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# Differential activation of chemically identified neurons in the caudal nucleus of the solitary tract in non-entrained rats after intake of satiating vs. non-satiating meals

Alison D. Kreisler, Elizabeth A. Davis, Linda Rinaman *

*University of Pittsburgh, Department of Neuroscience, Pittsburgh, PA, United States*

## HIGHLIGHTS

- Feeding-induced cFos activation was examined in non-meal-entrained rats.
- Liquid meals of 2–3% BW activated hindbrain NA neurons, especially the PrRP subset.
- Liquid meals of ~5% BW were required to activate GLP-1 neurons.
- NA activation increases proportionally with meal size.
- GLP-1 activation occurs only when rats consume a very large, unanticipated meal.

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## ABSTRACT

Satiety signals arising from the gastrointestinal (GI) tract and related digestive organs during food ingestion and digestion are conveyed by vagal sensory afferents to the hindbrain nucleus of the solitary tract (NST). Two intermingled but chemically distinct NST neuronal populations have been implicated in meal size control: noradrenergic (NA) neurons that comprise the A2 cell group, and glucagon-like peptide-1 (GLP-1)-positive neurons. Previous results indicate that A2 neurons are activated in a meal size-dependent manner in rats that have been acclimated/entrained to a feeding schedule in order to increase meal size, whereas feeding under the same conditions does not activate GLP-1 neurons. The present study was designed to test the hypothesis that both A2 *and* GLP-1 neuronal populations are recruited in non-entrained rats after voluntary first-time intake of an unrestricted, satiating volume of liquid Ensure. DBH-positive A2 neurons within the caudal visceral NST were progressively recruited to express cFos in rats that consumed progressively larger volumes of Ensure. Among these DBH-positive neurons, the prolactin-releasing peptide (PrRP)-positive subset was more sensitive to feeding-induced activation than the PrRP-negative subset. Notably, significant activation of GLP-1-positive neurons occurred only in rats that consumed the largest volumes of Ensure, corresponding to nearly 5% of their BW. We interpret these results as evidence that progressive recruitment of NA neurons within the caudal NST, especially the most caudally-situated PrRP-positive subset, effectively "tracks" the magnitude of GI satiety signals and other meal-related sensory feedback. Conversely, GLP-1 neurons may only be recruited in response to the homeostatic challenge of consuming a very large, unanticipated meal.



## 1. Introduction

Food intake is the product of meal size and meal number [1]. Meal number is regulated by cortical, limbic, striatal, and hypothalamic forebrain circuits, and is modulated by signals that convey information about environmental events, learned associations, reward, and energy status between meals {see [2,3] for reviews}. Conversely, meal size is controlled primarily at the level of the caudal brainstem [1], and is modulated by satiety signals arising from the gastrointestinal (GI) tract and related digestive organs during the ingestion and digestion of food; these signals are conveyed largely by vagal sensory inputs to the NST [4–10]. The NST relays feeding-related visceral sensory signals to the forebrain, as well as to brainstem pattern generators and pre-motor neurons that control the motoric components of feeding (i.e., licking, chewing, and swallowing [11,12]). Thus, NST neurons are critically involved in receiving and processing GI satiety signals that terminate ingestive consummatory behaviors, thereby limiting meal size [13].

NST neurons that receive and process satiety signals are neurochemically diverse [13]. However, recent studies have implicated two

* Corresponding author at: University of Pittsburgh, Dept. of Neuroscience, A210 Langley Hall, Pittsburgh, PA 15260, United States. Tel.: +1 412 624 6994; fax: +1 412 624 9198.
*E-mail address:* Rinaman@pitt.edu (L. Rinaman).

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intermingled but phenotypically distinct caudal NST neuronal populations in meal size control: noradrenergic (NA) neurons that comprise the A2 cell group, and preproglucagon-expressing neurons that are immunopositive for glucagon-like peptide-1 (GLP-1) [13–15]. Based on increased expression of the immediate-early gene product cFos, results in laboratory rats indicate that A2 and GLP-1 neurons are stimulated by experimental treatments that activate GI vagal sensory afferents with synaptic inputs to the caudal NST [16–20]. Such treatments include mechanical gastric distension [20] and systemic administration of cholecystokinin octapeptide (CCK) [17,18,21,22]. A2 neurons also are activated in a meal size-dependent manner by voluntary food intake in rats that have been acclimated/entrained to a feeding schedule in which repeating cycles of overnight food deprivation are followed by a predictable morning re-feeding period [16]. Conversely, the same repeating schedule of food deprivation followed by a large anticipated meal does not activate GLP-1 neurons [17], although GLP-1 neurons are activated in rats after a variety of interoceptive stressors [17]. Feeding schedule entrainment is associated with anticipatory physiological adjustments that serve to limit the homeostatic challenge of consuming large meals, thereby reducing the interoceptive stress that would otherwise be produced by the meal, and permitting increased meal size [2,23]. Since multiple lines of evidence support the view that central GLP-1 signaling suppresses food intake [5,24–26], the lack of GLP-1 neuronal recruitment in meal-entrained rats that consume a large anticipated meal may reflect homeostatic adjustments that minimize interoceptive stress. Indeed, attenuated feeding-induced activation of GLP-1 neurons may contribute to the progressively larger meals consumed by rats during meal entrainment [2,23]. In non-entrained rats, such anticipatory physiological adjustments are absent, and deprivation-induced food intake is more directly limited by GI distension and other sensory feedback generated by the acute homeostatic challenge of consuming a large unanticipated meal.

The present study was designed to test the hypothesis that both A2 *and* GLP-1 neuronal populations are recruited in non-entrained rats after voluntarily intake of a large, unanticipated meal. To challenge this hypothesis, we examined cFos activation among DBH- and GLP-1-positive caudal NST neurons in rats after deprivation-induced intake of unrestricted or restricted volumes of a palatable liquid diet (i.e., Ensure). We extended our analysis of feeding-activated neurons to include a specific caudal subset of DBH-positive A2 neurons that co-express prolactin-releasing peptide (PrRP) along with NA synthetic enzymes [27,28]. Central PrRP signaling is implicated in stress responses and control of energy balance [29–36], and PrRP neurons may participate in meal size regulation [37–39]. PrRP-positive neurons within the caudal NST are activated in experimentally naïve rats and mice after overnight food deprivation followed by re-feeding [40], although that report did not examine the potential relationship between the amount of food consumed and the extent of PrRP neuronal recruitment.

## 2. Material and methods

### 2.1. Animals and feeding protocol

Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Adult male Sprague–Dawley rats (Harlan; $n = 31$; 200–270 g BW) were individually housed in hanging wire cages in a temperature-controlled room with lights on from 07:00–19:00 h. Food (Purina rat chow #5001) and water were available ad libitum, except as noted for each experiment. Rats were acclimated to these conditions for at least 48 h before experiments were initiated. The test diet was liquid Ensure (Creamy Milk Chocolate or Homemade Vanilla; both 1.06 kcal/g; 14% protein, 64% carbohydrate, 22% fat by kcal; Abbott Nutrition, Columbus, OH). A pilot study revealed no flavor-related differences in 30-min intake by rats ($n = 12$) after overnight food deprivation, consistent with a previous report of similar ad libitum intake of chocolate vs. vanilla Ensure by male Sprague–Dawley rats [41]. All rats in the present study were pre-exposed overnight to a ball-tipped drinking spout attached to a graduated cylinder containing 10 ml of Ensure (in addition to chow and water) in order to reduce neophobic responses to the drinking spout and test diet during subsequent deprivation-induced feeding. Every rat consumed all of the available Ensure during this pre-exposure. In some cases, up to 5 ml of the 10 ml provided for overnight pre-exposure was inaccessible, either because the drinking spout leaked (as evidenced by a small volume of dried Ensure found on the pan beneath the wire cage floor the next morning), and/or because the inner metal tube extended a bit higher than the volume remaining at the bottom of the cylinder. Thus, pre-exposure volumes varied somewhat among rats, but did not vary systematically across experimental feeding groups.

Several days after Ensure pre-exposure, chow was removed from cages between 15:00 and 16:00 h; water was not removed. Twenty-four hours later, food-deprived rats were weighed and then given 30-min home-cage access to Ensure (the same flavor to which they had been pre-exposed) in one of three feeding conditions, or served as food-deprived/non-fed controls, as follows:

1. unrestricted access to Ensure (i.e., rats were given access to a volume in excess of any rat's 30-min intake);
2. 30% restricted (i.e., rats were given 70% of the average volume consumed in 30 min by rats in the "unrestricted access" group);
3. 50% restricted (i.e., rats were given 50% of the average volume consumed in 30 min by rats in the "unrestricted access" group); and
4. food deprived/non-fed controls (i.e., no Ensure).

Our pilot study confirmed that most food-deprived rats with unrestricted access to Ensure ($n = 10$) voluntary terminated intake within the first 15 min, whereas some rats consumed a few more mls during the subsequent 15-min period. However, at the end of 30 min, none were still actively consuming Ensure. Thus, rats with 30-min unrestricted access to Ensure were able to feed to satiety (i.e., as evidenced by voluntary termination of intake), whereas rats in the two restricted groups were limited to consuming smaller defined volumes during the same 30-min period. At the end of the 30-min feeding period, the amount of Ensure consumed by each rat was recorded and feeding bottles were removed.

### 2.2. Perfusion/tissue preparation

Rats were sacrificed 1 h after the end of the 30-min feeding period. This time point was selected to capture peak cFos activation in response to ingestion and satiation, since cFos protein immunolabeling peaks 60–90 min after neural stimulation and persists at peak levels for at least 30 additional minutes [42]. The rats were deeply anesthetized with pentobarbital sodium (*Fatal Plus*; 100 mg/kg BW, i.p., Butler Schein, Columbus, OH) and then transcardially perfused with 50–100 ml saline followed by 100 ml of 2% paraformaldehyde (PF; Sigma, St. Louis, MO) containing 1.5–2.0% acrolein (Polysciences Inc., Warrington, PA), followed by 100 ml of 2% PF. After perfusion, clamps were placed at the distal esophagus and proximal duodenum, stomachs were excised, and gastric contents removed and weighed. Brains were post-fixed overnight in situ in 2% PF at 4 °C, then removed from the skull, blocked, cryoprotected in 20% sucrose, frozen and sectioned at 35 μm using a sliding microtome. Sections were collected serially in six sets that each contained a complete rostrocaudal series of sections spaced by 210 μm. Sections were stored at −20 °C in cryoproservant solution [43] to await immunohistochemical processing.

### 2.3. Immunohistochemistry

Tissue sections were removed from cryopreservant, rinsed in 0.1 M phosphate buffer (PB, pH 7.2), pre-treated in 0.5% sodium borohydride