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# Xenin delays gastric emptying rate and activates the brainstem in mice

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ABSTRACT

Xenin, a 25-amino acid gastrointestinal peptide, inhibits feeding by acting through the central nervous system. Gastrointestinal hormones reduce food intake partly by activating the brainstem and inhibiting gastric emptying. Therefore, we hypothesized that xenin delays gastric emptying through the activation of the brainstem cells. To address this hypothesis, we examined the effect of intraperitoneal (i.p.) injection of xenin on gastric emptying rate and brainstem Fos expression in mice. Gastric emptying rate was reduced by about 93% in xenin-treated mice compared to saline-treated control mice. The i.p. xenin injection significantly increased Fos-immunoreactive cells in the nucleus of the solitary tract (NTS) of the brainstem, but not area postrema (AP) and dorsal motor nucleus of the vagus (DMV). These findings support the hypothesis that xenin-induced anorexia is at least partly due to delayed gastric emptying and the activation of the NTS cells.



Xenin is a 25-amino acid peptide that was initially identified in human gastric mucosa and subsequently found in other tissues and in other species [13,14]. Xenin is produced by a subpopulation of chromogranin A-positive endocrine cells in the duodenal and jejunal mucosa [2,14]. Similar to other anorectic gastrointestinal peptides, levels of circulating xenin increase after a meal, suggesting that xenin also regulates food intake by acting as a satiety factor [13]. Consistent with this hypothesis, it has been demonstrated that intracerebroventricular (i.c.v.) or intrahypothalamic administration of xenin reduces food intake [1,8,9,21,27]. Intraperitoneal (i.p.) injection of xenin also reduces food intake and increases Fos expression in a number of hypothalamic regions that are involved in the regulation of energy homeostasis [8,21]. In addition, xenin reduces food intake in animal models of obesity [21]. More recently, it was also reported that i.p. injection of xenin enhances glucose-dependent insulinotropic polypeptide (GIP)-mediated insulin secretion and improves hyperglycemia in mouse models with impaired insulinotropic action of GIP [38]. Xenin exerts these effects through the activation of non-ganglionic cholinergic neurons that innervate the pancreatic islet [38]. Taken together, these findings suggest that peripherally produced xenin reduces food intake at least partly by acting through the central nervous system (CNS) including the hypothalamus, and that enhancement of xenin action is a potential strategy to ameliorate obesity and type 2 diabetes. However, the mechanism by which xenin regulates food intake is not well understood.

Gastric emptying rate affects food intake and the anorectic effects of a number of gastrointestinal peptides such as cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) are associated with delayed gastric emptying [25,26,28]. Conversely, feeding-stimulatory effect of ghrelin is associated with accelerated gastric emptying rate [3]. Xenin injected i.c.v. at low doses increases gastrointestinal transit time in chicks, suggesting that the delayed gastric emptying contributes to the anorectic effect of centrally administered xenin [1,8,9,21,27]. However, it is unknown whether the same is true in mice when xenin is administered peripherally.

The dorsal vagal complex (DVC) of the brainstem includes area postrema (AP), nucleus of the solitary tract (NTS), and dorsal motor nucleus of the vagus (DMV), and participates in the mediation of gastrointestinal peptides-induced satiation [26,33]. Gastrointestinal signals including gastric distension and gastrointestinal hormones activate the DVC as indicated by the induction of Fos expression [26,33]. These findings led us to hypothesize that peripherally administered xenin reduces food intake partly by slowing gastric emptying through the activation of the cells in the DVC of the brainstem. In the present study, we addressed this hypothesis by examining the effect of intraperitoneal (i.p.) injection of xenin at a dose which can cause feeding suppression on gastric emptying rate and brainstem Fos expression as a marker for cell activation.

Male C57BL/6 mice were obtained from Charles River Laboratories (Montreal, QC) or from our animal facility. Mice were individually housed with free access to food and water under 12 h-light and 12 h-dark cycle (lights on at 06:00) throughout the experiment except during fasting. The University of Mani-

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**Table 1**
Estimation of gastric distention before i.p. injection of saline, xenin, or urocortin.

| | Saline ($n$ = 6) | Xenin ($n$ = 6) | Urocortin ($n$ = 4) | $P^*$ |
|---|---|---|---|---|
| 1-h food intake (g) | 0.85 ± 0.06 | 0.72 ± 0.08 | 0.80 ± 0.04 | 0.39 |
| Stomach weight (g) | 0.163 ± 0.005 | 0.148 ± 0.006 | 0.155 ± 0.006 | 0.24 |
| 1-h food intake (g)/stomach weight (g) | 5.20 ± 0.31 | 4.84 ± 0.52 | 5.22 ± 0.45 | 0.89 |

Values are means ± SEM ($n$ = 4–6/group).
* $P$ values by one-way ANOVA.

toba Protocol Management and Review Committee approved all procedures.

In the first study, we examine the effect of xenin on gastric emptying rate. The rate of gastric emptying was measured according to the method described previously [29]. Briefly, mice were fasted for 16 h and re-fed with pre-weighed food pellets for 1 h. After measuring 1-h food intake, mice were injected i.p. with saline, xenin (50 μg/g b.w., American Peptide Co., Sunnyvale, CA), or urocortin (3 nmol/mouse, Phoenix Pharmaceuticals, Belmont, CA). We chose 50 μg/g b.w. dose of xenin, because we found that the i.p. injection of xenin at this specific dose consistently reduced food intake in both *ad libitum* fed and 16-h fasted mice [8,21]. Urocortin was injected as a positive control, because this dose of urocortin is known to delay gastric emptying in mice [4]. Mice did not have access to food after the injection and were sacrificed by exposing to carbon dioxide 2 h after injection. The stomach was quickly exposed by laparotomy, ligated at both the pylorus and cardia, and removed. The weight of the stomach and the wet content of the stomach were immediately weighed. The rate of gastric emptying (%) was calculated by the following formula: Gastric emptying (%) = {1 − (wet weight of food recovered from the stomach/wet weight of food intake)} × 100. The wet weight of food intake was calculated by the following formula: Wet weight of food intake = $A \times (B/C)$, $A$ = dry weight of food intake, $B$ = average wet weight of gastric content after 1-h feeding, $C$ = average dry weight of food intake after 1-h feeding. $B$ and $C$ were determined in control mice by measuring both wet and dry weights of gastric contents which were collected 1 h after re-feeding. To estimate gastric distension before i.p. injection of drugs, food intake (g) during the 1-h feeding period was normalized to stomach weight (g).

In the second study, we examined the effect of xenin on the activity of the brainstem cells using Fos-immunoreactivity as a marker for cell activation. Because both feeding and prolonged fasting increase the levels of Fos protein in the brain areas which are involved in the regulation of food intake, we fasted mice for 6 h to minimize the possible effect of spontaneous feeding and prolonged fasting on Fos expression [6,35]. After 6-h fasting, mice were injected i.p. with saline or xenin (50 μg/g b.w.) at 1400 h and perfused with 4% paraformaldehyde 2 h later under avertin (5 mg/g b.w., i.p.) anesthesia. Brains were removed and post-fixed in 4% paraformaldehyde solution at room temperature and coronal sections (30 μm) were cut on a cryostat.

For immunohistochemical visualization of Fos-immunoreactive cells, tissue sections were washed in PBS followed by overnight incubation in a polyclonal rabbit antibody specific for c-Fos (1:20,000, Ab-5 Calbiochem, La Jolla, CA) in 0.3% Triton X-100 in PBS. Sections were washed in PBS followed by 1-h incubation in biotinylated goat anti-rabbit IgG antibody (1:200, Vector Laboratories, Burlingame, CA) in 0.3% Triton X-100 in PBS. After rinsing with PBS, sections were incubated in a solution of avidin and biotinylated peroxidase (Vector Laboratories). After washing in PBS, sections were developed for 5 min in a solution of 0.1% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.1 M Tris, pH 7.4, with 0.0025% $H_2O_2$. After rinsing in PBS, sections were mounted on slides followed by drying overnight and dehydration and coverslipping with VectaMount Permanent Mounting Medium (Vector Laboratories). All incubations were performed at room temperature.

Two sections at different anterior–posterior levels of the brainstem (approximately 7.3 mm and 7.5 mm posterior from the bregma) from each animal were processed for immunohistochemistry, according to the mouse brain atlas [30]. Photomicrographs were produced by capturing images using a digital camera under a 4× objective. We counted the number of Fos-immunoreactive cells in the AP, NTS, and DMV in the captured images in a blind fashion. These brainstem areas were identified according to the mouse brain atlas [30]. For each area, the sum of the number of Fos-immunoreactive cells on both sides of the two sections were calculated in each animal and used for the statistical analysis.

In the gastric emptying study, data were analyzed by a one-way ANOVA followed by Tukey–Kramer post hoc test. Immunohistochemistry data were analyzed by Student's $t$-test. Data represent means ± SEM. A $P$-value of less than 0.05 was considered significant.

To test the hypothesis that xenin reduces gastric emptying rate, we compared 2-h gastric emptying rate between xenin-injected mice and saline-injected control mice. Mice ate similar amounts of food during the 1-h re-feeding period prior to i.p. injection in all 3 groups (Table 1). Stomach weight was not distinguishable between the groups (Table 1). The i.p. injection of xenin significantly reduced the rate of gastric emptying by about 93% compared to the saline-treated group (Fig. 1). Urocortin significantly delayed gastric emptying compared to saline treatment. There was no statistical difference in gastric emptying rate between xenin-treated mice and urocortin-treated mice (Fig. 1).

It has been well demonstrated that ingestion of food affects gastrointestinal motility and the rate of gastric emptying, in turn, affects food intake [10]. The rate of gastric emptying is accelerated in animal models of obesity, indicating that rapid gastric emptying contributes to hyperphagia and increased body weight gain [4,5]. The anorectic effects of a number of gastrointestinal peptides are

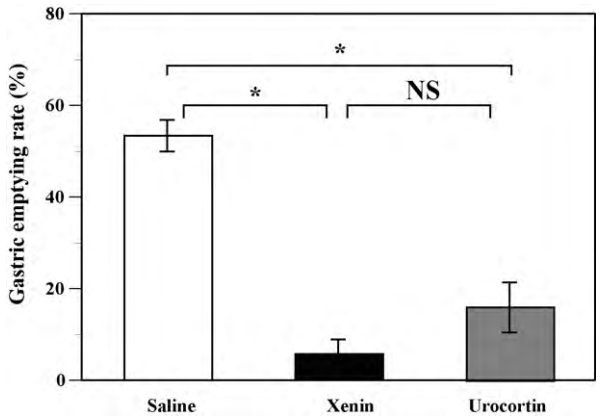


**Fig. 1.** Effect of i.p. administration of xenin on gastric emptying in mice. Mice were fasted overnight, refed for 1 h, and injected i.p. with saline, xenin (50 μg/g b.w.), or urocortin (3 nmol/mouse). Gastric emptying rates were measured 2 h after injection. Data are means ± SEM ($n$ = 4–6/group). *$P$ < 0.05 by Tukey–Kramer test. NS: not significantly different.

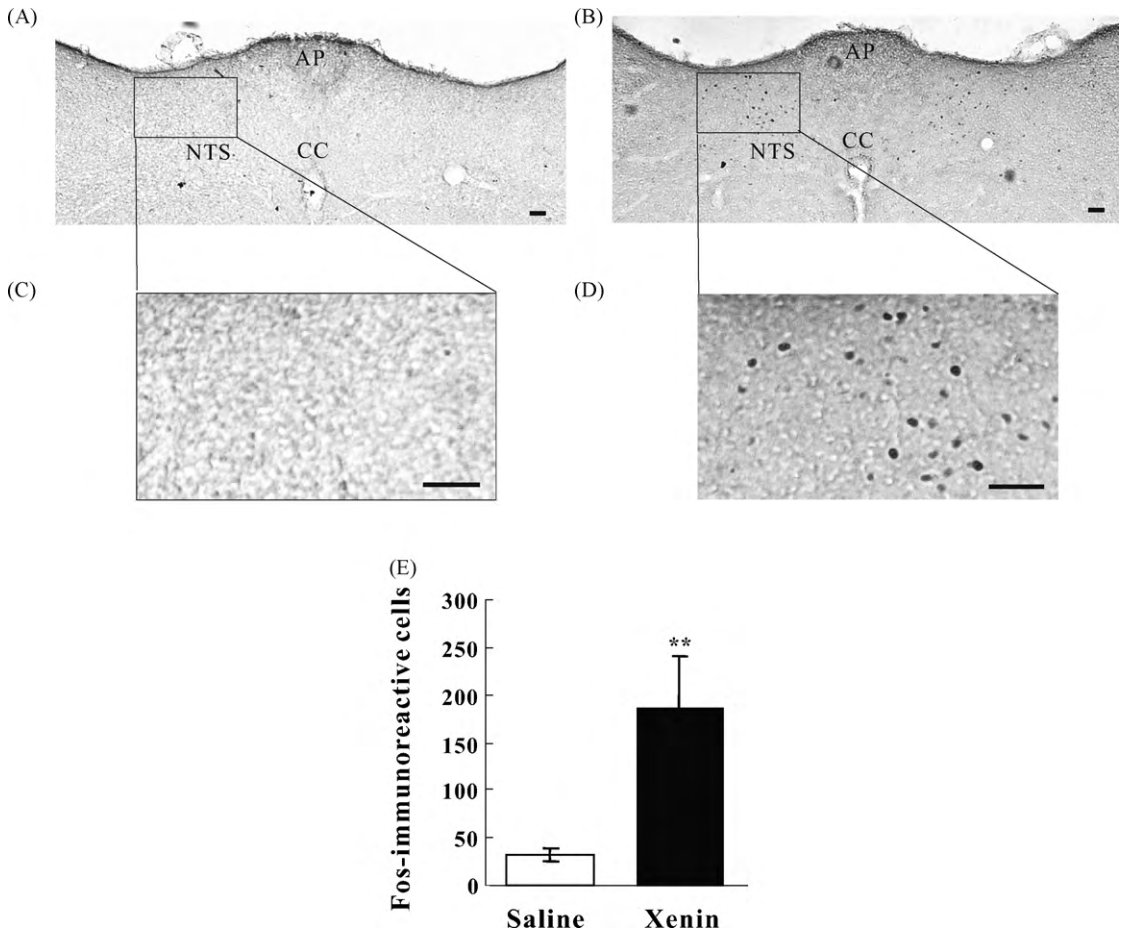


**Fig. 2.** Effect of i.p. administration of xenin on Fos-immunoreactivity in the mouse brainstem. Mice were fasted for 6 h, injected i.p. with saline or xenin (50 μg/g b.w.), and perfused 2 h later. (A and B) Representative photomicrographs of Fos-immunoreactive cells in the brainstem (approximately 7.5 mm posterior from the bregma) of mice treated with saline (A) or xenin (B). These images were captured using a digital camera under a 4× objective. (C and D) Higher magnification view of the boxed areas in A and B, respectively. (E) The number of Fos-immunoreactive cells in the NTS of mice treated with xenin or saline. Data are means ± SEM ($n = 3$/group). $^{**}P < 0.001$ (vs. saline by Student's *t*-test). Scale bar = 50 μm. AP, area postrema; CC, central canal; NTS, nucleus of the solitary tract.

partly mediated by the delayed gastric emptying [25,26,28]. Xenin injected i.c.v. at low doses increased gastrointestinal transit time in chicks suggesting that delayed gastric emptying contributes to xenin-induced feeding suppression [1,8,9,21,27]. Consistent with the findings in chicks, the present study demonstrated that i.p. injection of xenin significantly reduced gastric emptying in mice at the dose which produces a robust suppression of feeding during the first 1-2 h after injection [21]. There were no significant differences in 1-h food intake per gram of stomach weight between xenin-treated mice and saline-treated mice, suggesting that the degree of gastric distention was not different between the groups before i.p. injection of saline or xenin (Table 1). The temporal correlation between the anorectic effect of xenin and the inhibitory effect of xenin on gastric emptying supports the hypothesis that delayed gastric emptying contributes to the ability of peripherally administered xenin to inhibit food intake. Delayed gastric emptying results in greater gastric distention which leads to secretion of other anorectic peptides [19,36]. Thus, it is possible that increased secretion of other anorectic peptides mediate the feeding-suppressing effect of xenin. It should be also noted that there is no data available which deny the possibility that i.p.-injected xenin reduces food intake through its CNS actions independently of its effect on gastric emptying.

Xenin is structurally similar to neurotensin and there is a functional overlap between xenin and neurotensin [12]. Neurotensin delays gastric emptying rate and reduces food intake [7,16,22,23]. The feeding-suppressing effect of neurotensin is mediated via neurotensin receptor 1 (Ntsr1) [31]. We have recently found that i.c.v. injection of xenin fails to reduce food intake in Ntsr1-deficient mice, suggesting that the anorectic effect of xenin is also largely mediated via Ntsr1 [18]. Thus, it is reasonable to speculate that Ntsr1 mediates the effect of xenin on gastric emptying.

It has been suggested that the anorectic effect of xenin is mediated through the CNS activation [8,21,27]. However, the involvement of the brainstem in the mediation of xenin action has not been studied. To test the hypothesis that the effect of xenin on food intake and gastric emptying is mediated through the activation of brainstem cells, we compared Fos expression between xenin-treated mice and saline-treated control mice. There were no significant differences in average daily food intake prior to the experiment (saline: 4.58 ± 0.09 g, xenin: 4.67 ± 0.10 g, $P = 0.55$ by Student's *t*-test) and body weight (saline: 25.3 ± 0.8 g, xenin: 25.1 ± 0.4 g, $P = 0.82$ by Student's *t*-test). The i.p. injection of xenin significantly increased Fos-immunoreactive cells by 530% in the NTS compared with control saline injection (Fig. 2). Only a very limited number of Fos-immunoreactive cells were found in the DMV without a significant effect of xenin treatment (saline: 1 ± 1, xenin: 4 ± 2, $P = 0.24$ by Student's *t*-test, Fig. 2). No Fos-positive cells were found in the AP regardless of the treatment (Fig. 2).

Systemic administration of xenin increases *c-fos* mRNA and Fos protein expression in several hypothalamic regions which are

involved in the regulation of feeding [8,21]. These findings support the hypothesis that xenin reduces food intake at least partly by altering the activity of the hypothalamus. The DVC of the brainstem also plays a role in the regulation of food intake. Vagal afferent fibers are known to convey signals important for terminating food intake primarily to the NTS within the DVC. Activation of NTS by gut distension and ingestion of food via the vagal afferent neurons contributes to the termination of feeding [33]. Vagotomy blocks or attenuates the effect of gut hormones on food intake and DVC activation, indicating that gut hormones affect feeding by signaling to the brainstem via the vagal afferent neurons [26]. In the present study, we showed that the peripherally administered xenin activates NTS cells as represented by increased Fos expression. These findings are consistent with our hypothesis that the effect of xenin on gastric emptying and food intake is partly mediated through the activation of the cells in the NTS. The feeding-suppressing effect of xenin is partly mediated through Ntsr1 and neurotensin binding sites are present in the vagal afferent neurons, suggesting the possibility that xenin-induced NTS activation and feeding suppression is mediated through the direct action of xenin on the vagal afferent fibers [17,18]. In addition to the NTS, other DVC areas may also play a role in gut hormone-induced satiety [24], but we did not observe any significant effect of xenin treatment on the number of Fos-immunoreactive cells in AP and DMV. These data suggest that xenin-induced delay in gastric emptying is not mediated through the activation of AP and DMV cells. Strong evidence exists to suggest that food intake is regulated specifically by the NTS. Brainstem lesions including NTS attenuate the reduction of food intake by several gastrointestinal peptides [11,20,24]. Glutamate serves as a neurotransmitter or neuromodulator for the vagal afferent fibers innervating to the NTS, and microinjection of an antagonist of *N*-methyl-D-asparate (NMDA) receptor into the NTS increases food intake, but the same dose of the antagonist into other brainstem areas (i.e. DMV and the hypoglossal nucleus) does not increase food intake [37]. Taken together, our data suggest that peripherally administered xenin causes reduction in gastric emptying rate and food intake at least partly through the activation of the NTS cells possibly by a direct action of xenin on vagal afferent neurons.

There are reciprocal neural connections between the hypothalamus and the brainstem, and therefore the brainstem regulates food intake possibly by interacting with the hypothalamus. Because the i.p. injection of xenin induces Fos expression both in the hypothalamus and the brainstem, the effect of xenin on gastric emptying and food intake may be mediated through the hypothalamus-brainstem connection [8,21]. However, there is also evidence indicating that the brainstem plays a major role in mediating the effect of gastrointestinal peptides on gastric emptying and food intake. The i.p. injection of GLP-1 receptor agonist, exendin-4 (Ex-4) reduces gastric emptying rate and food intake in chronically supracollicular decerebrated rats to a similar extent as in control rats, indicating that the hypothalamus-brainstem connection is not necessary for the Ex-4-induced delay in gastric emptying and feeding suppression [15]. It has also been demonstrated that many dendrites of vagal gastric motorneurons extend from the DMV into the NTS and make synaptic contacts with vagal gastric primary sensory neurons in the NTS [32,34]. These findings support the possibility that the brainstem is sufficient to mediate the effect of gastrointestinal peptides on gastric emptying and food intake, and that the hypothalamus does not play a major role in this regulation. Thus, it is possible that xenin-induced delay of gastric emptying is mediated via the vagal afferent-brainstem-vagal efferent circuit. Although further studies are necessary to clarify whether the brainstem alone is sufficient or both the brainstem and the hypothalamus are required for xenin-induced reduction in gastric emptying rate and food intake, our data strongly suggest that the brainstem plays a key role in these xenin actions.

In conclusion, xenin reduces gastric emptying rate and activates a subset of cells in the NTS of the brainstem. Therefore, xenin-induced satiation could result in part from inhibition of gastric emptying and the activation of NTS cells. Our findings support the hypothesis that the enhancement of xenin action is a potential strategy to ameliorate obesity and obesity-associated metabolic perturbations.

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