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Mechanism of Hyperphagia Contributing to Obesity in Brain-Derived Neurotrophic Factor Knockout Mice

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Abstract

Global-heterozygous and brain-specific homozygous knockouts (KO's) of *brain-derived neurotrophic factor* (*BDNF*) cause late- and early-onset obesity, respectively, both involving hyperphagia. Little is known about the mechanism underlying this hyperphagia or whether BDNF loss from peripheral tissues could contribute to overeating. Since global-homozygous *BDNF*-KO is perinatal lethal, a *BDNF*-KO that spared sufficient brainstem BDNF to support normal health was utilized to begin to address these issues. Meal pattern and microstructure analyses suggested overeating of *BDNF*-KO mice was mediated by deficits in both satiation and satiety that resulted in increased meal size and frequency and implicated a reduction of vagal signaling from gut-to-brain. Meal-induced c-Fos activation in the nucleus of the solitary tract, a more direct measure of vagal afferent signaling, however, was not decreased in *BDNF*-KO mice, and thus was not consistent with a vagal afferent role. Interestingly though, meal-induced c-Fos activation was increased in the dorsal vagal motor nucleus (DMV) of *BDNF*-KO mice. This could imply that augmentation of vago-vagal digestive reflexes occurred (e.g., accommodation), which would support increased meal size and possibly increased meal number by reducing the increase in intragastric pressure produced by a given amount of ingesta. Additionally, vagal sensory neuron number in *BDNF*-KO mice was altered in a manner consistent with the increased meal-induced activation of the DMV. These results suggest reduced BDNF causes satiety and satiation deficits that support hyperphagia, possibly involving augmentation of vago-vagal reflexes mediated by central pathways or vagal afferents regulated by BDNF levels.

Keywords

Meal pattern; meal microstructure; receptive relaxation; satiation; satiety; vagal afferents

Controls of food intake are central to the regulation of energy balance. Their failure can result in overeating, which is a major factor in development of obesity (Hill and Peters,

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1998, Unger and Scherer, 2010). In fact, although it had long been thought obesity was mainly due to an energy-sparing metabolic defect (i.e. “slow metabolism”), recent research suggests that obese humans overeat and actually exhibit increased rather than decreased energy expenditure (Prentice, 2001). Brain-derived neurotrophic factor (BDNF) has numerous roles in neural development and function through activation of *trkB* and *p75* receptors, including potent anorexigenic activity (Noble et al., 2011). Consistent with this activity, glucose administration increases BDNF expression in the ventromedial hypothalamus (VMH; Unger et al., 2007), whereas food deprivation reduces its expression in the VMH and dorsal vagal complex (DVC; Xu et al., 2003, Bariohay et al., 2005). Also, central nervous system (CNS), intraperitoneal, or subcutaneous BDNF infusion decreases energy intake and body weight (Pelleymounter et al., 1995, Tonra et al., 1999, Kernie et al., 2000, Nonomura et al., 2001, Nakagawa et al., 2003, Bariohay et al., 2005, Wang et al., 2007, Spaeth et al., 2012). Moreover, in humans and mice *BDNF* and *trkB* mutations are associated with obesity that mainly results from overeating (Cai et al., 1999, Lyons et al., 1999, Kernie et al., 2000, Rios et al., 2001, Xu et al., 2003, Coppola and Tessarollo, 2004, Yeo et al., 2004, Gray et al., 2006, Gray et al., 2007, Han et al., 2008), and some contributing brain sites have been identified (Unger et al., 2007, Cordeira et al., 2010). Further, it appears that the hyperphagia resulting from reduced BDNF levels in the brain is due to effects on maintenance or function of neurons in the mature brain rather than on their development (Rios et al., 2001).

Several developing and mature peripheral tissues express BDNF, including those of the gastrointestinal (GI) tract (Lommatzsch et al., 1999, Fox, 2006, Fox and Murphy, 2008). The anorexigenic potential of BDNF produced by peripheral tissues, however, has not been explored. Vagal GI afferents are one possible pathway that could mediate the effects of reduced peripheral BDNF levels on feeding behavior. Developing and mature vagal afferents express *trkB* and *p75* (Ernfors et al., 1992, Wetmore and Olson, 1995, Anderson et al., 2006), and *BDNF* KO results in ~59% loss of vagal sensory neurons (Jones et al., 1994, Ernfors et al., 1994a) and disrupts development of vagal mechanoreceptors that supply the outer muscle wall of the stomach (Fox and Murphy, 2008, Murphy and Fox, 2010). Since vagal GI afferents carry the bulk of signals from gut-to-brain involved in regulating feeding (Smith, 1996, Schwartz et al., 2000), loss of BDNF from GI tract tissues in embryos and neonates could disrupt the development of vagal GI afferents and consequently alter this regulation. Additionally, loss of GI BDNF could alter the control of feeding in adults by disrupting vagal sensory transduction as demonstrated for slowly adapting mechanoreceptors (SAM's) innervating the skin (Carroll et al., 1998). SAM's comprise a large proportion of vagal GI afferents involved in feeding (Davison and Clarke, 1988, Zagorodnyuk et al., 2001). Based on meal pattern and microstructural analyses it was previously suggested that altered vagal satiation signaling did not contribute to the late-onset hyperphagia of global heterozygous *BDNF*-KO mice (Fox and Byerly, 2004). These are indirect measures of vagal afferent signaling, however, and since these mice had only a partial reduction of BDNF levels it is possible that alterations to their vagal afferents could have been largely compensated for by the remaining BDNF or other mechanisms. Therefore, it is possible that further reduction of peripheral BDNF levels beyond the decrease that occurs in global heterozygous mutants could be more effective at altering development or function of vagal GI afferents or perhaps other peripheral neural systems that could influence feeding behavior.

One reason BDNF-expressing neurons may regulate feeding behavior without having direct effects on metabolism is that they are strategically situated as the most distal of the known components in the chain of anorexigenic molecules, including leptin and melanocortins, that act in both the hypothalamus and DVC (Xu et al., 2003, Komori et al., 2006, Bariohay et al., 2009, Spaeth et al., 2012). Further, hypothalamic melanocortins appear to control feeding

and metabolism through separate pathways (Balthasar et al., 2005, Begriche et al., 2011) and BDNF neurons may only be activated by the pathway controlling feeding behavior. Thus, BDNF is the closest known anorexigenic molecules to the downstream brain regions involved in seeking and ingesting food and could therefore be utilized to identify connections to these downstream effectors, extending the range of potential target sites for developing treatments and preventions for obesity.

Mouse *BDNF*-KO models provide a potentially unique opportunity to investigate mechanisms underlying hyperphagia because their obesity appeared to be mediated entirely or nearly so by overeating. First, metabolic changes that occurred in these mice seemed for the most part to be secondary to increased food intake and weight gain (Kernie et al., 2000, Rios et al., 2001, Duan et al., 2003). Moreover, BDNF-deficient mice paradoxically exhibited behaviors such as increased locomotor activity that might be expected to increase energy expenditure and thus reduce body weight (Lyons et al., 1999, Kernie et al., 2000, Rios et al., 2001). In contrast, in almost all commonly studied rodent obesity models primary changes in feeding and metabolism parallel one another and interact, creating confounds in behavioral and physiological measures aimed at delineating mechanisms underlying hyperphagia (e.g., Bray and York, 1979). Second, BDNF-deficient mice exhibited hyperphagia beginning at early ages that preceded significant weight gain (Lyons et al., 1999, Kernie et al., 2000). Third, their overeating increased with age and body weight and was sufficiently large in magnitude to support the weight gain that occurred (Lyons et al., 1999, Kernie et al., 2000, Rios et al., 2001). Finally, pair feeding BDNF-deficient mice to the level of consumption of wild types prevented obesity from developing and feeding them intermittently reversed the obesity (Rios et al., 2001, Duan et al., 2003, Coppola and Tessarollo, 2004).

The goal of the present study was to take a first step toward identifying the mechanism of hyperphagia in *BDNF*-KO mice and for examining the potential contribution of peripheral BDNF. To achieve this, meal pattern and microstructure analysis of feeding behavior and the meal-induced signaling of vagal afferents from gut-to-brain were characterized in mice generated utilizing conditional gene targeting to globally reduce BDNF levels while sparing sufficient BDNF in the brainstem to maintain normal health.

EXPERIMENTAL PROCEDURES

Animals

Animals—*SM22a^{cre}* (also referred to as *transgelin^{cre}*, Tg(*Tagln-cre*)1Her/J; cat. no. 004746, JAX Labs, Bar Harbor, ME; Holtwick et al., 2002), *BDNF^{neo/+}* (Jones et al., 1994) or JAX Labs, cat. no. 002266 (Ernfors et al., 1994a), and *BDNF^{+/-lox}* (Gorski et al., 2003) mice were maintained at 23°C on a 12:12 hour (14:10 hour for breeding) light:dark schedule, lights on at 0500 with ad libitum access to tap water and Laboratory Rodent Diet 5001 (PMI Nutrition International, St. Louis, MO). When necessary to facilitate breeding, breeder pairs' diets were supplemented with irradiated in-shell sunflower seeds (Advanced Protocol Picolab Natural Sunflower Seeds #5LP8; Purina Mills; Jugloff et al., 2006). All procedures were conducted in accordance with *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 86-23, revised 1996) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

Rationale for use of *SM22a^{cre}* mice to target BDNF KO to smooth muscle—The *SM22a^{cre}* mouse strain was utilized because it meets several criteria required for testing the role of GI BDNF in vagal afferent development and feeding behavior. First, the *SM22a^{cre}* transgene targets cre recombinase expression to smooth muscle, one of the GI tissues that

expresses BDNF, without producing significant expression in other tissues (Lepore et al., 2005). Second, tests with a cre-dependent reporter strain provided evidence of high recombination efficiency in smooth muscle (Lepore et al., 2005). Third, recombination is initiated at an age when vagal GI afferents are developing - previous work demonstrated recombination occurred by E14.5 (Pan et al., 2011), and our analysis has extended this to earlier ages prior to arrival of vagal axons in the upper GI tract (see Results section “Assessment of *SM22a^{cre}*-mediated recombination”), which occurs on E12 (Rinaman and Levitt, 1993, Xiang and Burnstock, 2004, Murphy and Fox, 2007). Fourth, a smooth muscle specific KO of *BDNF* produced using this strain was predicted to be viable since the brainstem respiratory circuits thought to underlie full *BDNF*KO perinatal lethality should not be significantly altered (Katz, 2005). Finally, this strain has previously been successfully employed to target a gene KO – in fact this strain has been the most successful for knocking out genes of interest from smooth muscle (Frutkin et al., 2006).

Generation of *SM22a*–*BDNF*^{KO} mice—In the present study, *SM22a*–*BDNF*^{KO} mice were generated by combining three different mutations in individual mice, each mutation contributing to reducing BDNF levels in a unique manner. (1) *SM22a^{cre}* mice: Consistent with the reasonably large literature on the *SM22a* promoter and its regulation of cre recombinase expression, our initial analysis of *SM22a^{cre}*-mediated recombination suggested it was appropriate for targeting *BDNF*KO to smooth muscle. In a subsequent analysis, however, we found evidence of recombination in the adult brain (See Results sections “Assessment of *SM22a^{cre}*-mediated recombination” and “BDNF mRNA expression”). Thus, these mice (combined with *BDNF^{+/lox}* mice) caused loss of BDNF from GI smooth muscle of embryos and adults and from a small number of brain regions in the adult. (2) *BDNF^{+/lox}* mice: use of *BDNF^{+/lox}* mice was likely to have produced a reduction of BDNF levels in tissues that did not experience *SM22a^{cre}*-mediated recombination, probably as a result of disruption of the BDNF locus by insertion of the *lacZ* gene and 2 *loxP* sites. This was suggested by the substantial, but non-significant increase in body weight of the *SM22a^{+/+};BDNF^{neo/lox}* mice compared to *SM22a^{+/+};BDNF^{neo/+}* mice (see Results section “Body weight”), by the development of obesity in some homozygous *BDNF^{lox/lox}* mice (E.A. Fox and K.R. Jones, unpublished observations; Liao et al., 2012), by the occurrence in several tissues of greater decreases in BDNF mRNA than expected based on the mutations present (see Results section “BDNF mRNA expression”), and based upon evidence for reduced BDNF protein accumulation in *BDNF^{lox}* mice (Kaneko et al., 2012, Vigers et al., 2012). (3) *BDNF^{neo/+}* mice: These mice have global haploinsufficiency of *BDNF* and thus have about a 50% loss of BDNF from all tissues. Thus, the generation of mice harboring all 3 of these mutations and their associated reductions of BDNF expression provided a unique opportunity to examine the effects of a *BDNF*KO with sufficient BDNF remaining in brainstem respiratory circuits to support apparently normal health.

To generate these *SM22a*–*BDNF*^{KO} mice, *SM22a^{cre}* and *BDNF^{neo/+}* mice were crossed to generate *SM22a^{cre/+};BDNF^{neo/+}* mice, which were mated to *BDNF^{+/lox}* mice to obtain *SM22a*–*BDNF*^{KO} mice (*SM22a^{cre/+};BDNF^{neo/lox}* mice). This conditional KO strategy results in high efficiency of *BDNF* deletion in smooth muscle and avoids potential mitotic recombination that can occur in mice with *loxP* sites present on more than one chromosome. Offspring genotypes were determined by PCR analysis of DNA extracted from tail tips removed at weaning, or from the embryonic yolk sac or liver.

Analysis of *SM22a^{cre}*-mediated recombination—To map the spatial and temporal pattern of cre-mediated recombination produced by the *SM22a^{cre}* transgene, embryos at several developmental stages and 100 – 200 μ m slices of adult brain) were obtained from timed matings of *SM22a^{cre}* $\times$ B6;129S4-Gt(*ROSA*)26Sor^{tm1Sor/J} (*Rosa26*) reporter mice. When Cre recombinase acts in a cell of these reporter mice, a floxed DNA sequence that

blocks *lacZ* expression is removed and *lacZ* expression (β -galactosidase) is activated. Consequently, X-gal staining of β -galactosidase identifies these cells. Also, embryos and 100 – 200 μ m slices of adult brain from matings of *SM22a^{cre/+};BDNF^{neo/+}* $\times$ *BDNF^{+/-lox}* reporter mice – the same mating strategy used to generate *SM22a-BDNF^{KO}* mice – were employed to directly examine the spatial and temporal pattern of *SM22a^{cre}*-mediated recombination activity on the *BDNF^{+/-lox}* allele. Cre-mediated excision of floxed *BDNF* coding sequences in *BDNF^{+/-lox}* mice results in expression of the *lacZ* reporter gene brought under control of the *BDNF* promoters (Gorski et al., 2003). Thus, X-gal staining of β -galactosidase in these mice can identify cells in which loss of the *BDNF* coding sequences results in loss of *BDNF* expression. Noon of the day a copulatory plug was observed was designated embryonic day (E)0.5 (mated females were checked for plugs at 0730 hr). Since *BDNF* expression was observed in GI mesenchyme by E12 and in smooth muscle between E13 and birth (Fox, 2006, Fox and Murphy, 2008), offspring were harvested at E12, 13, 15, and 17 and stained with X-gal. Additionally, E10 embryos were harvested and examined to identify any unexpected early ectopic recombination that would persist in descendent lineages (e.g., Proweller et al., 2006). At each age, 4 - 6 bi-transgenic embryos from the *Rosa26* matings, and a smaller number of bi- and tri-transgenic embryos from the *BDNF^{+/-lox}* matings were examined. The majority of embryos examined at a given age were derived from different mating pairs.

Histochemical staining of β -galactosidase with X-gal

Embryos obtained from pregnant mice, or brain slices obtained from adults, were prepared for X-gal staining and immediately fixed. Embryos were fixed on ice for 30 minutes with 1% paraformaldehyde, 0.02% glutaraldehyde, 0.5 mM EGTA and 2 mM magnesium chloride in 0.1M sodium phosphate buffer, pH 7.4. Adult mice were given an overdose of methohexital sodium (120 mg/kg; Monarch Pharmaceuticals, Bristol, TN), perfused with saline for 5-10 min until the liver cleared and then with ice-cold 4% paraformaldehyde (PF) for 30 min. The brain was immediately removed, postfixed for 1 hour, and 100 – 200 μ m transverse sections prepared encompassing the entire brain and initial portion of the spinal cord. Embryos and brain slices were washed in three changes of buffer (2 mM magnesium chloride, 0.2% NP40, and 0.1% sodium deoxycholate in 0.1M sodium phosphate buffer, pH 7.4) on ice for 1 hour total, and stained overnight in the dark at 30°C in X-gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 0.1% X-gal in wash buffer; X-gal was dissolved at 2 mg/ml in dimethylformamide). Then embryos were postfixed for 48 hours in 4% PF at 4°C, washed with sodium phosphate buffered saline, pH 7.4 (PBS), and transferred to 10% buffered formalin at 4°C for a minimum of 5 days. Embryos were then embedded in paraffin, sectioned at a thickness of 8 μ m, and air-dried on gelatin-coated slides. Alternate ribbons of sections were counterstained with 0.1% neutral red, and all sections were dehydrated in a series of graded alcohols (70%, 95%, 2 $\times$ 100%; 2 minutes each), cleared in xylene (3 $\times$ 2 min), and coverslipped with Cytoseal (Richard Alan Scientific, Kalamazoo, Mich., USA). Adult brain slices were stored in 4% PF at 4°C.

RNA extraction, RT-PCR, and quantitative RT-PCR

SM22a-BDNF^{KO} (n = 11; one intestine sample had off-scale values and was not included in analysis, n = 10) and wild-type (*SM22a^{+/+};BDNF^{+/+}*, n = 8) embryos were harvested on E15, E16, or E17 as described for X-gal staining, and the esophagus-stomach, liver, and intestines were dissected. All tissues but the liver were homogenized in RLT Plus buffer (RNeasy Plus kit, Qiagen, Germantown, Maryland) and then RNA was extracted using kit instructions and stored at -80°C. Adult *SM22a-BDNF^{KO}* mice (n = 5; mRNA levels from almost all tissues from one animal were greater than 3 standard deviations from the means of the group and thus all of this animal's data were dropped; final n = 4) and wild-type littermates (n = 6) were sacrificed by cervical dislocation, the abdomen and thorax exposed

through a midline incision and the heart punctured and drained. Tissues, including frontal cortex, hypothalamus, heart, lung, liver, esophagus, stomach, small intestine, cecum, large intestine, mesentery and bladder, were harvested with sterile surgical instruments, stored briefly on ice, weighed, homogenized in Trizol (Invitrogen, Life Technologies) and then RNA was extracted and stored at -80°C . Each RNA sample (1 μg) was incubated with 1 unit of DNase1 (Invitrogen) to remove genomic DNA and then subjected to first-strand cDNA synthesis using 200 units SuperScript II or III reverse transcriptase (Invitrogen) with 500 ng of 12-18 mer oligo(dT) primers (Invitrogen) in 20 μl reactions and the product amplified by PCR. β -actin cDNA from each sample was amplified to assess the integrity of the isolated total RNA and reverse transcriptase was omitted in negative controls to confirm the removal of genomic DNA. For RT-PCR, amplification of 2 μl of cDNA from the first-strand reaction was performed using 22 units of *Taq* DNA polymerase (Invitrogen) in a 3 step protocol: 94°C for 3 min and 35 cycles with 94°C for 45 s, 55°C for 30 s, and 72°C for 90 s followed by 72°C for 10 min. Primer sequences employed were (Kawakami et al., 2002, Zermeno et al., 2009): *BDNF* forward: 5' GAA GAG CTG CTG GAT GAG GAC 3', *BDNF* reverse: 5' TTC AGT TGG CCT TTT GAT ACC 3', β -actin forward: 5' TGG TGG GTA TGG GTC AGA AGG ACT C 3', β -actin reverse: 5' CAT GGC TGG GGT GTT GAA GGT CTC A 3'. The methods and primers described previously for actin (used to normalize BDNF expression) and BDNF were followed except real-time PCR amplification was performed using an iCycler and the iQ SYBR Green Supermix (BioRad, Hercules, Calif., USA) and each primer set was optimized such that the correlation coefficient was 0.99–1.0 and the PCR efficiency was 95–100% (Cordeira et al., 2010).

Body weight, body composition, and food intake

Body weight—Growth curves were obtained by weighing mice to the nearest 100th of a gram once every 7 days from 6 - 30 weeks of age.

Body composition—Fat and lean body masses were determined using an EchoMRI whole-body composition analyzer (Houston, TX) in live mice without anesthesia (Taicher et al., 2003, Tinsley et al., 2004). The EchoMRI-900 (Echo Medical Systems, LLC, Houston, TX), a quantitative nuclear magnetic resonance instrument, was utilized to obtain precise measurements of body composition parameters, including total body fat, lean mass, body fluids, and total body water. Fat and lean mass were calculated as percent of total mass.

Meal pattern analysis—*SM22a*-*BDNF*^{KO} (n = 12), *SM22a*^{cre/+}; *BDNF*^{+lox} (n = 11), *SM22a*^{+/+}; *BDNF*^{neo/+} (n = 8), *SM22a*^{cre/+}; *BDNF*^{+/+} (n = 5), and wild-type (*SM22a*^{+/+}; *BDNF*^{+/+}, n = 5) mice 3-4 months of age were housed individually in plastic cages equipped with computerized pellet dispensers (Coulbourn Instruments, Allentown, PA). Three *SM22a*-*BDNF*^{KO}, one *SM22a*^{cre/+}; *BDNF*^{+lox}, and two *SM22a*^{+/+}; *BDNF*^{neo/+} mice hoarded pellets during data collection and therefore their data were not included in analyses, reducing the group sizes for *SM22a*-*BDNF*^{KO} (n = 9), *SM22a*^{cre/+}; *BDNF*^{+lox} (n = 10), and *SM22a*^{+/+}; *BDNF*^{neo/+} (n = 6) mice.

Diet—A balanced diet was employed (20 mg dustless precision pellets, Bio-Serv, Frenchtown, NJ). The caloric distribution in this diet is 22% protein, 66% carbohydrate, and 12% fat, with a caloric density of 3.623 kCal/gram. This is comparable to the maintenance diet in which the distribution is 28% protein, 60% carbohydrate, and 12% fat, with a caloric density of 3.04 kCal/gram.

Experimental protocol and apparatus—The balanced precision pellet diet was delivered using automated pellet dispensers and Graphic State software (v. 2.0; Coulbourn Instruments) as described previously (Fox and Byerly, 2004). Mice were adapted to the test

room and test cages for one week prior to testing. During that week animals received 3 limited pre-exposures to the test diet to prevent neophobia at the start of testing, each consisting of ten of the Bio-Serv precision pellets. Intake patterns were monitored 18 hours each day and animals were fasted the remaining 6 hours, during which time cage maintenance was performed and mice were weighed. Each daily session began at the start of the dark phase of the light cycle and extended 6 hours into the light phase, and meal pattern data were collected for 22 consecutive days. This interval provided time for adaptation to the diets and apparatus followed by at least 2 weeks of stable intake patterns. Mice of each genotype were always tested in parallel to control for any inadvertent variations in the testing conditions.

Meal criteria—Strict criteria were used to define a meal: Meal initiation was defined as a minimum of 7 pellet removals with less than 20 minutes elapsing between responses. Once a meal was initiated, meal termination was defined as the onset of a 20-minute interval with no intake. The criteria for meal onset (time interval between pellet removals and number of pellet removals) were determined by systematically varying them and examining the effect on meal number (Fox and Byerly, 2004). These data were used to identify the range of criteria that exhibited the greatest stability in estimates of meal numbers, and the specific set of criteria chosen was drawn from the middle of this range. These criteria were applied to the raw data using the Graphic State software to identify the times of onset and termination of each meal, which were used to calculate several meal parameters. These were considered to be good estimates based on the observation that mice consumed all or almost all of each pellet, as evidenced by the minute amount of spillage present on cage floors.

Meal microstructure—The first meal of each daily test session (defined as spontaneous food intake during the first 30 minutes after mice gained access to the food at the start of the session) was subjected to microstructural analysis to characterize changes in food intake rate over the course of this meal (Davis, 1998). Initial intake rate and changes in this rate across the 30 min feeding session were estimated by determining the amount of food consumed during each minute of the 30 min meal. The rationale for this approach has been discussed in detail (Fox and Byerly, 2004). Briefly, initial intake rate is mainly influenced by the strength of oropharyngeal stimulation, which drives ingestive behavior. In contrast, the rate of decrease in intake rate is mainly influenced by post-oral factors, especially vagal negative feedback signals activated by the accumulation of food in the GI tract.

Feeding-induced c-Fos activation in the DVC

Consumption of a larger-than-normal meal—A protocol modified from Rinaman et al. (Rinaman et al., 1998) was used to reliably induce voluntary consumption of a larger-than-normal meal to activate vagal GI afferents and their target neurons in the DVC. *SM22a-BDNF^{KO}* (*SM22a^{cre/+};BDNF^{neo/lox}*; n = 18) and control (*SM22a^{cre/+};BDNF^{+/+}*; n = 19) mice were each divided into 2 weight-matched groups. For 5 consecutive days mice were food-deprived overnight, exposed to a palatable diet for 1 hour each morning (Ensure Vanilla, 1.48 kcal/ml) followed by measurement of Ensure intake and body weight, and then fed chow for 3 hours each afternoon. On the fifth day, at the time Ensure was normally offered, one group was given Ensure (*SM22a-BDNF^{KO}* fed, n = 9; control fed, n = 10), and the other was given no food (*SM22a-BDNF^{KO}* fasted, n = 9; control fasted, n = 9). One hour after food was offered the amount consumed was measured. Mutant and wild-type mice of similar ages were run together to control for any inadvertent differences in lab environment or procedures that might have affected feeding behavior.

c-Fos response to consumption of a larger-than-normal meal—Thirty minutes after the last 1 hour presentation of ensure (or no food) mice were perfused with fixative as

described for adult mice used for X-gal staining of brain slices. Brains were removed and stored overnight (ON) at 4°C in the same fixative, switched to 25% sucrose in 0.1M PBS at 4°C for 48 h, and then 30 µm frozen cross-sections were cut through the longitudinal extent of the DVC and c-Fos was detected by immunohistochemistry (IHC). IHC procedures were performed at RT unless indicated. Every fifth section collected was stained with neutral red to aid identification of the borders of the NTS, AP, and DMV in adjacent c-Fos-stained sections. The remaining brain sections were washed in PBS, incubated 1 hour in 0.3% H₂O₂, washed in PBS, incubated in blocking solution for 30 min (1.5% normal goat serum, 0.5% triton X-100, 2% BSA), and then incubated 40-43 h in primary antibody (1:10,000; rabbit anti - c - Fos polyclonal #PC38, Calbiochem, EMD Chemicals, Gibbstown, NJ) at 4°C. These, as well as the secondary antibody employed in the present experiments were diluted with 1% BSA, 2% normal goat serum, and 0.3% Triton X-100. After washing with PBS, sections were incubated in secondary antibody for 45 min (biotin-conjugated goat anti-rabbit IgG (H+L); BA-1000; Vector Laboratories, Inc., Burlingame, CA.), and then washed again with PBS, incubated in ABC reagent for 45 min (Vectastain Elite ABC kit PK-6100; Vector Laboratories), and exposed to a color reaction, involving 0.06% diaminobenzidine tetrahydrochloride, 0.075% H₂O₂ and 0.06% NiCl in Tris-buffered saline pH7.6. Sections were washed in PBS, dehydrated and cleared in 10 min changes of 70, 95, and 100% EtOH, and 45 min xylene, and coverslipped with Cytoseal. Brain sections of mutant and wild-type mice were stained for c-Fos in parallel to control for any inadvertent variations in this procedure.

Quantification of c-Fos-like immunoreactivity (LIR)—Sections were mounted in the order collected from the caudal to rostral DVC. All sections with a completely intact DVC between the most caudal AP level and the obex (defined as the rostral-most section of the AP containing at least some AP tissue that extended completely across the caudal IVth ventricle) were used for quantification of c-Fos-LIR. This resulted in 6-12 sections used per mouse, and the average number of sections used did not differ between fed and non-fed control and fed and non-fed mutant groups (not shown). Area postrema (AP), nucleus of the solitary tract (NTS), and DMV borders used to assign c-Fos-LIR neuronal nuclei to one of these brain structures were determined by comparison of each c-Fos-stained section with an adjacent neutral red-stained section from the same brain. Sections compared were separated by a distance of 0-2 sections' thickness (0 – 60 µm) in the rostral or caudal direction. All stained elements approximating the size and shape of a cell nucleus were counted and recorded separately for each section unless staining was too faint to clearly distinguish from background staining. To normalize these counts, for each mouse the average number of cells with a c-Fos-LIR-labeled nucleus per section was calculated and used for statistical analysis. The baseline c-Fos activation (estimated as number of neuronal nuclei exhibiting c-Fos-LIR in non-fed mice) of old, obese mice (4-12 months of age) in the NTS, AP, and DMV was significantly reduced in *SM22a-BDNF^{KO}* mice compared to controls, and nearly so in the DMV of young, pre-obese (9-11 weeks of age) *SM22a-BDNF^{KO}* mice compared to controls (see Results section "C-Fos activation in response to a larger-than-normal meal"). Therefore, c-Fos activation in each fed mouse was expressed as a percent increase compared to baseline activation in non-fed mice. The baselines used to calculate these percent increases equaled the number of neurons/section with c-Fos-LIR averaged across all non-fed animals of the same genotype group.

Nodose ganglion cell counts

SM22a-BDNF^{KO} and wild-type adult mice were perfused with fixative as described for adult mice used for X-gal staining of brain slices. Right and left nodose ganglia were removed, stored overnight in fixative at 4°C, transferred to 25% sucrose in PBS and stored overnight at 4°C. Then 20 µm thick frozen sections were cut through the entire ganglion, all

sections were thaw mounted in sequence and stained with neutral red. For each ganglion, neuron profiles were counted at 200X magnification in equidistant sections that spanned the entire ganglion. The initial section counted was the first section that contained at least 10 neurons. Then every fourth section was counted. If a section was damaged, the next section in the sequence was counted in its place.

IHC staining of gastric IGLEs

We attempted to label the well characterized vagal mechanoreceptors with distinctive morphologies that innervate the outer muscle wall using anterograde transport of WGA-HRP injected into the nodose ganglion, a labeling method that permits quantification of these receptors (Fox et al., 2000). *SM22a-BDNF^{KO}* mice did not recover from this procedure. Therefore, the P₂X₂ receptor was stained using IHC to label intraganglionic laminar endings (IGLEs) that innervate the stomach wall (Castelucci et al., 2003). The stomachs of *SM22a-BDNF^{KO}* (*SM22a^{cre/+};BDNF^{neo/lox}*; n = 3) and control (*SM22a^{cre/+};BDNF^{+/+}*; n = 3) mice were removed after perfusion for the c-Fos experiments and stored in PBS at 4°C for 2 days. Separate wholemounts of the dorsal and ventral stomach walls were prepared by cutting each stomach along the greater and lesser curvatures, and then stretching each stomach half flat and pinning them, mucosa side up and storing them in 4% PF for 8 days. Next, each stomach half was placed in chilled PBS and sharp dissection used to remove the mucosa, submucosa, and circular muscle, exposing the myenteric plexus. Stomach wholemounts were washed in PBS, incubated in blocking solution (10% normal goat serum, 0.5% triton X-100, 2% BSA, and 0.1% sodium azide) at RT ON, and then in rabbit anti-P₂X₂ (1:250; Millipore, Cat. No. AB5244) at 4°C for 3 days. Primary and secondary antibodies were diluted with 1% BSA, 2% normal donkey serum, 0.3% Triton X-100, and 0.1% sodium azide. After washing with PBS, wholemounts were incubated in goat anti-rabbit rhodamine red-x (RRX; 1:100; Jackson ImmunoResearch Laboratories, Inc.) at RT for 2 hours, and then washed again with PBS, mounted, crushed for 30 min, air dried, coverslipped in glycerol, and sealed with nail polish. To control for non-specific staining by anti-rabbit-RRX, staining produced by the P₂X₂ antibody was compared with staining following its omission (incubation in primary antibody diluent alone was substituted) with all other protocol steps kept constant. IGLEs stained with P₂X₂ were quantified as previously described for WGA-HRP-labeled IGLEs except that fluorescence microscopy and 200X magnification were used (Fox et al., 2000).

Microscopy and imaging

X-gal- and c-Fos-stained tissue was examined with standard bright-field or differential interference contrast illumination and rhodamine fluorescence was visualized using a standard filter set (Leica DM5000 microscope; fluorescence filter cube L5). Photomicrographs were acquired directly with a video camera (Spot RT Slider; Diagnostic Instruments, Inc., Sterling Heights, MI). Rhodamine fluorescence was also visualized with a standard rhodamine filter set or a Texas red filter set (DSU-MRFPHQ) and optical sections, collected using z-increments of 1 µm were obtained with an Olympus BX-DSU spinning disk confocal attached to a BX61 motorized microscope (Olympus, Center Valley, PA) at 100 and 200X magnification. Confocal scanning and processing of three-dimensional image data were performed using Slidebook (v.5.0, Intelligent Imaging Innovations, USA).

Statistical analysis and graphical display of data

In all experiments and genotype groups, male and female mice were included and the numbers representing each sex in nearly every group studied were similar. Further, there were no significant sex differences within any genotype in body weight, fat in grams or percent body weight, or any meal pattern or microstructure measure (not shown). Therefore, data from males and females of the same genotype were combined for all analyses.

Changes in BDNF mRNA levels relative to those of the housekeeping gene, actin, as determined by quantitative RT-PCR, were calculated using the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001). ΔCT s (e.g. BDNF – actin) were calculated using the mean of duplicates for each sample and were subtracted from the average ΔCT for the defined control group. The $\Delta\Delta CT$ s were then used to calculate the percentage change relative to the control group. Differences in these relative mRNA levels in tissues from wild-type vs. *SM22a-BDNF^{KO}* mice were compared using t-tests. Nodose ganglion cell counts were compared between groups using t-tests. Body weights were compared using two-way analysis of variance (ANOVA) with genotype and age as independent variables. Tukey's honest significant difference (HSD) test was performed as a posthoc test to determine genotype differences. To determine the age at which the body weights of *SM22a-BDNF^{KO}* mice diverged from those of the control genotypes (wild types, *SM22a^{cre/+};BDNF^{+/+}* and *SM22a^{+/+};BDNF^{+lox}* groups), two-way ANOVA with genotype and age as independent variables was used to test for a genotype $\times$ age interaction. This was followed by Tukey's HSD posthoc test to make pairwise genotype comparisons at each age. Also, to determine genotype differences in asymptotic body weights, a one-way ANOVA with repeated-measures over weeks 20-30 and genotype as the independent variable was employed. If the main effect was significant, the same repeated measures ANOVA was performed to make pairwise comparisons between genotypes. Differences in body fat weight and in body fat weight as percent of body weight were examined using two-way ANOVA with genotype and age as independent variables. Tukey's HSD was performed as a posthoc test. Meal pattern and microstructure data, including food intake, each meal pattern parameter, and the decay of eating rate during the first meal were tested using one-way ANOVA with repeated-measures over days and genotype as the independent variable. If the main effect was significant, the same repeated measures ANOVA was performed to make pairwise comparisons between genotypes. Differences in food intake during minute one of the first meal and average body weight during the 22 days of collection of meal pattern data were examined using one-way ANOVA with genotype as the independent variable. No posthoc test was necessary as the main effect was not significant. Body weight measured at the start of training for the meal-induced c-Fos experiment and food intake assessed on the test day were compared between controls and *SM22a-BDNF^{KO}* mice using t-tests. Baseline c-Fos activation (c-Fos-LIR cell numbers/section in non-fed mice) of control and *SM22a-BDNF^{KO}* mice in each brain region were also compared using t-tests. Meal-induced activation of c-Fos in the DVC of fed mice was represented as percent increases compared to non-fed mice. Therefore, pairwise comparisons between *SM22a-BDNF^{KO}* mice and controls for each brain region were made using the non-parametric Mann-Whitney U test to avoid possible violations of assumptions of parametric tests. Finally, IGLE numbers/stomach half nodose ganglion neuron numbers in wild-type vs. *SM22a-BDNF^{KO}* mice were compared using t-tests. Values reported are means $\pm$ SEM. For all statistical tests, $p < 0.05$ was required for statistical significance. Statistica (v5.0, StatSoft, Tulsa, OK) was used for all statistical comparisons. Graphpad was used to construct all graphs (Graphpad Prism v4.0, Graphpad Software, Inc.). Photoshop CS software (v8.0 Adobe Systems, Mountain View, CA) was used to apply scale bars and text to digital photomicrographs, adjust their brightness and contrast and organize final figure layouts.

RESULTS

Characterization of BDNF KO

In the present study, *SM22a-BDNF^{KO}* mice were generated by combining three different mutations or transgenes in individual mice. These included (1) the *SM22a^{cre}* transgene, which targets cre recombinase to smooth muscle in embryos and adults, and to a small number of brain regions in adults, (2) a *BDNF^{+lox}* allele that is recombined by the cre

recombinase to remove BDNF coding sequences, and (3) a *BDNF*^{neo/+} mutation that results in global haploinsufficiency of *BDNF* (see “Generation of *BDNF* KO mice” in the Experimental Procedures section for further details).

Assessment of *SM22a*^{cre}-mediated recombination—Since BDNF expression in the embryo and early postnatal GI tract is largely restricted to smooth muscle (see below and Fox, 2006, Fox and Murphy, 2008), our aim was to target embryonic expression of cre recombinase, and thus the *BDNF* KO, to smooth muscle utilizing the promoter from the smooth muscle-specific *SM22a* gene. *Rosa26* reporter mice were employed to determine the total spatiotemporal tissue domain that exhibited *SM22a*^{cre}-mediated recombination in the embryonic GI tract. In *SM22a*^{cre/+};*Rosa26* embryos derived from *SM22a*^{cre/+} × *Rosa26* matings, mesenchyme (E12-E13; Figure 1A,C) and smooth muscle (E13-E17) of the outer wall of the intestines (Figure 1D-F) and a portion of the stomach (Figure 1A,B) were labeled for β-galactosidase at all ages examined. Also, GI mesentery and smooth muscle of blood vessels supplying the GI tract were labeled as early as E13 (Figure 1C). β-galactosidase staining was also observed in the intestinal mucosa at E17 (Figure 1D,E). This staining may have reflected the presence of endogenous β-galactosidase as previously described at E17 and postnatal day 4 (Fox and McAdams, 2010), although some likely represents recombination as it occurred in these tissues at younger ages, prior to the appearance of endogenous enzyme (e.g., stomach epithelium; Figure 1A). Importantly, no β-galactosidase expression was detected in brain parenchyma at any of the embryonic ages examined, whereas it was present in a small number of adult brain regions by 2 months of age (illustrated for offspring of *SM22a*^{cre} mice and *BDNF*^{lox} reporter mice; see below).

The spatial and temporal extent and specificity of *SM22a*^{cre}-mediated recombination activity on the *BDNF*^{+/lox} allele were assessed in *SM22a*^{cre/+};*BDNF*^{+/lox} and *SM22a*^{cre/+};*BDNF*^{neo/lox} embryos derived from matings of *SM22a*^{cre/+} × *BDNF*^{neo/lox} mice. This analysis identified tissues from which BDNF expression was lost as a result of this recombination in *SM22a*-*BDNF*^{KO} mice. *BDNF* is normally expressed in mid-to-late gestation embryos in smooth muscle of the outer wall of the LES, stomach, intestines, the lamina propria of the stomach wall, GI mesentery, and smooth muscle of blood vessels that supply the GI organs (Fox, 2006, Fox and Murphy, 2008). Examples of this GI *BDNF* expression are illustrated here by β-galactosidase staining in embryos derived from *BDNF*^{lacZ} mice ranging in age from E13-17 (Figures 2A,B,D and 3A,C,E).

In *SM22a*^{cre/+};*BDNF*^{+/lox} and *SM22a*^{cre/+};*BDNF*^{neo/lox} embryos, cre-mediated recombination occurred with a similar time course as observed in *SM22a*^{cre/+};*Rosa26* embryos. β-galactosidase was further restricted in spatial extent, however, to a subset of the GI tissues that normally produce BDNF, which included smooth muscle of GI blood vessels and portions of adjacent mesentery (for the stomach compare *BDNF* expression illustrated in Figure 2A,B,D with cre-mediated recombination shown in Figure 2C,E; for the intestines compare *BDNF* expression in Figure 3A,C,E with cre-mediated recombination in Figure 3B,D,F). The reduced spatial extent of staining in the GI tract of *SM22a*^{cre/+};*BDNF*^{+/lox} and *SM22a*^{cre/+};*BDNF*^{neo/lox} embryos compared to that in *SM22a*^{cre/+};*Rosa26* embryos was consistent with expression of *lacZ* after recombination of *BDNF*^{lox} being restricted by the *SM22a*^{cre} promoter (as in *SM22a*^{cre/+};*Rosa26* mice) and the *BDNF* promoters. Outside the GI tract, cre-mediated recombination occurred in smooth muscle of the aorta, the two main pulmonary arteries, and extracranial blood vessels (not shown). Similar to the embryo offspring of *SM22a*^{cre/+} and *Rosa26* mice, at the embryonic ages studied there was no evidence of recombination in the brain (e.g., E14; Figure 4A,B).

Overall, these results suggested the *SM22a*^{cre} transgene enabled targeted KO of *BDNF* alleles almost entirely restricted to the GI vasculature during development. They also

implied that this KO occurred sufficiently early in development to largely eliminate *BDNF* expression as vagal axons arrive in the upper GI tract (begins on E12) and prior to the formation of mechanoreceptor terminals (begins on E16; Murphy and Fox, 2007). Further, although we did not assess recombination in the adult GI tract, based on the overlap between expression of *BDNF* throughout the smooth muscle of the adult GI wall (Lommatzsch et al., 1999, Fox, 2006, Fox and Murphy, 2008) and the high efficiency *SM22a^{cre}*-mediated recombination in this tissue in adults (assessed using *Rosa26* cre reporter mice; Lepore et al., 2005), it is probable that *BDNF* coding sequences were largely eliminated from smooth muscle throughout the GI tract wall in adults, a tissue that receives substantial vagal mechanoreceptor innervation in mice (Fox et al., 2000). Further, consistent with the observations in *SM22a-cre;Rosa26* adults, there was a small amount of labeling in the adult brain of *SM22a-BDNF^{KO}* mice, suggesting that some *SM22a-cre*-mediated recombination occurred in this tissue. This labeling mainly occurred in a few highly circumscribed brain regions. Two strongly labeled areas included the hippocampus and a thin strip of anterior-lateral cerebral cortex oriented along the anterior-to-posterior axis (Figure 5A,B). Less dense labeling was observed in the VMH (Figure 5C). There were also scattered labeled cells in several brain regions including the tectum, midbrain central gray, medial prefrontal cortex and the cortex adjacent to the ventral hippocampus. No labeling was observed in the DVC or any region of pons, medulla or cerebellum.

BDNF mRNA expression—The cre reporter studies described above identified the developing and mature GI tissues that exhibited significant *SM22a^{cre}*-mediated recombination. Additionally, BDNF mRNA expression at E15, 16, and 17 was characterized in a block of tissue consisting of esophagus and stomach and a block consisting of small and large intestine and cecum to verify that in fact this recombination did result in significant reduction of BDNF levels in the gut of *SM22a-BDNF^{KO}* embryos. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of BDNF expression at E15, E16, and E17 yielded similar results, and thus were combined. Relative BDNF mRNA levels were significantly reduced (~90%) in the esophagus-stomach and intestines of *SM22a-BDNF^{KO}* embryos compared to wild types (relative mRNA level, % of wild type for esophagus-stomach: $7.48 \pm 1.5\%$; % of wild type for intestines: $10.23 \pm 1.9\%$; both $p < 0.0001$). Additionally, BDNF mRNA expression was assessed in adult tissues, including 2 brain regions and 10 viscera. All adult tissues examined from *SM22a-BDNF^{KO}* mice exhibited relative decreases in BDNF mRNA levels compared to wild types, ranging from 48% (bladder) to 1% (esophagus). These decreases were all significant except in the large intestine and bladder (Figure 6; dropping 2 outlier values from wild-type large intestine and bladder resulted in significant relative losses of BDNF mRNA that were similar in magnitude as that observed for the small intestine). There appeared to be a tendency for greater relative reductions in BDNF mRNA in more anterior organs as compared with more posterior ones.

Effect of *SM22a-BDNF^{KO}* on body weight and body fat

Body weight—The breeding strategy employed to generate *SM22a-BDNF^{KO}* mice resulted in offspring of 8 different genotypes. The growth curves of 7 of these groups are plotted in Figure 7A (the *SM22a^{cre/+};BDNF^{neo/+}* group was not included for clarity and because they were nearly identical in growth to *SM22a^{+/+};BDNF^{neo/+}* mice). *SM22a-BDNF^{KO}* mice began to gain weight at young ages, which developed into a dramatic early-onset obesity. By week 13 they weighed significantly more than *SM22a^{+/+};BDNF^{+/-lox}* ($p < 0.01$) and *SM22a^{cre/+};BDNF^{+/+}* (their most appropriate control group; $p < 0.01$) mice and by week 15 more than wild types ($p < 0.05$). The body weight of *SM22a-BDNF^{KO}* mice was greater than each of the other groups (*SM22a^{+/+};BDNF^{+/+}*, *SM22a^{cre/+};BDNF^{+/+}*, *SM22a^{+/+};BDNF^{+/-lox}*, *SM22a^{+/+};BDNF^{neo/+}*, *SM22a^{cre/+};BDNF^{+/-lox}*, and

SM22a^{+/+};*BDNF*^{neo/lox}) whether considered over the entire 6-30 week period that animals were weighed ($p < 0.0001$), or from 20 – 30 weeks of age, when the body weights of mice of all genotypes became asymptotic (all $p < 0.01$ except *SM22a*^{+/+};*BDNF*^{neo/lox}, $p < 0.05$). By week 30 the body weight of *SM22a*–*BDNF*^{KO} mice was 129% greater than for *SM22a*^{cre/+};*BDNF*^{+/+} mice, 106% greater than wild types, and 63-70% greater than global or targeted heterozygous KO mice (*SM22a*^{+/+};*BDNF*^{neo/+} and *SM22a*^{cre/+};*BDNF*^{+lox}). The tremendous increase in size of *SM22a*–*BDNF*^{KO} mice as well as their internal organs and intra-abdominal fat as compared to wild-type mice is illustrated in Figure 8.

As shown previously, global BDNF heterozygous mice (*SM22a*^{+/+};*BDNF*^{neo/+}) displayed a modest late (middle-aged)-onset obesity (29% increase in body weight compared to wild types at 30 weeks) with a large heterogeneity of weight gain as previously observed (Lyons et al., 1999, Kernie et al., 2000, Fox and Byerly, 2004). When considered over weeks 6-30 the body weight of global BDNF heterozygous mice was greater than wild-type, *SM22a*^{cre/+};*BDNF*^{+/+}, and *SM22a*^{+/+};*BDNF*^{+lox} control groups (all $p < 0.05$), but the large variability in weight gain precluded this from reaching significance from week 20-30 even when these three control groups were combined ($p = 0.084$; body weights of these three control groups did not differ significantly). A similar mild obesity was observed in heterozygous targeted KO mice (*SM22a*^{cre/+};*BDNF*^{+lox}). Their body weight was significantly greater than that of wild type, *SM22a*^{cre/+};*BDNF*^{+/+}, and *SM22a*^{+/+};*BDNF*^{+lox} control groups from weeks 6-30 (all $p < 0.0001$) and the three combined control groups from weeks 20-30 ($p < 0.05$; 31% increase in body weight compared to *SM22a*^{cre/+};*BDNF*^{+/+} controls at 30 weeks). Also during weeks 20 – 30, *SM22a*^{+/+};*BDNF*^{neo/lox} mice exhibited an increase in body weight compared to the global and targeted heterozygous KO's, but it was not significant (*SM22a*^{+/+};*BDNF*^{neo/+}, $p = 0.2$; *SM22a*^{cre/+};*BDNF*^{+lox}, $p = 0.12$, respectively).

Body fat—Body composition of wild-type and *SM22a*–*BDNF*^{KO} mice was assessed using NMR spectroscopy at 3 ages. These included the age at the start of meal pattern data collection (3 months), the oldest age body weight was routinely measured (7 months), and 9 months of age, as some of the KO mice appeared to continue to gain weight after 7 months (Figure 7B). By 9 months, the heaviest mouse weighed 72 g, of which 50% was fat. At 7 and 9 months (both $p < 0.001$), but not 3 months of age ($p = 0.059$), *SM22a*–*BDNF*^{KO} mice had significantly higher body fat than controls when considered as fat weight (Figure 7B), whereas fat as percent of body weight was significantly greater in the KO mice at all 3 ages (3 months: 218% increase, 22.04 ± 4.56 vs. $10.11 \pm 0.82\%$ of body weight, $p < 0.05$; 7 months: 379% increase, 40.2 ± 2.67 vs. $10.61 \pm 0.67\%$ of body weight, $p < 0.001$; and 9 months: 300% increase, 43.17 ± 3.14 vs. $14.41 \pm 1.84\%$ of body weight, $p < 0.001$). Additionally, this increase became progressively greater with age for *SM22a*–*BDNF*^{KO} mice, although it was only significant from 3 to 7 (weight: 282%; percent body weight: 182%; both $p < 0.001$) and 3 to 9 (weight: 339%; percent body weight: 196%; both $p < 0.001$) months.

Effect of *SM22a*–*BDNF*^{KO} on feeding behavior

Daily food intake—During meal pattern data collection (12-16 weeks of age), *SM22a*–*BDNF*^{KO} mice exhibited a marked hyperphagia as they consumed an average of 73% more food each day compared to *SM22a*^{cre/+};*BDNF*^{+/+} controls and 53% more than *SM22a*^{+/+};*BDNF*^{+/+} wild types and *SM22a*^{+/+};*BDNF*^{neo/+} mice (all $p < 0.0001$; Figure 7C). In fact, body weights of the *SM22a*–*BDNF*^{KO} mice and controls at these ages were similar when normalized to food intake (not shown), suggesting that all or most of the excess weight gain of *SM22a*–*BDNF*^{KO} mice - at least at 12-16 weeks of age - was due to hyperphagia. Consistent with this interpretation, pair-feeding heterozygous global BDNF

mutants (+/-) to wild-type littermates prevented late-onset obesity (Coppola and Tessarollo, 2004) and intermittent feeding reversed it (Duan et al., 2003).

Meal Patterns—For manipulations of the feeding regulatory system, changes in specific meal parameters can provide indications about the components of the system that have been altered. Therefore, as a first step toward gaining insight into the mechanism by which *SM22a*–*BDNF*^{KO} produced hyperphagia, their meal patterns were compared with those of several reference groups to identify changes. Further, these data were collected early in the dynamic phase of weight gain (3 months of age) of *SM22a*–*BDNF*^{KO} mice to minimize the impact of changes in their body weight on their feeding patterns. Although their body weight had begun to diverge from the control group, it was not yet significantly different in this cohort (Table 1). Eating patterns stabilized by day 7, therefore, data obtained from days 7–22 were analyzed. The two control groups, wild types (*SM22a*^{+/+};*BDNF*^{+/+}) and *SM22a*^{cre/+};*BDNF*^{+/+} mice did not differ significantly for any of the meal parameters, therefore, their data were combined for analysis. The averaged values for each parameter for each group that are not graphed are listed in Table 1. The results of this analysis indicated the hyperphagia exhibited by *SM22a*–*BDNF*^{KO} mice was mediated by increases in both meal size and meal frequency.

SM22a–*BDNF*^{KO} mice demonstrated a 31% increase in average meal size compared to controls ($p < 0.05$; Figure 9A; 37% increase vs. *SM22a*^{cre/+};*BDNF*^{+/+} mice, $p < 0.05$; not shown). The increase in meal size appeared to be due mainly to a non-significant increase in average intake rate of *SM22a*–*BDNF*^{KO} mice compared to controls (21%, $p = 0.28$; 46% vs. *SM22a*^{cre/+};*BDNF*^{+/+} mice, $p = 0.14$; Table 1). *SM22a*–*BDNF*^{KO} mice also showed a 26% increase in average meal frequency compared to controls ($p < 0.001$; Figure 10A). Consistent with this increase, the average intermeal interval (IMI) of *SM22a*–*BDNF*^{KO} mice was reduced by 20% compared to controls ($p < 0.05$; Figure 10B). Also, *SM22a*–*BDNF*^{KO} mice exhibited a 35% decrease in satiety ratio relative to control mice (satiety ratio is the ratio of meal size to the subsequent IMI, $p < 0.01$; Figure 10C). This decrease suggested the effectiveness of a given amount of food at producing satiety was reduced in the targeted KO mice, an effect that would have contributed to their decreased IMI and increase in meal frequency. As vagal afferents contribute to satiety signaling, this finding is consistent with effects of the *SM22a*–*BDNF*^{KO} on vagal afferents, although other pathways that signal satiety could have been affected. In contrast, global heterozygous mice (*SM22a*^{+/+};*BDNF*^{neo/+}) exhibited a 47% increase in average meal size compared to controls ($p < 0.05$; Figure 9A) due mainly to a near-significant increase in meal duration compared to controls ($p = 0.06$; Table 1). This increase was partially compensated for, however, by a non-significant 14% decrease in meal frequency that prevented hyperphagia ($p = 0.26$; Figure 10A).

Meal microstructure—The increased meal size of *SM22a*–*BDNF*^{KO} mice could be due to reduced gut-to-brain vagal negative feedback signals (satiation) or increased oropharyngeal positive feedback (e.g., food palatability). To investigate the relative contributions of these pathways, microstructure of the first 30 min of food intake, which followed 6 hour food deprivation each day from days 7–22 of the meal pattern data collection was examined. Both groups of targeted KO mice (*SM22a*–*BDNF*^{KO} and *SM22a*^{cre/+};*BDNF*^{+lox} groups) exhibited a highly unusual continuous intake over the entire 30 min of the first meal (Figure 9B). This contributed to a significant 145% increase in average food intake rate in *SM22a*–*BDNF*^{KO} mice compared to controls (control, 19 mg/min; *SM22a*–*BDNF*^{KO}, 45 mg/min) and precluded derivation of Weibull parameters that characterize the eating rate curves (Davis, 1998, Fox and Byerly, 2004). Instead, the amount of food consumed during the first minute was used to estimate initial intake rate and the amounts of food consumed during each minute from min 2–6 and min 7–30 were used to

estimate the early and late components of the decay of eating rate, respectively. The initial rate is mainly influenced by oropharyngeal stimulation, the early component of decay of eating rate by both oropharyngeal positive feedback and vagal GI negative-feedback signaling, and the late component by vagal GI negative feedback (Davis, 1998, Fox and Byerly, 2004). Although there were no significant differences in initial intake rates (min 1, $p = 0.12$; Figure 9B), the rapid rate of decay of food intake from min 2-6 was significantly reduced in the *SM22a-BDNF^{KO}* mice compared with targeted heterozygous KO and control mice, but not compared with *SM22a^{+/+};BDNF^{neo/+}* mice (average consumption per minute over min 2-6: *SM22a-BDNF^{KO}* 0.062 ± 0.005 g vs. *SM22a^{cre/+};BDNF^{+lox}* 0.038 ± 0.007 g, $p < 0.01$; vs. *SM22a^{+/+};BDNF^{neo/+}* 0.048 ± 0.007 g, $p = 0.087$; vs. control 0.041 ± 0.006 g, $p < 0.05$). Further, from the time point at which consumption rate leveled off in *SM22a-BDNF^{KO}* mice until the end of the meal (min 7-30), their decay of eating rate was significantly reduced compared to each of the other groups (average consumption per minute over min 7-30: *SM22a-BDNF^{KO}* 0.0393 ± 0.0017 g vs. *SM22a^{cre/+};BDNF^{+lox}* 0.026 ± 0.0018 g, $p < 0.01$; vs. *SM22a^{+/+};BDNF^{neo/+}* 0.0168 ± 0.0014 g, $p < 0.01$; vs. control 0.0103 ± 0.001 g, $p < 0.0001$). Decay of eating rate was also reduced for both global and targeted heterozygous KO's compared to controls (*SM22a^{+/+};BDNF^{neo/+}* and *SM22a^{cre/+};BDNF^{+lox}*, respectively, vs. controls). These reductions in decay of eating rate at min 2-6 and min 7-30 are consistent with decreased vagal negative feedback from gut-to-brain in *SM22a-BDNF^{KO}* mice, and therefore, with disruption of vagal satiation signaling contributing to their increased meal size. Nevertheless, to strengthen the case for involvement of vagal afferents in the feeding disturbances of *SM22a-BDNF^{KO}* mice it would be valuable to have more direct evidence that the development or signaling of these afferents were altered in these mice. Therefore, activation of the c-Fos immediate early gene in the target nuclei of vagal afferents in the brainstem of *SM22a-BDNF^{KO}* mice by meal-related stimuli was investigated to determine whether vagal afferent signaling was altered. Also, the number of vagal sensory neurons present in the nodose ganglia of *SM22a-BDNF^{KO}* mice was compared to the number in wild types to determine whether their survival or maintenance was altered.

Activation of nuclei within the DVC by meal-related stimuli

C-Fos activation in response to a larger-than-normal meal—If the altered feeding behavior of *SM22a-BDNF^{KO}* mice was due to reduced negative feedback signaling by vagal GI afferents, then meal-induced activation of NTS neurons, and possibly of AP or DMV neurons should have been reduced. A larger-than-normal meal was employed to activate as large a proportion of as many types of vagal afferents that innervate the upper GI tract as possible to increase the probability of identifying the altered sensory pathway(s). Further, large liquid meals have produced larger or more reliable increases in c-Fos activation in the NTS and AP as compared with smaller meals (Rinaman et al., 1998, Emond et al., 2001).

Examples of sections of the DVC sampled that were stained with neutral red are illustrated in a control and a *SM22a-BDNF^{KO}* mouse (Figure 11A,B, respectively) and representative c-Fos responses are shown in a control and an *SM22a-BDNF^{KO}* mouse from the non-fed groups (Figure 11C,D, respectively) and the fed groups (Figure 11E,F, respectively). Unexpectedly, baseline c-Fos activation (number of cells with nuclear c-Fos-LIR in non-fed groups) in the targeted KO's was reduced in old, obese (4-12 months of age) as compared with young, pre-obese (9-11 weeks of age) mice for all 3 brain regions studied (AP, 85%; DMV, 72%; and NTS, 76%; all $p < 0.05$; Figure 11G). Additionally, in the young, pre-obese targeted KO's there was a decrease in baseline c-Fos-LIR counts compared to controls in the DMV that was near-significant, or significant, depending on whether they were compared to only young controls (young controls vs. young targeted KO, $p = .065$), or to young and old

controls pooled (all controls vs. young targeted KO's, $p < 0.05$; young and old controls were not different; Figure 11G). Therefore, percent increases in meal-induced vs. baseline c-Fos-LIR cell nuclei counts were used to normalize responses, and data from young and old mice were analyzed separately (Figure 11H,I, respectively). In both young and old mice there was no effect on NTS activation, although in old mice there was a greater increase in activation in the NTS of KO's compared to controls that approached significance ($p = 0.057$; Figure 11H,I). These results did not support the hypothesis that vagal satiation signaling was reduced in *SM22a-BDNF^{KO}* mice. Interestingly though, the DMV in both young and old mice demonstrated significantly greater activation in targeted KO's compared to controls (young: $p < 0.01$, Figure 11H; old: $p < 0.05$, Figure 11I).

Body weight and food intake at time of c-Fos test—There was no difference in body weight or test meal size between young controls (*SM22a^{cre/+};BDNF^{+/+}*) and young *SM22a-BDNF^{KO}* mice, or in test meal size between old control and old *SM22a-BDNF^{KO}* groups (Table 2). For the old mice, however, body weight was increased by almost 100% in the targeted KO's as compared with controls (Table 2). Since the reduced baseline activation of the DMV in the young targeted KO mice and the increased meal-induced activation of this brain region in these mice occurred prior to development of obesity, these effects may represent expressions of the primary effect of the targeted KO. These results also suggest that the obesity of old *SM22a-BDNF^{KO}* mice, or associated factors, could have contributed to their reduced baseline c-Fos activation in the AP, NTS and DMV compared to young *SM22a-BDNF^{KO}* mice, or to their increased meal-induced c-Fos activation in the DMV compared to old control mice.

Assessment of effects of reduced GI BDNF on vagal GI afferents

Nodose ganglion cell counts—If the altered feeding behavior of *SM22a-BDNF^{KO}* mice was due to reduced satiation signaling by vagal GI afferents, the most straightforward prediction would be that this KO resulted in the failure of some vagal GI afferents to survive during development or to be maintained in adulthood. This would result in lower-than-normal numbers of neurons populating the adult nodose ganglion as observed with global homozygous *BDNF^{KO}*'s (Jones et al., 1994, Ernfors et al., 1994a). *SM22a-BDNF^{KO}* mice did exhibit a significant decrease in the number of neuronal profiles in the nodose ganglion compared to wild-types (9%; wild types: left = 1270 ± 20.87 , right = 1275 ± 47.71 ; total = 2546 ± 50.77 ; *SM22a-BDNF^{KO}* mice: left = 1165 ± 51.46 , right = 1159 ± 17.55 ; total = 2324 ± 46.9 ; comparisons of counts of right nodose, left nodose, or right and left combined all $p < 0.05$). Interestingly, this reduction was much smaller than that observed not only in global homozygous *BDNF^{KO}* mice (59%; *BDNF^{neo/neo}*), but also compared to heterozygous global *BDNF^{KO}* mice at birth (35%; *BDNF^{neo/+}*, ElShamy and Ernfors, 1997) or in adults (38%; M. Boynton and E.A. Fox, unpublished observations). Since *SM22a-BDNF^{KO}* mice have the global heterozygous *BDNF* mutation, they should exhibit at least a 35% loss of neurons from the nodose ganglion. Thus, the smaller-than-expected loss of vagal sensory neurons in *SM22a-BDNF^{KO}* mice raises the possibility that BDNF deficiency in GI smooth muscle resulted in increased survival of vagal sensory neurons.

Density and structure of vagal GI afferents—To determine whether the altered survival or maintenance, or possibly growth or differentiation of vagal afferents in *SM22a-BDNF^{KO}* mice involved those that supply the GI tract, we attempted to label and quantitatively compare vagal GI mechanoreceptors in controls and *SM22a-BDNF^{KO}* mice. Unfortunately, mutants did not recover from surgery for WGA-HRP injection into the nodose ganglion, which is the only method that labels virtually all receptors and therefore is the only method valid for making such quantitative comparisons. Thus, although *SM22a-BDNF^{KO}* mice appeared to have normal health even well into their second year, the effects

of their reduced BDNF levels in the brainstem, and possibly the greatly reduced levels in heart and lungs were probably revealed even in young mice when stressed by anesthesia and surgery that involved pressure placed on the nodose ganglion. As an alternative, IGLEs, one of the well characterized mechanoreceptors were successfully labeled in the esophagus, stomach and intestine utilizing a previously established marker, the purinergic receptor, P_2X_2 (Castelucci et al., 2003; not shown). Only IGLEs that innervated the stomach, however, were stained consistently enough to permit quantification. In contrast, numerous potential markers tested for identification of intramuscular arrays (IMAs), the other well characterized mechanoreceptors, failed. In preliminary experiments we determined that P_2X_2 IHC labeled 80% of the gastric IGLEs stained by anterograde transport of WGA-HRP injected into the nodose ganglion of C57Bl/6 mice (J.E. Biddinger and E.A. Fox, unpublished observations; Fox et al., 2000). Comparison between *SM22a*-*BDNF*^{KO} mice and controls (*SM22a*^{cre/+}; *BDNF*^{+/+} mice) revealed no difference in total gastric IGLE number (mutants 88.0 ± 11.14 vs. controls 93.33 ± 9.53). Moreover, qualitative comparisons of the morphology in *SM22a*-*BDNF*^{KO} mice (Figure 12A,C,E) and controls (Figure 12B,D) did not reveal any readily apparent differences. These results suggested that in the stomach wall, IGLE innervation that expresses P_2X_2 receptors developed and was maintained normally in *SM22a*-*BDNF*^{KO} mice. Nevertheless, these findings leave open the possibility that development of IGLEs that supply the intestine was altered.

DISCUSSION

Feeding behavior and vagal afferent innervation of *SM22a*-*BDNF*^{KO} mice were characterized to begin to gain insight into the mechanism underlying the hyperphagia that has been associated with various *BDNF*^{KO} models. *SM22a*-*BDNF*^{KO} mice exhibited an early-onset obesity and hyperphagia that were similar in time course and magnitude to brain-specific *BDNF*^{KO} mice (Rios et al., 2001). Additionally, meal pattern and microstructural analyses identified possible effects of the *SM22a*-*BDNF*^{KO} on vagal GI afferents. *SM22a*-*BDNF*^{KO} mice exhibited an increase in meal size, raising the possibility that they had a satiation, or meal termination deficit. The finding that *SM22a*-*BDNF*^{KO} mice demonstrated a decrease in the rate of decay of food consumption over the course of a scheduled meal bolstered this interpretation. The meal pattern analysis further showed that *SM22a*-*BDNF*^{KO} mice had increases in meal number and IMI and a decrease in satiety ratio, which could imply that they also had a satiety deficit – i.e. a given amount of food was less effective-than-normal at producing satiety, or at delaying the start of the next meal. Since vagal GI afferents mediate the bulk of satiation signaling and contribute to satiety signaling these deficits were consistent with reduced vagal afferent signaling from gut-to-brain.

In contrast, meal-induced c-Fos activation of the NTS, a more direct measure of vagal sensory signaling, was not reduced in pre-obese *SM22a*-*BDNF*^{KO} mice. This suggested their vagal GI afferent signaling to the brain was normal, and therefore, that reduced BDNF levels in the CNS probably caused their satiation and satiety deficits. Interestingly though, vagal motor neurons in the medial DMV column of *SM22a*-*BDNF*^{KO} mice exhibited increased meal-induced c-Fos activation. This could imply that augmentation of gastric vago-vagal digestive reflexes occurred (e.g., accommodation). Increased activation of these reflexes would reduce the intragastric pressure produced by ingesta, which would decrease vagal negative feedback signaling to the brain and thus increase meal size. Consequently, it is possible that amplification of gastric vago-vagal reflexes contributed to the hyperphagia and obesity of *SM22a*-*BDNF*^{KO} mice.

Possible mechanisms of reduced CNS BDNF levels on feeding behavior

Feeding patterns of *SM22α*–*BDNF*^{KO} mice are distinct from those of other obesity models—The pattern of feeding disturbances underlying the obesity of *SM22α*–*BDNF*^{KO} mice appears unique compared to most other genetic and diet-induced obesity models that involve overeating. In particular, *SM22α*–*BDNF*^{KO} mice exhibited moderate increases in both meal size and meal number early in the dynamic phase of weight gain that combined to support marked hyperphagia. Moreover, late-onset obesity in global heterozygous *BDNF* KO mice (*BDNF*^{neo/+} mice) fed a balanced diet is mediated entirely by increased meal number (Fox and Byerly, 2004). In contrast, hyperphagia associated with other obesity models has typically been mediated solely by large increases in meal size (Kissileff et al., 1979, Strohmayer and Smith, 1987, Ho and Chin, 1988, Farley et al., 2003, Atalayer and Rowland, 2011, Richard et al., 2011). In some instances the number of meals consumed each day remained normal, whereas in others it actually decreased. Moreover, when feeding patterns were examined prior to development of obesity they were similar to those obtained in obese mice (e.g., Richard et al., 2011). These different patterns of feeding disturbances in *SM22α*–*BDNF*^{KO} mice and other obesity models suggests either that part or all of the mechanisms underlying overeating differ, or that the interactions of parallel metabolic disturbances that are largely absent in *SM22α*–*BDNF*^{KO} mice (Lyons et al., 1999, Rios et al., 2001, Duan et al., 2003, Coppola and Tessarollo, 2004), but prominent in the other obesity models cited may have modified the effects of a similar underlying mechanism. This difference between *BDNF* KO's and other obesity models may reflect the role of BDNF as one downstream effector of leptin and melanocortins that is largely responsible for their inhibition of feeding, whereas, other downstream pathways may mediate leptin and melanocortin effects on metabolism and energy expenditure (Balthasar et al., 2005, Begriche et al., 2011).

Central effects of vagal GI afferent signaling may be decreased by reduced CNS BDNF levels—Microstructure analysis of the first daily meal of *SM22α*–*BDNF*^{KO} mice revealed a dramatic reduction in the rate of decay of feeding over the course of the meal. This resulted in large increases in eating rate and meal duration, suggesting vagal satiation signaling was reduced. In fact, their meal duration extended well beyond that of wild types, which lasted about 15 minutes, and showed little or no sign of abatement at the end of the 30 minute window examined. In contrast, the average daily meal duration of *SM22α*–*BDNF*^{KO} mice was normal, indicating that only their first meal was extended in duration. The first meal was the only one preceded by food deprivation, suggesting this contributed to its extended duration. A similar dramatic sensitivity of meal duration to food deprivation occurred with sham feeding animals – they ate continuously when deprived overnight (Antin et al [15] in Greenberg and Smith, 1996). In these animals vagal afferents were intact, but they were weakly activated during feeding due to reduced accumulation of food in the GI tract. The similar large effect of deprivation on meal duration in both sham feeding and *SM22α*–*BDNF*^{KO} animals raises the possibility that food deprivation also interacted with reduced vagal input in *SM22α*–*BDNF*^{KO} mice. It is unlikely that vagal satiation signaling from gut-to-brain itself, however, was altered in *SM22α*–*BDNF*^{KO} mice during the first daily meal. For instance, these mice exhibited meal-induced c-Fos activation in the NTS that was similar to controls, suggesting vagal GI afferent signaling in response to a meal was normal. This could imply that rather than having altered vagal signaling directly, reduced BDNF levels in the brain may have affected the transmission or processing of vagal input at CNS sites downstream of the NTS so that they had reduced inhibitory impact on the CNS circuit that drives feeding. Thus, the effects of food deprivation in *SM22α*–*BDNF*^{KO} mice may have interacted with the reduced transmission or processing of vagal input downstream of the NTS to produce an even greater reduction in the inhibition of feeding.

Vago-vagal digestive reflexes may be augmented by reduced CNS BDNF levels—Another possible mechanism by which reduced CNS BDNF levels in *SM22a-BDNF^{KO}* mice could contribute to their reduced satiation and satiety, and thus to their hyperphagia and obesity, was revealed by meal-induced c-Fos activation in the DVC. In particular, the motor neurons in the DMV of *SM22a-BDNF^{KO}* mice exhibited a reduction in baseline activity and an increase in meal-induced activation compared to controls. Moreover, the meal-induced activation was concentrated in the medial (gastric) DMV column, raising the possibility that gastric vago-vagal reflexes such as receptive relaxation (also referred to as accommodation) were augmented in *SM22a-BDNF^{KO}* mice. This reflex prepares the stomach to receive a meal without incurring large increases in intragastric pressure (Canon and Lieb, 1911; Grey, 1917). Activation of accommodation occurs when ingested food distends the esophagus or stomach and thus stretches or creates tension in the smooth muscle in the outer wall of these organs. These effects on the smooth muscle activate vagal mechanoreceptive afferents, which then stimulate vagal efferent pathways that relax the smooth muscle of the stomach wall (Abrahamsson and Jansson, 1973, Takahashi and Owyang, 1997, Wei et al., 1997, Rogers et al., 1999). Increased activation of such gastric vago-vagal reflexes in *SM22a-BDNF^{KO}* mice might have reduced the intragastric pressure resulting from a given amount of food consumed. For example, augmented receptive relaxation would increase relaxation of the stomach wall that occurs with the ingestion of food. Thus, more food would have to be ingested to increase intragastric pressure sufficiently to stimulate vagal gastric mechanoreceptors and generate satiation and satiety signals.

Previous findings in *c-Kit* and *steel* mutant mice are consistent with a relationship between the strength of the gastric accommodation reflex and meal size, as well as a possible relationship between altered vagal afferent signaling, accommodation and meal size (Fox et al., 2001, Fox et al., 2002, Chi and Powley, 2003). Mice from both of these strains had large, selective reductions in vagal sensory innervation of the forestomach, which is involved in the activation of the receptive relaxation reflex. This suggested their accommodation reflex was weaker-than-normal, and therefore, the stomach volume during a meal would be smaller-than-normal. Consistent with this, the meal size of *c-Kit* and *steel* mice was decreased compared to wild types. Further, as reviewed in Fox et al. (2002), analysis of several human clinical conditions, including diabetes, functional dyspepsia and esophageal reflux treated with fundoplication are consistent with a relationship between reduced meal size and decreased vago-vagal reflex activation of the gastric accommodation reflex. Note that although the effects on vagal afferents and meal size examined in *c-Kit* and *steel* mice were in the opposite direction of the augmentation of vago-vagal reflexes that we have suggested might occur in *SM22a-BDNF^{KO}* mice, they do add some validity to the possible contribution of enhancement of these reflexes to their feeding disturbances. Recent studies in humans have also implicated gastric accommodation as playing an important role in satiation that may contribute to long-term regulation of body weight (Janssen et al., 2011, Janssen et al., 2012).

Finally, regardless of whether or not augmented gastric accommodation occurred in *SM22a-BDNF^{KO}* mice, other factors certainly made important contributions to their hyperphagia. For example, in a previous study of the meal patterns and microstructure of heterozygous BDNF mutant mice (*BDNF^{neo/+}* mice), evidence was found, suggesting that enhancement of meal initiation and the oropharyngeal positive feedback that drives feeding contributed to their hyperphagia (Fox and Byerly, 2004). Thus, similar changes could also have been a factor in the overeating of *SM22a-BDNF^{KO}* mice.

An alternative interpretation of the c-Fos results based on the reduced baseline c-Fos activation in the DMV of *SM22a-BDNF^{KO}* mice is that fasting vagal motor activity was

decreased. This effect could have altered digestion efficiency in the direction of increased absorption and storage of ingested nutrients, which could have indirectly contributed to obesity. Importantly, because both the reduced baseline c-Fos activation and increased meal-induced c-Fos activation in the DMV of *SM22a-BDNF^{KO}* mice occurred before their weight gain increased relative to controls, these could represent primary effects of reduced BDNF that contributed to their hyperphagia.

After becoming obese, the reduced baseline c-Fos activity and larger percent increase in meal-induced c-Fos activation in the DMV of *SM22a-BDNF^{KO}* mice compared to controls was maintained. Additionally, obese *SM22a-BDNF^{KO}* mice exhibited reduced baseline c-Fos activity in the NTS, an effect that was probably secondary to obesity, but could have been critical to its maintenance. For example, the reduced baseline NTS activity in obese *SM22a-BDNF^{KO}* mice, since it occurred during fasting between meals, could have contributed to their satiety deficit if it was still present at these older ages. Reduced baseline NTS activity is consistent with decreased electrophysiological and behavioral responsiveness of vagal afferents to anorexigens, including CCK, and reduced excitability of vagal afferents in diet-induced obese animals (Paulino et al., 2009, Daly et al., 2011, Dockray and Burdya, 2011).

The contribution of *SM22a^{cre}*-mediated recombination in the adult brain to the hyperphagia and obesity of *SM22a-BDNF^{KO}* mice—The evidence of *SM22a^{cre}*-mediated recombination in the anterior-lateral cerebral cortex, hippocampus, and VMH of adult *SM22a-BDNF^{KO}* mice observed in the present study was a novel finding. This recombination could have resulted in a higher efficiency *BDNF^{KO}* in these structures compared to the *KO* in other brain regions. Although reduced BDNF in these brain areas could have contributed to the hyperphagia and obesity of *SM22a-BDNF^{KO}* mice, the available evidence suggests that this was not as likely for the cortical and hippocampal recombination as it was for that in the VMH. *Emx1-BDNF^{KO}* mice were generated in the same manner as *SM22a-BDNF^{KO}* mice, using the same *BDNF^{neo}* and *BDNF^{lox}* mouse strains as in the present study except that *Emx1^{ires-cre}* mice were used to target recombination (Gorski et al., 2003). Thus, these *Emx1-BDNF^{KO}* mice had partial reduction of BDNF throughout the animal due to a global *BDNF^{neo/+}* mutation (similar to *SM22a-BDNF^{KO}* mice) and loss of the other *BDNF* allele restricted to forebrain regions that included the hippocampus and virtually all of the cerebral cortex. *Emx1-BDNF^{KO}* mice, however, did not exhibit increased food intake or weight gain. This suggests the loss of BDNF from the adult cerebral cortex and hippocampus combined with the partial global loss of BDNF in *SM22a-BDNF^{KO}* mice did not contribute significantly to the hyperphagia and obesity of *SM22a-BDNF^{KO}* mice on their own.

Regarding the role of the VMH, while loss of BDNF from this brain region most probably contributed to the overeating and obesity of *SM22a-BDNF^{KO}* mice, it was not possible to determine the extent of its influence because it was confounded with possible effects of reduced BDNF levels throughout the CNS and in peripheral smooth muscle. Previous studies that targeted *BDNF^{KO}* to the VMH using cre-lox recombination failed to produce increased food intake or weight gain (e.g., R.B. Simerly, Saban Research Institute, University of Southern California, Personal communication). The *SF-1^{cre}* strain employed in these instances, however, does not target cre-mediated recombination to all of the VMH neurons that produce BDNF. In contrast, virally mediated *KO* of *BDNF* from the VMH and dorsomedial hypothalamus (DMH; Unger et al., 2007) produced increased food intake and weight gain. The magnitude of these changes was less than that observed in the present study (food intake and body weight increases reported by Unger et al. were 27 and 41%, respectively, and in the present study were 73 and 129%, respectively). These differences could have resulted, however, because the *KO* in the Unger et al. study was implemented in

the adult, whereas in the present experiment it was activated at earlier ages, sometime between birth and two months of age. Although we cannot determine the magnitude of the contribution of BDNF loss from the VMH to the hyperphagia and obesity in *SM22a-BDNF^{KO}* mice, this loss most likely interacted with reduced BDNF levels in other brain regions and possibly in peripheral tissues to produce these effects. Identifying the key brain sites that interact with the VMH to inhibit food intake and determining how they interact to achieve this will be essential for understanding BDNF's role in the inhibition of food intake by leptin and melanocortins and possibly other anorexigenic CNS pathways that might converge on CNS neurons utilizing BDNF.

Comparisons of food intake and body weight gain of *SM22a-BDNF^{KO}* mice and their component mice, which include targeted and global heterozygous BDNF mutants (*SM22a^{cre/+};BDNF^{+lox}* and *SM22a^{+/-};BDNF^{neo/+}* mice), indicated that the effects of loss of the majority of BDNF expression in *SM22a-BDNF^{KO}* mice produced a proportionally much greater effect on food intake and body weight as compared to loss of about half its expression in the heterozygous mutants. Both types of heterozygous *BDNF* mutants exhibited a final body weight increase (at 30 weeks of age) of about 30% compared to controls. These two weight gains if additive should have resulted in a 60% increase in weight gain, but this was less than half of the actual final weight gain of *SM22a-BDNF^{KO}* mice (129% increase). This suggests that up to a certain level the loss of BDNF can be partially compensated for, whereas loss of the majority of BDNF cannot be compensated for. Consistent with the hypothesis that compensation occurs with *BDNF* haploinsufficiency, global heterozygous *BDNF* mutants exhibited a significant increase in meal size, but this did not result in hyperphagia because they compensated, largely by reducing the number of meals consumed each day. Based on the role of BDNF in CNS plasticity (Noble et al., 2011), we propose that the greater decrease of BDNF levels in the CNS of *SM22a-BDNF^{KO}* mice as compared with the heterozygous mutants could have reduced the ability of CNS circuits to make all of the plastic changes necessary to compensate for the effects of the decrease in CNS BDNF levels on feeding and body weight.

One way decreased CNS BDNF levels may impact hyperphagia is by augmenting gastric vago-vagal reflexes as described above. BDNF is normally present in the DVC and some hypothalamic and forebrain regions that project to the DVC (Saper et al., 1976, van der Kooy et al., 1984, Gray and Magnuson, 1987, Conner et al., 1997). Therefore, these brain areas would have had reduced BDNF levels in *SM22a-BDNF^{KO}* mice. Since these pathways modulate vago-vagal reflexes at the level of the DVC, if their activity was modified by the reduced BDNF levels in the CNS of *SM22a-BDNF^{KO}* mice, this could have resulted in the altered baseline and meal-induced activation of DMV neurons.

BDNF levels in the CNS may also play a role in the effects of manipulations of food intake on vagal brainstem regulation of other autonomic reflexes, including resting and stress-induced heart rate. For instance, caloric restriction and intermittent feeding reduced heart rate in part by increasing vagal motor activation in the brainstem, whereas a high energy diet had opposite effects (Mager et al., 2006, Griffioen et al., 2012a, Griffioen et al., 2012b). A role for central BDNF was suggested by the finding that dietary energy restriction increases BDNF levels in several brain regions (Duan et al., 2001). Further, a mouse model of Huntington's disease that had reduced BDNF levels in several brain regions, including the brainstem, exhibited increased heart rate due in part to reduced vagal motor activation in the brainstem, an effect that was reversed by BDNF infusion into the lateral ventricles (Griffioen et al., 2012b).

Possible effects of peripheral loss of BDNF on vagal afferents in *SM22 α -BDNF^{KO}* mice

Although it is unlikely that peripheral loss of BDNF contributed significantly to the overeating and obesity of *SM22 α -BDNF^{KO}* mice, it still could have altered the development or function of vagal afferents. The reduction in vagal sensory neuron number we observed in *SM22 α -BDNF^{KO}* mice (9%), because it was much smaller than expected (by 26%), suggested these mice had an increase in survival of vagal afferents. Although this expectation of a larger decrease in nodose neuron survival was based on counts done at birth in *SM22 α -BDNF^{neo/+}* mice (35% decrease; Elshamy and Ernfors, 1997), it is unlikely that vagal sensory neurons were generated after birth in this mouse strain because a similar decrease (38%) was observed in adults (M. Boynton and E.A. Fox, unpublished observations). The apparent increase in vagal sensory neuron numbers in *SM22 α -BDNF^{KO}* mice suggests that BDNF produced in peripheral tissues innervated by vagal afferents may normally suppress vagal sensory neuron survival. Further, this raises the possibility that vagal afferent innervation of the GI tract was increased in *SM22 α -BDNF^{KO}* mice. The anatomical data we were able to obtain in these mice suggested that reduced GI BDNF did not impact the development of gastric IGLs that express P₂X₂ receptors. If this non-effect holds up for the additional 20% of gastric IGLs, then the apparent increase in survival of nodose ganglion neurons would have resulted in increased innervation by other vagal receptors, including IGL innervation of the esophagus or intestine, or IMA innervation of the stomach. Consistent with these possibilities, mice with only peripheral homozygous *SM22 α ^{cre}*-mediated KO of *BDNF* from smooth muscle were able to survive WGA-HRP injections into the nodose ganglion and exhibited a 35% increase in IGL innervation of the small intestine with no change in the stomach (Biddinger and Fox, 2012). Note that these targeted KO's were produced using *BDNF^{+/lox}* mice that did not have the *lacZ* gene inserted in the *BDNF* locus and appeared to have normal BDNF expression from this floxed allele (Rios et al., 2001). If vagal intestinal IGL innervation, or the sensory function of other vagal GI afferents were increased in *SM22 α -BDNF^{KO}* mice then these afferents could have been involved in mediating the increase in meal-induced c-Fos activation in the DMV and the implied vago-vagal reflexes as enhancement of esophageal, gastric, or intestinal vagal sensory pathways that activate these reflexes could have occurred (Wei et al., 1997, Rogers et al., 1999). Although suppression of neuron survival by BDNF is not typical, BDNF had a similar effect on Merkel mechanoreceptor innervation of the skin and sympathetic innervation of the necks of hair follicles (Fundin et al., 1997; Rice et al., 1998).

Other pathways/mechanisms contributing to hyperphagia and obesity of *SM22 α -BDNF^{KO}* mice

Similar to vagal afferents, it is unlikely any effects of loss of BDNF from peripheral tissues on sympathetic or enteric neurons contributed significantly to the overeating and obesity of *SM22 α -BDNF^{KO}* mice. Nevertheless it is possible that their development or function was altered and could have contributed to altered GI reflexes. At least some subdivisions of the sympathetic and enteric systems express trkB or BDNF and some of them modulate gut motility and feeding patterns (Coulie et al., 2000, Chai et al., 2003, Sclafani et al., 2003, Grider et al., 2006, Boesmans et al., 2008, Fu et al., 2011). For example, since some myenteric neurons express trkB receptors (Lommatzsch et al., 1999), the *SM22 α -BDNF^{KO}* could have altered development or function of the myenteric nervous system, which provides local regulation of many of the same GI functions influenced by vago-vagal reflexes, including motility and exocrine and endocrine secretion.

Another potential effect of GI BDNF deficiency is altered function of enteroendocrine cells in the GI epithelium. These cells respond to nutrient stimulation by secreting hormones, including ghrelin, CCK, and GLP-1, that influence GI function and feeding behavior in part by activating vagal afferents (Raybould, 2010). Therefore, if secretion of these hormones

were altered in *SM22a-BDNF^{KO}* mice, they could have affected GI reflexes or feeding behavior in a manner that contributed to their obesity or hyperphagia. BDNF is expressed in mature GI epithelium, however, its high and low affinity receptors, trkB and p75, respectively, do not appear to be expressed in this tissue (Lommatzsch et al., 1999). Nevertheless, since the axons of vagal mucosal afferents grow along blood vessels en route to the mucosa during development, it is possible that loss of BDNF from vascular smooth muscle could influence their development, and thus their responsiveness to hormones secreted by enteroendocrine cells.

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ABBREVIATIONS

ANOVA	analysis of variance
AP	area postrema
BDNF	brain-derived neurotrophic factor
ca	celiac artery
cc	central canal
c-Fos-LIR	c-Fos-like-immunoreactivity
CNS	central nervous system
cr	crypt
DMH	dorsomedial hypothalamus
DMV	dorsal motor nucleus of the vagus nerve
DVC	dorsal vagal complex
ep	epithelium
e	esophagus
GI	gastrointestinal
HSD	Tukey's honest significant difference
IHC	immunohistochemistry
IGLE	intraganglionic laminar ending
IMA	intramuscular array
IMI	intermeal interval
KO	knockout
lp	lamina propria
les	lower esophageal sphincter
mNTS	medial subnucleus of the NTS

mes att	mesenteric attachment
NTS	nucleus of the solitary tract
ON	overnight
PBS, RT-PCR	reverse transcription polymerase chain reaction
pH 7.4	sodium phosphate buffered saline
PF	paraformaldehyde
SAM	slowly adapting mechanoreceptor
s	stomach
v	vagus nerve
VMH	ventromedial hypothalamus

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HIGHLIGHTS

- Our aim was to gain insight into the mechanism of hyperphagia of BDNF KO mice
- Feeding behavior and vagal afferent innervation of BDNF KO mice were characterized
- Meal size and number were increased, suggesting deficits in satiation and satiety
- Increased vagal motor activation by a meal suggested accommodation reflex augmented
- Augmented accommodation reflex may contribute to reduced satiation and satiety

Figure 1.

Recombination of *loxP* sites in *Rosa26* embryos mediated by *SM22a-cre* expression in the embryo, represented by β -galactosidase (stained here with X-gal) was largely restricted to smooth muscle (sm) or mesenchyme (mesch) in the walls of the stomach, intestine and GI blood vessels (bv) as well as the associated mesentery (mes). A-F. Photomicrographs and photomontages of X-gal-stained (blue stain) sections counterstained with neutral red (except panel B was only stained with X-gal). Sections shown are from the stomach at E13 (A) and E15 (B), or from the intestines at E13 (C) and E15 (F), and the small intestine (D) and large intestine (E) at E17. B. Scale bars = 100 μ m. Abbreviations: cr, crypt; ep, epithelium; mes att, mesenteric attachment.

Figure 2.

SM22a-cre-mediated recombination in *BDNF^{lox}* mice was restricted mainly to sites of *BDNF* expression in vascular smooth muscle of blood vessels supplying the GI tract shown here in the region of the junction of the esophagus and the stomach. Photomicrographs and photomontages similar to those described in Figure 1 illustrate β -galactosidase expression in the esophagus-stomach in *BDNF^{lacZ}* mice, representing *BDNF* expression (A,B,D), and in *BDNF^{lox}* mice, representing recombination of *loxP* sites (C,E). Sections shown are from ages E13 (A), E14 (C), E15 (B) and E17 (D,E). A-E. *BDNF* expression occurred in the smooth muscle (or mesenchyme) of the walls of the stomach, LES and blood vessels supplying the GI tract and associated mesentery (A,B,D). *BDNF* expression also occurred in the mucosa, but was restricted to the lamina propria of the antrum and corpus of the stomach (A,B,D). In contrast, *cre*-mediated recombination was restricted mainly to smooth muscle of GI blood vessels (C,E), but also occurred to a limited extent in the associated mesentery (not shown). Blood vessels that exhibited *BDNF* expression or *cre*-mediated recombination in the region of the esophagus-stomach junction were often closely opposed to the anterior vagal trunk or its branches (A,C). Scale bars = 100 μ m. Abbreviations as for Figure 1 and: ca, celiac artery; e, esophagus; lp, lamina propria; les, lower esophageal sphincter; s, stomach; v, vagus nerve.

Figure 3.

SM22a-cre-mediated recombination in *BDNF^{lox}* mice was restricted mainly to sites of *BDNF* expression in vascular smooth muscle of blood vessels supplying the GI tract shown here in the intestines. Photomicrographs and photomontages similar to those described in Figure 1 illustrate β -galactosidase expression in the intestines and associated mesentery in *BDNF^{lacZ}* mice, representing *BDNF* expression (A,C,E), and in *BDNF^{lox}* mice, representing recombination of *loxP* sites (B,D,F). A-F. Sections shown are from ages E13 (A), E14 (B,D), E15 (C) and E17 (E,F). *BDNF* expression occurred in the smooth muscle (or mesenchyme) of the walls of the intestines at some sites of mesentery attachment and of the blood vessels supplying the GI tract and associated mesentery (A,C,E). In contrast, cre-

mediated recombination was restricted mainly to smooth muscle of GI blood vessels and also occurred to a limited extent in the associated mesentery (B,D,F) and the smooth muscle of the wall of the intestine adjacent to the mesentery attachment. Scale bars = 100 μ m. Abbreviations as for Figures 1 and 2.

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Figure 4.

SM22a-cre-mediated recombination in *BDNF^{lox}* mice did not occur within the embryonic brain. Photomontages of oblique sections through the heads of E14 embryos that were stained with X-gal and counterstained with neutral red are shown in A (focused more on the forebrain) and B (focused more on hindbrain and cerebellum). These images illustrate the lack of β -galactosidase expression, and therefore the absence of cre-mediated recombination of *loxP* sites in the brains of *BDNF^{lox}* mice. Also, no evidence of recombination was observed in the brains of E17 embryos, or in *Rosa26* embryos at any of the ages examined (not shown). Scale bars = 200 μ m.

Figure 5.

SM22α-cre-mediated recombination in *BDNF^{lox}* mice occurred within a small number of adult brain regions. Photomontages (A,B) and a photomicrograph (C) of coronal brain slices taken from adult *SM22α-BDNF^{KO}* mice that were stained with X-gal. A. This image illustrates a cross-section through the strip of cortex that was stained with X-gal and extended rostrally and caudally from this level. B. The dorsal hippocampus is illustrated here, but a similar density of staining was observed in cell bodies of the ventral hippocampus. C. X-gal staining in the VMH shown here in the rostral half of the nucleus, which contained stronger staining than the caudal half that is typically associated with satiety. Recombination occurred in a similar pattern in the *Rosa26* adult brain, but it was denser and in some brain regions encompassed larger areas than in the *BDNF^{lox}* brain as expected since it was not restricted by the *BDNF* promoters (not shown). Scale bars in A and B = 400 μm, and in C = 200 μm.

Figure 6.

BDNF mRNA expression was assessed in adult tissues of *SM22α-BDNF^{KO}* and wild-type mice, including 2 brain regions and 10 viscera. The relative decreases in percent BDNF mRNA levels in mutants compared to wild types are plotted in this histogram.

Figure 7.

SM22α-BDNF^{KO} mice exhibited early-onset obesity associated with hyperphagia. A. Growth curves plotted from 6-30 weeks of age. Groups plotted include *SM22α-BDNF^{KO}* (*SM22α^{cre/+};BDNF^{neo/lox}*, n = 9), *SM22α^{+/+};BDNF^{neo/lox}* (n = 11), *SM22α^{cre/+};BDNF^{+/-lox}* (n = 12), *SM22α^{+/+};BDNF^{neo/+}* (n = 7), *SM22α^{cre/+};BDNF^{+/+}* (n = 7), *SM22α^{+/+};BDNF^{+/-lox}* (n = 8), or wild type (*SM22α^{+/+};BDNF^{+/+}*, n = 5). Note that some group sizes from 6-12 weeks of age were less than stated because the initial *SM22α-BDNF^{KO}* mice discovered to be obese and some of their littermates were included in this plot, even though they were first weighed after 6 weeks of age. *SM22α-BDNF^{KO}* mice began to gain weight rapidly from 2 months (8 weeks) of age, surpassing the average body weights of control groups by 13-15 weeks of age. B. Fat weight in grams as derived from NMR analysis of body composition for wild types and *SM22α-BDNF^{KO}* mice at 12, 30 and 38 weeks of age. *SM22α-BDNF^{KO}* mice were composed of significantly greater amounts of fat at all ages examined, and their fat weight increased significantly from 12-30 weeks of age. C. Food intake was measured daily for the 22 days of meal pattern collection at 3-4 months of age. Here the averages over the last 16 days when meal pattern parameters had stabilized were plotted for the 4 groups tested. *SM22α-BDNF^{KO}* mice exhibited a 73% increase in average daily food intake compared to the control group, and showed increases of similar magnitude compared to both the heterozygous smooth-muscle targeted (*SM22α^{cre/+};BDNF^{+/-lox}*) and global heterozygous BDNF mutant (*SM22α^{+/+};BDNF^{neo/+}*) groups.

Figure 8.

SM22α-BDNF^{KO} mice (left of each image) exhibited an enormous increase in growth relative to control mice shown here in two female littermates at approximately 18 months of age. This growth included their overall size (A), the size of their abdominal organs and fat (B). Stomachs were dissected out of these non-fasted mice for direct comparison (C).

Figure 9.

Altered meal pattern and microstructure of $SM22\alpha$ - $BDNF^{KO}$ mice suggested they had a deficit in satiation, or meal termination signaling. The group names in the graph legend are the same as for Figure 6. A. $SM22\alpha$ - $BDNF^{KO}$ mice exhibited a 31% increase in average meal size compared to the control group, and global heterozygous BDNF mutants ($SM22\alpha^{+/+};BDNF^{neo/+}$) showed a 47% increase in meal size, whereas the heterozygous smooth-muscle targeted ($SM22\alpha^{cre/+};BDNF^{+/lox}$) were similar to controls. B. During the first daily meal (30 min) at dark onset after a mild fast (6 hr), $SM22\alpha$ - $BDNF^{KO}$ mice demonstrated a reduced rate of decay of food intake compared to the $SM22\alpha^{+/+};BDNF^{neo/+}$, $SM22\alpha^{cre/+};BDNF^{+/lox}$, and control groups, suggesting satiation and possibly vagal negative-feedback signaling were disrupted. During the late phase (min 7-30), intake was maintained in $SM22\alpha$ - $BDNF^{KO}$ mice and in the heterozygous targeted mutants, albeit at a slightly lower level, but not in the other groups.

Figure 10.

Altered meal frequency, IMI and satiety ratio of $SM22\alpha$ - $BDNF^{KO}$ mice suggested they had a deficit in satiety signaling - signaling generated in response to factors activated by a meal that delay the onset of the subsequent meal. A. $SM22\alpha$ - $BDNF^{KO}$ mice showed a 26% increase in the average number of meals consumed daily compared to controls, whereas heterozygous targeted KO mice ($SM22\alpha^{cre/+};BDNF^{+/lox}$) were similar to controls, and global heterozygous KO's ($SM22\alpha^{+/+};BDNF^{neo/+}$) exhibited a non-significant 14% reduction. B. $SM22\alpha$ - $BDNF^{KO}$ mice exhibited a small, but significant decrease in average IMI compared to controls, whereas all other groups showed smaller decreases that were not different from controls. C. The satiety ratio of $SM22\alpha$ - $BDNF^{KO}$ mice, or ratio of the size of a meal to the following IMI, was decreased by 35% compared to controls, and was also reduced compared to the heterozygous targeted mutants ($SM22\alpha^{cre/+};BDNF^{+/lox}$), but not the global heterozygous mutants ($SM22\alpha^{+/+};BDNF^{neo/+}$). This suggested a given amount of food was less effective at producing satiety in $SM22\alpha$ - $BDNF^{KO}$ mice.

Figure 11.

Meal-induced c-Fos activation was increased in the DMV of young (9-11 weeks of age), pre-obese and old (4-12 months of age), obese *SM22 α -BDNF^{KO}* mice. Cross sections through the DVC of controls (A,C,E) and *BDNF^{KO}* mice (B,D,F). A,B. Sections taken from AP levels of the DVC sampled and stained with neutral red are illustrated. C,D,E,F. Sections taken from AP levels of the DVC sampled and stained for c-Fos-LIR are shown. Arrowheads indicate the approximate medial and lateral borders of the gastric (medial) column of DMV neurons and they point to the approximate ventral border of the DMV. Few cells showed c-Fos-LIR in the non-fed groups (C,D). Increased numbers of c-Fos-LIR neuronal nuclei were observed in the mNTS, DMV, and AP of the fed groups (E,F). In the

DMV this increase was concentrated in the gastric column of neurons at mid – caudal AP levels. G. In non-fed groups, the average number of c-Fos-LIR neuronal nuclei stained per section was reduced in old, obese compared to young, pre-obese *SM22a- $BDNF^{KO}$* mice in the AP, mNTS and DMV. In contrast, this basal or fasting c-Fos-LIR activation level remained stable as controls aged. This difference in basal activation necessitated using percent increases for comparisons and separate analyses for young and old mice. H. At young ages (9-11 weeks), only the DMV exhibited a change as *SM22a- $BDNF^{KO}$* mice had a greater percent increase in c-Fos-LIR in the DMV than did controls. Importantly, the body weight of *SM22a- $BDNF^{KO}$* mice was not yet significantly greater than controls (Table 2), minimizing the possibility that the effect of the *SM22a- $BDNF^{KO}$* on activation of the DMV was confounded by effects of obesity. I. In contrast, at older ages (4-12 months), after significant obesity had developed in *SM22a- $BDNF^{KO}$* mice, the difference in the DMV of young mice was maintained, and additionally they seemed to exhibit a greater percent increase of c-Fos-LIR in the NTS compared to controls, but this difference just failed to reach significance ($p = 0.057$). Scale bar in A = 100 μ m, applies to all images. Abbreviations: AP, area postrema; cc, central canal; DMV, dorsal motor nucleus of the vagus nerve; mNTS, medial subnucleus of the nucleus of the solitary tract.

Figure 12.

The morphology of gastric IGLEs that express P_2X_2 receptors is normal in *SM22a-BDNF^{KO}* mice. IGLEs and myenteric neurons immunostained for P_2X_2 receptor-LIR in the myenteric plexus within the smooth muscle wall of the stomach of *SM22a-BDNF^{KO}* and wild-type adult mice were scanned in 3 dimensions with a confocal microscope and these scans were projected into 2 dimensions. A. Digital photomontage that illustrates a group of IGLEs in the antrum of an *BDNF^{KO}* mouse. B-E. Higher magnification confocal scans of IGLEs from the corpus (B,C) and forestomach (D,E) of wild-type (B,D) and *SM22a-BDNF^{KO}* (C,E) mice. Arrowheads (A), IGLEs; arrows (A,B,D), P_2X_2 -expressing myenteric

neurons, some partially obscured by the overlying IGLE terminal puncta. Scale bars = 25 μm (A) or 50 μm (B-E).

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Table 1

Meal pattern parameters that did not exhibit any significant differences between groups (mean $\pm$ SEM; *SM22 α -BDNF^{KO}* (n = 9), *SM22 α ^{cre/+}; BDNF^{+/-lox}* (n = 10), *SM22 α ^{+/-}; BDNF^{neo/+}* (n = 6), and controls (*SM22 α ^{cre/+}; BDNF^{+/-}* plus wild types *SM22 α ^{+/-}; BDNF^{+/-}*, n = 10). Each measure was averaged daily. The tabled values are based on the average of these daily values over the last 16 days of behavioral testing (days 7-22). Body weight (grams), Meal duration (min), and Intake rate (mg/min).

	<u>Control</u>	<u><i>SM22α^{+/-}; BDNF^{neo/+}</i></u>	<u><i>SM22α^{cre/+}; BDNF^{+/-lox}</i></u>	<u><i>SM22α-BDNF^{KO}</i></u>
Body weight	25.43 $\pm$ 0.06	23.83 $\pm$ 0.16	28.42 $\pm$ 0.19	37.73 $\pm$ 0.49
Meal duration	19.74 $\pm$ 6.7	48.58 $\pm$ 8.65	30.27 $\pm$ 6.7	18.19 $\pm$ 7.07
Intake rate	24.42 $\pm$ 3.0	17.71 $\pm$ 4.36	20.22 $\pm$ 3.0	29.6 $\pm$ 3.2

Table 2

Food intake and body weight at the time of testing in the meal-induced c-Fos activation experiment. (mean $\pm$ SEM; *SM22 α -BDNF^{KO}*; young n = 10, old n = 8) and controls (*SM22 α ^{cre/+}; BDNF^{+/+}*; young n = 11, old n = 8).

	Control		<i>SM22α-BDNF^{KO}</i>	
	<u>Young</u>	<u>Old</u>	<u>Young</u>	<u>Old</u>
Food intake	3.4 $\pm$ 0.3 (n=6)	3.6 $\pm$ 0.2 (n=4)	3.7 $\pm$ 0.7 (n=5)	3.6 $\pm$ 0.5 (n=4)
Body weight (fed)	25.5 $\pm$ 4.0 (n=6)	25.3 $\pm$ 0.1 (n=4)	28.0 $\pm$ 4.7 (n=5)	$\pm$ 51.3 $\pm$ 16.2* (n=4)
Body weight (non-fed)	23.8 $\pm$ 2.1 (n=5)	24.4 $\pm$ 2.8 (n=4)	26.5 $\pm$ 2.9 (n=5)	$\pm$ 53.1 $\pm$ 2.1* (n=4)