The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis

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Submitted 17 June 2003; accepted in final form 27 August 2003

Riediger, T., D. Zuend, C. Becskei, and T. A. Lutz. The anorectic hormone amylin contributes to feeding-related changes of neuronal activity in key structures of the gut-brain axis. Am J Physiol Regul Integr Comp Physiol 286: R114–R122, 2004. First published September 4, 2003; 10.1152/ajpregu.00333.2003.—Amylin is a peptide hormone that is cosecreted with insulin from the pancreas during and after food intake. Peripherally injected amylin potently inhibits feeding by acting on the area postrema (AP), a circumventricular organ lacking a functional blood-brain barrier. We recently demonstrated that AP neurons are excited by a near physiological concentration of amylin. However, the subsequent neuronal mechanisms and the relevance of endogenously released amylin for the regulation of food intake are poorly understood. Therefore, we investigated 1) amylin’s contribution to feeding-induced c-Fos expression in the rat AP and its ascending projection sites, and 2) amylin’s ability to reverse fasting-induced c-Fos expression in the lateral hypothalamic area (LHA). Similar to amylin (20 μg/kg sc), refeeding of 24-h food-deprived rats induced c-Fos expression in the AP, the nucleus of the solitary tract, the lateral parabrachial nucleus, and the central nucleus of the amygdala. In AP-lesioned rats, the amylin-induced c-Fos expression in each of these sites was blunted, indicating an AP-mediated activation of these structures. Pretreatment with the amylin antagonist AC-187 (1 mg/kg sc) inhibited feeding-induced c-Fos expression in the AP. Food deprivation activated LHA neurons, a response known to be associated with hunger. This effect was reversed within 2 h after refeeding and also in nonrefed animals that received amylin. In summary, our data provide the first evidence that feeding-induced amylin release activates AP neurons projecting to subsequent relay stations known to transmit meal-related signals to the forebrain. Activation of this pathway seems to coincide with an inhibition of LHA neurons.

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While stimulation of the LHA results in hyperphagia, animals with LHA lesions become hypophagic and show blunted feeding responses to hypoglycemia and neuroglucopenia (6). Neurons of the LHA are directly excited by a decrease of the glucose concentration (34), but they also have been shown to be indirectly modulated in response to changes in blood glucose, most likely by ascending projections from the brain stem (35). In line with these electrophysiological studies, it has been demonstrated immunohistologically that insulin-induced hypoglycemia activates neurons in the LHA, including cells expressing the orexigenic peptide orexin (8), which is upregulated during fasting and hypoglycemia (9). Interestingly, the hypoglycemia-induced c-Fos expression could only be observed in fasted but not in freely feeding rats, indicating an inhibitory effect of prandial signals on neuronal activity in the LHA. As further suggested by our recent studies, amylin serves in fasted but not in freely feeding rats, indicating an inhibitory effect of prandial signals on neuronal activity in the LHA. As further suggested by our recent studies, amylin inhibitory effect of prandial signals on neuronal activity in the LHA. As further suggested by our recent studies, amylin

To test this hypothesis, we analyzed whether fasting-induced c-Fos expression in the LHA might be reversed by refeeding and by peripheral amylin injection in nonreared animals.

MATERIALS AND METHODS

Male adult Wistar rats were used, which had ad libitum access to standard laboratory rat chow (890 25 W16, Provimi Kliba, Gossau, Switzerland) and water, except during food deprivation as indicated below. All animals were housed individually and maintained in a temperature-controlled room on an artificial 12:12-h dark-light cycle (21 ± 1°C, lights on at 6:00 AM). Rats were adapted to the housing conditions and to handling for at least 2 wk before the start of the experiments.

Surgery. The surgical procedure to lesion the AP was adapted from McGlone et al. (29) and is the same as described in our previous studies (27). After exposure, the AP was blotted with cotton ear swabs (29) and is the same as described in our previous study (40). In preliminary experiments it had been confirmed that a subcutaneous injection of amylin (5 µg/kg) because the anorectic effect of amylin is known to be attenuated by AP lesion (28). APX rats, which responded to the amylin injection by a reduction of food intake of >10% compared with the intake under control conditions, were excluded from the studies. For histological confirmation of the lesion, slices from the entire lesioned area were obtained. Only those animals were included in which the AP was totally ablated and the surrounding NTS tissue appeared largely intact.

c-Fos expression induced by exogenous amylin. All treatments were conducted during the first 4 h of the light phase. For the immunohistochemical analysis of amylin-induced c-Fos expression, the APX and nonlesioned rats received a subcutaneous injection of amylin (20 µg/kg sc, Peninsula Laboratories, Belmont, CA) or saline (controls), respectively. This dose of amylin represents an intermediate dose compared with dose ranges characterized in earlier studies (45, 46). In preliminary experiments it had been confirmed that a subcutaneous injection of 20 µg/kg amylin and an intraperitoneal dose of 5 µg/kg, which has been used to characterize amylin’s anorectic action, produce similar c-Fos responses in the AP. One-hundred twenty minutes after amylin or control administration, animals were deeply anesthetized (pentobarbital sodium, 80 mg/kg ip) and transcardially perfused with ice-cold sodium phosphate buffer (PB, 0.1 M, pH 7.4), followed by 4% paraformaldehyde in PB. Brains were postfixed for 2 h in 4% paraformaldehyde and cryoprotected by incubation in 20% sucrose in PB (48 h at 4°C). Coronal brain sections (20 µm thick)

containing the AP, the NTS, the LPB, and the CeA (according to the brain map of Paxinos and Watson (36)) were cut using a cryostat and were thaw-mounted on adhesive slides (SuperFrost Plus, Faust, Switzerland). After air-drying and rehydration in phosphate-buffered saline containing 0.1% Triton X-100 (PBS-T, pH 7.4), sections were incubated for 1 h at room temperature using a 1.5% dilution (0.3% PBS-T) of normal donkey serum (Milan Analytica AG, La Roche, Switzerland) to block unspecific binding sites. For detection of c-Fos expression, sections were incubated for 48 h at 4°C using a 1:1,000 dilution of a rabbit polyclonal antiserum directed against c-Fos (Ab5, Calbiochem-Novabiochem, Bad Soden, Germany). After washing in 0.1% PBS-T, sections were incubated for 75 min at room temperature with Cy3-conjugated goat anti-rabbit immunoglobulin (Dianova, Hamburg, Germany). Sections were rinsed in PBS and mounted with Citifluor mounting medium (PBS/glycerol 1:1 vol/vol; Citifluor Products, Kent, UK). Digitalized photographs were taken on a fluorescence microscope (Zeiss Axioskop).

c-Fos expression induced by refeeding and endogenous amylin. To investigate amylin’s contribution to feeding-induced alterations of neuronal activity, four groups of weight-matched rats were food deprived for 24 h starting at the beginning of the dark phase. Thirty minutes before the subsequent dark onset, two groups of rats were pretreated with AC-187 (1 mg/kg, Amylin Pharmaceuticals, San Diego, CA) or injected with saline (control). Rats were then either re rer at dark onset for 2 h (AC-187/refed; saline/refed) or remained fasted for additional 2 h (AC-187/fasted; saline/fasted). In our previous study, this dose of AC-187 effectively blocked the c-Fos expression in AP neurons elicited by peripheral amylin injection (40). The amount of ingested food during refeeding was determined by subtracting the weight of the remaining pellets (including spills) after refeeding from the total weight of the presented chow. c-Fos immunoreactivity in the brains was analyzed as described above.

Effect of refeeding and amylin on c-Fos expression in the LHA. The influence of refeeding and peripherally applied amylin on fasting-induced c-Fos expression in the LHA was investigated with the same fasting/refeeding paradigm as described above. Three experimental groups of rats were fasted for 24 h. At dark onset, two groups of rats were injected with amylin (20 µg/kg sc) or saline, respectively. Food was withheld for additional 2 h. The third group of rats was re fed at dark onset for 2 h. After the 2-h refeeding/treatment period, rats were euthanized, and brains were processed for c-Fos immunoreactivity. In addition to these experimental groups, a nonfasted ad libitum-fed control group of rats was also included, which received a control injection of saline.

Data collection and analysis. For quantification of the c-Fos responses, c-Fos-immunoreactive (c-Fos-IR) cells were counted in each animal from all slices of the AP, the NTS (at the level of the AP), and from 10 to 15 consecutive slices of the LPBN (between bregma −9.3 and −9.6). In the CeA (between bregma −2.3 and −3.0) and the LHA (between bregma −1.8 and −2.1), every third slice was evaluated. In the LHA, c-Fos-IR cells were counted in the dorsal aspect of the LHA located between the fornix and the internal capsule. The cells counts from each brain region were averaged for each animal. The group means ± SEs were calculated by the averaged cell counts from each animal in the respective treatment group (n = number of animals).

For pairwise comparisons, statistical differences between the treatment groups were analyzed by Mann-Whitney rank sum test. Statistical differences between more than two experimental groups were analyzed by one-way ANOVA, followed by Student-Newman-Keuls post hoc test. Differences were considered significant with P < 0.05.

RESULTS
c-Fos expression induced by endogenous amylin. In accordance with our previous study (40), the peripheral injection of amylin elicited a strong c-Fos response in the AP, while
c-Fos-IR neurons were almost absent under control conditions. The basal c-Fos expression was similarly low in the NTS, LPBN, and CeA, but was strongly increased in amylin-treated rats. Figure 1 shows representative photomicrographs of saline-treated controls and amylin-treated rats. In the LPBN, amylin-induced c-Fos expression was confined to the external part. c-Fos-IR nuclei in the CeA were localized at a rostrocaudal level between bregma −2.20 and −3.00.

In APX rats, which had been confirmed to be insensitive to amylin’s anorectic action (results not shown), amylin failed to produce c-Fos responses in any of the investigated structures. Figure 2 illustrates representative sections of the AP/NTS region of a nonlesioned amylin-treated rat compared with the corresponding region of an amylin-injected APX rat. In the APX animal, the nervous tissue of the AP was totally destroyed, while the NTS appeared largely intact. Although
c-Fos-IR nuclei were abundant in the NTS of the nonlesioned animal, no amylin-induced c-Fos expression was observed in the NTS of the APX rat. Similar results were obtained in the LBPN and CeA (immunostainings not shown).

As shown by the quantitative analysis of Fig. 3, amylin significantly increased the mean numbers of c-Fos-positive nuclei in the nonlesioned group compared with saline-treated controls. Amylin-treated APX rats displayed significantly lower numbers of activated cells in the investigated brain structures than amylin-injected nonlesioned animals. The mean numbers of c-Fos-IR neurons in the amylin-treated APX rats were consistently reduced to similar values compared with unstimulated nonlesioned rats.

**c-Fos expression induced by refeeding and endogenous amylin.** All refed rats included in this study ingested similar amounts of food during the 2-h refeeding period. The average food intake of the saline-treated and the AC-187-treated rats was almost identical [3.6 ± 0.8 g/100 g body wt (saline, n = 7) vs. 3.5 ± 0.6 g/100 g body wt (AC-187, n = 4)].

Refeeding led to a similar pattern of neuronal activation as described above for the amylin-induced c-Fos expression. While basically no c-Fos staining was observed in the AP, NTS, LBPN, or CeA of fasted saline-treated rats, refed rats injected with saline displayed high numbers of c-Fos-positive neurons in these sites (Fig. 4). It is noteworthy that the localization of activated neurons in the parabrachial nucleus (PBN) of refed rats was not only confined to the external part of the LPBN as in amylin-treated rats but comprised also medial regions of the PBN (Fig. 4). As shown in the quantification of the average c-Fos responses (Fig. 5), the mean number of activated neurons in the AP, NTS, LBPN, and CeA after refeeding was significantly higher than the very low level of c-Fos immunoreactivity observed under fasting conditions.

Pretreatment with the amylin receptor antagonist AC-187 partly blocked the refeeding-induced c-Fos expression in the AP. In Fig. 6, immunostained AP/NTS regions of fasted and refed animals that received a control injection of saline or AC-187 are presented. In AC-187-treated refed animals, the c-fos response in the AP was reduced, while the number of positively labeled cells in the NTS appeared to be unaffected by the antagonist. In accordance with our previous study with freely feeding rats (40), the c-Fos expression induced by AC-187 in fasted animals was similar to the low basal activity observed in saline-treated controls, underlining the lack of specific or unspecific receptor activation by the antagonist per se under these experimental conditions.

The antagonistic effect of AC-187 on refeeding-induced c-Fos expression in the AP is quantified in Fig. 7. The average number of activated neurons was reduced by 53% in animals that received AC-187 before refeeding. In contrast to its effect in the AP, AC-187 did not block refeeding-induced activation in the other investigated structures, because the numbers of c-Fos-IR cells were not significantly affected by AC-187 in any of these nuclei (data not shown).

**Effect of refeeding and amylin on c-Fos expression in the LHA.** Twenty-four-hour food deprivation led to an activation of neurons in the LHA. The c-Fos-IR neurons that were activated by fasting were located in a discrete region in the dorsolateral subregion of the LHA bordering the internal capsula (between bregma −1.80 and −2.10). In Fig. 8, the topographical distribution of the responsive neurons is presented in a low-magnification photomicrograph of the entire LHA and in a higher magnification of the respective area. As shown by Fig. 8, almost no activated cells were observed in the corresponding region in refed animals, indicating that the ingestion of food reversed the fasting-induced c-Fos expression in the LHA. Interestingly, the same reversal of neuronal activity was mimicked by peripheral injection of amylin in rats that were not allowed to refeed (Fig. 8) and were thus devoid of any other meal-related signal possibly interfering with the action of amylin.

On the quantitative scale, amylin significantly reversed the fasting-induced activation to a similar degree as refeeding the animals. The mean numbers of c-Fos-positive cells did not differ significantly between both groups and were 63% (amylin) and 70% (refeeding) lower than the average number of activated neurons in the saline-injected fasted group (Fig. 9). Although the number of c-Fos-IR neurons was lowest in the ad libitum-fed control group (83% lower than fasted/saline), there was no significant difference compared with the fasted/amylin-injected or the fasted/refed group.

**DISCUSSION**

Our current investigations demonstrating that peripherally applied amylin acts via the AP to stimulate an ascending pathway extends earlier qualitative studies analyzing the effect of amylin on c-Fos expression in rats with lesions of the AP and the adjacent NTS (46). The amylin-induced c-Fos expression in the NTS, LPBN, and CeA was attenuated in APX rats, underlining the primary receptive function of the AP for peripheral amylin. This observation is in close correspondence with our previous work showing that amylin is ineffective to reduce food intake in APX rats (27, 28) and with electrophys-
iological studies demonstrating a dose-dependent excitatory effect of amylin on AP neurons (40). As furthermore shown by the latter investigations, amylin appears to activate AP neurons via formation of the intracellular second messenger cGMP. In line with evidence that amylin directly activates AP neurons via cGMP formation, we could demonstrate that amylin-dependent cGMP-formation colocalizes with the expression of the calcitonin receptor (42). The calcitonin receptor represents the core component of functional amylin receptors when co-expressed with receptor activity modifying protein (RAMP)-1 or RAMP-3 (12, 32).

The abovementioned evidence strongly suggests that the AP is the primary target of circulating amylin. Nonetheless, there are also studies demonstrating anorectic effects of centrally applied amylin (2, 10). According to previous studies (28), it appears likely that circulating amylin modulates short-term feeding behavior via a direct effect on brain areas different from the AP. The fact that amylin is transported across the blood-brain barrier (3) and the widespread distribution of amylin binding sites in the brain (48) may, however, be interpreted as an indication that amylin may also target other structures. In addition to the brain areas investigated in the present study, peripheral amylin has also been reported to activate neurons in the bed nucleus of the stria terminals (BNST) (46). In line with the observations of the present study, the amylin-induced c-Fos in this brain site is also secondary to an activation of AP neurons.

Similar to exogenously applied amylin, refeeding caused substantial neuronal activation in the AP, NTS, LPBN, and CeA. However, the amylin-induced c-Fos expression was confined to the external part of the LPBN, while in reed animals a high number of activated neurons was also abundant in the medial portion of the PBN. Although the amylin-induced activation in the LPBN is clearly secondary to activation of AP neurons, it remains unclear whether these cells were excited via direct monosynaptic projections from the AP (49) or were activated by relaying NTS neurons connecting the AP with the LPBN (37). Irrespective of the question of which of these pathways is involved in the amylin-mediated activation, the amylin-responsive LPBN neurons are most likely part of an
The enteroreceptive pathway known to be activated by gastrointestinal signals promoting satiety via stimulation of vagal afferents (16, 21, 53). Principally, the NTS and PBN seem to contain functionally and topographically distinct subsets of neurons, which receive enterosensory and gustatory inputs (15, 21). Our observation that, in contrast to amylin-injected rats, lateral (presumably enteroreceptive) and medial (presumably orosensory) subpopulations of PBN neurons are activated in refed rats is in line with the aforementioned considerations, since both vagal and gustatory pathways are activated in response to feeding.

The role of hindbrain mechanisms controlling food intake has been intensively investigated for the anorectic effect of amylin. Our results show that feeding-associated amylin release substantially contributes to neuronal activation of AP neurons in refed rats. Pretreatment with the amylin receptor antagonist AC-187 significantly reduced the refeeding-induced c-Fos response by 53%, indicating that feeding-associated amylin release substantially contributes to neuronal activation of AP neurons in refed rats.
CCK. It is generally accepted that CCK, which is postprandially released from mucosal I cells, induces a short-term satiety effect by stimulation of vagal afferents carrying CCK-A receptors (20, 44, 47, 50). In contrast to amylin, which exerts its anorectic effect via the AP, the CCK-induced inhibition of food intake is independent of the AP but requires an intact medial and commissural NTS as the main terminal field for the gastric branch of vagal afferents (15). In a recent study, the contribution of endogenously released CCK in postprandial satiety and c-Fos expression was confirmed in CCK-A receptor-deficient rats (OLETF rats) and by using the selective CCK-A receptor antagonist MK329 (18). Animals with blocked CCK signaling showed a reduced level of meal-induced c-Fos immunoreactivity in the NTS, indicating that postprandial neuronal activation in the brain stem partly depends on endogenous CCK.

By a blockade of amylin binding sites with the amylin receptor antagonist AC-187, we used a similar approach to investigate whether feeding-associated amylin release contributes to neuronal activation in the AP and subsequent relay centers. Consistent with our observation that AP neurons are excited by exogenous amylin, pretreatment with AC-187 partly blocked the feeding-induced c-Fos expression in AP neurons of refed rats. The fact that AC-187 did not completely prevent activation of AP neurons could reflect a stimulatory effect of other feeding-related signals coactivating AP neurons together with amylin. These factors could for example include a postprandial increase in blood glucose or a local release of the anorectic glucagon-like peptide 1 (GLP-1) from enteroceptive NTS neurons (23). Supportive of this issue, glucose and GLP-1 specifically activate amylin-sensitive AP neurons as demonstrated by our electrophysiological studies (38, 41).

In contrast to antagonizing the feeding-induced activation in the AP, AC-187 was ineffective at blocking meal-related c-Fos expression in the NTS, the LPBN, and the CeA, although these structures are evidently stimulated subsequent to amylin-dependent excitation of AP neurons (see above). The inability of AC-187 to block c-Fos expression in the latter areas after refeeding may be explained by converging afferent input from the vagus nerve at the level of the NTS. We thus propose that feeding-related signals transmitted via vagal afferents (e.g.,

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**Fig. 8.** Immunohistochemical c-Fos stainings of 20-μm-thick coronal sections of the rat LHA after 24 h of food deprivation, refeeding, and amylin injection (20 μg/kg sc). Fasted rats showed a high density of c-Fos-IR nuclei in a discrete dorsolateral area of the lateral hypothalamic area (LHA). Top left: low-magnification photomicrograph displaying the topographical localization of activated cells in the LHA (f, fornix; opt, optic tract). Top right: higher magnification of the dorsolateral region of the LHA bordering the internal capsule and displaying the highest density of fasting-induced c-Fos expression (between bregma −1.80 and −2.10). Bottom: no c-Fos expression was observed in the dorsolateral LHA of refed rats (left) and nonrefed rats receiving an injection of amylin (right), indicating that feeding and amylin reverse fasting-induced activation in the LHA.

**Fig. 9.** Quantification of c-Fos-IR nuclei in the LHA of fasted, refed, nonrefed/amylin-injected (20 μg/kg sc), and ad libitum-fed rats. Bars represent group means (means ± SE, nos. of animals/group are indicated below bars). The fasting-induced c-Fos expression in the LHA was significantly reversed in the refed and the nonrefed/amylin-injected group. Under both experimental conditions, the mean number of c-Fos-IR cells was ~75% lower compared with the number of activated neurons in fasted/saline-injected rats. Both groups did not differ significantly from the ad libitum-fed group. Bars with different lowercase letters are significantly different (P < 0.05).
CCK, gastric distension, etc.) activate NTS neurons, which are synaptically coactivated by amylin-excited AP neurons. As a consequence, feeding-induced c-Fos expression in the NTS appears to be unaffected by the blockade of amylin signaling because the abolished excitatory input from the AP to the NTS is masked by a strong vagal activation of NTS neurons.

The persistence of neuronal activation in the NTS-LPBN-CeA axis of refed/AC-187-treated rats is in accordance to the unaltered feeding response of these animals compared with saline-treated controls. Under similar experimental conditions, AC-187 did not affect food intake subsequent to food deprivation (54). It should be emphasized, however, that these results do not rule out a function of endogenous amylin as a postprandial satiety factor. As demonstrated by feeding studies with amylin knockout mice, amylin has a permissive effect on CCK and bombesin-induced satiety, since anorectic effects of these peptides were reduced in these animals (30).

Although the effect of amylin on its primary target neurons in the AP has been well characterized, the involvement of higher brain centers, especially hypothalamic structures, is poorly understood. There are currently no studies demonstrating an effect of peripheral amylin on neuronal activity in the hypothalamus, which is intimately interconnected with the brain stem-amygdala axis activated by amylin. As demonstrated by our recent study, peripherally administered amylin downregulates the expression of orexin in the LHA (4). Moreover, neurons of the LHA, partly including those expressing orexin, are activated in response to hypoglycemia and food restriction (8, 22). Hence, it appears likely that feeding-related signals and amylin in particular reduce feeding at least partly by inhibiting neuronal activity in the LHA. Our current data on the feeding- and amylin-induced reversal of fasting-induced c-Fos expression in the LHA underline the relevance of this mechanism. It has to be stressed that amylin administration alone (without refeeding) is sufficient to reverse fasting-induced LHA activation. Thus exogenously applied amylin seems to effectively mimic the ingestion of food as far as the inhibitory impact of LHA neurons is concerned. It can be excluded that this inhibitory amylin-dependent influence on LHA neurons is due to a direct effect of amylin on these cells because the LHA is devoid of amylin binding sites (48). For this reason we propose that the LHA receives inhibitory input from amylin-activated neurons of the AP-CeA pathway. The LHA is densely innervated by projections from the CeA (5) but also from the LPBN (7) and the NTS (37). It remains to be determined if any of these projections contributes to the observed inhibition of LHA neurons. In a recently published electrophysiological in vivo study, glucose-inhibited neurons of the LHA were inhibited subsequent to electrical stimulation of the gastric vagal trunks (58). Because gastrointestinal vagal afferents transmit mechanical, chemical, and hormonal satiety signals to the NTS, this observation parallels our current finding of a feeding-induced inhibition of the LHA. Although our current study clearly identified LHA neurons, which are inhibited in response to food intake and amylin administration, the phenotype of these neurons remains unknown. Mappings of orexin immunoreactivity have identified orexin-expressing cell bodies predominantly in more caudal parts of the LHA (33). Therefore, double stainings detecting both amylin-induced c-Fos and orexin will have to be combined with the paradigm of our present study to answer the question of whether orexin-expressing LHA neurons are involved in the observed responses.

Perspectives

In summary, our study demonstrates an excitatory effect of endogenously released amylin on neurons of the AP. The amylin-mediated stimulation of AP neurons is transmitted rostrally via synaptic activation of neurons in the NTS, the LPBN, and the CeA. The activation of this pathway in response to feeding appears to coincide with an inhibition of neurons in the LHA, which are activated during fasting. We thus propose that a prandial amylin secretion together with vagal signals from the gastrointestinal tract promotes satiety at least partly by shutting down the activity of LHA neurons, which is considered to correlate with hunger. Future histological studies should aim at identifying the phenotype of the feeding- and amylin-responsive neurons of the brain stem-LHA pathway. Furthermore, in analogy to feeding experiments with APX animals, the involvement of the LPBN and the CeA in amylin-induced satiety needs to be confirmed by lesion studies.

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