Mice with chronically increased circulating ghrelin develop age-related glucose intolerance

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Reed JA, Benoit SC, Pfluger PT, Tschoëp MH, D’Alessio DA, Seeley RJ. Mice with chronically increased circulating ghrelin develop age-related glucose intolerance. Am J Physiol Endocrinol Metab 294: E752–E760, 2008.—Ghrelin is a gut peptide that stimulates food intake and increases body fat mass when administered centrally or peripherally. In this study, ghrelin was overexpressed in neurons using the neuron-specific enolase (NSE) promotersequences and mouse ghrelin cDNA (NSE-Ghr). Ghrelin expression in NSE-Ghr brain tissues was increased compared with wild-type mice. Ghrelin expression was also increased to a much smaller extent in liver of these mice, but mRNA levels in stomach or duodenum did not differ from wild-type mice. Body weight and composition was analyzed in two lines of NSE-Ghr mice, one line with increased circulating bioactive ghrelin (L43) and one line without (L73). No increases in body weight, food intake, or fat mass were found. Energy expenditure was measured in L43 mice and did not differ from wild-type controls, whereas locomotor activity was increased in NSE-Ghr mice. Young NSE-Ghr mice had normal glucose tolerance; however, L43 NSE-Ghr mice, but not L73 mice, developed glucose intolerance at 32 wk of age. Despite the impaired glucose tolerance in L43 mice, insulin levels did not differ from those of wild-type mice. These findings suggest a role for ghrelin in age-associated impairments of glucose homeostasis.

food intake; adipose; obesity

GHRELIN IS A 28 AMINO-ACID PEPTIDE WITH A UNIQUE EIGHT-CARBON OCTANOYL GROUP MODIFICATION TO THE NH₂-TERMINAL THIRD AMINO ACID (17). Important roles for ghrelin include increasing food intake and body fat mass as well as stimulating growth hormone release and gastric motility (8, 16, 26). Other studies (2) have demonstrated a role for ghrelin in ACTH, cortisol, and prolactin release. Since the octanoyl modification appears to be required for all biological functions of ghrelin in vivo, the nonoctanoylated form, des-octanoyl ghrelin, has been thought (13) to be biologically inactive.

Ghrelin was originally isolated from the stomach, and this tissue has the highest levels of ghrelin expression (16). However, ghrelin is also expressed at lower levels in the small intestine and has been detected (17) at very low levels in many other tissues, including large intestine, pituitary, lung, placenta, ovary, testis, kidney, hypothalamus, and various endocrine tumors. The only known receptor for ghrelin is the growth hormone secretagogue receptor (GHS-R), which is expressed in several parts of the central nervous system (CNS; ventral tegmentum, substantia nigra, raphe nucleus, hippocampus, brainstem, and several hypothalamic nuclei, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamic nucleus (VMN), anteroventral preoptic nucleus, anterior hypothalamic nucleus, suprachiasmatic nucleus, anterolateral hypothalamic nucleus, and tuberomammillary nucleus (4, 10, 31)]. Among these brain regions, ghrelin binding was of highest intensity at the presynaptic axon terminals of the ARC and PVN neurons (7). In the ARC and VMN, GHS-R and neuropeptide Y (NPY) were colocalized in some cells, suggesting that ghrelin stimulation of NPY neurons may underlie, at least in part, the orexigenic effect of ghrelin (24, 27).

Exogenous administration of ghrelin acutely causes an increase in food intake, and chronic administration results in profound increases in body fat (12, 20, 26). Despite the clear evidence that exogenous ghrelin influences the regulation of energy balance, the evidence for an important role of endogenous ghrelin in the regulation of body weight has been less clear. There have been several reports of targeted loss-of-function mutations of ghrelin or the ghrelin receptor. Ghrelin-deficient (Ghr−/−) and ghrelin receptor-deficient mice had normal growth rates, food intake, and body composition when fed a standard chow diet (23, 29, 32). GHS-R−/− or Ghr−/− mice fed a high-fat diet beginning at 4 or 6 wk of age, respectively, were protected from diet-induced obesity in two reports (29, 32). These findings contrasted with the unchanged susceptibility to diet-induced obesity found when high-fat diet was introduced at 20 wk of age (23).

One possible reason for the relatively small phenotypes in these loss-of-function mouse models is the plasticity of the hypothalamic circuits that are key targets of leptin action. Ghrelin activates agouti-related protein (AgRP)/NPY neurons in the ARC, and several lines of evidence (6, 7, 15, 25) support the hypothesis that this activation is a key part of how exogenous ghrelin exerts its effects on food intake and energy balance. Like the ghrelin knockouts, both AgRP and NPY knockout mice have little, if any, body weight phenotype when fed standard chow diet (22, 29). However, recent evidence (5, 9) points to the possibility that the absence of a major effect may be the result of developmental compensation of these hypothalamic circuits. When AgRP/NPY neurons are ablated late in life, mice show a profound decrease in food intake and pronounced weight loss. However, if that same ablation is executed early in development, the mice go on to have normal food intakes and body weight (19). Thus, it is plausible that the
relatively minor phenotypic effect of loss-of-function mutations of ghrelin or the ghrelin receptor is a consequence of developmental compensation.

Several transgenic mice with increased peripheral expression of ghrelin have failed to produce models of hyperphagia or obesity. Despite several studies showing that exogenous des-octanoyl ghrelin did not affect food intake or body weight, Asakawa et al. (3) reported that transgenic mice with cytomegavirus-driven ghrelin expression had very high levels of plasma des-octanoyl ghrelin in association with a decrease in body weight, food intake, and adipose mass, delayed gastric emptying, and a decrease in linear growth. Ariyasu et al. (1) found that mice bearing a transgene with chicken β-actin promoter directing expression of preproghrelin had high levels of plasma des-octanoyl ghrelin and were smaller than controls but otherwise unremarkable. Studies by Iwakura et al. (14) using rat insulin II promoter or rat glucagon promoter to drive ghrelin expression in the pancreatic islets found no significant alteration in body weight or food intake and no significant changes in pancreatic function or histology, including insulin secretion and glucose metabolism in either mouse.

Some studies (16, 17) have shown that very low levels of ghrelin are expressed in the hypothalamus, but the significance of that expression remains unclear. Lall et al. (18) used the rat growth hormone-releasing hormone promoter to drive expression of human GHS-R in mice. The resulting growth hormone-releasing hormone-GHS-R mice showed increased hypothalamic GHS-R expression and postweaning growth rate, but adult lean body mass was comparable with nontransgenic controls. The most significant change to body composition was a reduction in adipose mass, particularly in females.

Our initial goal for these experiments was to develop a model in which ghrelin expression would be increased across all tissues and a model in which it would be increased only in the CNS to compare ghrelin function selectively in these different tissues. Like others (1), however, we found that we could not increase bioactive ghrelin levels using the cytomegavirus promoter. Interestingly, however, we found that there was a doubling of bioactive ghrelin in the circulation of one line of mice in which we were attempting to increase CNS ghrelin levels using the neuron-specific enolase (NSE) promoter. Although that made it impossible to ascribe a selective CNS function to any resulting phenotype, it did become the first successful genetic model of increased bioactive ghrelin. This article then focuses on a comparison of multiple lines of mice in which ghrelin is successfully overexpressed using the NSE promoter. We report here a comparison between two lines of NSE-ghrelin (NSE-Ghr) mice that are similar in brain expression of the transgene but differ in terms of circulating bioactive ghrelin. Overexpression of ghrelin in the CNS alone did not increase food intake or body fat and did not change energy expenditure. However, aged mice with increases in both CNS and circulating ghrelin developed impaired glucose tolerance.

EXPERIMENTAL PROCEDURES

Mice. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Pronuclear injections were performed at the Transgenic Animal Core at the University of Cincinnati, using FVB/N as the genetic host. Animals were bred and housed in microisolator cages in the Vivarium at the Genome Research Institute, University of Cincinnati. Wild-type controls derived from F1 nontransgenic littermates were used for all studies. All studies were performed on male mice, and NSE-Ghr mice were heterozygotes.

NSE-Ghr construct. The coding region of the mouse ghrelin or motilin-related peptide (GenBank accession no. XM_132807) was amplified using forward primer 5′-CCAGGCAATTCCAGGTCACT and reverse primer 5′-TCAGGAAAGGAACGCAGG to produce a 430-bp PCR product. The product was introduced 3′ to the NSE promoter sequences in a mammalian expression vector. Orientation and sequence identity were confirmed by sequencing the NSE-Ghr construct with primers flanking the insertion site. The plasmid construct was excised from vector sequences, gel purified, and used for pronuclear injection into fertilized FVB/N oocytes.

Genotyping. Tail DNA was isolated for use as PCR template. Transgenic pups were identified by PCR screening using forward primer 5′-CCCCGGCTGAGTCGCGAT and reverse primer 5′-TGTCATCCCCAGCATGCGTG, as indicated by dark gray arrows in Fig. 1A. PCR products were analyzed by agarose gel electrophoresis, with a 450-bp band diagnostic for integration of the NSE-Ghr transgene. Three distinct lines of NSE-Ghr-positive mice were established, identified as L43, L54, and L73.

Serum ghrelin measurements. Mice were deeply anesthetized using pentobarbital sodium. Blood was collected from the inferior vena cava and allowed to clot. Serum for total ghrelin was assayed without additives. For active ghrelin measurement, each 100 μl of serum was treated according to the manufacturer’s package insert protocol. All serum samples were stored at −20°C until time of assay. Total and bioactive ghrelin were measured by radioimmunoassay using the RIA kit from Phoenix Pharmaceuticals (Belmont, CA) following the manufacturer’s protocol.

Fig. 1. Generation of mice with neuron-specific overexpression of ghrelin. A: the neuron-specific enolase (NSE) promoter sequences were used to drive expression of mouse ghrelin cDNA (NSE-Ghr). The ghrelin protein-coding region was flanked by intronic (i) sequences and followed by a polyadenylation (pA) signal at the 3′ end of the construct. Genotyping was performed using primers located in regions indicated by dark gray arrows. Black arrows indicate approximate location of primer sites for initial RT-PCR screening for neuronal expression. Dark gray arrows indicate location of primer sites for real-time PCR measurements. B: endogenous and/or transgenic ghrelin expression in NSE-Ghr and wild-type (WT) brains was measured by quantitative real-time PCR (Q-PCR). Light gray arrows in A indicate the approximate location of primers. The assay did not distinguish between endogenous and transgene-derived transcripts. Ghrelin transcripts were barely detectable in total RNA from hypothalamus (HYPO) and cortex but were abundant in both tissues from line 43 (L43) and L73 NSE-Ghr mice (data are means ± SE; n = 6–8).

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active serum ghrelin were measured by RIA using commercially available ghrelin ELISA kits (Linco Research, St. Charles, MO) that detect human, rat, and mouse ghrelin.

**Quantitative gene expression.** Mice were deeply anesthetized with pentobarbital sodium, and blood was collected from the inferior vena cava in 1-ml syringes. Stomach, duodenum, liver, and brain were harvested. Total RNA was isolated as described above and verified by L32 amplification products in agarose gel before any quantitative real-time PCR (Q-PCR) reactions were run. Endogenous and transgenic ghrelin mRNA were amplified with forward primer 5’-TCACGGTTCAATGTGCTCCCTTC and reverse primer 5’-GCCTGTCCGTGTTACTTGTG, with an annealing temperature of 64.3°C. L32 was amplified from every sample for use as an endogenous control, using forward primer 5’-GCCAGGAGACGACAAAAAT and reverse primer 5’-AATCTCTTTGCTCCCTGATCC, with an annealing temperature of 61.2°C. Mouse ghrelin mRNA Q-PCR primer sites in transgene construct are indicated by light gray arrows in Fig. 1A. Each primer set was optimized with a temperature and concentration gradient such that the correlation coefficient was 0.99–1.0 and the PCR efficiency was 90–100% within the range of expected expression level. Q-PCR was performed in triplicate using an iCycler and the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), with two-step amplification (95°C for 10 s and annealing temperature for 30 s) for 40 cycles. For data analysis, the average threshold cycle (CT) of each set of triplicates was calculated. To normalize the data, the ΔCT was calculated for each sample by subtracting the average CT of L32 from the average CT of the gene of interest. For relative quantitation, the ΔCT was averaged for the wild-type control group and subtracted from the ΔCT of each experimental sample to generate the ΔΔCT. The ΔΔCT was then used to calculate the approximate fold change (2△△CT).

**Food intake, total body weight, and body composition measurements.** Mice were housed individually and fed ad libitum with standard chow provided in hoppers. Fill weight of each hopper was recorded and weighed weekly to obtain weekly intake. Mice were weighed weekly to determine body weight. Water was provided at all times. MRI was performed at various time points to determine fat and lean body composition, using an Echo MRI whole body composition analyzer (EchoMedical Systems, Houston, TX). Each mouse was tested in duplicate, and readings were accepted if they differed by ≤10%. Total lean, fat, and fluid mass were also required to differ by ≤10% from total body weight.

**Indirect calorimetry and quantification of locomotor activity.** After 48 h of adaptation, mice were individually monitored for another 48 h in LabMaster chambers (TSE Systems, Bad Homburg, Germany) that allow simultaneous measurement of metabolic performance, home cage activity (light beam breaks), and drinking and feeding behavior, calorimetric recording of O2 and CO2 concentration for quantification of O2 consumption (V̇O2) and CO2 production (V̇CO2), air flow, and temperature. Locomotor activity and respiratory exchange ratio were calculated for the light and dark phases.

**Glucose tolerance tests.** Mice were placed in clean cages to be fasted for ~20 h prior to testing. The tip of the tail was nicked to obtain blood samples for glucose and insulin measurements. Glucose was measured immediately, using Freestyle glucometers and test strips. For insulin measurement, ~40–50 μl of whole blood was collected in heparin-coated microhematocrit tubes and sealed at one end with clay. After collection of baseline samples was complete, mice received an intraperitoneal injection of 1.5 mg glucose/g body wt in a volume that was 1% of body weight. Blood samples for insulin assay were kept on ice until plasma, and cells were separated by spinning the capillaries in a microhematocrit centrifuge. Capillaries were scored and broken at the plasma-cell interface, and plasma was placed in microtubes for storage at −20°C. Insulin was measured in 5- or 10-μl triplicates of plasma using an Ultra-Sensitive Rat Insulin ELISA kit (Crystal Chem, Downer’s Grove, IL), which also detects mouse insulin.

**RESULTS**

**NSE-Ghr-positive mice.** The NSE promoter and mouse ghrelin cDNA sequences (NSE-Ghr) were incorporated into a transgene designed to direct expression of ghrelin in neuronal cell types (Fig. 1A). This construct was developed to generate mice with high levels of central ghrelin expression. NSE-Ghr-positive mice were screened by PCR amplification of the 3’ end of the promoter sequences and 5′ end of the ghrelin cDNA within the transgene construct (Fig. 1A, dark gray arrows). Two lines of NSE-Ghr transgene-positive mice, identified as L43 and L73, expressed NSE-Ghr transcripts in whole brain that were detected by RT-PCR (Fig. 1A, black arrows). A third line, L54, did not appear to express the transgene. Transgenic pups were indistinguishable from nontransgenic littermates and appeared to grow at a normal rate. All mice used in this study were males and heterozygous for the NSE-Ghr allele.

**NSE-Ghr expression in brain tissues.** To determine whether the NSE-Ghr transgene was expressed in neuronal tissues, we measured ghrelin mRNA in total RNA extracts from cortex and hypothalamus as representative of brain tissue. Q-PCR primers were designed to detect both transgenic and endogenous ghrelin (Fig. 1A, light gray arrows). Ghrelin mRNA levels in NSE-Ghr L43 and L73 brain tissues were 80- to 150-fold higher than those in wild-type controls (Fig. 1B). Since endogenous ghrelin expression is not detectable above background levels in cortex and is found only at very low levels in hypothalamus (16, 17), it seems reasonable to conclude that the high levels of ghrelin mRNA in the L43 and L73 brains are overwhelmingly due to expression of the NSE-Ghr transgene. One key issue that is not addressed by these measurements is the extent to which the increased transgene results in increased levels of octanoylated ghrelin. Unfortunately, this issue is difficult to address since the available assays for octanoylated ghrelin require large amounts of fluid, making cerebrospinal fluid sampling problematic. Furthermore, tissue homogenates are also not easily applied to these assay systems.

**Ghrelin mRNA expression in wild-type FVB/N tissues.** Because the NSE-Ghr mice have increased levels of central ghrelin expression, it was important to determine whether ghrelin expression in the stomach or duodenum was affected. To answer this question, we first determined relative amounts of ghrelin expression along the axis of the stomach, as well as duodenum and liver, in the wild-type background strain (FVB/N) for the NSE-Ghr mice. We dissected wild-type mouse stomachs and isolated total RNA from the fundus, body, and antrum regions (Fig. 2A). Endogenous ghrelin mRNA was measured by Q-PCR, with expression in the stomach body set at 1 (Fig. 2B). Expression was highest in the body of the stomach, accounting for ~70% of ghrelin synthesis within the stomach of wild-type mice. Expression of ghrelin in duodenum was ~9–10% of that in the gastric body. Endogenous transcripts were also detected in wild-type hypothalamus, cortex, and liver, but at a concentration of 0.01% or less than those found in the gastric body.

**Ghrelin mRNA expression in NSE-Ghr tissues.** We compared ghrelin expression in stomachs of wild-type and NSE-Ghr L43 mice (Fig. 2C). Levels in the stomach fundus and duodenum did not differ from those of wild-type mice. Ghrelin mRNA expression was only 2–3% in L43 NSE-Ghr brain and liver and 0.25% that of stomach, but these levels were signif-
significantly increased compared with those of wild-type mice (Fig. 2C, inset). Levels measured in L73 tissues were similar to all of those found in L43 (not shown). Thus, NSE-Ghr transgenic mice had increased hepatic as well as central ghrelin expression.

Serum ghrelin. Because ghrelin acts as a hormone, it was critical to determine whether expression of the transgene resulted in increased circulating levels of total or octanoylated ghrelin. Fasted or fed FVB/N mice were killed 1 h after the onset of darkness, and blood was collected to determine whether this was an appropriate time point for measuring serum ghrelin levels and the range of ghrelin levels under different circumstances. Blood was also collected from wild-type C57Bl/6 mice so that we could compare our results with those previously reported in the literature (12, 24). Serum ghrelin levels in fed FVB/N mice were significantly lower than those of wild-type control mice (Fig. 3A). Results were similar in C57Bl/6 mice from 16 to 32 wk of age (Fig. 4, C and D). By 20 wk, body weights of L43 mice were significantly lower than those of wild-type controls, whereas those of L73 mice were comparably lower than in the L43 mice but did not reach statistical significance. NMR analysis was performed at 16, 20, and 32 wk to determine relative amounts of fat and lean tissue. There were no significant differences in body composition between transgenic and wild-type groups at 16 and 20 wk (data not shown).

Energy expenditure and activity. Since the changes in food intake were relatively modest, we also measured energy expenditure and activity in L43 NSE-Ghr mice. The L43 mice had a small but statistically significant increase in overall...
activity measured via running wheels and tended to have an increased total distance traveled in the running wheels (Fig. 5A). However, total energy expenditure as measured by calorimetry did not differ between L43 mice and controls (Fig. 5B).

Glucose tolerance. Glucose was administered via intraperitoneal injection to fasted mice to assess glucose tolerance at 12 wk of age. L43 and L73 mice did not differ from wild-type controls during the 2-h glucose excursion (data not shown). When the test was repeated at 32 wk of age, glucose tolerance was impaired in L43 mice compared with wild-type control mice (Fig. 6A), whereas blood glucose levels in NSE-Ghr L73 mice did not differ from wild-type control mice (Fig. 6B). Fasting glucose levels did not differ between the groups, but the area under the curve (AUC) was increased in L43 mice (Fig. 6A, inset) compared with L73 and wild-type mice (Fig. 6B, inset). Insulin levels in L43 NSE-Ghr mice did not differ from wild-type controls at any time points measured during the intraperitoneal glucose tolerance test (Fig. 6C). Plasma insulin in L73 and control mice was measured at 0 and 15 min but did not differ (data not shown).

**DISCUSSION**

The goal of this study was to determine the consequences of chronically increased ghrelin action via transgenic overexpression in neurons. The use of the NSE promoter to target neurons increased ghrelin mRNA levels in extracts of brain and also liver. This was associated with substantially elevated blood levels of octanoylated ghrelin in one line of NSE-Ghr mice. This represents the first successful genetic manipulation that increases circulating levels of the key form of ghrelin that is believed to act on the receptor to increase ghrelin signaling (13). Despite numerous previous studies in humans and mice (20, 26) demonstrating that raising circulating ghrelin concentrations with exogenous administration of the peptide stimulated food intake, the NSE-Ghr mice actually consumed slightly less food and were slightly more active than sex-matched wild-type controls. Body and adipose mass of NSE-Ghr mice were also slightly decreased compared with controls, possibly as a result of increased physical activity.

Ghrelin has received considerable attention as a circulating orexigenic factor, and the potency of this action has made the ghrelin-signaling system a plausible pharmacological target. Indeed, repeated administration of exogenous ghrelin to rodents causes hyperphagia and weight gain (12, 20, 26), suggesting that factors that increase endogenous secretion of the peptide could contribute to obesity. The results of the current study present a challenge to this model. Despite chronically increased circulating ghrelin, these mice did not eat more than controls and in fact appeared to be in slightly negative energy balance compared with wild-type mice. We cannot rule out developmental compensation for the transgene effects or disruption of qualitative aspects of ghrelin physiology, such as central to peripheral hormone ratios or diurnal patterns of secretion, to contribute to the absence of a body weight phenotype in the L43 line. However, it seems clear from these data that simply raising plasma ghrelin over the lifespan of a mouse is not sufficient to cause obesity.

Despite the lack of the predicted obesity phenotype, NSE-Ghr mice with high circulating levels of bioactive ghrelin developed age-related glucose intolerance, suggesting a novel and potentially pathophysiologically relevant consequence of elevated ghrelin. Disruption of glucose homeostasis was an unexpected finding in the mice overexpressing bioactive ghrelin. These animals had decreased fat mass and normal fasting insulin and glucose concentrations, making it unlikely that they had significant insulin resistance based on adiposity. However, in response to the administration of glucose, mice with high levels of circulating ghrelin had peak glucose levels ~100 mg/dl higher than the controls and an overall 25% increase in glycemia, based on the AUC. In light of the substantial difference in glycemia persisting across the 2-h glucose tolerance
test, the equivalent insulin levels in the transgenic mice likely represent impaired insulin secretion in the L43 mice.

The transgenic technology used here does have a significant drawback. Making a conclusion about the gene that is overexpressed can be confounded by the potential for the transgene to disrupt other genes, because it is randomly incorporated into the DNA. Typically this issue is addressed by comparing multiple lines of transgenic animals, where there is a very low probability that the same genes are disrupted in separately injected embryonic stem cells. In this case, however, only a single line showed two important phenotypes: elevated bioactive ghrelin in the plasma and impaired glucose tolerance. We cannot rule the possibility that both of these effects are not the result of ghrelin overexpression but rather two independent effects of the transgene. However, it would appear to be more parsimonious to conclude that the effect on glucose tolerance is a result of increased circulating bioactive ghrelin levels since these findings are consistent with recent observations in ob/ob mice that were crossed with Ghr−/− mice. In the Ghr−/− ob/ob mice there were no significant changes in adiposity compared with ob/ob mice, but the diabetic phenotype normally observed in ob/ob mice was markedly improved with the elimination of ghrelin action. In this case, the rescue of the diabetic consequence of leptin deficiency was due to enhanced ghrelin-stimulated insulin secretion in Ghr−/− ob/ob mice, particularly when measured 15 min after glucose injection. Taken together with the observations in Ghr−/− ob/ob mice, our data support a modulatory role for neural and/or circulating ghrelin in β-cell function that contributes to altered glucose homeostasis.

Increased neural and plasma ghrelin did not appear to affect endogenous expression of ghrelin in the gut. We found similar levels of ghrelin mRNA accumulation in stomach and duodenum from NSE-Ghr and wild-type control mice. Our quantitative assays did not differentiate between endogenous and transgenic transcripts, so it remains possible that endogenous expression was decreased to compensate for ectopic expression of the NSE-Ghr transgene in stomach tissues. However, if this was the case, it seems more likely that expression would have been uniform throughout the stomach rather than mirroring the endogenous distribution with varying levels between antrum vs. body or fundus.

Whether endogenous ghrelin is normally made in the brain has been controversial (7, 16, 29). We detected similar levels of ghrelin mRNA in hypothalamus and liver from wild-type mice. However, these levels were very low compared with those achieved in brains of NSE-Ghr mice or the levels normally expressed in stomach and intestine. It is possible that the increased hepatic expression of ghrelin mRNA in transgenic mice is due to neural expression in the liver, although neural ganglia are scarce in the liver parenchyma. Alternatively, there may have been some ectopic expression of the transgene in liver cells.

Serum ghrelin was measured in ad libitum-fed mice 1 h after dark, when endogenous ghrelin in wild-type mice was at its lowest levels. Fig. 4. Feeding behavior, body weight (BW), and adiposity in WT and NSE-Ghr mice. Food intake was measured in age-matched WT and L43 mice. A: average weekly intake did not differ significantly, but cumulative intake was significantly decreased in L43 mice compared with control mice. B: when cumulative food intake for the 4-wk period is expressed as g food/g BW, WT and L43 mice did not differ (data are means ± SE; n = 8–10, *P < 0.05). C and D: BW was monitored in cohorts of mice from 8 until 31 wk of age. L43 NSE-Ghr mice weighed significantly less than WT mice at 20 wk, and the difference in BW persisted to termination of the experiment at 31 wk (data are means ± SE; n = 8–10, *P < 0.05). WT and L73 NSE-Ghr mice differed only at 8 wk (data are means ± SE; n = 5–10, *P < 0.05). E and F: body composition of mice at 31 wk of age was analyzed by whole body MRI. L43 and L73 mice tended to have slightly less fat as a percentage of total BW, but the difference did not reach statistical significance for either line.
lowest, so that we could more sensitively detect the contribution due to expression of the NSE-Ghr transgene. The level of total ghrelin expression in the L43 and L73 lines 1 h into the dark phase was similar to that of fasted wild-type mice. It is not clear from these studies whether the transgenic animals retain the diurnal pattern of ghrelin secretion found in wild-type mice. An alternative would be continuous release with relatively unchanging concentrations. In either case, it appears that the L43 animals have excessive levels of octanoylated ghrelin for at least one-half of the day. The source of the increased bioactive ghrelin in the L43 NSE-Ghr mice is unclear. It is possible that the increased plasma levels represent spillover from neural release in the brain; however, this does not explain why L43 had an increased level of the octanoylated form. Alternatively, the transgene may be expressed in peripheral neurons and secreted into the peripheral circulation in organs such as the liver, where we also detected an increase in ghrelin transcripts. It is also possible that undetected expression of the transgene in some other tissue contributes to the increase in plasma ghrelin concentration. An alternative hypothesis, that increased central ghrelin signaling upregulates endogenous ghrelin expression in the gut, is unlikely since gastric expression levels and patterns did not differ between NSE-Ghr and wild-type mice.

Since little is known about the enzymes that process the ghrelin precursor protein, it remains unclear whether there are changes in the processing of NSE-Ghr transcripts. Although transcript levels were similar in the stomach and duodenum of NSE-Ghr and wild-type mice, it is possible that there are differential amounts of processing. Nevertheless, it is interesting to note that, although several transgenic mice in which ghrelin is driven by other promoters fail to increase circulating octanoylated ghrelin, NSE-Ghr mice do have an approximately fivefold increase in octanoylated ghrelin levels. Zhang et al. (30) recently reported an alternate product of the ghrelin

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Fig. 6. Glucose tolerance test following intraperitoneal (ip) injection of glucose (ipGTT) was performed on WT and NSE-Ghr mice at 32 wk of age. A: although fasting glucose levels did not differ, blood glucose measurements show impaired glucose tolerance in L43 mice compared with controls at 15–60 min after ip administration of 1.5 mg dextrose/g BW. Area under the curve (AUC) was significantly increased in L43 mice compared with WT mice (inset) (data are means ± SE; n = 8, *P < 0.05). B: blood glucose measurements in L73 mice show that glucose tolerance did not differ from that in controls at 15–120 min after ip administration of 1.5 mg dextrose/g BW. AUC did not differ between L73 and WT mice (inset) (data are means ± SE; n = 5–10). C: insulin was measured in serum collected from WT and L43 mice during the ipGTT. Results at all time points were similar in all mice (data are means ± SE; n = 6–8).
transcript, which they called obestatin. Obestatin was reported to have an anorexigenic effect, although this remains controversial (11, 21). Obestatin is cleaved from a portion of the prepro-protein closer to the carboxy-terminal region. This region is contained within the NSE-Ghr transgene and may be available for processing into obestatin. Consequently, we cannot rule out the possibility that at least some aspects of the resulting phenotypes in these mice are the product of simultaneous increases in both ghrelin and obestatin.

In summary, we have produced the first successful gain-of-function genetic model of increased circulating bioactive ghrelin in transgenic mice by overexpressing murine ghrelin with the NSE promoter. Despite the fivefold increase in circulating levels of bioactive ghrelin, such mice show no increases in food intake, body mass, or fat mass and no decreases in energy expenditure. Although body weight is normal, mice with increased circulating ghrelin have impaired glucose tolerance at 32 wk of age. These data support a role for circulating ghrelin levels in age-associated impairment in glucose tolerance and open up the possibility that ghrelin receptor antagonists might be useful in preventing such impairments.

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