Noradrenergic neurons of the area postrema mediate amylin’s hypophagic action

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Potes CS, Turek VF, Cole RL, Vu C, Roland BL, Roth JD, Riediger T, Lutz TA. Noradrenergic neurons of the area postrema mediate amylin’s hypophagic action. Am J Physiol Regul Integr Comp Physiol 299: R623–R631, 2010. First published June 10, 2010; doi:10.1152/ajpregu.00791.2009.—Circulating amylin inhibits food intake via activation of the area postrema (AP). The aim of this study was to identify the neurochemical phenotype of the neurons mediating amylin’s hypophagic action by immunohistochemical and feeding studies in rats. Expression of c-Fos protein was used as a marker for neuronal activation and dopamine-β-hydroxylase (DBH), the enzyme-catalyzing noradrenaline synthesis, as a marker for noradrenergic neurons. We found that ∼50% of amylin-activated AP neurons are noradrenergic. To clarify the functional role of these neurons in amylin’s effect on eating, noradrenaline-containing neurons in the AP were lesioned using a saporin conjugated to an antibody against DBH. Amylin (5 or 20 μg/kg sc)-induced anorexia was observed in sham-lesioned rats with both amylin doses. Rats with a lesion of >50% of the noradrenaline neurons were unresponsive to the low dose of amylin (5 μg/kg) and only displayed a reduction in food intake 60 min after injection of the high amylin dose (20 μg/kg). In a terminal experiment, the same rats received amylin (20 μg/kg) or saline. The AP and nucleus of the solitary tract (NTS) were stained for DBH to assess noradrenergic lesion success and for c-Fos expression to evaluate amylin-induced neuronal activation. In contrast to sham-lesioned animals, noradrenaline-lesioned rats did not show a significant increase in amylin-induced c-Fos expression in the AP and NTS. We conclude that the noradrenergic neurons in the AP mediate at least part of amylin’s hypophagic effect.

AMYLIN IS CO-SECRETED WITH insulin by pancreatic β-cells in response to nutrient ingestion (8, 26). At near physiological plasma concentrations amylin effectively decreases food intake in rats and is considered a physiological satiation signal (4, 15). Likewise, the amylin analog pramlintide causes weight loss in obese subjects that is accompanied by sustained reductions in 24-h food intake, portion sizes, fast food intake, and binge eating tendencies (5, 37).

Convergent evidence from pharmacological studies using site-specific injections of amylin antagonists and targeted lesion studies clearly implicate the area postrema (AP) as the primary site of amylin’s hypophagic action (18, 19, 25). A direct excitatory action of amylin upon AP neurons has been confirmed by electrophysiological studies in which amylin increased formation of the intracellular second messenger cGMP (30). Subsequent to AP activation, the adjacent nucleus of the solitary tract (NTS) and the lateral parabrachial nucleus (LPB) are activated by peripheral amylin and seem to be involved in amylin’s hypophagic properties (7, 18, 19, 31, 34).

The neurochemical phenotype(s) of the AP neurons mediating amylin’s inhibitory effect on eating are unknown. Immunohistochemical studies identified catecholamine immunoreactive fibers and cell bodies in the AP and NTS (1, 2). Immunohistological phenotyping studies suggest that the catecholaminergic population of AP neurons is mainly noradrenergic (2, 10, 13). Some processes showing noradrenergic immunoreactivity appear to form reciprocal connections between the AP and the medial NTS (2). Furthermore, a large number of projections from the AP and NTS to the LPB are noradrenergic (12, 24).

In the present study, we sought to determine whether amylin-activated neurons in the AP are noradrenergic and to elucidate the relative contribution of these neurons in mediating amylin’s actions. First, we used double immunohistochemistry to quantify the number of amylin-activated neurons (c-Fos protein expression) that were positive for dopamine-β-hydroxylase (DBH). DBH is the key enzyme involved in the conversion of dopamine to noradrenaline and is an accepted marker for noradrenergic neurons. To clarify the functional role of these neurons in amylin’s ability to reduce food intake we used double immunohistochemistry to determine whether administration of the dopamine receptor antagonist, apomorphine (1 mg/kg ip), would be attenuated.

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MATERIALS AND METHODS

Animals and Housing

The experiments were performed in part at Amylin Pharmaceuticals, San Diego, CA, (study 1) and in part at the University of Zurich, Switzerland (study 2). Care of rats and all procedures were within the Institutional Animal Care and Use Committee guidelines at Amylin Pharmaceuticals, (in accordance with Animal Welfare Act Guidelines), or approved by the Veterinary Office of the Canton Zurich, Switzerland. Male adult Sprague-Dawley rats were maintained in a temperature-controlled room (21 ± 1°C) on an artificial 12:12-h dark-light cycle. They had free access to food (described below) and water throughout the experiments, unless noted otherwise, and were adapted to the housing conditions and handling for at least 2 wk before the experiments.

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Sixteen male Sprague-Dawley rats were obtained from Charles River Laboratories (CRL:CD rats; Wilmington, MA). Rats were maintained on standard rodent chow (cat. no. LM-485; Harlan Teklad) and housed two rats per cage. The average body weight at the time of perfusion was 300 ± 2 g. Rats were fasted for 24 h and then injected with one of two doses of amylin (5 μg/kg or 20 μg/kg ip; Peptinysynth, Torrance, CA; n = 4/group) or saline (n = 4) at dark onset. Two hours later, rats were fully anesthetized with isoflurane and were perfused with saline followed by cold 4% paraformaldehyde. Tissue was postfixed overnight and cryoprotected in 30% sucrose for 24 h. Brains were frozen on dry ice and stored at −80°C until sectioning, when five series of 30-μm thick sections were cut on a freezing microtome. Tissue was processed for DBH and c-Fos.

Immunohistochemistry was performed on floating sections. For c-Fos/DBH double fluorescence, blocking was performed with 0.3% Triton-1% BSA. Rabbit polyclonal antibody against c-Fos protein (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibody against DBH (model MAB308, lot LV1556118; Chemicon) were used in dilutions of 1:4,000 and 1:2,000, respectively. Sections were incubated at 4°C for 48 h and then incubated with goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 secondary antibodies (Molecular Probes, Eugene, OR) at 1:500 for 2 h at room temperature in the dark. After a thorough washing in PBS, sections were mounted immediately and coverslipped with ProLong Gold antifade reagent (Molecular Probes). Fluorescent images were captured by using a Leica LSM 710 confocal microscope and Zen 2008 software. Analysis of positive cells in each region was conducted by an investigator blinded to treatment groups. Colocalization was assessed manually by counting c-Fos-positive nuclei and DBH-positive cell bodies, and the percentage of cells of the two types that were double labeled was determined. The percentage of c-Fos-positive and DBH-positive cell bodies in any region was measured daily starting 5 days before surgery and until the day of the first feeding trial. Body weight was measured daily starting 5 days before surgery and until the day of the first feeding trial (15 days after the last surgeries). Body weight was again assessed on each trial day and on the day of perfusion. Cumulative 24-h food intake was measured 1 day before surgery and then daily from day 3 after surgery until the day of the first feeding trial. Food intake was also assessed on the day before each feeding trial. Body weight and 24-h food intake were measured 1 h before dark onset. These parameters were analyzed to observe whether the lesion itself would have an effect on baseline food intake and body weight, as this was the case in a study using DSAP but targeting the NTS (32).

Feeding trials with amylin. The rats were habituated to subcutaneous injections by injecting saline 2 and 4 days before the first trial. Feeding trials were performed at dark onset using a crossover design with at least 3 days of recovery between trials. The half-life of amylin in the circulation is about 13 min (42, 45), and the hypophagic effect of an acute injection of amylin typically lasts for no more than 2–4 h (16, 17); therefore, carryover effects from the previous trial could be excluded. Food was removed 2 h before dark onset; rats received injections of either saline (control) or amylin (5 or 20 μg/kg sc; Bachem, Dubendorf, Switzerland) just before dark onset. Food was then returned, and cumulative intake was measured 30 and 60 min after injection (±0.1 g) and corrected for spillage.

Immunohistochemistry for DBH and c-Fos. Five days after the last feeding trial, the rats were 24-h fasted and subcutaneously injected with either saline or 20 μg/kg amylin ( sham: n = 6 per group; DSAP: n = 9 per group) at dark onset. Two hours later, the animals were deeply anaesthetized (pentobarbital sodium, 80 mg/kg ip; Kantsapotheke Zürich, Switzerland) and transcardially perfused with ice-cold sodium phosphate buffer (0.1 M, pH 7.2), followed by 4% paraformaldehyde in phosphate buffer. Brains were postfixed for 2 h and cryoprotected in 20% sucrose/phosphate buffer (48 h at 4°C). Three series of 20-μm coronal brain sections containing the AP, NTS, and LPB [according to Paxinos and Watson (27)] were cut in a cryocutome (model CM3050S; Leica Nussloch, Germany) and thaw-mounted on adhesive glass slides (SuperFrost Plus, Menzel, Germany).
One series of sections was immunohistochemically stained for DBH to label the noradrenaline neurons. These sections were used to assess the extent of the noradrenaline neuron lesion and to verify that the lesion was restricted to the AP. After air drying for 1 h at room temperature and rehydration in PBS (pH 7.4) containing 0.1% Triton X-100 (PBST), sections were incubated in 1% BSA (Sigma-Aldrich) in 0.3% PBST for 2 h, followed by mouse monoclonal anti-DBH primary antibody (1:2,000 in BSA/PBST; model MAB308, lot LV1390388, Chemicon) for 48 h at 4°C. The sections were then incubated in 1:400 biotinylated goat anti-mouse IgG (1:400 in 0.3% PBST, model BA-9200, lot T0206; Vector Labs) for 1 h at room temperature. After incubation for 1 h in 1:100 avidin-biotin complex (Vectastain ABC kit; Vector), DBH-labeled cells were stained by incubation in 3,3′-diaminobenzidine-tetrahydrochloride (DAB) chromogen solution prepared in PBS (0.05% DAB, 0.009% H₂O₂, Sigma-Aldrich). The slides were rinsed in PBST, dehydrated in graded alcohol, immersed in xyol, and cover-slipped with Entellan (Merck, Germany).

Another series of sections was immunohistochemically processed for c-Fos protein to evaluate the effect of lesioning AP noradrenaline neurons on amylin-induced neuronal activation. Brain sections were blocked in donkey normal serum (1.5% in 0.3% PBST; Jackson ImmunoResearch) for 2 h and then incubated in the primary rabbit anti-c-Fos IgG fraction of antiserum against peptide corresponding to the NH₂-terminal region of c-Fos (1:10,000 in 0.3% PBST; model F7799, lot 124K4881; Sigma-Aldrich) for 48 h at 4°C. A biotin conjugated donkey anti-rabbit IgG (H+L) secondary antibody was used (1:1,000, model 711–065-152, lot 68226; Jackson ImmunoResearch) for 90 min at room temperature. After incubation for 1 h in 1:100 ABC, c-Fos was visualized by incubation for 7 min in nickel-cobalt-enhanced DAB solution in Tris-HCl (0.05% DAB, 0.008% H₂O₂, 0.08% NiCl₂, and 0.01% CoCl₂; Sigma-Aldrich). The slides were mounted as previously described. Brain sections were analyzed at 10- and 20-fold magnification using a microscope equipped with a digital camera (Axioskop; Carl Zeiss, Feldbach, Switzerland). All images and figure panels were prepared in Corel Draw 12 or in Adobe Photoshop CS. Small adjustments of brightness and contrast were made in some pictures for adequate reproduction quality. Quantification of the total number of DBH and of c-Fos immunoreactive neurons in every third section of the AP and postsubpontine NTS (bregma −13.68 to −14.28) was performed at the microscope with the ×20 objective, resulting in 6–7 slices per area and rat. The investigator was blinded to the groups (lesion, treatment). Group means were calculated from the averaged cell counts per animal. The percentage of noradrenaline cell lesion and of noradrenaline cells remaining was calculated; 100% corresponded to the mean number of DBH positive cells in sham-lesioned rats. An animal was considered to be noradrenaline lesioned if there were <50% remaining DBH-positive neurons in the AP, no lesion due to DSAP spread into the NTS, and no mechanical lesion due to the injection. These criteria were chosen based on previous pilot studies suggesting that the noradrenaline lesions produced in the AP do not nearly reach 100%, and on studies targeting noradrenaline neurons in the NTS that suggested that at least a 50% lesion was necessary to observe a feeding effect (32, 33).

The DBH fiber density in the LPB region from bregma level −8.76 to −9.36 (27) was evaluated semiquantitatively to determine the LPB area affected by DSAP injection; this allowed identification of the localization of the injection site and the size of the diffusion area. DBH fiber densities were qualitatively rated as +, presence of DBH fibers; ++, dense; ++++, very dense; +++++, extensive DBH fibers.

**Antibody Specificity**

The specificity of the primary antibodies was demonstrated by the respective suppliers and was confirmed by our own controls. Specifically, the anti-c-Fos antibody from Santa Cruz used in study 1 was tested by Western blot analysis. The staining from anti-c-Fos antibody from Sigma used in study 2 was shown to be specifically inhibited with c-Fos immunizing peptide. The mouse monoclonal anti-DBH-IgG specificity was demonstrated by the supplier (Chemicon, Millipore) by immunohistochemistry staining pattern/morphology in rat spinal cord and dorsal root ganglia. We observed specific DBH-positive staining in the expected A1 (ventrolateral medulla), A2 (dorsomedial medulla including the AP and NTS) regions in our stainings, but not in surrounding brain regions that do not contain noradrenergic neurons (gracile nucleus, hypoglossal nucleus) (3). The specificity of the secondary antibodies was tested by the suppliers by immunoelectrophoresis. They were tested by ELISA or were solid-phase adsorbed to ensure minimal cross-reactivity with serum proteins from other species. The lack of unspecific tissue binding was further verified by control immunostainings omitting the primary antibody.

**Statistical Analyses**

All data are expressed as means ± SE. In study 1, one-way ANOVA followed by Newman-Keuls post hoc tests was employed to compare between groups. In study 2, daily body weight gain and 24-h food intake data was analyzed by two-way repeated-measures ANOVA comparing noradrenaline-lesioned with sham-lesioned rats, and Bonferroni post hoc tests were used to detect differences between the two groups at each individual day. The number of DBH cells in the AP and NTS, body weight, and 23-h food intake on the day of each trial and of perfusion, were compared by an unpaired Student’s t-test. The 23-h food intake before each trial was compared between trials by one-way ANOVA to confirm that animals had similar baseline food intake in all trials. Data from the feeding trials were analyzed by two-way repeated-measures ANOVA for the effect of noradrenaline lesion and treatment, and Bonferroni post hoc pairwise comparisons to detect significant differences between groups. For statistical analysis of differences in amylin-induced c-Fos expression, a two-way ANOVA followed by Bonferroni post hoc tests were performed. Pearson correlation analysis was conducted to evaluate the correlation between the percentage of remaining DBH-positive cells in the AP and the number of c-Fos positive neurons. For all statistical tests a P value <0.05 was considered significant.

**RESULTS**

**Study 1: Phenotypical Characterization of Amylin-Activated Neurons by Double Immunohistochemistry**

Amylin significantly increased the number of c-Fos-positive cells in the AP compared with saline, and animals treated with amylin at the 20 μg/kg dose had a significantly greater number of c-Fos-positive cells than animals treated with 5 μg/kg amylin (Fig. 1). Double-labeling analyses revealed that ~50% of specifically amylin-activated cells from both doses (22 ± 1 cells, amylin 5 μg/kg and 37 ± 8 cells, amylin 20 μg/kg) within the AP were also DBH-positive (11 ± 5 cells, amylin 5 μg/kg and 19 ± 8 cells, amylin 20 μg/kg); furthermore, the level of colocalization was significantly higher than in saline-injected rats. From the total DBH-positive population of neurons, 17% and 23% were c-Fos-positive after amylin 5 and 20 μg/kg, respectively, vs. 0% after saline. The total number of DBH-positive cells was similar in all three treatment groups.

**Study 2**

Role of noradrenaline in amylin’s hypophagic action. Sixteen DSAP-treated animals that did not show mechanical damage in the AP were included in the studies. According to our criteria, 10 of these animals had lesions of >50% noradrenaline neurons in the AP and were hence considered
noradrenaline lesioned (Fig. 2A); six animals had lesions of < 50% (small lesions). Using this technique, we were unable to achieve a complete ablation of noradrenaline neurons. The mean percentage of the remaining noradrenaline neurons in the 10 animals with noradrenaline lesion was 44 ± 1% (range between 37 and 50%). That is, the mean noradrenaline lesion success was 56 ± 1%. The mean number of DBH-positive neurons in the AP of the 10 noradrenaline-lesioned rats was significantly lower than in the sham-lesioned group (Fig. 2B). These same rats showed no reduction in the number of DBH-positive neurons in the adjacent NTS (Fig. 2, B, E, and F). Analysis of LPB DBH fiber density in the 10 animals with noradrenaline lesions revealed that these animals had, on average, dense (++) to very dense (+++) DBH terminals compared with the 12 sham-lesioned rats who displayed very dense (+++) or extensive (++++) DBH fibers in the LPB region from bregma level –8.76 to –9.36. The DBH staining also allowed estimating the center of DSAP injections, which were shown to be restricted to bregma levels between –8.88 and –9.24 in all lesioned animals.

Body weight increased significantly in both groups of rats over the observation time (Fig. 3A; P < 0.001), but body weight gain did not differ between noradrenaline-lesioned and sham-lesioned rats (effect of lesion: P = 0.13; interaction lesion × time: P = 0.32). Two-way ANOVA revealed an effect of time on 24-h food intake, i.e., daily food intake increased progressively over the observation time (Fig. 3B; P < 0.001). However, 24-h food intake did not differ between noradrenaline-lesioned and sham-lesioned rats on any day before or after surgery (effect of lesion: P = 0.61; interaction lesion × time: P = 0.28). There was no significant body weight difference between groups on the day of each feeding trial [trial 1: sham 393 ± 7 g, noradrenaline lesioned 406 ± 7 g, P > 0.05, not significant (NS); trial 2: sham 419 ± 7 g, noradrenaline lesioned 435 ± 9 g, P > 0.05, NS; trial 3: sham 435 ± 8 g, noradrenaline lesioned 450 ± 9 g, P > 0.05, NS]. Body weights on the day of perfusion did not differ significantly ( sham 454 ± 8 g, noradrenaline lesioned 467 ± 10 g, P > 0.05, NS). Twenty-three-hour food intake just before each feeding trial also did not differ significantly between groups (trial 1: sham 31.0 ± 0.9 g, noradrenaline lesioned 32.3 ± 0.9 g, P > 0.05, NS; trial 2: sham 32.3 ± 1.0 g, noradrenaline lesioned 33.6 ± 0.7 g, P > 0.05, NS; trial 3: sham 30.9 ± 1.1 g, noradrenaline lesioned 32.3 ± 0.8 g, P > 0.05, NS) nor between the three trials.

Two-way ANOVA revealed a significant effect of 5 μg/kg amylin treatment on 30 min (P < 0.05) and 60 min (P < 0.01) cumulative food intake. Although this analysis did not detect a

Fig. 1. A–B: double immunohistochemical stainings of amylin-induced c-Fos expression and dopamine-β-hydroxylase (DBH) in the area postrema (AP). The c-Fos-positive nuclei are green; the red cytoplasmic staining corresponds to DBH. B: high-power magnification picture of the indicated AP area. Arrow 1, single labeled c-Fos positive nuclei; arrows 2, single labeled DBH neurons; arrows 3, double-labeled neurons. C: quantification of c-Fos, DBH, and double-positive neurons in the AP of animals injected with either saline (white columns), 5 μg/kg (black columns) or 20 μg/kg amylin (striped columns). Data are expressed as group means ± SE (n = 4); *significantly different from vehicle; †significantly different from amylin 5 μg/kg (P < 0.05).
significant effect of lesion or interaction of treatment with lesion, post hoc analysis revealed that amylin (5 μg/kg) only reduced eating in sham-lesioned rats (30 min: \( P < 0.01 \), 60 min: \( P < 0.05 \), saline vs. amylin) but not in animals with an AP noradrenaline lesion (Fig. 4). Cumulative 30- and 60-min food intake was similar in saline-treated, sham- and noradrenaline-lesioned controls, respectively. The higher dose of amylin (20 μg/kg) reduced eating in sham-lesioned rats at both time points (30 min: \( 3.9 \pm 0.3 \) g saline vs. \( 2.8 \pm 0.5 \) g amylin, \( P < 0.05 \); 60 min: \( 4.1 \pm 0.3 \) g vs. \( 3.1 \pm 0.4 \) g, \( P < 0.05 \)). Two-way ANOVA revealed an effect of amylin treatment at both time points (\( P < 0.01 \)). In noradrenaline-lesioned rats, this dose of amylin induced a significant reduction of eating only at the 60-min time point (30 min: \( 3.8 \pm 0.3 \) g saline vs. \( 3.0 \pm 0.4 \) g amylin 20 μg/kg, \( P > 0.05 \) NS; 60 min: \( 4.1 \pm 0.4 \) g vs. \( 3.1 \pm 0.4 \) g, \( P < 0.05 \)).

**Role of the noradrenergic system in amylin’s effect on c-Fos expression.** Amylin significantly increased the number of c-Fos-positive AP neurons in sham-lesioned rats (Fig. 5, A and C). Although c-Fos positive cells were still evident following amylin administration in noradrenaline-lesioned rats, this difference failed to reach statistical signifi-

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**Fig. 2.** A–F: noradrenergic (NA) neuron labeling in rats injected with anti-dopamine-β-hydroxylase-saporin (DSAP) or IgG-saporin (sham). A: categorization of the animals targeted in the AP with DSAP, into NA-lesions above or equal to 50% or below 50%, calculated from the average number of DBH-positive cells in sham-lesioned rats. B: mean number of remaining DBH-positive neurons in the AP and nucleus of the solitary tract (NTS) of the 10 animals with NA-lesions ≥50% (white bars) and the 12 sham-lesioned rats (black bars, ± SE); ***\( P < 0.001 \). C and E: DBH immunohistochemical staining of the AP (C) and NTS (E) of a sham-lesioned animal. D and F: DBH staining of the AP (D) and NTS (F) of a NA-lesioned animal injected with DSAP. Scale bar = 100 μm.
Moreover, amylin-treated, noradrenaline-lesioned rats clearly displayed a significantly lower number of c-Fos-positive neurons in the AP than sham-lesioned rats ($P < 0.001$). Two-way ANOVA revealed a significant effect of lesion (noradrenaline lesioned vs. sham, $P < 0.01$) and treatment (amylin vs. saline $P < 0.001$), and also a significant interaction of treatment with lesion ($P < 0.05$).

We generally observed low c-Fos expression in the NTS. Nonetheless, two-way ANOVA revealed a significant effect of amylin ($P < 0.01$). Amylin increased c-Fos expression only in sham- but not in noradrenaline-lesioned rats (sham: $0.3 \pm 0.1$ saline vs. $6.4 \pm 2.3$ amylin, $P < 0.01$; noradrenaline lesioned: $0.4 \pm 0.3$ saline vs. $2.6 \pm 1.4$ amylin, $P > 0.05$, NS).

To test whether the lesion extent had an influence on the number of neurons activated after amylin, a correlation analysis was conducted. In this analysis, all DSAP-injected animals that were treated with amylin were included regardless of the lesion success. There was a positive correlation between the number of c-Fos-positive neurons in the AP after amylin and the number of remaining DBH-positive neurons (expressed as percentage of the average number of DBH cells in sham-lesioned rats) that were detected in the same animal (Fig. 6; $P < 0.05$); this indicates that rats with larger noradrenaline lesions and less remaining DBH-positive neurons also displayed lower numbers of amylin-activated AP neurons.

**DISCUSSION**

The present studies implicate amylin-activated noradrenergic neurons within the AP in mediating amylin’s hypophagic effects. A large proportion of AP neurons that were activated by peripheral amylin were noradrenergic (50%). A specific lesion of about half of the total AP noradrenergic population with the toxin DSAP was sufficient to abolish the hypophagic effect of $5 \mu g/kg$ amylin. Lesion of the AP noradrenergic neurons also reduced amylin’s ability to induce c-Fos expression in AP and NTS neurons.

Amylin induced a significant and dose-dependent increase in the number of c-Fos-positive AP neurons consistent with previous reports (23, 31, 34). The number of DBH/c-Fos double-labeled neurons was higher in amylin than in saline-injected rats. Interestingly, the relative percentage of double-labeled neurons in relation to the total number of c-Fos-positive cells was similar at both amylin doses (50%). Of note, there was no difference in the total number of DBH-positive neurons between groups; therefore, the number of neurons coexpressing DBH and c-Fos in the different conditions was not influenced by differences in the level of DBH labeling.

To lesion the AP noradrenergic neurons, we injected DSAP into the AP and bilaterally into the LPB. The AP is exposed to and communicates with the ventricular system and the blood; therefore substances injected into the AP may partially be washed out by the cerebrospinal fluid or by the blood circulation. Neuroanatomical tracing and noradrenaline immunohistochemical mapping studies suggest that the AP sends dense noradrenergic projections to the LPB (22, 24, 36), while the LPB itself has no noradrenergic cell bodies (Ref. 11 and C. S. Potes and V. F. Turek, unpublished observations). Projections from the AP to the LPB, either directly or via the NTS, seem...
Fig. 5. Amylin induced c-Fos expression in the AP in rats with a sham (A) or with ≥50% NA-lesion in AP neurons (B). C: quantification of amylin-induced c-Fos expression in the AP in sham (n = 6) and NA-lesioned rats (n = 5). There was an attenuated amylin induced c-Fos response in NA-lesioned rats. Data are expressed as group means ± SE. Bars with different letters are significantly different (P < 0.05). Scale bar = 100 μm.

to be involved in the transmission of amylin signaling and mediation of its hypophagic effect (7, 29, 31). Thus, we chose to inject DSAP into the LPB to increase the proportion of retrogradely lesioned AP noradrenaline neurons. DSAP injections into the LPB reduced the DBH-fiber density in the targeted LPB region known to receive AP projections [−8.76 to −9.36 according to bregma (27)]. We suggest that the failure to completely eliminate LPB DBH fibers may be, in part, to the small amount of injected DSAP or because terminals of the observed fibers may reside in regions outside the target area.

In this study, we specifically lesioned AP noradrenergic neurons, and did not observe any signs of lesion of NTS noradrenaline neurons after DSAP treatment. Some reports suggest that the AP receives some noradrenaline and adrenergic fibers from the NTS (2). Our observation that there was no detectable lesion of noradrenaline neurons in the NTS after DSAP injections into the AP suggests that only few NTS noradrenergic neurons project to the AP. This is consistent with studies showing only a weak innervation of the AP by A2 neurons (1, 36). Although there are noradrenaline projections from the NTS to the LPB (12, 24), our LPB DSAP injections did not reduce the noradrenaline population of the subpos-tremal NTS. Previous tracing studies showed that there are many NTS projections to the caudal LPB (−9.24 to −9.60 relative to bregma), while the AP terminals localize more within the DSAP targeted area [bregma −8.88 to −9.24 (9, 29, 39, 41)]. Furthermore, it seems that only 17% of all NTS neurons projecting to the entire parabrachial nucleus are catecholaminergic (12). It is therefore possible that the small DSAP targeted area of the LPB receives mainly AP noradrenergic projections, while the more caudal LPB and the remaining parabrachial nuclei, which were not targeted by DSAP in our experiments, receive more NTS noradrenergic projections. As the extant literature characterizing the presence of noradrenaline projections from AP and NTS to the LPB does not provide enough topographical details about the projections pattern of noradrenaline neurons in the LPB, further studies are warranted (12, 22, 24).

The saporin portion of DSAP leads to neurodegeneration via inactivation of the 60s subunit of the ribosomes, thus prohibiting protein synthesis (38). DSAP produces noradrenaline cell death within 1–2 wk (6, 20, 21). Therefore, from the beginning of the second wk after DSAP treatment, the effects of noradrenaline lesion on baseline measurements of spontaneous food intake and body weight could occur. In contrast to noradrenaline lesions in the NTS (32), which resulted in decreased body weight gain from day 7 postsurgery, we observed no difference in body weight change and spontaneous daily food intake throughout the entire observation period between sham and AP noradrenaline-lesioned animals.

In the present study, a lesion of only about half of the total AP noradrenergic population abolished the inhibitory effect of 5 μg/kg amylin on feeding; at the high dose (20 μg/kg) amylin slightly reduced eating but only at the 60-min time point after injection. This may have been mediated by the remaining noradrenaline neurons in the AP or by amylin-sensitive neurons of a different phenotype. As indicated above, the high dose of amylin activated a larger number of noradrenergic AP neurons, even though the percentage of DBH/c-Fos double-labeled neurons was similar at both amylin doses. This suggests that the high dose of amylin may have recruited more noradrenergic AP neurons that survived after DSAP injection (~44% noradrenaline neurons remaining) than the low dose; this may explain why the high dose of amylin still reduced eating in rats with a noradrenaline lesion. Nevertheless, our study highlights the importance of the noradrenergic system of the AP in the mediation of amylin’s effect on food intake. It is
worth mentioning that the six rats with noradrenaline lesions smaller than 50% showed a significant reduction in food intake only at the 60-min time point (data not shown). This suggests that rats having small noradrenaline lesions are partly responsive to amylin. Furthermore, the lower sensitivity to amylin in rats with small noradrenaline lesions in the AP underscores the importance of the noradrenaline system in mediating amylin’s hypophagic effect.

The specific AP noradrenaline lesion also reduced amylin’s ability to induce c-Fos expression in both the AP and the NTS. The reduction observed in the AP was presumably due to the lesion of noradrenaline neurons that represent the primary targets for amylin. This notion is further supported by the correlation between the number of amylin-activated neurons and the number of remaining noradrenaline neurons in the AP. This means that rats with larger noradrenaline lesions, and hence less remaining DBH-positive neurons, also displayed lower numbers of amylin-activated AP neurons. The amylin-induced activation of NTS neurons is known to be secondary to a direct activation of AP neurons (31, 35). Therefore, the attenuation of amylin-induced c-Fos in the NTS in noradrenaline-lesioned rats was most likely a direct consequence of a reduction in the excitatory input from the AP, the primary site for amylin’s action (14).

Our studies do not allow for differentiation between the roles of direct noradrenergic AP-LPB vs. indirect AP-NTS-LPB projections mediating the hypophagic effect of amylin. AP noradrenergic neurons are known to project to the NTS (2), but direct catecholaminergic projections from the AP to the parabrachial nucleus have also been proposed to be involved in conveying hormonal and enteroceptive signals from the hindbrain to the forebrain (24). In conclusion, we provided evidence for an important functional role of AP noradrenergic neurons in the mediation of amylin’s hypophagic effect. Recognizing which neurotransmitter is involved in amylin’s signaling at the brain stem is important to define future treatment targets and predict possible side effects for the use of amylin agonists as therapeutic agents.

**Future Perspectives**

It will be interesting to employ the same approach used here to investigate the role of the noradrenaline system in other reported amylin effects that are presumed to be mediated via the AP, such as the inhibition of gastric emptying and of pancreatic glucagon secretion, increase in energy expenditure, modulation of CCK anorexia, etc. The present study suggests that noradrenergic transmission is involved in propagating amylin’s hypophagic action. By employing complementary techniques such as microdialysis, it may be possible to formally clarify whether amylin induces noradrenaline release from AP neurons and whether these terminals reside within the NTS and/or LPB. Intraparenchymal injections of adrenergic receptor antagonists within the NTS and the LPB would provide further confirmation for a region-specific role of noradrenaline for amylin-induced inhibition of food intake.

It will be also valuable to further characterize the neurochemical nature of the remaining nonnoradrenergic amylin-activated AP neurons and their possible function with respect to amylin’s centrally mediated effects.

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**DISCLOSURES**

The authors disclose a conflict of interest (such as defined by AJP policy). V. F. Turek, R. L. Cole, C. Vu, B. L. Roland and J. D. Roth were all employees and stockholders of Amylin Pharmaceuticals, at the time these studies were conducted.

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