats' behavior (time spent in and entries to inner zone) was measured after intra-VTA sCT (0.04 μ g) or vehicle (100nM aCSF). Reduced time in the inner zone is thought to indicate increased anxiety-like behavior (Crawley, 1985; Sestakova et al., 2013). However, no significant changes occurred in the percentage of time spent in the inner zone (Figure 4A; $F_{1,10} = 1.06$, $p = 0.33$) or the number of entries to the inner zone (Figure 4B; $F_{1,10} = 0.39$, $p = 0.55$) in sCT-treated versus vehicle-treated animals. To ensure that the lack of any anxiogenic effects of VTA sCT was not specific to behavior in the open field test, social interaction was used as another method to analyze anxiety-like responses after VTA amylin receptor activation. Social interaction is another well-established measure of anxiety-like behavior in rodents (Cheeta et al., 2000; File and Hyde, 1978; File et al., 2000; File and Seth, 2003), with decreased interaction thought to reflect increased anxiety-like responses (File and Seth, 2003). In a social interaction test, VTA injection of sCT produced no change in the dependent variables assessed in the paradigm: percentage of time during the test period spent interacting with the stimulus animal (Figure 4C; $F_{1,11} = 0.21$, $p = 0.66$), the number of social interaction bouts (Figure 4D; $F_{1,11} = 1.85$, $p = 0.20$), the mean duration of each interaction bout (Figure 4E; $F_{1,11} = 2.82$, $p = 0.12$), or the latency to the first interaction (Figure 4F; $F_{1,11} = 0.99$, $p = 0.34$). Collectively, these results suggest that VTA amylin receptor activation does not produce anxiogenic effects, at least in these paradigms.

3.6. Effects of VTA amylin receptor activation on food selection in a free-choice diet paradigm depend on baseline food preferences

The present studies examining intake of pure macronutrient solutions suggest that VTA amylin receptor activation has more rapid and possibly more potent suppressive effects for intake of fat compared to intake of carbohydrate (sucrose). However, these experiments

examined intake of solutions available for a relatively brief access period each day. To evaluate the impact of VTA amylin receptor activation on food choice when animals had consistent access to fat or sucrose, rats were maintained on a free-choice diet in which fat (vegetable shortening), 30% sucrose, and standard rodent chow were available ad libitum.

First, to ensure that animals were consistently eating from all three energy sources, baseline intakes of each food were measured prior to experimental testing. Because the foods are different caloric densities (vegetable shortening: 9.167 kcal/g; 30% sucrose: 1.2 kcal/ml; chow: 3.35 kcal/g), intake of each food was converted to kcal to allow for accurate comparisons. Interestingly, we noted that most rats consistently derived the highest percentage of their 24h caloric intake from either shortening or sucrose. The baseline intakes of these “fat-preferring” and “sucrose-preferring” rats are shown in Figure 5A–B. On average, the rats in each group consume the majority of their daily calories from their preferred food (main effect of food type in fat-preferring rats, $F_{2,10}=17.73$, $p=0.0005$, intake of fat compared to chow or sucrose, $p<0.05$; main effect of food type in sucrose-preferring rats, $F_{2,10}=11.80$, $p=0.002$, intake of sucrose compared to chow or fat, $p<0.05$). However, there are no differences between fat-preferring and sucrose-preferring rats in terms of total daily caloric intake (Figure 5C; $F_{1,10}=0.06$, $p=0.81$) or daily body weight change (Figure 5D; $F_{1,10}=0.01$, $p=0.92$).

Given this interesting pre-existing difference we observed in macronutrient preference, we investigated the role of VTA amylin signaling on food intake and body weight gain in fat- or sucrose-preferring rats maintained on free-choice diet. Animals received a unilateral VTA pretreatment of the amylin receptor antagonist AC187 (0.1µg) or vehicle (100nl aCSF) followed by systemic administration of sCT (5µg/kg, IP) or vehicle (1ml/kg 0.9% NaCl), and intakes of each energy source were measured. The dose of AC187 was selected to have no effect on total energy intake or body weight gain over the 24h test period (Mietlicki-Baase et al., 2015a; Mietlicki-Baase et al., 2013). As any potential amylin-based pharmacotherapy for the treatment of obesity would likely be administered systemically, IP administration of sCT was used to begin to establish possible clinical relevance of these effects, while pharmacologically blocking VTA amylin receptors to determine the specific contribution of VTA amylin signaling to these effects. The data from fat-preferring rats are shown in Figure 6, while the results in sucrose-preferring animals are in Figure 7.

In fat-preferring rats, statistically significant interactions were observed for AC187 $\times$ food $\times$ time ($F_{6,30}=2.46$, $p=0.047$) and sCT $\times$ food $\times$ time ($F_{6,30}=7.07$, $p=0.00009$). Planned comparisons revealed that, consistent with our fluid macronutrient data, intake of fat was most rapidly suppressed by sCT, with significant reductions in shortening intake in sCT-treated rats beginning at 3h post-injection and persisting throughout the remainder of the 24h test (Figure 6A; veh/sCT or AC187/sCT, $p<0.05$ versus veh/veh). Chow intake (Figure 6B) and sucrose intake (Figure 6C) were also suppressed by sCT in fat-preferring rats (veh/sCT or AC187/sCT versus veh/veh, $p<0.05$), but these effects were not observed until 6h or even 24h post-injection. Interestingly, VTA amylin receptor blockade with AC187 significantly attenuated the suppression of shortening intake at 6h and 24h post-injection (veh/sCT versus AC187/sCT, $p<0.05$), but had no impact on the suppression of chow intake or sucrose intake by sCT in fat-preferring rats. As expected, total energy intake was suppressed by peripheral

sCT (Figure 6D; sCT $\times$ time interaction, $F_{3,15}=13.11$, $p=0.0002$; planned comparisons between veh/veh and veh/sCT, $p<0.05$ from 3–24h; between veh/veh and AC187/sCT, $p<0.05$ from 6–24h), as was body weight gain over the 24h post-injection (Figure 6E; AC187 $\times$ sCT interaction, $F_{1,5}=10.80$, $p=0.02$; veh/veh versus veh/sCT or AC187/sCT, $p<0.05$). VTA pretreatment with AC187 also partially blocked these effects at 24h (for both total kcal and body weight gain, veh/sCT versus AC187/sCT, $p<0.05$).

Effects on overall energy intake and body weight gain were similar in sucrose-preferring rats. Systemic delivery of sCT reduced total caloric intake (Figure 7D; sCT $\times$ time interaction $F_{3,15}=22.69$, $p=0.000008$; planned comparisons between veh/veh and veh/sCT or AC187/sCT, $p<0.05$ from 3–24h) and 24h body weight gain (Figure 7E; main effect of AC187, $F_{1,5}=11.44$, $p=0.02$; main effect of sCT, $F_{1,5}=14.51$, $p=0.01$; AC187 $\times$ sCT interaction, $F_{1,5}=5.89$, $p=0.06$; planned comparisons, veh/veh versus veh/sCT, $p<0.05$). Again, these effects were blocked by AC187 in the VTA (total caloric intake, planned comparisons between veh/sCT and AC187/sCT, $p<0.05$ at 24h; body weight gain, planned comparisons between veh/sCT and AC187/sCT, $p=0.053$). However, different patterns of effects were observed for the impact of peripheral sCT on intake of the various food choices, as well as the ability of VTA amylin receptor blockade to influence intake of each food. Here, intake of shortening was again quickly reduced by sCT (Figure 7A; sCT $\times$ food $\times$ time, $F_{6,30}=3.81$, $p=0.006$; planned comparisons between veh/veh and veh/sCT or AC187/sCT, $p<0.05$ from 3–24h). However, an early reduction in sucrose intake also occurred (Figure 7C; planned comparisons between veh/veh and veh/sCT or AC187/sCT, $p<0.05$ from 3–24h). Chow intake was decreased by sCT beginning at 6h post-injection (Figure 7B; planned comparisons between veh/veh and veh/sCT, $p<0.05$ from 6–24h; veh/veh versus AC187/sCT, $p<0.05$ at 24h). In contrast to the specific ability of VTA amylin receptor antagonism to attenuate suppression of fat intake in fat-preferring rats, a broader effect of AC187 is observed in sucrose-preferrers, such that the suppression of 24h intake of each of the foods by sCT is partially reversed by VTA AC187 (for all 3 foods, planned comparisons between veh/sCT and AC187/sCT, $p<0.05$ at 24h). Together, these results support previous data indicating that VTA amylin receptor activation has an important role in the control of food intake and body weight, and consistent with other studies in this paper, suggest that suppression of fat intake occurs relatively rapidly after amylin receptor activation. Additionally, these data hint at an intriguing role of individual differences in the role of VTA amylin receptor signaling in the control of food intake and food choice, with more selective effects on fat intake observed in fat-preferring individuals as opposed to the broader effects on multiple types of foods observed in rats that prefer sucrose.

4. Discussion

The ability of amylin to promote negative energy balance through actions in the mesolimbic reward system is just beginning to be investigated, and we still lack a comprehensive understanding of its mechanisms of action. Given the interest in amylin-based pharmacotherapies as potential treatments for obesity (Sadry and Drucker, 2013), it is extremely important to fully elucidate the behavioral effects of amylin, not only for energy balance control but also for other motivated behaviors. The experiments presented here reveal that VTA amylin signaling preferentially suppresses fat intake versus sucrose intake,

supporting a novel ability of mesolimbic amylin receptor activation to selectively affect macronutrient intake and selection. Furthermore, these studies support the hypothesis that VTA amylin receptor activation has broader effects on other types of motivated ingestive behavior, such as stimulated water intake. Importantly, the effects of VTA amylin receptor activation on energy balance control and motivated ingestive behavior appear to occur independent of anxiety-like behaviors.

4.1. VTA amylin receptor activation potentially reduces fat intake

Previous research from our group has demonstrated the ability of VTA amylin receptor activation to suppress intake of palatable foods such as HFD and sucrose (Mietlicki-Baase et al., 2015b; Mietlicki-Baase et al., 2013), but had not examined the possibility that mesolimbic amylin signaling might selectively reduce intake of an isolated macronutrient when animals were given a choice of palatable fat or carbohydrate solutions. Two-bottle choice tests demonstrated greater effects of VTA sCT on fat intake compared to sucrose intake. Indeed, VTA sCT suppressed fat intake either exclusively (10% Intralipid®/10% sucrose) or more rapidly and durably than sucrose intake (20% Intralipid®/10% sucrose). The neurobiological mechanisms underlying this effect are unclear, but may relate to the consequences of the orosensory input from the stimuli. Palatable gustatory stimuli, including both sucrose and fat, elicit dopamine release in the nucleus accumbens (Hajnal et al., 2004; Liang et al., 2006; Roitman et al., 2008). At least for sucrose, this occurs in a concentration-dependent manner (Hajnal et al., 2004; Norgren et al., 2006). We have shown previously that random delivery of sucrose pellets elicits a rapid increase in phasic dopamine in the nucleus accumbens core, and that the longer-term hypophagic effects of VTA amylin signaling are mediated in part by a reduction in accumbens core dopamine signaling (Mietlicki-Baase et al., 2015b). As the dopaminergic response to sucrose in the core is rapid, yet suppression of sucrose intake in the present studies was delayed, this raises a few possibilities. First, it is possible that the fat solution elicited greater dopamine release in the nucleus accumbens than did sucrose, and that VTA sCT may have more potent intake-suppressive effects on a stimulus that produces greater dopamine release. Second, it may be that these consequences of VTA amylin receptor activation are mediated by changes in levels of dopamine, or even another second-order downstream neurotransmitter in a different nucleus. The comparative effects of different concentrations of Intralipid® and sucrose on accumbens dopamine release, and the ability of VTA amylin signaling to suppress this release, should be addressed in future studies.

Alternatively, post-ingestive effects could mediate differences in the effects of VTA amylin on fat and sucrose intake. Fat ingestion and carbohydrate ingestion exert very different effects on insulin and blood glucose levels, with carbohydrates producing greater increases in these measures than fat (Bottger et al., 1973; Shin et al., 2009). Additionally, we have shown in the present experiments that VTA sCT suppressed intake of saccharin, a non-nutritive sweetener. Saccharin intake also has rapid effects on plasma insulin levels but does not produce significant changes in blood glucose (Tordoff and Friedman, 1989). It is possible that VTA amylin receptor activation may generally have more potent intake-suppressive effects on stimuli that produce less drastic changes to insulin/blood glucose (e.g., fat, saccharin).

Another possibility involves the effects of the various macronutrients/tastants on the release of other gut and pancreatic hormones. Oral gavage of glucose produces changes in gut hormones such as glucagon-like peptide-1 that are not recapitulated by oral gavage of non-nutritive sweeteners (Fujita et al., 2009). Therefore it may be that the particular constellation of changes in blood glucose, insulin, and other gut/pancreatic hormones influences the ability of VTA amylin receptor activation to suppress intake of the solution. This possibility may relate to changes in amylin itself as a result of ingestion of the macronutrient solutions. For example, if fat ingestion produced more rapid increases in peripheral amylin release than carbohydrate, this could “boost” the amylin signal to the brain. However, studies that have examined macronutrient-specific effects on plasma amylin are extremely limited and have produced varying results (Eller et al., 2008; Martin et al., 2014; Martin et al., 2010). A key consideration in these effects is the particular carbohydrate or fat used in the test. Different types of carbohydrates or fats can produce diverging neurobiological consequences (Dela Cruz et al., 2015; Hryhorczuk et al., 2016) highlighting the need to conduct further studies investigating whether the effects observed in the present studies are reproduced with a variety of palatable fats and carbohydrates.

Current data from the free-choice diet paradigm support the notion that VTA amylin receptor activation more rapidly suppresses fat intake compared to sucrose intake, but also reveal an unexpected effect of individual differences in baseline food preference. Baseline food intake data revealed subgroups of rats that consistently preferred to get the majority of their daily energy intake from either shortening or sucrose. Intriguingly, no overall differences in daily caloric intake or body weight gain were observed between groups; the difference was solely in the source of energy. However, intake of each food was differentially affected by the experimental treatments in fat-preferring and sucrose-preferring animals. In both groups, intakes of all three foods (shortening, chow, and sucrose) were reduced by peripheral sCT, with the difference of an earlier suppression of sucrose intake in sucrose-preferring rats. Furthermore, while VTA AC187 treatment selectively attenuated the shortening intake-suppressive effect of sCT in fat-preferring rats, broader effects were observed in sucrose-preferring animals, where VTA amylin receptor blockade significantly attenuated the sCT-induced suppression of all food types. These results highlight the critical but often overlooked variable of individual differences in food intake studies, and suggest that baseline food preference may interact with VTA amylin receptor signaling to produce differential effects on the intakes of specific foods. It is important to note that VTA AC187 did not completely block the intake-suppressive effects of peripheral sCT. This is likely due to the ability of a systemically administered amylin receptor agonist to activate amylin receptors in a variety of CNS nuclei, such as the area postrema (Lutz et al., 1998) and lateral dorsal tegmental nucleus (Reiner et al., 2017). Nevertheless, the fact that VTA-specific amylin receptor blockade attenuated the hypophagic effects of peripheral sCT underscores the critical contribution of VTA amylin signaling in the overall anorectic effects of a systemic amylin receptor agonist.

4.2. Amylin receptor activation in the VTA reduces intake of motivationally relevant stimuli without producing anxiety-like effects

Our results show for the first time that VTA amylin receptor activation is able to suppress intake of non-nutritive but motivationally relevant ingestants. Injection of sCT directly into the VTA reduced intake of saccharin, a non-nutritive sweetener. As saccharin still represents a gustatory stimulus, we also tested the effects of VTA sCT on water intake. Interestingly, amylin receptor activation in the VTA had no effect on unstimulated water intake when food was unavailable, but significantly suppressed drinking stimulated by intracerebroventricular injection of AngII. Central AngII is thought to increase the motivational value of water (Rolls et al., 1972), supporting the hypothesis that VTA amylin receptor activation suppresses intake of motivationally relevant ingesta. As mentioned earlier, VTA amylin receptor activation decreases dopaminergic signaling in the accumbens core (Mietlicki-Baase et al., 2015b). Dopamine has well-established roles in reward-related processes (Baik, 2013; Meye and Adan, 2014; Vucetic and Reyes, 2010), and the results observed for saccharin intake and stimulated drinking may also be mediated by a blunting of dopamine by VTA amylin. Dopamine in the accumbens can reflect the changing motivational value of a stimulus relative to physiological state. For example, dopamine in response to the taste of salt is higher when an animal is sodium-depleted (Cone et al., 2016). VTA amylin signaling may impinge on this process of dopaminergic stimulus encoding to impact intake. Interestingly, changes in the dopamine response to salt as a function of repletion/depletion state appear to be downstream of activity in the lateral hypothalamus (LH) (Cone et al., 2016). The LH projects monosynaptically to the VTA (Phillipson, 1979; Saper et al., 1979) and produces amylin (Li et al., 2015). Therefore, it is possible that LH-derived amylin acts at VTA amylin receptors to reduce the rewarding value of a stimulus by suppressing dopamine, consequently suppressing intake. This is a fascinating possibility that needs to be addressed empirically in future experiments. Furthermore, it will be informative to more extensively evaluate the effects of VTA amylin signaling on mixtures of foods/sweeteners. Our previously published finding that VTA sCT suppresses intake of a high-fat/high-sugar diet (Mietlicki-Baase et al., 2015b), supporting the idea that intake of mixed foods can also be reduced by amylin receptor activation. Given the current findings that VTA sCT reduces intake of fat solutions and, separately, of saccharin, it would be interesting to assess the intake-suppressive effects of VTA amylin receptor activation on a saccharin-sweetened fat solution.

Anxiety-like responses can cause a reduction in feeding (Blasio et al., 2014; Strongman, 1965), making it important to separate hypophagia from alterations in anxiety. Moreover, as amylin-based compounds are thought to be leading candidates for the development of new anti-obesity pharmaceuticals (Jorsal et al., 2016; Sadry and Drucker, 2013), it will be critical to ensure that such pharmacotherapies do not cause anxiogenic or other mood-altering effects that would limit or prevent their use. Surprisingly, very few papers have investigated any potential anxiety-like effects of amylin or amylin receptor agonists. From the limited body of literature available, amylin agonists do not seem to have anxiogenic effects in rats. Injection of sCT into the periaqueductal gray of rats had no impact on anxiety or corticosterone levels (Aboufatima et al., 1999), and systemic administration of amylin likewise did not change plasma corticosterone (Koopmans et al., 1991; Smeltzer et al.,

2012). Publications on human patients taking the amylin analog pramlintide also report no anxiety-like effects (Ravussin et al., 2009) and inconsistent effects on plasma cortisol levels (Amiel et al., 2005; Schmitz et al., 1997). Our data from social interaction and open field testing presented here are consistent with these findings, and indicate that VTA amylin receptor activation in rats is not anxiogenic. Nevertheless, anxiety-like responses to peripheral or central/intraparenchymal amylin receptor agonists are not well-studied, and represent an area of research with potentially important implications for amylin-based pharmacotherapies.

The studies presented in this paper used sCT, a well-established amylin receptor agonist (Lutz et al., 2000), as a pharmacological tool to activate amylin receptors in the VTA. Although sCT has extremely high affinity for (Beaumont et al., 1993) and ability to activate amylin receptors (Christopoulos et al., 1999; Hay et al., 2006), it also can activate the calcitonin receptor alone (without RAMP heterodimerization) (Christopoulos et al., 1999; Hay et al., 2006). However, research from our group has shown that delivery of amylin itself into the VTA of rats suppresses feeding (Mietlicki-Baase et al., 2015a), and in other reward-relevant nuclei such as the lateral dorsal tegmental nucleus, the effects of direct injection of sCT are largely recapitulated by direct injection of amylin itself (Reiner et al., 2017). These results suggest that, although sCT may have the capacity to activate other calcitonin-family receptors, the energy balance-relevant effects are likely attributable to amylin receptor activation.

4.3. Conclusions

Collectively, the data presented here reveal that VTA amylin signaling suppresses palatable food intake as well as other motivated ingestive behaviors. These results add to the growing body of literature showing that signals studied in the context of feeding and body weight regulation can affect a variety of motivated behaviors through actions in the mesolimbic reward system (Schmidt et al., 2016; Shirazi et al., 2013). Our findings provide evidence for a novel role of VTA amylin receptor signaling in macronutrient intake and preference, supporting the role of mesolimbic amylin signaling in energy balance control. The finding that fat intake is more potently affected by VTA amylin signaling than is sucrose intake suggests that an amylin-based pharmacotherapy for obesity might be able to be coupled with specific dietary recommendations to produce greater weight loss. However, the results of the free-choice diet experiments demonstrate an intriguing effect of baseline food preferences on the ability of amylin to suppress intake of particular foods. With the growing interest in individualized medicine for obesity and related comorbidities (Camilleri and Acosta, 2016; Kaul and Ali, 2016; Reddon et al., 2016), these results also underscore the importance of recognizing preexisting individual differences when identifying novel treatment strategies for obesity.

Acknowledgments

We thank Holly Dow for her advice on the social interaction study. Technical assistance for these experiments was provided by Brianne Jeffrey, Rinzin Lhamo, and Gregory Sousa.

Funding: This work was supported by the National Institutes of Health [grant numbers DK103804 (EGM-B), DK105858 (DJR), DA039308 (MEW), DA037897 and DA039393 (HDS), DK096139 and DK105155 (MRH)].

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Highlights

- VTA amylin receptor activation more potently reduces intake of fat than of sucrose
- VTA amylin signaling reduces saccharin intake and stimulated water intake
- Effects of VTA amylin on macronutrient intake depend on baseline food preferences
- VTA amylin receptor activation does not induce anxiety-like responses

Figure 1. VTA amylin receptor activation rapidly decreases fat intake, with later suppression of sucrose intake

A representative coronal rat brain section (40μm) showing the placement of VTA injection is shown in (A), with the black arrow indicating ink within the VTA. In a two-bottle test (B), VTA injection of the amylin receptor agonist sCT (0.04μg) reduced intake of both 20% Intralipid® and 10% sucrose, with a more rapid effect on intake of the fat solution (n=7). When separate one-bottle acceptance tests were conducted with the same concentrations of Intralipid® (C; n=9) and of sucrose (D; n=6), intra-VTA sCT (0.04μg) reduced intake of the Intralipid® solution but had no effect on sucrose intake. Data are shown as mean ± SEM. The key applies to panels B–D. *, p<0.05 compared to Intralipid®/vehicle treatment; &, p<0.05 compared to sucrose/vehicle treatment.

Figure 2. Amylin receptor activation in the VTA has robust suppressive effects on fat intake compared to sucrose intake for isocaloric or equally consumed solutions

When rats ($n=7$) had access to a 10% Intralipid® solution (A), a significant time $\times$ drug interaction was observed such that VTA sCT ($0.04\mu\text{g}$) suppressed intake of the fat solution at all times tested. In contrast, when rats were provided an isocaloric (1 kcal/ml) 25% sucrose solution (B), a main effect of sCT ($0.04\mu\text{g}$) was observed with no significant time $\times$ drug interaction. The percentage by which intake was suppressed by sCT was greater for Intralipid® than for sucrose, although this was not statistically significant (C). In a separate two-bottle test (D; $n=11$), in which the concentrations of Intralipid® (10%) and sucrose (10%) were chosen such that they were equally consumed under vehicle conditions, VTA administration of sCT ($0.04\mu\text{g}$) selectively reduced fat intake with no effect on volume of sucrose ingested. Data are shown as mean $\pm$ SEM. *, $p<0.05$ compared to vehicle (drug $\times$ time interaction); [^], main effect of sCT ($p<0.05$); §, different from Intralipid®/vehicle ($p<0.05$).

Figure 3. VTA injection of sCT decreases intake of other motivationally relevant fluids

Intake of a non-nutritive solution, saccharin, was significantly reduced by VTA sCT (0.04 μ g) (A; n=8), as was water intake stimulated by lateral ventricle administration of the dipsogenic hormone angiotensin II (AngII; B; n=8). To test whether all fluid intake is suppressed by VTA administration of sCT, water intake was evaluated after intra-VTA sCT injection (0.04 μ g) when food was (C; n=10) or was not (F; n=10) available. When food was available after sCT injection, intakes of both water (C) and chow (D) were decreased. However, there was no change in the ratio of water intake to food intake over the 24h test (E). In contrast, when only water was available after VTA sCT injection, drinking was unchanged (F). Together these results suggest that the suppression of water intake observed when food is available is likely secondary to the reduction in chow intake. Data are shown as mean $\pm$ SEM. *, $p < 0.05$ compared to vehicle treatment; §, different from all other groups by pretreatment (vehicle or sCT) $\times$ treatment (vehicle or AngII) interaction.

Figure 4. Anxiety-like behavior is not increased by VTA amylin receptor activation

Rats were evaluated for anxiety-like behaviors after VTA sCT administration (0.04 μ g) in two separate paradigms. Activity in an open field apparatus was monitored (n=5–7/drug condition); no differences were detected in the percentage of time spent in the inner zone (A) or in the number of entries into the inner zone (B). In a social interaction test (n=5–8/drug condition), VTA sCT did not significantly affect the percentage of time spent engaging in social interaction (C), the number of bouts of interaction (D), the average duration of each social interaction bout (E), or the latency to interact (F). These results indicate that VTA amylin receptor activation is not producing anxiety-like responses. Data are shown as mean $\pm$ SEM. The key applies to all panels.

Figure 5. Rats on a free-choice diet can be classified as “fat-preferring” or “sucrose-preferring”
 When rats are given ad libitum access to a free-choice diet consisting of three foods (vegetable shortening, chow, and 30% sucrose solution), some animals get the majority of their daily kilocalories from the shortening (“fat-preferring”, A; n=6), while for other rats their energy intake is derived primarily from the 30% sucrose solution (“sucrose-preferring”, B; n=6). Although these differences in food selection are robust, there are no concomitant alterations in total daily caloric intake (C) or weight gain (D). Data are shown as mean $\pm$ SEM. *, $p < 0.05$ compared to all other foods. Key for type of food applies to panels A and B; key for food preference applies to panels C and D.

Figure 6. VTA amylin signaling in fat-preferring rats impacts intake of fat, but not of chow or sucrose

When fat-preferring rats ($n=6$) on the free-choice diet are given a peripheral injection of sCT ($5\mu\text{g/kg}$) just before lights off, intakes of shortening (A), chow (B), and 30% sucrose (C) are reduced, as are total energy intake (D) and 24h body weight gain (E). An intra-VTA pretreatment with the amylin receptor antagonist AC187 ($0.1\mu\text{g}$) attenuates the suppression of shortening intake, but not the suppression of chow or sucrose intake. This dose of AC187, which has no effect on total energy intake or body weight gain on its own, also partially blocked these negative energy balance effects of IP sCT. Data are shown as mean $\pm$ SEM. The key applies to all panels. *, $p<0.05$ compared to vehicle/vehicle; §, vehicle/sCT versus AC187/sCT, $p<0.05$.

Figure 7. VTA amylin signaling in sucrose-preferring rats has broader effects on food intake

For sucrose-preferring rats ($n=6$) on the free-choice diet, intakes of all foods (shortening, A; chow, B; 30% sucrose, C) are reduced by an IP injection of sCT ($5\mu\text{g/kg}$). These effects are all attenuated at 24h by an intra-VTA pretreatment with the amylin receptor antagonist AC187 ($0.1\mu\text{g}$). Total energy intake and 24h body weight gain are also suppressed by IP sCT, and these effects are likewise attenuated at 24h by VTA amylin receptor blockade. Data are shown as mean $\pm$ SEM. The key applies to all panels. *, $p<0.05$ compared to vehicle/vehicle; \$, vehicle/sCT versus AC187/sCT, $p<0.05$; #, vehicle/sCT versus AC187/sCT, $p=0.053$.