INTRODUCTION

The lateral parabrachial nucleus (LPBN) in rodents as well as primates is a key relay for visceral signals from the caudal hindbrain to forebrain areas associated with appetite control. LPBN receives axonal inputs from different brain areas, such as the area postrema (AP) and nucleus of the solitary tract (NTS), and LPBN neurons project to numerous forebrain regions, including the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST).

Noradrenaline signaling in the LPBN mediates amylin's and salmon calcitonin's hypophagic effect in male rats

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Abstract
The LPBN (lateral parabrachial nucleus) plays an important role in feeding control. CGRP (calcitonin gene-related peptide) LPBN neurons activation mediates the anorectic effects of different gut-derived peptides, including amylin. Amylin and its long acting analog sCT (salmon calcitonin) exert their anorectic actions primarily by directly activating neurons located in the area postrema (AP). A large proportion of projections from the AP and the adjacent nucleus of the solitary tract (NTS) to the LPBN, are noradrenergic (NA), and amylin-activated NA<sup>AP</sup> neurons are critical in mediating amylin's hypophagic effects. Here, we determine the functional role of NA<sup>AP</sup> amylin activated neurons to activate CGRP and non-CGRP LPBN neurons. To this end, NA was specifically depleted in the rat LPBN through a stereotaxic microinfusion of 6-OHDA, a neurotoxic agent that destroys NA terminals. While amylin (50 μg/kg) and sCT (5 μg/kg) reduced eating in sham-lesioned rats, no reduction in feeding occurred in NA-depleted animals. Further, the amylin-induced c-Fos response in the LPBN and c-Fos/CGRP colocalization were reduced in NA-depleted animals compared to controls. We conclude that AP → LPBN NA signaling, through the activation of LPBN CGRP neurons, mediates part of amylin's hypophagic effect.

KEYWORDS
6-OHDA, CGRP, CTR, DBH, NA

1 INTRODUCTION

The lateral parabrachial nucleus (LPBN) in rodents as well as primates is a key relay for visceral signals from the caudal hindbrain to forebrain areas associated with appetite control.

Abbreviations: 6-OHDA, 6-hydroxydopamine; AMY, amylin receptor; AP, area postrema; BNST, bed nucleus of the stria terminalis; CCK, cholecystokinin; CGRP, calcitonin gene related peptide; CeA, central amygdala; CNO, clozapine N-oxide; CTA, conditioned taste avoidance; CTR, calcitonin receptor; DA, dopamine; DBH, dopamine-β-hydroxylase; DSAP, immunotoxin anti-DBH saporin; HN, hypoglossal nucleus; IHC, Immunohistochemistry; KPBS, potassium phosphate-buffered saline; LC, locus coeruleus; LiCl, lithium chloride; LPBN, lateral parabrachial nucleus; LPS, lipopolysaccharide; MRI, magnetic resonance imaging; NA, noradrenaline; NGS, normal goat serum; NTS, nucleus of the solitary tract; PB, Phosphate Buffer; PBS, Phosphate-Buffered Saline; PFA, paraformaldehyde; RAMP, receptor activity modifying proteins; SEM, standard error of the mean; sCT, salmon calcitonin; SubC, subcoeruleus; Veh, vehicle.

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Amylin is a 37-amino-acid peptide, produced by pancreatic β-cells and co-secreted with insulin in response to a meal. The best investigated function of amylin is to reduce eating by inducing satiation (i.e., the promotion of meal-ending processes) via direct action on AP neurons. For this reason, amylin is considered promising as pharmacotherapy for obesity.

The amylin receptor (AMY) is composed of the core calcitonin receptor (CTR), which heterodimerizes with one or several receptor activity modifying proteins (RAMP1, 2, and 3). RAMPs are a critical component of the amylin receptor, they promote receptor specificity and enhance amylin’s affinity to the CTR core. Our recent study showed the role of RAMP1 or RAMP3 in mediating amylin’s ability to induce neural activation and its various metabolic effects. We showed that the RAMP1 subunit as a mediator of fat storage and utilization, while the RAMP3 subunit contributes to glucose homeostasis and amylin’s anorectic effect. Finally, the presence of at least one RAMP subunit (1 or 3) is both necessary and sufficient for amylin-induced c-Fos in the AP, when CTR is present.

Amylin’s satiation effect is mainly mediated by the primary activation of neurons in AP, where the density of AMY is high. AP neurons then signal to other downstream nuclei, such as the LPBN. Indeed, amylin increases the expression of the marker of neuronal activation c-Fos in the AP and LPBN, and the effect in the LPBN is dependent on an intact AP. AP neurons are directly excited by not only exogenous, but also endogenous amylin: the blockade of amylin binding sites with the amylin receptor antagonist AC-187 reduced the feeding-induced c-Fos expression in AP of amylin binding sites with the amylin receptor antagonist AC-187 reduced the feeding-induced c-Fos expression in AP neurons of refeed rats. Salmon calcitonin (sCT) is structurally similar to amylin and acts via AMY or via CTR alone, and dose comparisons with amylin have demonstrated a more potent, long-lasting effect mediated by its irreversible binding to AMY. The electrolytic lesion of LPBN reduces amylin’s anorectic effect and abolishes the neural activation, reflected by the lack of the c-Fos expression, in LPBN projection sites but not in the AP. A large proportion of projections from AP and NTS to the LPBN are noradrenergic (NA). Amylin and sCT activate NA neurons in the AP/NTS regions and approximately 44% of the amylin-induced and 47% of the sCT-induced c-Fos-positive neurons were also positive for dopamine-β-hydroxylase (DBH), the key enzyme for NA synthesis and recognized marker of NA neurons. Indeed, a specific lesion of about half of the total AP NA population with the immunotoxin anti-DBH saporin (DSAP) was sufficient to abolish the hypophagic effect of amylin in rats and also reduced amylin’s ability to induce c-Fos expression in AP neurons. However, while the phenotype of amylin activated neurons in the AP has been shown to be mainly NA, the phenotype of AP-activated LPBN neurons responsible for the mediation of amylin’s effect, remains unknown.

Previous studies identified a group of neurons in the LPBN that express calcitonin gene-related peptide (CGRP), which are activated by a meal and which lead to a decrease in food intake when chemogenetically activated with clozapine N-oxide (CNO). The chemogenetic inhibition of CGRP neurons with CNO attenuated anorexia following injection of toxins, such as lithium chloride (LiCl) and lipopolysaccharide (LPS), and showed a trend toward an attenuation of the appetite suppressive effects of amylin and cholecystokinin (CCK). In line with these observations, acute amylin administration activates CGRPLPN neurons in mice. Finally, it has been shown that NANTS neurons make monosynaptic contact with CGRPLPN neurons and directly activate CGRPLPN neurons to promote anorexia.

Thus, the aim of this study was to assess the phenotype of amylin activated LPBN neurons, especially to confirm the CGRPergic phenotype and to uncover the specific role of NA signaling from the AP to the LPBN. In other words, we wanted to define in detail whether a specific NA AP to LPBN depletion affects amylin’s and sCT’s anorectic effects and the activation of CGRP neurons in the LPBN.

## MATERIALS AND METHODS

### 2.1 Animals

The experiments were performed at the University of Zurich, Switzerland. All procedures were approved by the Veterinary Office of the Canton Zurich, Switzerland. Male adult Sprague-Dawley rats (Charles River Laboratories, France), with a body weight at arrival between 226 and 250 g, were maintained in a temperature-controlled room (21 ± 1°C) on an artificial 12:12-h dark-light cycle (light off at 11 hours). The animals were fed with a standard chow diet (Diet 3436, Provimi Kliba AG, Kaiseraugst, Switzerland; energy content: 3.15 kcal/g, 65.4% energy from carbohydrates, 12.3% from fat, and 22.4% from protein as percent of total energy content). Rats had free access to food (except for the period of food deprivation described below) and water throughout the experiments. Animals were handled and adapted to the housing conditions for at least 1 week before the experiments.

### 2.2 Study 1: Characterization of amylin receptor complex on NA neurons in the AP

Male Sprague-Dawley Rats (n = 5) were deeply anesthetized and were perfused intracardially with cold 0.1 M of phosphate buffer (PB) for 1.5 minutes followed by 4% of paraformaldehyde (PFA) in PB for 2.5 minutes. Brains
were removed and postfixed overnight in 4% of PFA and cryoprotected in 20% of sucrose for 24 hours. Brains were frozen on dry ice and stored at −80°C until sectioning. Four series of 25 μm thick coronal sections containing the AP, from bregma level −14.28 to −13.68, were cut on a cryostat (CM3050S, Leica Biosystems, Germany), mounted on Superfrost Plus slides (Thermo Fisher Scientific, Reinach, Switzerland) and cryoprotected in 50% of PB, 30% of ethylene glycol, 20% of glycerol, and subsequently stored at −20°C.

DBH/CTR double Immunohistochemistry (IHC) was performed on free-floating sections. Tissue was washed with 0.1 M PBS- 0.1% triton, and then, blocked in 0.3% triton- 2.5% normal donkey serum (NDS) in phosphate-buffered saline (PBS) at room temperature for 2 hours. Sections were then incubated at room temperature for 20 hours with rabbit anti-calcitonin receptor primary antibody (1/400, Abcam ab11042, Cambridge, United Kingdom). CTR immunostaining, the main signaling component of the amylin receptor, is a good indicator of the presence of the amylin receptor as already showed before but not all cells expressing CTR express RAMP. Unfortunately, we were not able to find a system to stain for all three RAMPs. After washing, sections were incubated with donkey anti-rabbit Alexa Fluor CY3 (1:200, Jackson Immunoresearch, Cambridge, United Kingdom) secondary antibody for 2 hours at room temperature. Tissue was then blocked in 0.3% triton-2.5% NDS in PBS at room temperature for 2 hours. Sections were then incubated at room temperature for 20 hours with dopamine-β-hydroxylase (DBH) mouse monoclonal primary antibody (1:1’000, Chemicon/Millipore MAB308, Burlington, MA, USA). After rinsing, sections were treated with secondary antibody, donkey anti-mouse 488 (1:200, Jackson Immunoresearch) for 2 hours at room temperature. After a thorough washing in PBS, the sections were counterstained with DAPI (0.25 μg/mL, 5 minutes), mounted immediately and coverslipped with vectashield antifade mounting medium (Vectorlabs, Burlingame, CA, USA).

Before starting the experiment, we validated the specificity of DBH and CTR primary antibodies in the hindbrain by assessing that their expression was restricted to areas known to express DBH and CTR (Figure S1C-E). Furthermore, negative controls in which sections were incubated with normal serum (ie, without the primary antibody) were conducted. Furthermore, a recent paper of our group showed the specificity of the CTR antibody: it was possible to colocalize DBH and CTR (Figure S1C-E). Tissue was processed for c-Fos/CGRP to quantify the number and the phenotype of amylin-activated neurons. c-Fos/CGRP double immunofluorescence was performed on free-floating sections. The blocking was performed with 0.4% triton - 4% normal goat serum (NGS) in PBS for 2 hours at room temperature. Sections were incubated at 4°C for 48 hours with c-Fos (1:500, Cell signaling 2250) and mouse anti-CGRP (1:1000, UCLA/CURE Digestive Research Center, Los Angeles, CA, USA) antibodies, and then, incubated with goat anti-rabbit Alexa Fluor 488 and goat anti-mouse CY3 (1:200, Jackson Immunoresearch) for 2 hours at room temperature. As above, sections were counterstained with DAPI, mounted and coverslipped with vectashield. 3D fluorescent images were captured using a Leica CLSM SP8 confocal microscope with a 64X magnification and analyzed with Imaris x64 software. Colocalization was assessed manually after thresholding all the images similarly in ImageJ by counting c-Fos nuclei colocalized with CGRP and DAPI.

2.3 | Study 2: Phenotypical characterization of amylin-activated neurons in the LPBN by double IHC

Male Sprague-Dawley (n = 16) rats, weighing between 300 and 350 g at the time of the sacrifice were maintained on standard rodent chow and group-housed by three to four rats per cage. Animals were fasted for 12 hours during the light phase and injected with one dose of amylin (50 μg/kg i.p. Bachem, Bubendorf, Switzerland; n = 8/group) or saline (n = 8/group) at dark onset. Ninety minutes later, rats were anesthetized and perfused, and the brains were postfixed and frozen as described above. Four series of 25 μm thick coronal sections containing the AP and the LPBN, from bregma level −14.28 to −13.68 and −9.72 to −8.64, respectively, were cut on a cryostat, cryoprotected, and subsequently stored at −20°C as described above.

Before starting the immunostaining, we validated the specificity of CGRP primary antibody in the hindbrain (Figure S1A,B). Tissue was processed for c-Fos/CGRP to quantify the number and the phenotype of amylin-activated neurons. c-Fos/CGRP double immunofluorescence was performed on free-floating sections. Thet blocking was performed with 0.4% triton - 4% normal goat serum (NGS) in PBS for 2 hours at room temperature. Sections were incubated at 4°C for 48 hours with c-Fos (1:500, Cell signaling 2250) and mouse anti-CGRP (1:1000, UCLA/CURE Digestive Research Center, Los Angeles, CA, USA) antibodies, and then, incubated with goat anti-rabbit Alexa Fluor 488 and goat anti-mouse CY3 (1:200, Jackson Immunoresearch) for 2 hours at room temperature. As above, sections were counterstained with DAPI, mounted and coverslipped with vectashield. 3D fluorescent images were captured using a Leica CLSM SP8 confocal microscope with a 64X magnification and analyzed with Imaris x64 software. Colocalization was assessed manually after thresholding all the images similarly in ImageJ by counting c-Fos nuclei colocalized with CGRP and DAPI.

2.4 | Study 3: Role of NA in mediating amylin’s and sCT’s hypophagic action

Thirty-two male Sprague-Dawley rats, weighing between 300 and 350 g at the time of stereotaxic surgery and 430 to 450 g at the time of sacrifice, were used. Rats were maintained on standard rodent chow ad libitum, except for the periods of food deprivation described below. One week after the surgery, rats were single-housed in BioDAQ cages (Research
Diets, New Brunswick, NJ, USA) to allow automated measurements of food intake and meal patterns.

### 2.4.1 Surgeries

NA terminals in the LPBN were lesioned using 6-hydroxydopamine (6-OHDA). 6-OHDA is formed from dopamine (DA) as an autoxidation product or metabolite. The local stereotaxic microinfusion of 6-hydroxydopamine causes severe loss of NA with little evidence of nonspecific tissue reaction or destruction that could be detected by conventional light microscopic methods. NA nerve-endings take up 6-OHDA causing the destructive effect because the neurotoxin auto-oxidizes and induces the production of reactive oxygen species, producing a long-term depletion of NA and comparable loss of activity of its synthesizing enzymes.

Solutions of 6-OHDA were freshly prepared prior to surgeries. 6-OHDA hydrochloride (6-OHDA.Cl; H4381, Sigma-Aldrich, St. Louis, Missouri, United States) was dissolved in sterile saline (0.9%) solution containing the antioxidant ascorbic acid (1 mg/mL), pH 7.4 at a concentration of 1.25 μg/μL, and 2 μL per side was injected. Since 6-OHDA is light and heat sensitive, exposure to light was avoided.

Rats were anesthetized with a mixture of ketamine (40 mg/kg in 0.9% NaCl s.c.; Ketanarkon, Streuli Pharma) and medetomidine hydrochloride (0.8 mg/kg in 0.9% NaCl s.c.; Medetor, Virbac, Glattbrugg, Switzerland) and pretreated with pargyline hydrochloride (50 mg/kg i.p.; P8013, Sigma-Aldrich) that prevents the oxidation of 6-OHDA and prolongs its effective half-life after injection.

Before starting the surgery, antibiotics (Baytril: 10 mg/kg in 0.9% NaCl s.c.; Bayer, Leverkusen, Germany) were administered prophylactically. After the surgical procedures animals were treated with an anti-inflammatory drug (Metacam 1.5 mg/kg s.c.; Boehringer Ingelheim, Biberach, Germany) and Revertor (3.5 mg/kg; Virbac), a selective α2 adrenergic receptor antagonist to reverse the sedative effects of medetomidine. Following surgery, animals were group-housed and monitored daily.

Sixteen rats were bilaterally injected with 5 μg of 6-OHDA into the LPBN at two different levels (2.5 μg of 6-OHDA in 2 μL per each side, 1.25 μg/μL per level), using a NanoFil syringes 33 GA beveled (World Precision Instrument, Friedberg, Germany) according to Paxinos and Watson stereotaxic coordinates, from bregma level between −9.16 and −9.30 (taking bregma as reference, level 1: dorsoventral: −6.9 mm, lateral-medial: ±2.2 mm, rostro-caudal: −9.4, level 2: dorsoventral: −7 mm, lateral-medial: ±2.5 mm, rostro-caudal: 9). Sixteen sham-operated animals were injected in the LPBN with an equivalent volume of vehicle (Veh; sterile saline 0.9%, ascorbic acid 1 mg/mL pH 7.4). The surgeries and injections (6-OHDA or Veh) were done in random order over a period of 6 days. The first feeding trial was performed 21 days following 6-OHDA-lesion surgery, when the extent of NA depletion was expected to be maximal and stable. At the end of amylin trials, rats were sacrificed and perfused. The LPBN and the AP were immunostained for DBH to confirm the efficacy of the NA lesions.

### 2.4.2 Food intake and body weight measurements

Body weight was measured weekly before and after the surgery, then, it was measured daily in the first period (1 week) in the BioDAQ system. Then, body weight was again measured on each trial day, 1 hour before dark onset, and on the day of perfusion. Meal patterns in sham and LPBN NA-lesioned groups (n = 16/group) were analyzed using the BioDAQ Food Intake Monitor (Research Diet, New Brunswick, NJ, USA). During the experiment, meal pattern data were collected under two conditions; first, eating was recorded for 24 hours in ad libitum-fed rats and second, 24-hours food intake data were collected after a 12-hours fasting during the light phase, that is, food was presented at dark onset. Individual meals were defined with an inter-meal interval of ≥900 seconds and a meal size of ≥0.23 g.

### 2.4.3 Feeding trials with amylin and sCT

The rats were habituated to intraperitoneal (i.p.) injections by injecting saline for 3 to 5 days before the first amylin feeding trial. Feeding trials were performed at dark onset in a randomized, crossover manner, so that each animal was examined under all treatment conditions with at least 3 days of recovery between trials. Food was removed at light onset; rats received injections of either saline (control), amylin (50 or 100 μg/kg i.p.; H-9475, Bachem), or sCT (5 μg/kg i.p.; 4033011.0001, Bachem) just before dark onset. Specifically, the dose of sCT was chosen based on our prior work that showed that 5 μg/kg are sufficient to induce anorexia at 1, 2, 4, and 24 hours. Food was then returned, and meal patterns were recorded automatically in BioDAQ cages. The same animals were used for all the trials (saline, amylin, and sCT).

### 2.4.4 Body composition

At the end of the study, body composition was measured postmortem by a magnetic resonance imaging (MRI) based approach to quantify total fat and lean mass. The animals were individually placed in a plastic holder, the holder was inserted into a tubular space in the EchoMRI (Echo Medical System, Houston, Texas, USA) and the scanning started.
Scanning was repeated two times per animal and the results were delivered as numeric values ready to analyze.

### 2.4.5 Immunohistochemistry for DBH and for CGRP/c-Fos

Animals were fasted for 12 hours and injected with amylin (50 μg/kg i.p.) or saline at dark onset (n = 8 per treatment/surgery). Ninety minutes later, rats were anesthetized with pentobarbital, perfused, and brains were removed and processed as described above. Brains were cut in four series of 25 μm. One series of sections was stained for DBH to label the NA neurons in AP and the NA terminals in LPBN. These sections were used to assess the extent of the NA depletion and to verify that it was restricted to the LPBN. IHC was performed on free-floating sections. c-Fos/DBH double immunofluorescence: blocking was performed with 0.4% Triton X-4% normal goat serum (NGS) in PBS for 2 hours at room temperature. Sections were incubated at 4°C for 48 hours with rabbit polyclonal antibody against c-Fos protein (1:500, Cell signaling 2250) and mouse monoclonal antibody against DBH (1:1000, Chemicon/Millipore MAB308). After washing, sections were incubated with goat anti-rabbit Alexa Fluor 488 (1:200, Jackson Immunoresearch) and goat anti-mouse Alexa Fluor 555 (1:250, Invitrogen) secondary antibodies for 2 hours at room temperature. After a thorough washing in PBS, the sections were counterstained with DAPI (0.25 μg/mL, 5 minutes), mounted immediately and coverslipped with vectashield antifade mounting medium (Vectorlabs). Another series of sections was stained for c-Fos/CGRP to verify whether noradrenaline reaches CGRP neurons. IHC was performed on free-floating brain sections and the c-Fos/CGRP double immunofluorescence staining was performed using the same protocol as above. Images were captured by using Zeiss fluorescent microscope with a 20X magnification and analyzed with ImageJ software. Colocalization was assessed manually after thresholding all the images similarly in ImageJ by counting c-Fos positive nuclei colocalized with CGRP neurons.

### 2.5 Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). In study 2, unpaired Student t test was used to quantify the amylin activated neurons in LPBN, the total number of c-Fos positive cells and the degree of colocalization with CGRP in LPBN were assessed. In study 3, the number of DBH cells in the AP, DBH, and CGRP fiber density in LPBN, activated CGRP neurons in response to amylin, fat, and lean mass in sham and NA-depleted animals were compared by an unpaired Student t test. Data from basal food intake, feeding trials, body weight, and body weight gain were analyzed by two-way repeated measures ANOVA for the effect of NA-depletion and treatment followed by Sidak's multiple comparison test to detect significant differences between groups. For all statistical tests a P value < .05 was considered significant.

### 3 RESULTS

#### 3.1 CTR is expressed on NA AP neurons

Our previous work showed that NA in AP neurons is crucial in mediating amylin's ability to reduce food intake and induce neural activation. Given the potential involvement of NA signaling in mediating the energy balance effects of amylin AP signaling, we hypothesized that AMY is expressed on AP NA neurons. Therefore, to analyze the expression and colocalization of the AMY on AP NA neurons, we performed double-label IHC for CTR and DBH in the AP. As shown in Figure 1, 48% of AP NA neurons express CTR. This suggests that amylin can act through a direct activation of a subpopulation of AP NA neurons, confirming the potential mechanism by which NA AP is essential for amylin's effects.

#### 3.2 Amylin activates CGRP neurons

To confirm amylin's ability to activate CGRP neurons, an immunohistochemical approach was used and c-Fos was quantified in CGRP neurons in male rats after an acute amylin administration (50 μg/kg) (Figure 2A-L). In line with previous reports, amylin significantly increased the number of c-Fos positive cells by 17-fold in the AP (P < .001; data not shown) and by sixfold in the LPBN compared to Veh controls (P < .01; Figure 2M). The number of CGRP cells colocalized with DAPI was also quantified and showed no difference between vehicle and amylin-treated rats (Figure 2N). Colocalization analysis revealed that 38% of amylin-activated cells within the LPBN are CGRP-positive per LPBN section (Figure 2O).

#### 3.3 NA signaling through LPBN is involved in mediating amylin's and sCT's anorectic effect

To clarify the functional role of NA signaling to the LPBN, NA was specifically depleted in LPBN of male Sprague-Dawley rats, through a stereotaxic microinfusion of 6-OHDA. The successful and specific DBH depletion was confirmed by IHC at the end of the experiment because chemically depleted-rats showed on average a six-fold
reduced DBH fiber density in the LPBN (Figure 3G-I); the lesion was specific to the LPBN since no change was found in the AP in respect to the number of amylin-induced c-Fos positive cells or to the number of DBH positive cells (Figure 3A-F); hence, the NA depletion in the LPBN noradrenergic terminals did not damage the noradrenergic neurons in the AP.

To test amylin’s and sCT’s ability to reduce food intake in NA LPBN-depleted animals, food intake was measured after acute saline (control), amylin (50 or 100 µg/kg i.p), or sCT (5 µg/kg i.p.) administration. Amylin-induced anorexia was observed in sham-lesioned rats at 1 hour postinjection with a 23% decrease in eating compared to saline-injected sham rats (sham: Veh vs Amylin (50 µg/kg), \( P < .05 \); Figure 4A). Amylin’s hypophagic effect was no longer present at 22 hours (Figure 4B), whereas sCT’s anorectic effect in sham-operated rats was still present until 22 hour post-injection (Figure 4D-E). In NA LPBN-depleted rats, amylin’s and sCT’s anorectic effects were blunted compared to sham-operated rats (Figure 4A-F).

Further, the first meal size was reduced in sham animals compared to NA-depleted animals after amylin (Figure 4C) and after sCT treatment (Figure 4D). In addition, it seems that in general, vehicle-treated NA-depleted rats displayed a decrease in the first meal size but the effect was nonsignificant. Average ad libitum meal pattern was analyzed during the dark phase (12 hours), the light phase (12 hours) and 24 hours and no differences were identified (Table 1). Average meal patterns were also analyzed at 1 and 12 hours after amylin or sCT injections (Tables 2 and 3) and other than total food intake, no other alteration after NA depletion was observed. Overall, NA-depleted rats gained more weight compared to sham rats during the whole experimental period (Figure 5 A-C). Body composition analysis revealed that NA-depleted animals had more total fat and lean mass expressed as percent of final body weight compared to the sham animals (\( P < .05 \); Figure 5E,G), suggesting that the increase in body weight in LPBN NA-depleted rats is due to an increase in lean and fat mass.
At the end of the experiment, an IHC analysis was performed to evaluate if LPBN NA-depletion reduced the LPBN neural activation and if this was specific to CGRP neurons (Figure 6A-L). Thus, while amylin administration (50 µg/kg i.p.) significantly activated c-Fos in sham-operated rats and increased the number of c-Fos/CGRP double-labeled neurons (Figure 6N; see also Figure 2H), a reduction of the total number of c-Fos positive cells and of specific CGRP neurons activation by 40% and 58%, respectively, was observed in NA-depleted animals compared to sham animals (sham: Veh vs Amy, \( P < .01 \); Figure 6A-N), without a significant change in total CGRP fiber density (Figure 6O). Colocalization analysis revealed that in the sham group, 35% of amylin-activated cells within the LPBN were CGRP-positive (47 ± 10 cells; \( P < .0001 \); Figure 6N) while in the NA-depleted group only
25% of amylin-activated cells within the LPBN were CGRP-positive (18 ± 3 cells; Figure 6N).

To evaluate if NA depletion damaged other neuronal cells in this particular area, we quantified the number of c-Fos+ non-CGRP cells and there was no difference between sham and NA-depleted animals treated with vehicle, and between sham and NA-depleted animals treated with amylin, resp. Instead, there was a significant difference in the c-Fos+ CGRP+ neurons between sham and NA-depleted group treated with amylin. This means that the NA depletion had a specific role in reducing the CGRP neural activation mediated by amylin, and that NA depletion did not reduce the activation of other neurons (non-CGRP) induced by amylin (Figure S2).

### 4 DISCUSSION

In this study, we first clarify the functional role of NA in AP-mediated amylin signaling by showing that approx. 50%
FIGURE 4  NA LPBN depletion reduced amylin’s and sCT’s ability to reduce food intake. A, Effect of vehicle (0.9% NaCl), amylin (50 µg/kg and 100 µg/kg; i.p.) on 1 hour cumulative food intake in 12 hours fasted sham (n = 16) or NA-depleted (n = 16) rats. B, Effect of vehicle and amylin on 22 hours cumulative food intake in 12 hours fasted sham or NA-depleted rats. C, Effect of amylin on first meal size in dark phase. D, Effect of sCT (5 µg/kg; i.p.) on 1 hour cumulative food intake in 12 hours fasted rats. E, Effect of sCT on 22 hours cumulative food intake in 12 hours fasted rats. F, Effect of sCT on first meal size in dark phase. The same animals received all the treatments: a single vehicle treatment was compared with amylin and sCT treatments. Data are expressed as mean ± SEM. Data were analyzed with two-way ANOVA followed by post hoc t test, *P < .05; **P < .01; ***P < .001

TABLE 1  Ad libitum meal patterns (food intake, meal number, meal size, meal duration) during the dark phase (12 hours), the light phase (12 hours) and 24 hours

|                     | Dark phase (12 hours) | Light phase (12 hours) | 24 hours |
|---------------------|-----------------------|------------------------|----------|
|                     | Sham                  | NA-depleted            | Sham     | NA-depleted | Sham | NA-depleted |
| Total food intake (g) | 22.9 ± 1.07           | 23.3 ± 0.88            | 5.03 ± 0.61 | 5.07 ± 0.44 | 26.9 ± 0.76 | 26.8 ± 0.84 |
| Meal number         | 5.75 ± 0.3            | 6.02 ± 0.49            | 1.67 ± 0.18 | 1.50 ± 0.13 | 6.96 ± 0.29 | 7.02 ± 0.48 |
| Meal size (g)       | 4.22 ± 0.21           | 4.25 ± 0.31            | 2.51 ± 0.25 | 3.23 ± 0.27 | 4.07 ± 0.19 | 4.01 ± 0.25 |
| Meal duration (min)  | 197 ± 10.01           | 207 ± 11.5             | 36.8 ± 4.87 | 39.5 ± 4.01 | 225 ± 10.3  | 232 ± 13.5 |

Note: Data are expressed as mean ± SEM; n = 16/group. Data are analyzed with two-way ANOVA followed by post hoc t test.
of NA neurons express CTR. We next demonstrate that a significant portion (~1/3) of amylin activated neurons in the LPBN in rats are CGRPergic. Then, by evaluating the direct AP projection site, we observe the activation of LPBN neurons (CGRP and non-CGRP neurons) with a specific NA depletion in the rat LPBN and show that approx. 35% of CGRP neurons receive NA input from the AP. Our study extends the current knowledge on the neuronal substrate and pathways engaged by amylin to induce anorexia.

Previous studies in mice showed that amylin activated CGRP-PBN neurons and that chemogenetic inhibition of CGRP-PBN neurons partially decreased amylin's ability to reduce food intake in male mice.23 With an IHC approach and in line with previous reports, we confirmed that systemic acute amylin administration in male rats increased the number of c-Fos positive cells in the AP and in the LPBN, compared to Veh controls, and that ~40% of amylin activated neurons were CGRP positive. It is known that many projections from the AP/NTS to the LPBN are noradrenergic.18,19 A recent study27 showed that NA/NTS neurons activation decreases feeding and that these neurons send direct excitatory input to CGRP-PBN neurons; in addition, activation of these neurons reduced eating without inducing aversive anxiety behavior. Furthermore, previous work from our research group showed that lesioning part of NA-containing AP neurons with DSAP was sufficient to abolish the hypophagic effect of amylin,21 and that complete AP ablation reduced amylin's anorectic effect and amylin's ability to induce c-Fos in AP projections sites, including the LPBN.6

Building up on these results, we hypothesized that systemic acute amylin administration increases neural activity in LPBN neurons through AP → LPBN NA signaling. DBH IHC revealed the presence of NA terminals in LPBN, corroborating previous observations.37,38 The NA fibers were localized in the same area as CGRP-PBN neurons, suggesting that following amylin stimulation, AP noradrenergic neurons activate CGRP-PBN neurons via direct synaptic contact. We now confirmed the functional role of NA NTS neurons in mediating amylin's effect on eating though LPBN neurons activation. When NA was specifically

### TABLE 2

|                     | Vehicle | Amylin (50 µg/kg) | Amylin (100 µg/kg) | sCT (5 µg/kg) |
|---------------------|---------|-------------------|--------------------|--------------|
|                     | Sham    | NA-depleted       | Sham               | NA-depleted  |
| **First hour of the dark phase** |         |                   |                    |              |
| Total food intake (g) | 4.95 ± 0.58 | 4.58 ± 0.45 | 2.95 ± 0.64 | 4.50 ± 0.67 | 3.25 ± 0.51 | 4.65 ± 0.64 | 3.06 ± 0.64 | 4.95 ± 0.64* |
| Meal number         | 0.94 ± 0.06 | 1.19 ± 0.09 | 0.88 ± 0.09 | 0.81 ± 0.10 | 0.94 ± 0.06 | 0.94 ± 0.06 | 0.94 ± 0.06 | 0.94 ± 0.15 |
| Meal size (g)       | 4.43 ± 0.58 | 3.99 ± 0.43 | 3.25 ± 0.56 | 3.65 ± 0.65 | 3.59 ± 0.55 | 4.23 ± 0.68 | 3.67 ± 0.66 | 3.82 ± 0.65 |
| Meal duration (min) | 23.1 ± 3.12 | 24.5 ± 2.97 | 21.6 ± 3.69 | 22.1 ± 4.23 | 23.5 ± 3.07 | 26.9 ± 4.45 | 17.1 ± 1.75 | 20.4 ± 3.50 |

Note: Data are expressed as mean ± SEM; n = 8/group. Data are analyzed with two-way ANOVA followed by post hoc t test.

*P < .05 (sham vs NA-depleted).

### TABLE 3

|                     | Vehicle | Amylin (50 µg/kg) | Amylin (100 µg/kg) | sCT (5 µg/kg) |
|---------------------|---------|-------------------|--------------------|--------------|
|                     | Sham    | NA-depleted       | Sham               | NA-depleted  |
| **12 hours of the dark phase** |         |                   |                    |              |
| Total food intake (g) | 28.6 ± 1.06 | 30.8 ± 0.91 | 29.9 ± 0.98 | 30.6 ± 0.89 | 29.6 ± 0.84 | 31.2 ± 0.95 | 20.7 ± 2.44 | 30.3 ± 1.49** |
| Meal number         | 6.44 ± 0.47 | 6.87 ± 0.42 | 7.06 ± 0.41 | 6.88 ± 0.52 | 7.31 ± 0.55 | 6.94 ± 0.51 | 5.69 ± 0.58 | 6.31 ± 0.87 |
| Meal size (g)       | 4.23 ± 0.21 | 4.49 ± 0.21 | 4.09 ± 0.15 | 5.01 ± 053 | 3.96 ± 0.19 | 4.46 ± 0.24 | 3.54 ± 0.52 | 3.80 ± 0.47 |
| Meal duration (min) | 229 ± 22.2 | 232 ± 13.8 | 251 ± 33.4 | 239 ± 14.9 | 243 ± 32.5 | 243 ± 16.1 | 193 ± 40.3 | 211 ± 10.8 |

Note: Data are expressed as mean ± SEM; n = 8/group. Data are analyzed with two-way ANOVA followed by post hoc t test.

**P < .01 (sham vs NA-depleted).
depleted in the LPBN (but not in the AP) of male Sprague-Dawley rats, amylin, and sCT were no longer able to reduce eating. An acute amylin administration determined the strongest decrease in food intake in the first hour post administration, while sCT had a more potent and long-lasting effect, probably related to its irreversible binding to the CTR receptor. Amylin and sCT both activate AMY neurons, mostly present in AP, and also in NTS, and sCT also activates the CTR neurons in AP/NTS. Our studies do not allow us to conclude whether the effects seen here originated specifically in AMY neurons, or whether sCT may utilize two different neural pathways, through AMYAP and CTRAP/NTS neurons.

Interestingly, CGRP neurons are not just involved in mediating anorexia and satiation, but these neurons appear to mediate conditions that threaten energy homeostasis, ranging from mild satiation in response to a large meal, to visceral malaise due to nausea or inflammation, to pain produced by heat or foot shock. A recent report showed that the optogenetic stimulation of CGRP-LPBN neurons is sufficient to induce conditioned taste avoidance (CTA) and that the genetic inhibition or silencing of CGRP LPBN neurons attenuated LiCl-mediated CTA. Recently, high doses of peripheral sCT (150 μg/kg) have been shown to induce conditioned taste avoidance (CTA); however, we and others have shown in multiple studies that systemic (<300 μg/kg) or icv (<100 pmol) amylin or that lower doses of systemic sCT (<11 μg/kg) do not cause aversive or sickness-like behaviors indicative of a nausea/emesis, thus, suggesting that the anorectic effects of amylin are not mediated by malaise.

Together, the previous studies and our current results may suggest that a specialized subpopulation of CGRP neurons is involved in the control of feeding behavior and in the response to satiating peptides such as amylin and sCT. As already mentioned above, part of peripheral sCT’s effect seems to be mediated by the activation of non-CGRPLPBN neurons, through the direct activation of CTR in the NTS. Thus, it is possible to consider that also other LPBN neurons, that are not CGRPeric, may be involved in the mediation of amylin effects. We can confirm in our study that the portion of non-CGRPLPBN amylin activated neurons is significant (~60%) and together it suggests that CGRP neurons are probably not the only pathway mediating amylin’s and sCT’s responses. Hence, it is possible that amylin and sCT responses may be mediated by two different pathways, one from the AMYAP and one from CTRNTS. Further studies are needed to characterize the phenotype and the functional role of these non-CGRPLPBN neurons involved in mediating amylin effect.

In conclusion, the present study confirmed the central role of the LPBN in propagating amylin’s and sCT’s hypophagic action, and particularly the importance of AP → LPBN NA signaling in the mediation of this process, through the activation of LPBN (CGRP and non-CGRP) neurons (Figure 7). Further studies are required to expand our immunohistochemical data, for example, with an in vivo

![Figure 5](image-url)
**Figure 6** NA LPBN depletion reduced c-Fos+ cells and c-Fos + CGRP neurons. Representative images of c-Fos (red) and CGRP cells (green) in the LPBN of sham and NA-depleted rats 90 minutes after vehicle or amylin administration (50 μg/kg; i.p.). A-C, Sham animals treated with vehicle. D-F, Sham animals treated with amylin. G-I, NA-depleted animals treated with vehicle. J-L, NA-depleted animals treated with amylin. M, Quantification of total LPBN c-Fos+ cells per section. N, Quantification of c-Fos + CGRP neurons in LPBN (highlighted by arrows). O, Quantification of CGRP fiber density, measured as estimated density of immunoreactivity (Integrated Density). Data are expressed as means ± SEM. Scale bar represents 100 μm. Data in M, N, O are analyzed with two-way ANOVA followed by Tukey test, **P < .01, ****P < .0001

**Figure 7** Hypothetical model of AP to LPBN amylin noradrenaline signaling. Amylin and sCT primarily activate noradrenergic neurons in AP that project to LPBN neurons (CGRP and non-CGRP) and physiologically reduce body weight and food intake. NA (red triangles) depletion in the LPBN through the use of 6-OHDA increase body weight, fat, and lean mass and decrease amylin and sCT effect.
calcium imaging tool to confirm the neural activation of CGRP-LPNB neurons mediated by systemic amylin and sCT administration. Furthermore, the specific role of CGRP as a LPBN-intrinsic neuropeptide and neurotransmitter, in the mediation of amylin’s and sCT’s effects to control food intake and to stimulate neural activity in LPBN projection’s sites, for example, located in the CeA and BNST, remains to be investigated.

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DISCLOSURE

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS

L. Boccia, C. Le Foll, and T.A. Lutz designed the experiments; L. Boccia performed the experiments; L. Boccia and C. Le Foll analyzed the experiments and wrote the manuscript; C. Le Foll and T.A. Lutz reviewed this manuscript.

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