Proteins Activate Satiety-Related Neuronal Pathways in the Brainstem and Hypothalamus of Rats

Rodolphe Faipoux, Daniel Tomé, Sylvette Gougis, Nicolas Darcel, and Gilles Fromentin*

UMR914 Nutrition Physiology and Ingestive Behavior, INRA, AgroParisTech, CRNH-IleDeFrance, F-75005 Paris, France

Abstract

Our objective was to study the relationship between the satiety induced by high-protein meals and the activation of brain areas involved in the onset of satiety. In rats, we used immunohistochemistry to monitor brain centers activated by a meal by receiving information from the gastrointestinal tract or via humoral pathways. In the nucleus of the solitary tract (NTS), the acute or chronic intake of high-protein meals led to increased activation of the noradrenergic/adrenergic pathways involved in cholecystokinin-induced satiety. In the arcuate nucleus of the hypothalamus, the melanocortin pathway was also more strongly activated after the acute or chronic intake of high-protein meals. Moreover, the glucagon-like peptide 1 pathway arising from the NTS, which is triggered, among other behaviors, during nonphysiological anorexia, was not activated by high-protein meals, supporting the lack of aversive behavior associated with this diet. Taken together, these results show that the ability of high-protein meals to inhibit food intake occurs alongside the activation, in nutrient-sensitive brain areas, of several specific neuronal populations involved in satiety. J. Nutr. 138: 1172–1178, 2008.

Introduction

Dietary proteins are potent inducers of satiety and inhibitors of food intake in both rats and humans (1–6), but the brain areas and neuronal populations responsible for these effects are still not fully understood.

The involvement of vagal afferent pathways in satiety-related neuronal populations is not as easily distinguishable. We therefore preferred to use satiety throughout this article for clarity and to avoid confusion between 2 phenomena that are not so easily distinguishable.

1 Supported by Institut National de la Recherche Agronomique (Paris, France) and Institut National Agronomique Paris-Grignon (Paris, France).
2 Author disclosures: R. Faipoux, D. Tomé, S. Gougis, N. Darcel, and G. Fromentin, no conflicts of interest.
3 Supplemental Table 1 is available with the online posting of this paper at JN.nutrition.org.
4 Abbreviations used: ARC, arcuate nucleus of the hypothalamus; BSA, bovine serum albumin; CCK, cholecystokinin; CTA, conditioned taste aversion; dβH, dopamine-β-hydroxylase; GLP-1, glucagon-like-peptide 1; a-MSH, α-melanocortin stimulating hormone; NAVA, noradrenaline/adrenaline; NPY/AgRP, neuropeptide Y/Agouti related protein; NTS, nucleus of the solitary tract; P14, P55A and P55C, 14% P/E and 55% P/E acute and chronic total milk protein diets, respectively; PB, phosphate buffer; POMC, proopiomelanocortin.
5 In this article, we did not discriminate between satiation and satiety, even if central activation of nucleus of the solitary tract neurons are rather related to satiation and hypothalamic to satiety. However, projections between these 2 areas tend to blur this distinction. We therefore preferred to use satiety throughout this article for clarity and to avoid confusion between 2 phenomena that are not so easily distinguishable.
6 To whom correspondence should be addressed: E-mail: fromenti@agroparistech.fr.
high-protein meal and which neural populations were recruited within these specific areas. The response to the high-protein meal was determined in rats after 2 d (acute condition) or 21 d (chronic condition) of consumption of the daily high-protein meal.

Materials and Methods

Animals and diets. Adult male Wistar rats (n = 32) from Harlan were housed in individual cages at 22 ± 2°C under a 12-h reverse light/dark cycle (0900, 2100, lights on at 2100). All experimental procedures used during the study complied with the guidelines issued by the French National Animal Care Committee and were approved by the Regional (Île de France Sud) Animal Care and Ethical Committee. As for the composition of the diets used, standard protein diets were modified versions of the AIN-93M diet (2). All diets were moistened (1:2 ratio of powder:water for P55 and P14 diets when used as a meal, 1:1 for P14 diet when used during the ad libitum consumption period) to minimize spillage. Food intake was determined by the difference in food cup weight before and after each experimental period, corrected for spillage, the amount of water added, and evaporation. All rats had free access to water throughout the experimental period. For the first 10 d of the experiment (prefeeding period), rats were adapted to laboratory conditions and conditioned to a 2-meal pattern (as described below) with the P14 diet (baseline food). The last day of the prefeeding period is referred to as d 0.

Experimental design. The rats were divided into 4 groups (n = 8). Two separate experiments were conducted: 1 to monitor the effect of the high-protein meal during transition (2 d) and the other 1 after habituation (21 d). During the prefeeding period, rats received daily at 900 the first meal consisting of 30-min access to 3 g of P14 diet (43.8 kJ) (1,2), followed 90 min after the beginning of the first meal by ad libitum consumption of P14 diet for the remainder of the dark period (Fig. 1). In each experiment, at d 1, 1 group (P14) was on the same feeding pattern as during the prefeeding period, whereas 1 P55 group (P55A and P55C for transition and habituation experiment, respectively) received daily at 0900 the first meal consisting of 30-min access to 3 g of P55 (43.8 kJ), followed 90 min after the beginning of the first meal by ad libitum consumption of P14 diet until 2100. Therefore, instead of consuming a 14% protein on energy and 76% carbohydrates on energy diet throughout the day, these rats consumed an equivalent diet of 19% protein on energy and 71% carbohydrates on energy. In the transition experiment, P55A (acute effect) and P14 groups were killed after 2 d of treatment. In the habituation experiment, P55C rats (chronic effect) and P14 groups were killed after 21 d of treatment. Results for food intake are expressed in kJ and as the ratio between the actual consumption during the measured period of time and the mean consumption over the same period of time for the 4 consecutive days before d 1 (considered as basal daily consumption). We checked complete 3-g meal consumption each day of the experimental period and measured food intake after the first hour of ad libitum consumption of P14. Moreover, water intake during the 3-g meal period was measured by weighing the water bottle before and after the meal each day.

Tissue collection. To study the dynamics of Fos protein expression in the central nervous system (24), rats were killed with a lethal injection of pentobarbital sodium (90 mg/kg, intraperitoneal) 90 min after the beginning of their first meal. The 60-min period without food allowed us to visualize brain activations that were only dependent on the 3-g calibrated meal. The thoracic cage was opened and rats were perfused transcardially via a 16-gauge needle placed in the left ventricle with 500 mL of saline followed by 1000 mL 4% phosphate-buffered (PB) (pH = 7.4) paraformaldehyde and 0.2% picric acid (Sigma) to improve neurotransmitter retention (25). The brains were removed and left overnight in 15% sucrose in PB for cryoprotection then stored in 30% sucrose in PB with sodium azide (to prevent bacterial contamination).

Immunohistochemical staining. Brains were frozen (−40°C), sectioned in 20-μm-thick sections in a cryostat at −24°C (Leica), and floating sections were collected in PB in serially ordered sets. For each rat, 4 series were collected in each brain area (identified using the Paxinos and Watson stereotaxic atlas). Sections of the brainstem were collected from −14.5 to −13.3 mm relative to Bregma (12 sections), corresponding to the part of the NTS that is common with the area postrema and a part of caudal NTS where most of the GLP-1 cell bodies are present (26). The hypothalamus areas, sections were collected from −4.5 to −2.1 mm relative to Bregma (24 sections), covering the entire ARC. Each complete set of sections was processed for double labeling immunohistochemistry using the ABC complex/diaminobenzidine tetrahydrochloride method for c-Fos staining and the ABC complex/SG method for neuronal phenotype staining. Briefly, sections were mounted on slides, dried overnight, and frozen (−20°C). After moisturizing in PBS, slices were incubated for 60 min at room temperature in 2% bovine serum albumin (BSA), 0.5% Triton X-100 in PBS (PBS-BSA). After appropriate washing in PBS (as after each incubation), sections were incubated for 24 h with goat anti c-Fos antibody at room temperature (for antibody specifications and dilutions see Supplemental Table 1). Sections were placed for 3 h at room temperature with a biotinylated secondary antibody (Vector Laboratories) diluted 1:200 in PBS-BSA. To quench endogenous peroxidase, slices were treated with 1% hydrogen peroxide for 30 min and then with Elite Vectastain ABC reagent (1 h at room temperature) to enhance bound secondary antibody. Antibody complexes were then revealed by a reaction for 5–10 min with dianobenzidine tetrahydrochloride (Sigma) (with 0.01% hydrogen peroxide) until a Fos-like brown-black staining appeared. Fos staining was followed by neuronal phenotype staining [dopamine-β-hydroxylase (dPH) or GLP-1 for brainstem sections and α-MASH for hypothalamic sections]. Briefly, sections were washed and incubated in PBS-BSA for 60 min before incubation for 72 h at 4°C in proper primary antibody serum at an appropriate dilution in PBS-BSA (see Supplemental Table 1). After incubation with biotinylated secondary antibody, staining was
amplified with the ABC reagent as described above and complexes were revealed by reaction for 10–15 min with the Elite Vectastain SG kit (Vector Laboratories) until blue-gray cytoplasmic staining appeared. After washing and drying overnight, sections were cleared in a 100% ethanol bath (2 min) followed by 2 baths of xylene (5 and 10 min, respectively), and coverslipped with Depex (BDH). To check for staining variability between days, the series contained matched sections from all experimental groups.

**Quantitative analysis of staining.** The sections for analysis were magnified under a Zeiss computer-assisted microscope. Pictures were obtained and analyzed using imaging software (Axiovision v 4.5, Zeiss). The total number of Fos-positive neurons in each section was determined, as was the total number of specific phenotype-positive neurons. Finally, double-labeled neurons were counted. Fos-positive neurons were counted when exhibiting dark-brown nuclei, and phenotype-positive neurons were counted when dark-blue/gray ring cytoplasm were clearly identified. The results of neuronal activation are presented as averaged results for each 200-μm segment of the studied area (averaging 2 counted sections per rat per segment) except for GLP-1 staining, where 400-μm segments were used because of the limited number of neurons exhibiting GLP-1 expression.

**Statistics.** Results are expressed as means ± SEM (n = 6–8 per group for all data). Differences between groups were determined by a Student’s t test (SAS version 6.11). If statistical differences were found, further differences between groups were determined for each segment of the area studied.

Differences were considered significant at P < 0.05. Because of the impact of environmental conditions on Fos expression in the brain, P55 groups were only compared with a P14 group that was fed during the same period of time and not between each other (acute vs. chronic).

### Results

**Food intake after a high-protein load.** Rats ate the entire 3-g load each day of the experimental period. Water intake was measured during the meal and P14 groups (1.5 ± 0.4 mL) did not differ from P55 groups (1.1 ± 0.5 mL) in water consumption during this period. When compared with those getting P14, rats receiving a P55 load consumed less food during the first hour of ad libitum consumption of P14, during transition as well as after habituation. Rats ate less during their 1-h P14 intake after 2 d (3-g meal + 1-h P14 intake: 108.1 ± 5.8 kJ) or 3 wk (131.8 ± 11.7 kJ) of P55 meals than P14 groups (at d 2: 132.6 ± 7.3 kJ; at d 21: 173.2 ± 12.1 kJ; P < 0.05). Moreover, paired comparison with the last day of the prefeeding period showed that P55 meals decreased 1-h P14 intake by 19.9% (111.0 ± 4.0% for the P14 group vs. 88.9 ± 3.7% for the P55A group; t test; P < 0.01).

**Brainstem response to the high-protein meal at d 2 and d 21.** Ingestion of the high-protein meal at d 2 induced an increase in Fos protein expression in the NTS (Figs. 2A,3A). Indeed, the number of Fos positive neurons was increased in the most rostral part of the NTS studied in P55A rats compared with P14 rats (67.5 ± 10.6 vs. 37.8 ± 14.4 at −13.6 mm from Bregma and 78.3 ± 17.3 vs. 41.8 ± 10.0 at −13.4 mm from Bregma; P < 0.05 for both segments). Moreover, a P55A meal increased the number of double-labeled Fos and dβH positive neurons (Figs. 2A,3A) in the same area (18.8 ± 3.9 vs. 9.3 ± 3.2 at −13.6 mm from Bregma for the P55A and P14 diets, respectively; P < 0.05), whereas total number of dβH-containing neurons did not increase (77.1 ± 5.6 vs. 64.7 ± 6.0 for P55A and P14 groups, respectively). Moreover, the P55A and P14 rats did not differ in the number of double-labeled Fos and GLP-1 positive neurons (Fig. 3A) in any of the areas studied.

Ingestion of the high-protein meal at d 21 led to results similar to those obtained in the acute condition. Indeed, rats in the P55C group also increased in Fos protein expression...
Central activation by protein meals

**Discussion**

A reduction in energy intake induced by dietary protein may result from 2 distinct mechanisms, i.e. satiety and nonphysiological anorexia. The present results show that a high-protein meal induced a reduction in food intake during a subsequent meal in parallel to an activation of neuronal populations involved in the induction of satiety in both the NTS and the ARC. By contrast, the NTS neuronal pathway related to nonphysiological anorexia was not significantly recruited by the high-protein meal, which is consistent with a lack of aversive response related to a high-protein diet.

The present findings regarding satiety-related brain neuronal pathways are in line with previous behavioral observations that showed a high-protein diet induced satiety but did not induce CTA (13,15). These results are also generally in agreement with those of a previous c-Fos study on the effects of a high-protein diet on NTS activation (7). In addition, this study has broadened previous observations by identifying precise groups of neurons (and, more particularly, noradrenergic neurons) associated with the satiety induced by a high-protein meal (27).

These neurons (usually described as A2 neurons) send projection to the paraventricular nucleus of the hypothalamus (28–31). However, retrograde lesions of specific NA/A neurons from the paraventricular nucleus of the hypothalamus do not affect CCK-induced anorexia (32) and damaged one-half of the A2 neurons (32,33), whereas complete lesions of the A2 group dampens the CCK-induced anorexia in a neuron number-dependent manner (8). Therefore, one-half of A2 neurons, which are not triggered by orexigenic stimulus like glucoprivation (34) and that send projections to other hypothalamic areas (8) or to the ventrolateral medulla (30), are responsible for CCK-induced anorexia. We did not discriminate in our study which part of the A2 neurons were activated by high-protein meals, but triggering a potent anorectic pathway as CCK’s could explain part of the effect of high-protein meals in reducing energy intake.

These results are consistent with the increased activation of the melanocortin pathway within the ARC induced by high-protein meals. Neurons in this area are principally regulated by 2 types of signals: 1) circulating nutrients and/or hormones; and 2) other brain areas involved in the regulation of food intake, especially NPY/AgRP neurons in the ARC. Variations in the levels of postprandial circulating hormones induced by a high-protein meal are still a matter of debate, and postgestive satiety could be mediated by modulation of the release of hormones such as ghrelin, GLP-1, CCK, or peptide YY (35), as well as by insulin and leptin. The role of hormones thus cannot be ruled out and this hypothesis needs to be verified, especially for leptin, which can act directly on POMC neurons in the ARC (19,36) and could therefore explain the increased activation of these neurons. An alternative explanation for increased POMC neuron activation is a decrease in NPY neuron activity. Indeed, our results showed that non-POMC neurons were significantly
less activated with high-protein meals. Because arcuate neurons are mainly POMC or NPY (37), it could be hypothesized that NPY neurons are less activated after high-protein meals. Considering the tonic inhibitory action of NPY neurons on POMC neurons, a reduction in the activity of NPY neurons would lead to increased activation of POMC neurons (18,38). It seems likely that the greater difference observed in the activation of POMC neurons between standard and high-protein meals during transition than after habituation was only partially induced by the increase in protein amount per se but is also due to the change in type of food, even if feeding behavior in both conditions were very similar.

The activation of neuronal populations involved in satiety within the NTS and ARC after the acute and chronic consumption of high-protein meals highlighted the fact that dietary protein-induced satiety is related to the activation of multiple anorexigenic pathways. In this paradigm, the vagus nerve is only 1 of the mechanisms involved in protein-induced satiety explaining the failure to suppress protein-induced hypophagia through vagotomy (16,39,40). Activation of the NAVA neurons within the NTS suggests that the vagus nerve is indeed activated by a high-protein meal and conveys anorexigenic signals. In addition, even without the activation of brainstem or vagal inputs, high-protein diets promote satiety through an enhancement of melanocortin neuron activity within the ARC. High-protein meals are thus able to mobilize several pathways to promote satiety. These multiple targets of dietary proteins can be explained by the different pre- and postabsorptive specificities of protein. A higher protein content increases gastric volume (without any additional water intake in our study) and delays gastric emptying (41) via CCK pathways (42). Because gastric distension induces Fos in the NTS (43) and activates noradrenergic neurons in this area (44,45), with a volume consistent with what was used in our study (i.e. 9 mL for the 3-g meal), a longer-lasting increase in gastric volume would induce activation of the brainstem noradrenergic population.

Furthermore, the delivery of peptone or dietary peptides requires peripheral and central CCK receptors to induce satiety (46,47). It has previously been demonstrated that luminal peptides induce CCK release from endocrine cells through activation of the peptide transport system Pept1 (48). If the protein effect on brainstem is mediated by peptides arising from digestion and the release of CCK that binds to peripheral CCK-A receptors via the vagus nerve, this would explain why c-fos studies using free amino acid solutions failed to induce brainstem activation (49,50). If free amino acid solutions do not trigger NTS activation, they are nonetheless able to induce satiety (30) in the same way as intact protein. Following the intestinal absorption of free amino acids, the rise in blood amino acid levels would therefore directly activate central areas sensitive to circulating nutrients, like the ARC, and enable them to detect blood nutrients due to its proximity to the 3rd ventricle and its weaker blood-brain barrier (51). A high-protein meal increased plasma concentration of amino acids like leucine, sensed by the mammalian target of rapamycin, and activated POMC neurons (52). An alternative is the direct amino acid nutrient activation of VMH and POMC neurons in the ARC (53,54).

Another objective of this study was to determine whether high-protein meals failed to activate neuronal pathways usually involved in CTA by studying the activation of brainstem GLP-1 population, and therefore our results support previous studies showing that high-protein–induced anorexia was not CTA related (13,15). The brainstem GLP-1 neurons are involved in satiety, especially in conveying signals regarding moderate gastric distension (55), but these neurons are also known to be activated during CTA. Indeed, the central administration of GLP-1 induces CTA in rats (56) and the brainstem GLP-1 pathway is essential for or activated by many aversive stimuli like LiCl (9,57), lipopolysaccharide (58) aversive doses of CCK (10), or noxious gastric distension (55). These neurons send direct projections into the parabrachial nucleus (59) involved in taste aversion learning (14) and direct/indirect projections into the central nucleus of the amygdala (60), also involved in CTA formation (61). The lack of difference in activation of GLP-1 neurons within the NTS between high- and standard-protein meals therefore provided further evidence that the high-protein meal did not induce CTA in rats. High-protein–induced anorexia would therefore result from greater satiety and the activation of related neural pathways.

In this study, we show that high-protein meals induce increased activation of 2 major brain areas involved in the control of food intake. Whether other areas are involved in detecting high-protein meals, including the area postrema or the anterior piriform cortex (which is able to detect indispensable amino acid deficiency) remains to be further determined. Moreover, variations in other hypothalamic circuits, such as orexin-containing neurons in the lateral hypothalamic area or neural pathways within the paraventricular nucleus of the hypothalamus, still need to be studied, as does the relationship between the hypothalamic area and the brainstem, especially via melanocortinergic connections (62,63). A question generally raised regarding reductions in food intake with dietary nutrients is whether this behavior will slowly fade over several days and repeated intakes. The present results show that with respect to the neuronal pathways involved in satiety, repeated intakes did not suppress an increase in the activation of these pathways, especially in the brainstem.

Literature Cited


