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Increased short-term food satiation and sensitivity to cholecystokinin in neurotrophin-4 knock-in mice

Michael M. Chi,¹ Guoping Fan,² and Edward A. Fox¹

¹*Behavioral Neurogenetics Laboratory and Ingestive Behavior Research Center, Department of Psychological Sciences, Purdue University, West Lafayette, Indiana 47907; and* ²*Department of Human Genetics, University of California-Los Angeles School of Medicine, Los Angeles, California 90095*

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Chi, Michael M., Guoping Fan, and Edward A. Fox. Increased short-term food satiation and sensitivity to cholecystokinin in neurotrophin-4 knock-in mice. *Am J Physiol Regul Integr Comp Physiol* 287: R1044–R1053, 2004. First published August 5, 2004; doi: 10.1152/ajpregu.00420.2004.—Neurotrophin-4 (NT-4) knockout mice exhibited decreased innervation of the small intestine by vagal intraganglionic laminar endings (IGLEs) and reduced food satiation. Recent findings suggested this innervation was increased in NT-4 knock-in (NT-4KI) mice. Therefore, to further investigate the relationship between intestinal IGLEs and satiation, meal patterns were characterized using solid and liquid diets, and cholecystokinin (CCK) effects on 30-min solid diet intake were examined in NT-4KI and wild-type mice. NT-4KI mice consuming the solid diet exhibited reduced meal size, suggesting increased satiation. However, compensation occurred through increased meal frequency, maintaining daily food intake and body weight gain similar to controls. Mutants fed the liquid diet displayed a decrease in intake rate, again implying increased satiation, but meal duration increased, which led to an increase in meal size. This was compensated for by decreased meal frequency, resulting in similar daily food intake and weight gain as controls. Importantly, these alterations in NT-4KI mice were opposite, or different, from those of NT-4 knockout mice, further supporting the hypothesis that they are specific to vagal afferent signaling. CCK suppressed short-term intake in mutants and controls, but the mutants exhibited larger suppressions at lower doses, implying they were more sensitive to CCK. Moreover, devazepide prevented this suppression, indicating this increased sensitivity was mediated by CCK-1 receptors. These results suggest that the NT-4 gene knock-in, probably involving increased intestinal IGLE innervation, altered short-term feeding, in particular by enhancing satiation and sensitivity to CCK, whereas long-term control of daily intake and body weight was unaffected.

intraganglionic laminar endings; meal pattern; satiety; small intestine; vagus

THE SENSORY DIVISION of the vagus nerve is indispensable for central nervous system regulation of homeostasis. Vagal afferents that innervate the gastrointestinal (GI) tract contribute to this regulation through the generation and transmission of signals that modulate GI function and ingestive behavior. Intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs) are the two morphologically distinct classes of vagal mechanoreceptors that supply the muscle wall of the GI

tract in rats and mice (18, 40). IGLEs were recently demonstrated to be at least one of the transduction sites of vagal mechanoreceptors activated by both muscle stretch and contraction (45). Moreover, based on the ubiquitous distribution of IGLEs and their interactions with myenteric ganglia, a role for IGLEs in transduction and coordination of GI motility patterns, such as those involved in mixing and propulsion of food, has been postulated (40).

In addition to their roles in GI function, it is thought that IGLEs signal the brain about GI muscle stretch and tension and the associated accumulation and movement of food in the GI tract, which contributes to control of meal size or short-term satiety (21, 40). Included in this negative feedback signaling role, vagal afferents, which may include IGLEs, play an important role in integrating hormonal and neural satiation signals that contribute to meal termination (4). The most established hormone of this class is cholecystokinin (CCK), which is released from enteroendocrine cells of the duodenal mucosa in response to nutrient stimulation (31).

It has been proposed that the extrinsic sensory innervations of different organs or organ compartments have different roles in regulating GI function and food intake that are conferred by their unique tissue properties (e.g., 20). However, testing this proposal for vagal afferents has not been feasible because individual vagal subdiaphragmatic branches each contain motor and sensory axons (26, 39) and innervate multiple organs (1), precluding manipulation of vagal sensory receptors supplying a single organ. The potential to overcome this limitation was provided by neurotrophin-4 (NT-4) knockout mice that exhibited a reduced number of vagal sensory neurons (7, 23) and had a substantial loss of IGLEs that was restricted to the intestine (15). The absence of these receptors in NT-4 knockout mice was associated with a reduction of specific components of short-term satiety or satiation (15). Direct comparison of these effects with those produced by more extensive vagal afferent loss suggested that the intestinal IGLE pathway transmits a specific subset of vagally mediated negative feedback signals generated during ingestion (see discussion in Ref. 15). The alterations of satiation observed in NT-4 knockout mice were also consistent with previous studies that have implicated vagally mediated negative feedback signals from the small intestine in the control of short-term satiety, including signals

Address for reprint requests and other correspondence: E. A. Fox, Dept. of Psychological Sciences, 703 Third St., Purdue Univ., West Lafayette, IN 47907 (E-mail: au_gc@psych.purdue.edu).

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activated by intestinal distension that are probably transduced by mechanoreceptors (34, 38, 44).

Recently, NT-4 knock-in (NT-4KI) mice were found to have changes in the number of vagal sensory neurons compared with controls (12), which raised the possibility that the NT-4-dependent subset of these neurons might have increased in number. Consistent with this, preliminary experiments have suggested the NT-4 gene knock-in is associated with a substantial enlargement of IGLEs restricted to the intestine (14; E. A. Fox, R. J. Phillips, and T. L. Powley, unpublished observations; Fig. 1). These structures could represent either increased numbers of IGLEs that combine to form large clusters or increased size of individual IGLEs. Therefore, NT-4KI mice may be valuable for further investigation of the relationship between intestinal IGLE signaling and specific components of

satiation. For example, an important prediction would be that increased intestinal IGLE innervation should result in the opposite behavioral effect as a loss of these IGLEs. This would be an enhancement of satiation, which would ideally be represented by appropriate changes in meal parameters such as a decrease in meal size, meal duration, or intake rate. To investigate this prediction, NT-4KI and wild-type mice were subjected to a meal pattern analysis; and to try to reveal the broadest possible spectrum of changes in intake pattern as well as to establish the generality of any results, diets differing in both viscosity (liquid and solid) and nutrient content were employed. In fact, different components of satiation were altered in NT-4 knockout mice tested with these same two diets (15). In addition, to further examine the relationship between the putative alteration of intestinal IGLEs and satiation, we characterized the effect of exogenous CCK on suppression of short-term food intake in NT-4KI and wild-type mice because this effect is mediated largely, if not entirely, by abdominal vagal afferents (31). Moreover, this test is especially relevant for assessing the possibility of altered small intestinal innervation because this portion of the GI tract is thought to be among the initial sites of action of CCK released from mucosal enteroendocrine cells of the upper small intestine during a meal (8, 33). Additionally, the CCK-1 receptor antagonist devazepide was used to assess the specificity of any food intake suppression observed after exogenous CCK-8 injections. Therefore, should NT-4KI mice exhibit enhancement of satiation, if the underlying mechanism involves altered vagal afferent signaling, then the short-term satiety induced by CCK should also be increased, and this effect should be blocked by devazepide.

MATERIALS AND METHODS

Experiment 1: Comparison of Meal Patterns in NT-4KI and Wild-Type Mice: Solid Diet

Animals. For experiments 1, 2, and 3 mice were obtained from our breeding colony and were genotyped by polymerase chain reaction as previously described (12). In experiment 1, male ($n = 7$) and female ($n = 7$) heterozygous mutant mice (NT-4KI/+) and their male ($n = 9$) and female ($n = 8$) wild-type littermates (+/+), weighing 15–25 g (females) and 19–28 g (males) and 3–6 mo of age, were used. For experiments 1, 2, and 3, mice were maintained at 22°C on a 12:12-h light/dark cycle with lights on at 0600 and off at 1800 (see exception in experiment 3). Mice had ad libitum access to tap water and were weighed on a daily basis. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Purdue University Animal Care and Use Committee.

Meal patterning analysis. Mice were housed individually in plastic cages equipped with computerized pellet dispensers (Coulbourn Instruments, Allentown, PA). They were adapted to the cages, exposed to the test pellets (20-mg dustless precision food pellets; Bioserv, Frenchtown, NJ; Table 1) and accustomed to the feeding stations for a minimum of 1 wk before the 30-day test phase. Food was available for 19 h per day, with a 5-h period of no food during the light period to weigh the mice, reset the machines for the following run, and provide a defined start time for each day's feeding measures. Feeding stations were used to monitor and dispense the test pellets. An infrared beam was monitored every 100 ms by a computer to determine the state of a pellet in the food magazine. Each time a pellet was removed a new pellet was dispensed, the time was recorded, and these data were exported from the Coulbourn Graphic State software to an Excel (Microsoft, Redmond, WA) macro to be analyzed. The 19-h runs,

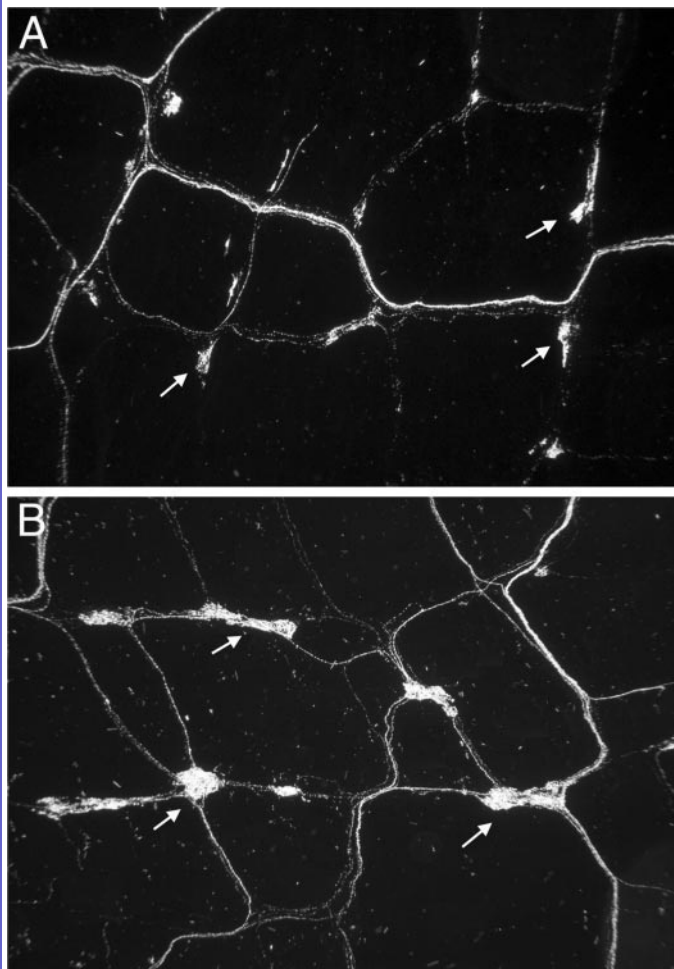


Fig. 1. Preliminary studies in wild-type (A) and neurotrophin-4 knock-in (NT-4KI; B) mice identified enlarged intraganglionic laminar endings (IGLEs) restricted to the small intestine of NT-4KI mice (14; E. A. Fox, R. J. Phillips and T. L. Powley, unpublished observations). IGLEs (light gray; some indicated by arrows) in whole mounts of the muscle wall of the duodenum (not visible in the dark background) are illustrated in these dark-field photomicrographs at low magnification (approximately $\times 80$). Associated vagal sensory axon bundles traversing the myenteric plexus are also labeled. Anterior in A is to the right and in B is to the bottom, with the long axis of the majority of IGLEs in both panels oriented parallel to circular muscle fibers. IGLEs were labeled after nodose ganglion injection with wheat germ agglutinin-horseradish peroxidase, tissue processing, and photomicrography as described previously (15).

Table 1. *Caloric distribution of the diets used throughout the experiments*

	Chow	Pellets	Isocal
Protein	28	22	13
Fat	12	12	37
Carbohydrates	60	66	50

Values are in %.

starting at 1430 and ending at 0930 the following day, were broken down into 1-min bins. This time frame allowed for the examination of the 3.5 h of the light cycle preceding and following the 12-h dark cycle. The start of a meal was defined as three pellets consumed in a 7-min period and the end of a meal was defined as the beginning of a 20-min period of no eating, criteria based on determinations made in a previous study (5).

Data representation and analysis. Meal parameters were determined by averaging 5-day blocks for each mouse. The averages for all mice in a group were then averaged for analysis. Data were analyzed using one-way repeated-measures ANOVAs, with group as the independent variable and each of the meal parameters examined individually as the dependent variables. Total daily intakes and body weight gains were also analyzed using one-way repeated-measures ANOVAs. In all cases, the main effect of group was used to determine significance. Data from one mouse were dropped from the male wild-type group because it hoarded pellets, resulting in a final group size of eight.

For *experiments 1, 2, and 3*, graphs were made using Graphpad Prism 4.0 (Graphpad Software; San Diego, CA), statistical analyses were performed using Statistica 6.0 (StatSoft; Tulsa, OK), and significance was determined to be any *P* value <0.05.

Experiment 2: Comparison of Meal Patterns in NT-4KI and Wild-Type Mice: Liquid Diet

Animals. After the completion of *experiment 1*, all male and female heterozygous mutant (NT-4KI/+) and wild-type mice were returned to maintenance on standard chow available ad libitum for a minimum of 2 wk. They then began adaptation to the liquid diet and liquid dippers before being tested in the present experiment. Although there was potential for prior experience with the solid diet to influence subsequent patterns observed with the liquid Isocal diet, it is probable that any such effect would be minimal. First, the chow maintenance diet and solid pellet diet are similar in macronutrient composition (Table 1). Furthermore, in previous experiments that tested the same two diets separated by a 2-wk washout period during which animals were fed chow, meal patterns of several different strains of mutant and wild-type mice were unaffected by the order of presentation of these diets (e.g., 15). Additionally, in the present experiments, mice were tested for an extended period of time, permitting adjustments to the liquid diet and associated apparatus. In *experiment 2*, male (*n* = 7) and female (*n* = 7) heterozygous mutant mice (NT-4KI/+) and their male (*n* = 9) and female (*n* = 8) wild-type littermates (+/+), weighing 19–25 g (females) and 20–30 g (males) and 6–9 mo of age, were used. The additional male wild-type mouse used in *experiment 2* was the mouse that had been dropped from *experiment 1* because of pellet hoarding. Also, one female knock-in mouse used in *experiment 1* that did not overcome neophobia to the liquid diet was replaced with a naive female knock-in mouse.

Meal patterning analysis. A similar procedure to *experiment 1* was followed, with the main differences being the use of the liquid diet (Table 1) and the cages equipped with liquid dippers (Coulbourn Instruments, Allentown, PA). The dippers consisted of a metal arm with a 0.08-ml cup attached to the end. The cup rested in a tray of Isocal (Mead Johnson, Evansville, IN) until the mouse broke the infrared beam by placing its head in the food magazine, at which time

the arm was lifted to allow the mouse to drink for 5 s. The arm was then returned to the tray. A computer recorded the times of each event to be analyzed in a manner similar to *experiment 1*.

Data representation and analysis. Intakes were expressed as number of dipper activations, not amounts consumed, because of the variability in the amount of Isocal consumed per cup, as discussed previously (5). The start of a meal was defined as five cup deliveries in a 9-min period, and the end of a meal was defined as the beginning of a 20-min period of no eating, criteria based on determinations made in a previous study (5).

Experiment 3: Effects of CCK-8 on Short-Term Food Intake in NT-4KI and Wild-Type Mice

Animals. Naive male (*n* = 6) and female (*n* = 6) heterozygous mutant mice (NT-4KI/+) and their male (*n* = 5) and female (*n* = 5) wild-type littermates (+/+), weighing 18–25 g (females) and 22–31 g (males) and 5–7 (females) and 7–9 (males) mo of age, were used. Mice were presented daily with jars of 20-mg pellets for 30 min at 0800 until consistent intakes were achieved. Mice were then tested every 2–3 days with either CCK-8 (catalog no. H-2080; Bachem) or the saline vehicle (0.9% sodium chloride; Abbott Laboratories, North Chicago, IL). On test days, mice that had been fasted overnight (females, 16 h; males, 21 h) were injected intraperitoneally with CCK (0.5, 1, 2, and 4 μg/kg) or saline in randomized order, with a minimum of one saline injection test intervening between doses of CCK-8. Males were maintained on a different light cycle (lights on at 2400 and off at 1200) that had been changed to accommodate other experiments, and thus deprivation time was increased with the goal of keeping it as close as possible to the females' schedule while avoiding testing in the dark phase. Injections were given 5 min before access to the pellet jars for 30 min. On completion of the test, the animal's maintenance diet was returned for at least one full day until the next deprivation period. Each dose was repeated twice in randomized order.

After the entire set of CCK tests was complete, devazepide was used to assess the specificity of the CCK-1 receptors in mediating the food intake suppression observed after exogenous CCK-8 injections. Devazepide (a gift from ML Laboratories) was diluted in normal saline containing 1% DMSO and brought to a concentration of 300 μg/kg. On test days, mice that had been fasted (females, 16 h; males, 21 h) were given the devazepide or vehicle control 15 min before the CCK (4 μg/kg) or saline injection. During each test, mice were exposed to one of three combinations of injections: vehicle followed by saline (Veh/Sal), vehicle followed by CCK (Veh/CCK), or devazepide followed by CCK (Dev/CCK). Five minutes after these CCK or saline injections, they were given access to pellet jars for a 30-min intake test. All combinations of injections were given twice in randomized order.

Data analysis. For the CCK tests, the means of the intakes for each mouse after saline and CCK injections were determined and a corresponding percent suppression was calculated. Amounts of intake after saline and CCK administration and the corresponding percent suppressions were averaged for each mouse for each dose. Results were analyzed using two-way ANOVAs, with group and treatment as the independent variables and intake as the dependent variable. Fisher's protected least significant difference test was performed for post hoc examination of doses at which a significant treatment effect occurred. At these doses, comparisons of intakes were made within groups to determine differences in suppression of intake after CCK vs. saline injections. Data from one female control and one male control were dropped because these mice hoarded pellets, reducing their respective group sizes from five to four.

For the devazepide tests, the means of the intakes for each mouse after each combination of injections (Veh/Sal, Veh/CCK, Dev/CCK) were determined and a corresponding percent suppression was calculated compared with vehicle/saline. Results were analyzed using

Table 2. The 12-h dark cycle meal parameters, solid and liquid diets

Parameter	Solid Diet		Liquid Diet	
	Wild type	NT-4KI	Wild type	NT-4KI
Meal number	9.62±0.33	13.00±0.23*	12.18±0.24	9.74±0.22*
Meal duration, min	4.16±0.35	3.46±0.21	4.92±0.25	7.85±0.33*
Meal size†	11.25±0.43	8.23±0.23*	17.38±0.54	21.69±0.70*
IMI, min	79.72±4.20	46.57±0.94*	45.02±0.80	42.33±0.94
Satiety ratio‡	7.06±0.19	5.90±0.14*	2.81±0.07	2.16±0.07*
1st 30 min†	3.86±0.27	4.52±0.19	10.55±0.32	9.79±0.29
Rate§	3.16±0.10	2.72±0.09	3.84±0.87	2.93±0.06*

Values are means ± SE. IMI, intermeal interval. Parameters were calculated daily for 30 days and averaged in 5-day blocks for all mice (solid diet: wild type $n = 16$, NT-4KI $n = 14$; liquid diet: wild type $n = 17$, NT-4KI $n = 14$). *Significant difference ($P < 0.05$) vs. wild type. †Solid diet: pellet number; liquid diet: number of dips. ‡Solid diet: min/pellet number; liquid diet: min/number of dips. §Solid diet: pellet number/min; liquid diet: number of dips/min.

one-way ANOVAs with treatment as the independent variable and percent suppression as the dependent variable. Comparisons were made within group to determine differences in suppression of intake after vehicle or devazepide pretreatment.

RESULTS

Several parameters were assessed daily for 30 days in NT-4KI and wild-type mice fed nutritionally complete solid (*experiment 1*) or liquid (*experiment 2*) diets. These measures included first meal size (intake during initial 30 min in the light phase of daily feeding session after a 5-h fast), meal size, meal duration, meal frequency, intermeal interval (IMI), satiety ratio, intake rate, total intake, and body weight. Mice were studied for 30 days to ensure that stable food intake patterns were examined so that the influence of any potential differences in learning ability between mutants and controls would be minimized (42).

There were no sex differences for any of the parameters examined in the NT-4KI group or among wild-type mice in *experiments 1* or *2* ($P > 0.05$), and, therefore, within each experiment the data from males and females in each of these groups were pooled for analysis. Because mice consume the majority of their food during the dark phase, in both *experiments 1* and *2*, the feeding patterns were analyzed for the dark phase alone, as well as for the dark and light phases combined.

Experiment 1: Comparison of Meal Patterns in NT-4KI and Wild-Type Mice: Solid Diet

Meal parameters. During the dark phase, mutant mice displayed changes in feeding compared with wild types that were indicative of greater satiation, as they ate smaller meals

[26.5%; $F(1,26) = 8.5426$, $P < 0.01$], mainly as a consequence of nonsignificant reductions in meal duration and intake rate [16.8%; $F(1,26) = 0.6139$, $P > 0.40$; 14.0%; $F(1,26) = 1.8490$, $P = 0.19$, respectively]. All meal parameters are shown in Table 2. Additionally, compared with wild types, mutant mice exhibited a much shorter delay between meals [IMI; 41.6%; $F(1,26) = 10.7785$, $P < 0.01$], suggesting postmeal satiety was reduced, and a small decrease in satiety ratio [16.4%; $F(1,26) = 60.180$, $P < 0.05$], implying that a given amount of food was slightly less effective at producing postmeal satiety than it was in controls (satiety ratio is the ratio of IMI to the preceding meal size). Moreover, the NT-4KI mice ate meals more frequently than controls [35.1%; $F(1,26) = 12.9303$, $P < 0.01$], which maintained similar daily intakes to the wild-type mice (see below) and thus may have been a compensatory response to reduced meal size.

Analysis of meal parameters over the total 19-h cycle resulted in a nearly identical pattern of effects to those observed during the dark phase, although the magnitudes of these effects were slightly reduced, resulting in loss of the significant difference in satiety ratios (Table 3).

Cumulative food intake and body weight gain. In contrast to their altered pattern of short-term food intake, NT-4KI mice ate similar total daily amounts of pellets to wild types throughout the experiment [$F(1,26) = 0.000$, $P > 0.99$; Fig. 2A]. Furthermore, although the mutant mice had slightly lower average body weights on test day 1 [wild type = 22.48 ± 0.71 g; NT-4KI = 20.51 ± 0.92 g; $F(1,26) = 9.318$, $P < 0.01$], both groups had similar changes in body weight throughout the experiment [$F(1,26) = 2.4250$, $P > 0.10$; Fig. 2B]. These results suggest that long-term mechanisms of feeding and body

Table 3. Total 19-h cycle meal parameters, solid and liquid diets

Parameter	Solid Diet		Liquid Diet	
	Wild type	NT-4KI	Wild type	NT-4KI
Meal number	14.66±0.39	18.61±0.32*	18.27±0.28	15.54±0.29*
Meal duration, min	3.77±0.21	3.27±0.17	4.24±0.15	6.16±0.19*
Meal size†	10.15±0.29	7.77±0.18*	15.77±0.34	18.13±0.43*
IMI, min	76.01±2.68	53.72±0.78*	49.57±0.69	48.96±0.74
Satiety ratio‡	7.55±0.13	7.13±0.11	3.30±0.07	2.86±0.07*
1st 30 min†	7.15±0.56	5.07±0.34	12.67±0.74	11.95±0.66
Rate§	3.04±0.10	2.64±0.08	3.93±0.07	3.06±0.06*

Values are means ± SE. Parameters were calculated daily for 30 days and averaged in 5-day blocks for all mice (solid diet: wild type $n = 16$, NT-4KI $n = 14$; liquid diet: wild type $n = 17$, NT-4KI $n = 14$). *Significant difference ($P < 0.05$) vs. wild type. †Solid diet: pellet number; liquid diet: number of dips. ‡Solid diet: min/pellet number; liquid diet: min/number of dips. §Solid diet: pellet number/min; liquid diet: number of dips/min.

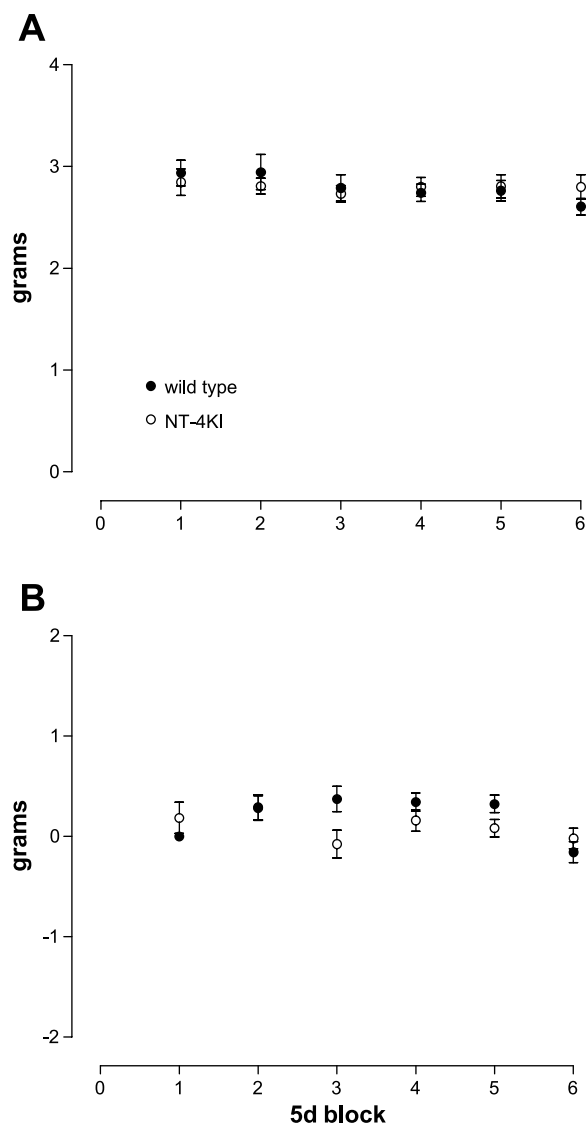


Fig. 2. *A*: daily food intake for wild-type ($n = 16$) and NT-4KI ($n = 14$) mice on the solid diet in 5-day (5d) time blocks (means \pm SE). Both groups ate similar amounts, and each group ate consistent amounts throughout the experiment. *B*: daily body weight gains in 5-day time blocks were not different between groups, indicating that any differences in meal parameters were not due to differences in body weight compared with the weights at the start of the experiment.

weight regulation were not altered by the *NT-4* gene knock-in. To investigate whether the slightly reduced average body weight of the mutants contributed to the meal pattern changes, outliers were dropped from both groups to closely match their weight ranges, and then meal parameters were recalculated. This eliminated the average body weight difference [wild type = 23.75 ± 0.49 g, $n = 12$; NT-4KI = 22.57 ± 1.1 g, $n = 8$; $F(1,18) = 3.297$, $P > 0.25$] and the meal parameters for these weight-matched subgroups were very similar to those of the complete groups reported in Table 2 (e.g., wild type and NT-4KI, respectively: meal size = 11.2 ± 0.5 and 8.25 ± 0.23 pellets, IMI = 71.2 ± 3.4 and 47.6 ± 1.5 min, meal number = 10.17 ± 0.37 and 13.24 ± 0.3), suggesting body weight differences did not contribute significantly to the meal pattern effects.

Experiment 2: Comparison of Meal Patterns in NT-4KI and Wild-Type Mice: Liquid Diet

Meal parameters. During the dark phase, mutant mice consuming the liquid diet had an altered meal pattern suggestive of increased satiation, as their rate of consumption was slower than for controls [23.7%; $F(1,27) = 18.287$, $P < 0.001$]. All meal parameters are shown in Table 2. In contrast, however, NT-4KI mice also exhibited an increase in meal size [24.8%; $F(1,27) = 5.6193$, $P < 0.05$] that was due to an increase in meal duration [59.6%; $F(1,27) = 10.8830$, $P < 0.01$]. Additionally, NT-4KI mice demonstrated a decrease in meal frequency [20.0%; $F(1,27) = 11.6920$, $P < 0.01$] that resulted in similar total daily intakes for mutants and controls and thus may have been a compensatory response to the increased meal size. Moreover, despite the increased meal size of NT-4KI mice, they exhibited similar IMIs as controls [$F(1,27) = 1.652$, $P > 0.20$], resulting in smaller satiety ratios [23.1%; $F(1,27) = 9.715$, $P < 0.01$]. This suggests that a given amount of liquid diet consumed by the mutants was not as effective at producing satiety as in controls.

The pattern of meal parameter effects over the total 19-h cycle was identical to that described above for the dark phase, and although the magnitudes of these effects were similar in some instances, in others they were slightly reduced when calculated for the entire cycle (Table 3).

Cumulative food intake and body weight gain. Similar to experiment 1, control and mutant mice maintained on the liquid diet did not differ in their total daily food intakes [$F(1,27) = 1.252$, $P > 0.25$; Fig. 3A], and although the NT-4KI mice had slightly lower body weight on test day 1 [wild type = 24.55 ± 0.66 g; NT-4KI = 21.98 ± 0.57 g; $F(1,27) = 22.883$, $P < 0.01$], weight gains were similar throughout testing [$F(1,27) = 0.001$, $P > 0.95$; Fig. 3B]. These results suggest a lack of effect of the *NT-4* gene knock-in on long-term control of food intake and body weight. As in experiment 1, weight-matched mutant and control subgroups with similar average body weights [wild type = 23.91 ± 0.53 g, $n = 12$; NT-4KI = 23.11 ± 0.55 g, $n = 9$; $F(1,19) = 1.218$, $P > 0.30$] had meal parameters that were very similar to those of the complete groups reported in Table 2 (e.g., wild type and NT-4KI, respectively: intake rate = 3.7 ± 0.09 and 2.92 ± 0.07 dips/min, meal duration = 5.45 ± 0.33 and 8.14 ± 0.43 min, meal size = 18.4 ± 0.7 and 22.6 ± 1.0 dips), suggesting body weight differences did not contribute significantly to the altered meal parameters exhibited by the NT-4KI mice.

Experiment 3: Effects of CCK-8 on Short-Term Food Intake in NT-4KI and Wild-Type Mice

CCK tests. Results for CCK tests for male and female mice were analyzed separately due to the different durations of their deprivation periods before CCK injection (see MATERIALS AND METHODS), because length of deprivation has been shown to modulate CCK suppression of short-term consumption (25). Injections of CCK resulted in suppression of 30-min food intake in both sexes and in both NT-4KI and wild-type mice. However, mutants suppressed their intake at lower CCK doses than wild types, and at doses that produced suppression in both groups, it was greater for the NT-4KI group. For the females (Fig. 4A), two-way ANOVAs indicated that neither mutants nor controls suppressed their intakes at the lowest dose [0.5

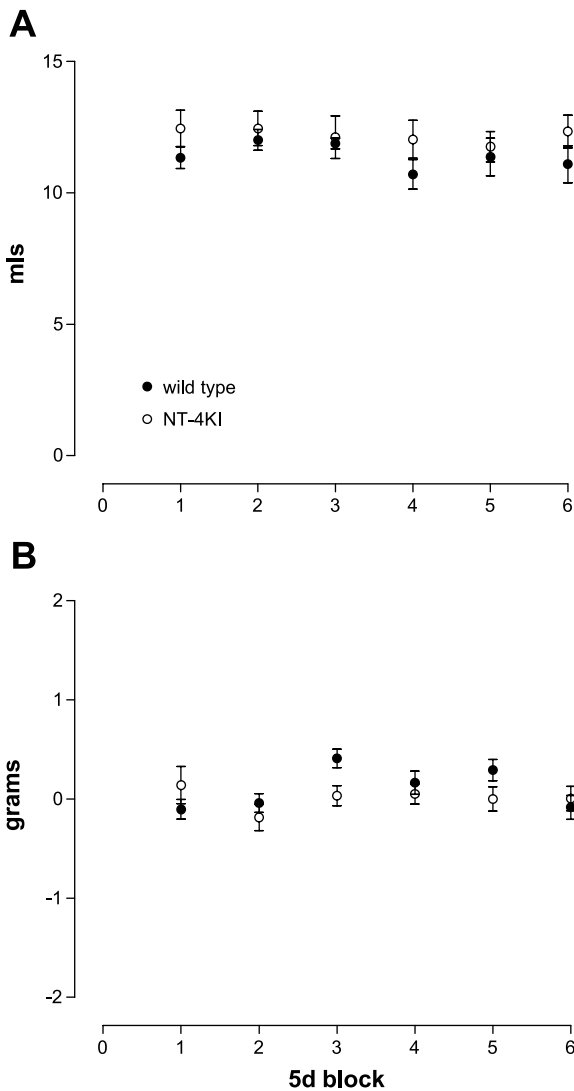


Fig. 3. *A*: daily food intake for wild-type ($n = 17$) and NT-4KI ($n = 14$) mice on the liquid diet in 5-day time blocks (means \pm SE). As with the solid diet, both groups ate similar amounts and each group ate consistent amounts throughout the experiment. *B*: daily body weight gains in 5-day time blocks also did not differ between groups, and any differences in body weight were due to the differences in initial weight at the start of the experiment.

$\mu\text{g}/\text{kg}$; $F(1,18) = 0.0386$, $P > 0.80$], and although NT-4KI mice exhibited some suppression of their intake at the 1.0 $\mu\text{g}/\text{kg}$ dose, the effect of CCK treatment was not significant [$F(1,18) = 2.107$, $P = 0.11$]. Both groups showed suppression of intake at the 2.0 $\mu\text{g}/\text{kg}$ dose, and the CCK effect was significant [$F(1,18) = 9.5869$, $P < 0.01$], but post hoc analysis revealed that only the suppression for the mutants was significant (NT-4KI: $P < 0.01$; wild type: $P > 0.40$). At the highest dose examined (4 $\mu\text{g}/\text{kg}$), both mutants and controls again suppressed their intakes, with the mutants appearing to asymptote. The CCK treatment effect for this dose was significant [$F(1,16) = 25.674$, $P < 0.01$], and the post hoc test indicated suppression of intake was significant for both NT-4KI mice and wild types (NT-4KI: $P < 0.01$; wild type: $P < 0.05$).

Male mice showed similar trends as the females, but each dose of CCK appeared less effective in producing suppression of 30-min intake in male controls and mutants than it was for

the respective female groups. This difference could have been due to several factors, including the older age of the males, a sex difference, or the greater level of deprivation experienced by the males (25, 32, 37). However, the directions of age and sex differences have generally been different from that observed here, whereas increasing deprivation has typically reduced the effectiveness of CCK, consistent with the present data. For the males (Fig. 4*B*), neither NT-4KI mice nor controls showed suppression of intake after injections of the 0.5 [$F(1,16) = 2.8727$, $P > 0.10$] or 1.0 [$F(1,16) = 2.6896$, $P > 0.12$] $\mu\text{g}/\text{kg}$ doses. At the 2.0 $\mu\text{g}/\text{kg}$ dose the effect of CCK treatment was significant [$F(1,16) = 8.5525$, $P < 0.01$], but only NT-4KI mice exhibited significant suppression of intake (NT-4KI: $P < 0.01$; wild type: $P > 0.35$). Similarly, at the 4.0 $\mu\text{g}/\text{kg}$ dose the CCK treatment effect was significant [$F(1,16) = 16.095$, $P < 0.01$], and although wild types demonstrated decreased intake that approached significance, only mutants showed a significant suppression of intake (NT-4KI: $P < 0.01$; wild type: $P = 0.075$).

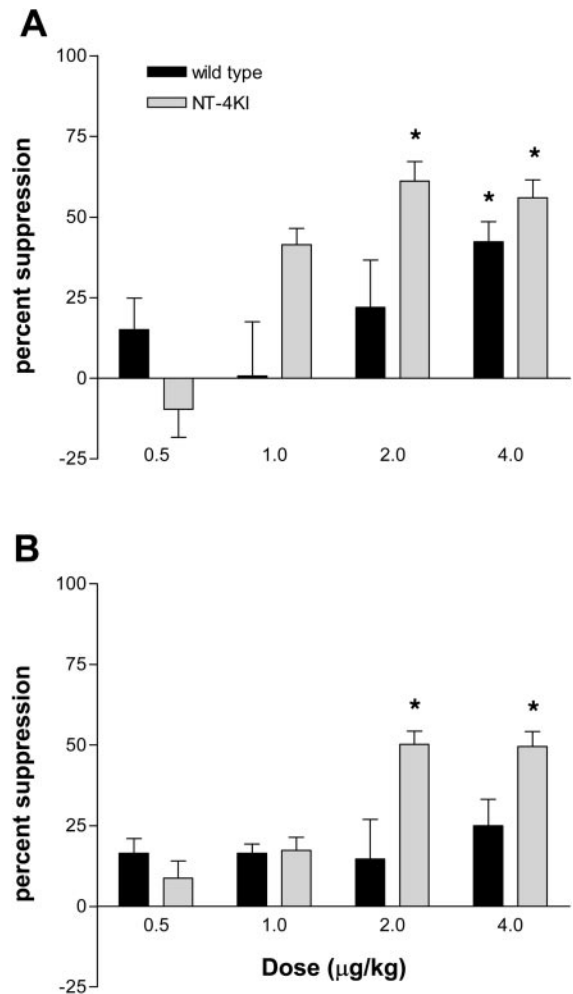


Fig. 4. Percent suppression of intake (mean difference in intake after CCK and saline trials as a percentage of mean intake after saline trials) for female wild-type ($n = 4$) and NT-4KI ($n = 6$) mice (*A*) and male wild-type ($n = 4$) and NT-4KI ($n = 6$) mice (*B*) for each of the 4 doses used. NT-4KI mice consistently showed greater sensitivity to CCK, with significant suppressions in intake for females and males at the 2.0 and 4.0 $\mu\text{g}/\text{kg}$ doses. *Significant difference between intakes ($P < 0.05$) within group after CCK and saline administration.

Because the wild-type males did not reach the asymptotic suppression level of the mutants (50–60%) with the 4.0 $\mu\text{g}/\text{kg}$ CCK injection, larger doses (8 and 16 $\mu\text{g}/\text{kg}$) were tested to determine whether CCK could produce the same maximum level of suppression in wild-type mice as it had with lower doses in NT-4KI mice. At the 8 $\mu\text{g}/\text{kg}$ dose, both control and mutant suppressions increased compared with the 4 $\mu\text{g}/\text{kg}$ response, but the controls still did not achieve 50–60% suppression in intake [mutant: $67.15 \pm 10.35\%$; $F(1,10) = 23.997$, $P < 0.01$; wild type: $37.76 \pm 8.7\%$; $F(1,6) = 1.991$, $P > 0.20$]. Moreover, the increase for the mutants appeared to be due to illness, as indicated by a hunched posture and a significant delay before initiating consumption, whereas the behavior of controls appeared normal for the testing conditions. Thus only wild types were tested with 16 $\mu\text{g}/\text{kg}$ CCK, and, without inducing apparent signs of illness, this dose did elicit a suppression of intake similar in magnitude to the largest observed in NT-4KI mice [$51.26 \pm 7.75\%$ suppression; $F(1,6) = 6.7771$, $P < 0.05$].

Devazepide tests. Satiety induced with peripheral CCK injections has typically been attributed to the activation of CCK-1 receptors because it is blocked by the CCK-1 receptor antagonist devazepide (e.g., 30). To ascertain whether the increased sensitivity to CCK injections in NT-4KI mice might also be mediated by CCK-1 receptors, the effects of the 4 $\mu\text{g}/\text{kg}$ CCK dose, which was the largest dose administered to mutants that did not produce signs of illness, was tested with devazepide or devazepide vehicle pretreatment. Both female and male mice suppressed their 30-min intakes of the pelleted test diet after Veh/CCK injections compared with Veh/Sal, and this suppression was significantly attenuated by pretreatment with devazepide (Dev/CCK) for both controls and mutants [females: $F(1,6) = 4.6605$, $P < 0.05$; $F(1,10) = 15.8889$, $P < 0.01$, respectively; males: $F(1,6) = 6.3068$, $P < 0.05$; $F(1,10) = 5.3279$, $P < 0.05$, respectively; Fig. 5, A and B]. This indicated that the suppressions of food intake obtained from the previous CCK tests were specific to the CCK-1 receptors. For the females, the degree of attenuation after Dev/CCK varied between groups, but it was not significantly different [$F(1,8) = 1.7735$, $P > 0.20$]. The degree of suppression with Veh/CCK injections in mutants and controls varied compared with the same 4 $\mu\text{g}/\text{kg}$ CCK dose given alone (compare Figs. 4B and 5B); however, these differences in intake were not significant for females or males [$F(1,16) = 2.3713$, $P > 0.10$; $F(1,16) = 0.2265$, $P > 0.60$, respectively].

DISCUSSION

Comparison of feeding behavior in NT-4KI and wild-type mice revealed that short-term controls of food intake were altered in a manner suggesting that specific components of satiety were enhanced. In parallel, additional changes in meal parameters occurred, which compensated for this enhancement of satiety to maintain similar daily food intake and body weight gain in mutant and control mice, implying that long-term controls of feeding remained intact. Moreover, different subsets of meal parameters were altered when mice consumed a solid or a liquid diet, suggesting diet properties such as viscosity or nutrient content interacted with short-term regulatory controls to determine the pattern of meal taking. Additionally, consistent with enhancement of satiety mediated by

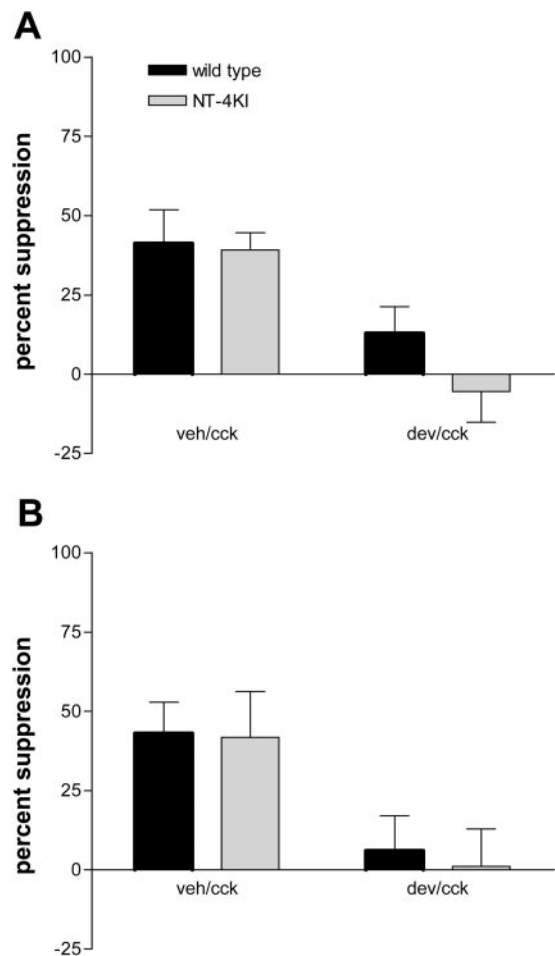


Fig. 5. Percent suppression of intake during the first 30 min after devazepide trials for female wild-type ($n = 4$) and NT-4KI ($n = 6$) mice (A) and male wild-type ($n = 4$) and NT-4KI ($n = 6$) mice (B) of experiment 3. Both groups showed a significant attenuation of suppression when administered devazepide before CCK (dev/cck vs. veh/cck), indicating the specificity of the effect was due to the CCK-1 receptors.

vagal afferents, exogenous CCK produced increased suppression of solid diet intake in NT-4KI mice compared with wild types.

The selectivity for enhancement of satiety in the knock-ins was most clear for patterns of consumption of the solid pellet diet and was represented by a decrease in meal size resulting from the combined effects of decreasing trends in both of the parameters that determine meal size: intake rate and meal duration. This reduction in meal size of NT-4KI mice was compensated for by an increase in meal frequency, resulting in comparable total daily intake to wild types. In contrast, there was less selectivity for increased satiety when mice were tested on the liquid diet. Whereas some parameters changed in a manner that could be interpreted as increased satiety, others might have different implications. In particular, increased satiety was suggested by the lower intake rate of the mutants relative to controls. There was also a parallel increase in meal duration, which probably represented a compensatory adjustment to the decreased intake rate. However, this change was substantial and actually overcompensated for the decreased intake rate, resulting in an increase in meal size. Therefore, it



is also possible that this increase in meal duration was associated with a decrease in satiation or that it resulted from altered vago-vagal reflexes (see below). Additionally, a decrease in meal number compensated for the increased meal size to maintain similar daily intakes to wild types.

Interestingly, in contrast with the effects suggesting NT-4KI mice exhibited enhanced satiation within a meal, they had small decreases in satiety ratio, implying that a given amount of diet may have produced slightly less postprandial satiety in NT-4KI mice than it did in controls. Satiation, which partly determines meal size, depends more on the preabsorptive effects of food, whereas postprandial satiety, which partly determines the delay between meals, is dependent on both pre- and postabsorptive effects of food (6, 41). Therefore, it is possible that the effect of the *NT-4* gene knock-in on satiation was different, although not mutually exclusive from its effect on postprandial satiety. For example, if the smaller, more frequent meals of the NT-4KI mice were associated with a reduced efficiency of nutrient absorption, then these mice might develop an increase in appetite more rapidly than normal after a meal and thus exhibit reduced postprandial satiety. This interpretation is consistent with the lack of correlation between meal size and the subsequent intermeal interval that has been observed (6).

Specificity of the Effects of the NT-4 Gene Knock-In

Before considering the potential relationship between increased intestinal IGLE innervation and the altered short-term feeding and CCK sensitivity observed in NT-4KI mice, it is important to address the specificity of effects of the *NT-4* gene knock-in in these mice. Heterozygous NT-4KI mice are viable and fertile, exhibiting only a mild behavioral phenotype probably due to altered cutaneous sensory function and they show increased development of functional synapses in cultured newborn hippocampal neurons (12). Either or both of these effects of the *NT-4* gene knock-in might have been responsible for the changes in meal patterns observed. However, possible mechanisms that could convert these deficiencies into the selective alterations of feeding observed are not readily apparent because the relationships of these deficiencies to the physiological systems that control food intake are tenuous. In contrast, the preliminary evidence for effects of the *NT-4* gene knock-in on the GI tract, specifically on its intestinal IGLE innervation, provides candidate mechanisms that might account for the behavioral changes observed. Thus it seems most parsimonious to hypothesize that the altered IGLEs contributed to the changes in meal patterns. Moreover, because the increased intestinal IGLE innervation may be the only GI alteration produced by the knock-in, this change and its proposed effects on vagal afferent functions described below appear to provide a valuable means for determining the roles of vagal intestinal afferents in a manner that has not previously been possible.

Possible Roles of Vagally Mediated Negative Feedback Signaling and Vago-Vagal Reflexes in Altered Short-Term Feeding

Although the increased IGLE innervation of the small intestine in NT-4KI mice was clear in preliminary studies, hypotheses about the potential roles of this altered innervation in the changes in feeding patterns should remain provisional until it is

more fully characterized and reported. Additionally, because quantitative comparisons of mechano- and chemoreceptor innervation of the duodenal mucosa have not been feasible, we cannot rule out the possibility that these endings are also altered in NT-4 knockout or NT-4KI mice (15). If such alterations occurred, they could contribute to the changes in vagal negative feedback signaling (9) and vago-vagal reflexes (24, 29) in NT-4KI mice discussed below.

There are essentially two general mechanisms by which changes in the vagal IGLE innervation of the small intestine could contribute to the meal pattern changes observed. First, IGLEs may transduce muscle stretch or tension produced by distension or peristaltic contractions in the duodenum that occur in association with food accumulation and passage, generating negative feedback signals that contribute to meal termination (21, 40). Abdominal vagotomy, treatment with the selective unmyelinated sensory neurotoxin capsaicin, and selective celiac afferent vagotomy have been demonstrated to prevent the suppression of either normal or sham feeding that resulted from negative feedback activated by intestinal stimuli, suggesting this feedback is in large part vagally mediated (27, 38, 43, 44). Moreover, a potential role for intestinal IGLEs was suggested by decreased satiation in NT-4 knockout mice, which have a loss of IGLEs restricted to the small intestine (15). Additionally, *c-Kit* and steel mutant mice that have a reduced IMA supply to the forestomach but normal IGLE innervation of the stomach and intestine, ate discrete meals, suggesting satiation occurred and that IGLEs, because they were intact, probably produced some of the distension signals that contributed to meal termination (5, 16). Therefore, an increase in small intestinal IGLE innervation might result in generation of increased negative feedback signaling in response to a given amount of muscle stretch or tension, which could lead to reduced meal size.

A second more indirect means by which increased IGLE innervation may alter short-term food intake is through alterations of IGLE-mediated GI reflex functions. Duodenal distension activates receptive relaxation of the proximal stomach, which decreases intragastric pressure, supporting an increase in gastric fill and favoring a slowing of gastric emptying (10, 11, 22). Moreover, this reflex was reduced by cooling of the abdominal vagus or by capsaicin treatment, suggesting vagal afferents contributed (11, 22). Consequently, increased vagal sensory innervation of the small intestine could result in increased activation of this reflex in response to a given amount of distension. Thus, to the extent that intestinal IGLEs signal the duodenal distension that activates receptive relaxation of the forestomach, increased IGLE signaling could have enhanced this reflex, an effect that would favor consumption of larger meals.

Therefore, the two most probable effects of increased intestinal IGLE innervation, decreased meal size due to increased negative feedback and increased meal size due to increased receptive relaxation of the proximal stomach, would oppose one another and thus might cancel each other out, or, alternatively, the characteristics of the diet may lead one of these effects to dominate. It appeared that when animals were fed the solid diet, the negative feedback effect governed the response because meal size was consistently and substantially reduced. In contrast, when animals consumed the liquid diet, there were indications that both pathways might have contributed because



intake rate was decreased (possibly representing increased negative feedback), but meal duration and meal size were increased (possibly indicating reduced intragastric pressure or reduced satiation).

The differences in properties of the liquid and solid diets that may have contributed to their different effects on meal parameters include viscosity and macronutrient proportions. For example, the rate of transit of liquid diets is more sensitive to pressure gradients within the GI tract, whereas solid diets depend more on motility reflexes such as peristaltic contractions to advance through the GI tract. Therefore, in NT-4KI mice the solid diet may have progressed through the different stomach compartments and into the intestine at a normal rate because the peristaltic reflex was probably intact. In contrast, the weaker than normal pressure gradient between the forestomach and antrum, a consequence of increased forestomach relaxation, could have permitted the liquid diet to collect in the forestomach for a greater than normal period of time, contributing to the increased meal duration and meal size observed. Additionally, the different viscosities of the liquid and solid diets would result in different patterns of GI mechanoreceptor activation and thus in different strength and duration of altered negative feedback signaling and GI reflexes in NT-4KI mice, potentially contributing to the differences in meal pattern changes that occurred with the solid and liquid diets.

Differences in macronutrient proportions of the diets could also have been important. For example, high-fat/high-carbohydrate diets similar to Isocal used in the present study, whether in liquid or solid form, typically support hyperphagia by increasing meal size (13, 41). This may be a consequence of the palatability of these diets, their high energy density, or the reduced satiating potency of fat relative to protein and carbohydrate (19, 41). Thus, the different patterns of activation of chemoreceptors produced by the varied nutrient contents of the solid and liquid diets may also have contributed to the differences in meal pattern changes observed.

Possible Mechanisms of Increased CCK Sensitivity in NT-4KI Mice

NT-4KI mice exhibited significant suppression of 30-min food intake at lower CCK doses than wild types, suggesting they were more sensitive to CCK. Additionally, the CCK-induced suppression of feeding was attenuated by the CCK-1 receptor antagonist devazepide in both NT-4KI mice and wild types, consistent with the studies demonstrating vagal mediation of CCK-induced satiation through activation of CCK-1 receptors (4). This finding also implies that the effects of CCK on food intake were not due to a nonspecific effect of systemic delivery of CCK.

A large body of evidence suggests that most or all of the effect of peripherally administered CCK on food satiation is mediated by sensory receptors innervating the stomach and proximal small intestine via the abdominal branches of the vagus nerve (31). Although CCK receptors have not been localized within vagal afferent nerve terminals (2, 28, 36), electrophysiological recordings from vagal afferents have shown that load-sensitive mechanoreceptors with receptive fields in either the stomach or duodenum can be both sensitized and activated by CCK infusions (33, 35). However, it remains to be determined whether these are direct effects on vagal

afferents or whether intermediary cell types are involved. The particular vagal afferents that mediate the effect of CCK have also remained unclear. Recently, c-Kit mutants that have a selective loss of IMAs from the forestomach (17) were shown to suppress food intake in response to CCK, which suggested that other vagal sensory receptors such as IGLEs or mucosal afferents may have contributed to this suppression (3, 5). Additionally, CCK is secreted from the mucosa of the upper small intestine, and CCK injections into the artery supplying the duodenum inhibit food intake at lower doses than injections into the jugular vein, suggesting afferents innervating this region of the GI tract contribute to the effect of CCK on meal size (8). Thus to the extent that IGLEs are among the mechanoreceptors responding to CCK, increased IGLE innervation of the small intestine would provide increased numbers of individual nerve terminals available for activation by CCK, an alteration that could have supported the greater sensitivity of NT-4KI mice to this hormone.

In summary, NT-4KI mice, which have shown increased intestinal IGLE innervation in preliminary studies, exhibited an increase in satiation after both solid and liquid diets, as predicted based on previous results obtained with NT-4 knockout mice. Long-term mechanisms, however, were maintained as daily intake and body weight gains were unaffected. In addition, the mutant mice showed an increased sensitivity to exogenous CCK, which was subsequently attenuated with the antagonist devazepide. Taken together, these results provide further support for the roles that IGLEs play in promoting satiation, as well as demonstrate the potential of genetically engineered mice in addressing questions of energy balance and homeostasis that were previously not possible.

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