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Targeted Deletion of the *Vgf* Gene Indicates that the Encoded Secretory Peptide Precursor Plays a Novel Role in the Regulation of Energy Balance

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Summary

To determine the function of VGF, a secreted polypeptide that is synthesized by neurons, is abundant in the hypothalamus, and is regulated in the brain by electrical activity, injury, and the circadian clock, we generated knockout mice lacking *Vgf*. Homozygous mutants are small, hypermetabolic, hyperactive, and infertile, with markedly reduced leptin levels and fat stores and altered hypothalamic proopiomelanocortin (POMC), neuropeptide Y (NPY), and agouti-related peptide (AGRP) expression. Furthermore, VGF mRNA synthesis is induced in the hypothalamic arcuate nuclei of fasted normal mice. VGF therefore plays a critical role in the regulation of energy homeostasis, suggesting that the study of lean VGF mutant mice may provide insight into wasting disorders and, moreover, that pharmacological antagonism of VGF action(s) might constitute the basis for treatment of obesity.

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Introduction

Obesity and anorexia are common disturbances of energy homeostasis that result from imbalances between energy intake and expenditure. Within the brain, the hypothalamus plays a pivotal role in the regulation of food intake and energy output, perhaps most dramatically demonstrated through the effects that particular hypothalamic lesions have on appetite and body weight (Schwartz et al., 1995; Levin and Routh, 1996). A number of hypothalamic neurotransmitters and neuropeptides have been implicated in the central control of energy homeostasis, including catecholamines, serotonin, neuropeptide Y (NPY), melanocyte-stimulating hormone (α-MSH), corticotropin-releasing hormone (CRH), agouti-related peptide (AGRP or ART), melanin-concentrating hormone (MCH), and hypocretin/orexin, which illustrates the complexity of this system (Flier and Maratos-Flier, 1998; Woods et al., 1998). However, the study of mutant mice with distinct metabolic abnormalities has contributed significantly to our understanding of the control of body weight. A number of genetically obese mouse strains have been analyzed, including those that fail to synthesize leptin (*Lep*^{ob}/*Lep*^{ob}) and those that express abnormal hypothalamic–leptin receptors (*Lepr*^{db}/*Lepr*^{db}). These studies indicate that the binding of leptin to its hypothalamic receptor is an important metabolic control point (Elmquist et al., 1998; Friedman and Halaas, 1998). In the fasted state, falling leptin levels signal the hypothalamic pituitary–gonadal (HPG) axis to suppress reproduction, and energy is conserved through a decrease in basal metabolism, while in the fed state, elevated leptin levels result in decreased food intake and thermogenesis. The contribution of other hypothalamic neuropeptide circuits to the regulation of feeding and metabolism has been demonstrated in part through the analysis of additional obese strains of mice such as agouti and mice that overexpress the agouti-related protein (AGRP or ART) or fail to express the melanocortin 4 (MC4) receptor (Huszar et al., 1997; Ollmann et al., 1997; Shutter et al., 1997).

Genetically lean mice, however, are much less common, but their study should offer mechanistic insight into obesity, wasting disorders, and the hypothalamic response to starvation. Here, we describe the characterization of lean mice that result from targeted disruption of the *Vgf* gene. VGF was originally identified on the basis of its rapid, robust, and relatively selective regulation by nerve growth factor in PC12 cells (Levi et al., 1985; Salton et al., 1991; Hawley et al., 1992). Anatomic studies have revealed that VGF is widely expressed by neurons in the developing and adult central and peripheral nervous systems and is particularly abundant in the adult hypothalamus (van den Pol et al., 1989, 1994; Snyder and Salton, 1998; Snyder et al., 1998b). VGF expression in the brain is induced rapidly by neuronal activity (Lombardo et al., 1995; Snyder et al., 1998a), injury (Mahata et al., 1993; Snyder et al., 1998a), and by light and the circadian clock (Wisor and Takahashi, 1997). The *vgf* gene encodes a dibasic amino acid–rich

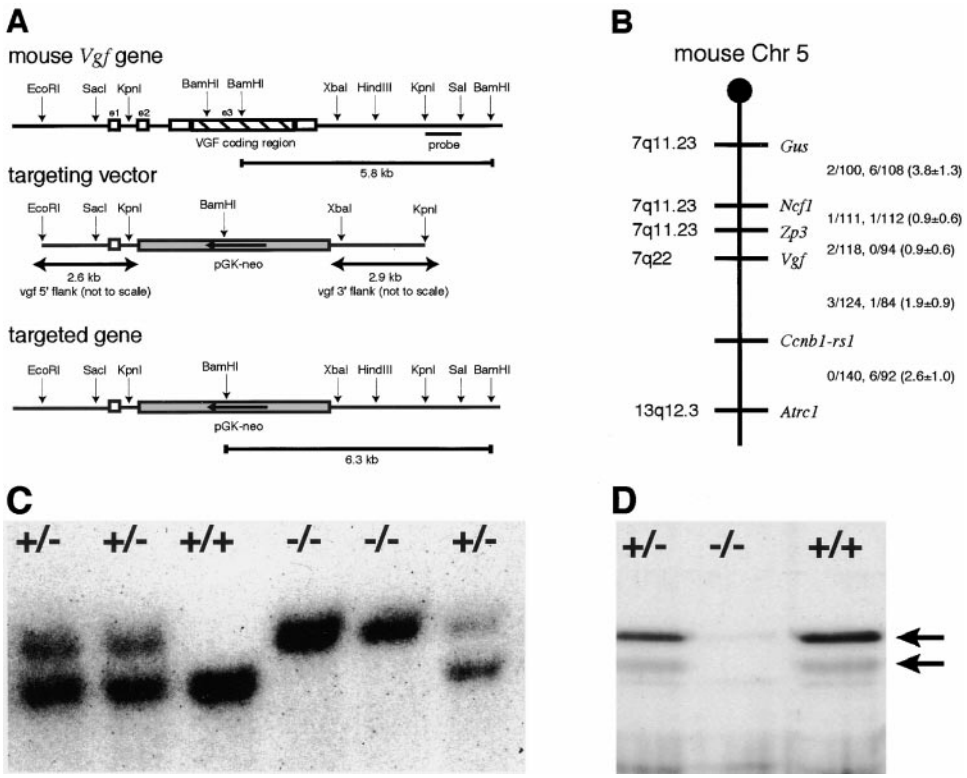

Figure 1. Targeting and Chromosomal Localization of the *Vgf* Locus

(A) Targeting vector for positive selection of homologous recombinants at the *Vgf* locus. The three exons (e1, e2, and e3) of *Vgf* are represented by the open rectangles with the VGF coding sequence hatched, and the PGK-*neo* cassette is represented by the shaded rectangle. BamHI digests will liberate 6.3 kb and 5.8 kb fragments from the targeted and untargeted alleles, respectively, which can be visualized by using the indicated 1 kb KpnI–SalI fragment immediately 3′ to the sequence contained within the targeting vector.

(B) Chromosomal location of the murine *Vgf* gene on chromosome 5. To the right of the map are given recombination fractions for adjacent loci. The first fraction represents data from Cross 1 ([NFS/N or C58/J × *Mus musculus musculus*]F1 × *Mus musculus musculus*), and the second fraction from Cross 2 ([NFS/N × *Mus spretus*]F1 × *Mus spretus* or C58/J). In the first cross, the NFS/N and C58/N allele of *Vgf* was scored as an EcoRI fragment of >28 kb, and in the second cross, *Vgf* was identified as PvuII fragments of 1.0 kb in *Mus spretus* and 1.3 kb in NFS/N. The numbers in the parentheses represent recombinational distances ± standard errors. To the left are given the map locations of the corresponding human homologs.

(C) Southern blot of genomic DNA isolated from pups in a litter born to heterozygous (+/−) VGF mutant parents. Tail DNAs from wild type (+/+), heterozygous mutants (+/−), and homozygous mutants (−/−) were digested with BamHI and probed with the 3′ KpnI–SalI *Vgf* genomic fragment.

(D) Western blot using rabbit anti-rat VGF antisera (Salton et al., 1995); protein extracts of forebrain dissected from heterozygous mutant (+/−), homozygous VGF mutant (−/−), and wild-type (+/+) mice; and enhanced chemiluminescence detection. VGF polypeptide, a doublet migrating with an apparent size of ~85–90 kDa, is indicated by the arrows.

polypeptide that is cleaved in a cell-type-specific manner (Possenti et al., 1989; Salton, 1991; Liu et al., 1994; Trani et al., 1995) and undergoes regulated release from dense core secretory vesicles (Possenti et al., 1989; Benson and Salton, 1996). These features suggest that VGF or VGF-derived peptides may be biologically active. The studies of homozygous VGF-deficient mice that follow indicate that these mice are remarkably hypermetabolic and hyperactive, have reduced body mass and peripheral fat stores, and display a profile of hypothalamic neuropeptide gene expression that would be expected in a fasted animal. Consistent with a critical regulatory role for VGF in energy homeostasis, in situ hybridization studies demonstrate that VGF mRNA levels are induced in the hypothalamic arcuate nuclei of normal mice in response to fasting. VGF therefore plays a significant and nonredundant role in the control of body weight and basal metabolism, and as such, could represent a novel target for pharmacologic intervention to prevent human obesity.

Results

Generation of VGF-Deficient Mice

To construct the targeting vector, the mouse *Vgf* gene was cloned from an 129/SvJ genomic library. The intron/exon organization and coding and regulatory sequences of mouse (Figure 1A), rat (Salton et al., 1991), and human (Canu et al., 1997a, 1997b) *vgf* genes are highly conserved. The *Vgf* locus was mapped to mouse chromosome 5 in a region of homology to human chromosome 7 (Figure 1B), in agreement with recent localization of the human *VGF* gene to chromosome 7 (Canu et al., 1997a). To generate VGF-deficient mice, a targeting vector was constructed in which the entire *Vgf* coding sequence

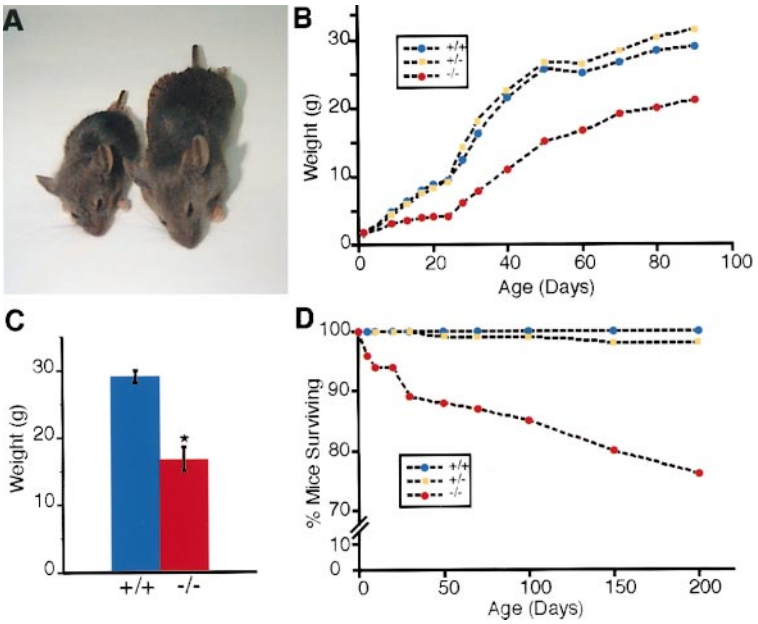

Figure 2. Phenotype of VGF Mutant Mice

(A) Size comparison of a typical 4-week-old homozygous VGF mutant male (left) with a wild-type littermate (right).

(B) Comparison of the weights (g) of representative homozygous and heterozygous VGF mutant and wild-type littermates during the first 90 days of life.

(C) Mean weights (g) of 12- to 14-week-old wild-type (+/+) and homozygous VGF mutant (−/−) mice (mean ± SE; asterisk, $p < 0.05$; $n = 15$).

(D) Survival of homozygous and heterozygous VGF mutant and wild-type mice ($n = 30$).

was replaced with the phosphoglycerate kinase– (PGK-) driven neomycin resistance cassette (Figure 1A). Following transfection and selection of R1 embryonic stem (ES) cells, Southern analysis indicated that 2 of the 750 selected G418-resistant clones had undergone targeted deletion of *Vgf*. Cells from the two targeted ES cell lines were injected into C57BL/6 blastocysts to generate chimeric founder mice, and germline transmission of the targeted allele was obtained (Figure 1C). Null mutation of the targeted allele was confirmed by Northern (data not shown) and Western (Figure 1D) analyses of total RNA and protein, respectively, isolated from the brains of 4-week-old control, heterozygous, and homozygous mice.

Phenotypic Characterization of Mice Lacking VGF

Homozygous VGF mutant mice were obtained from heterozygote crosses, in either 129/SvJ or C57BL/6 backgrounds, at the normal Mendelian frequency. At birth, VGF-deficient pups were indistinguishable from heterozygous or wild-type littermates. However, by postnatal day 3 (P3), VGF mutant pups weighed 10%–20% less, and by the third week of postnatal life, 40%–60% less than normal or heterozygous littermates (Figures 2A and 2B), irrespective of litter size. After weaning, VGF mutant mice maintained body weights 50%–70% those of wild-type (Figures 2B and 2C). Wild-type littermates that were nursed for restricted periods of time to decrease their weights into the range of age-matched, VGF-deficient pups throughout weaning were indistinguishable at 4 months of age in weight and fertility from normally reared wild-type mice (data not shown).

Histologic examination of the upper and lower gastrointestinal tracts and fluoroscopic analysis of upper gastrointestinal motility were similar for mutant and normal 2-week-old and adult mice. Survival of VGF mutant mice was reduced in comparison with heterozygote or wild-type mice (Figure 2D), particularly during the postnatal period prior to weaning. Sensory, proprioceptive, and olfactory function of VGF-deficient mice appeared normal.

VGF Mutant Mice Are Lean and Have Reduced Leptin Levels and the Profile of Neuropeptide Gene Expression that Is Commonly Seen in Fasted Mice

In addition to being smaller than normal mice, VGF mutant mice also had a significantly elevated basal metabolic rate, as measured by O_2 consumption (Figure 3A) and CO_2 output. Although hypermetabolic, VGF-deficient mice had reduced serum T4 thyroid hormone levels (wild-type: 5.04 ± 0.2 μg/dl, $n = 8$; −/−: 3.65 ± 0.3 μg/dl, $n = 8$), slightly decreased pituitary thyroid–stimulating hormone (TSH) content (see Figure 9), and normal core body temperatures (data not shown). Sympathetic activity, as gauged by measurement of heart rate and adrenal norepinephrine and epinephrine levels, also appeared normal (data not shown). Peripheral fat stores, however, were dramatically reduced in VGF mutant mice (Figure 3B). Total body fat in the VGF-deficient mice was reduced and represented a smaller fraction of total body composition than did fat in normal mice (Figure 3C), while the contribution of total protein to body mass was similar in VGF mutant and control mice. Fecal fat levels were similar in normal and VGF-deficient mice (LabCorp, Burlington, North Carolina), suggesting comparable gastrointestinal tract fat absorption. Expression of leptin mRNA in the epididymal fat pad was significantly reduced in VGF mutant mice (Figure 3D), as were circulating leptin levels (wild-type: 2.70 ± 0.63 ng/ml, $n = 15$; −/−: 0.23 ± 0.06 ng/ml, $n = 18$; $p < 0.001$).

Under conditions in which fat stores and leptin levels are reduced in a normal animal to such a degree, a compensatory increase in food intake and/or decrease in energy output would be expected. Daily food intake in ad lib fed VGF mutant mice was similar to that of normal littermates (Figure 3E), representing a significant

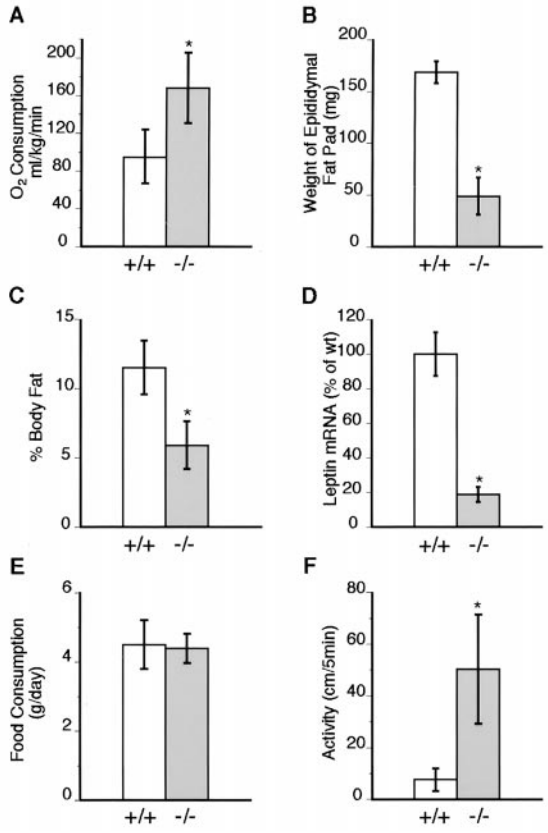

Figure 3. Examination of Metabolic Rate, Peripheral White Adipose Stores, Leptin Expression, Food Intake, and Activity in VGF Mutant Mice

(A) As an indicator of metabolic rate, oxygen consumption was quantified and averaged over a 24 hr period with Accuscan metabolic cages and is expressed as mean ± SE (asterisk, $p < 0.05$; $n = 6$).

(B) Weights of epididymal fat pad, dissected from VGF mutant and wild-type mice, are expressed in mg (mean ± SE; asterisk, $p < 0.05$; $n = 12$).

(C) Body composition analysis. The relative contribution of fat to total body composition, expressed as a percentage of body mass, for wild-type and VGF mutant mice is shown (mean ± SE; asterisk, $p < 0.05$; $n = 12$).

(D) Leptin mRNA levels in white adipose tissue. Samples (7 μg) of RNA isolated from wild-type or VGF mutant epididymal fat pad were subjected to Northern blot analysis. Leptin mRNA levels were quantified by densitometric scanning and are expressed as a percentage of wild-type control (mean ± SE; asterisk, $p < 0.05$; $n = 12$).

(E) Daily food intake in ad lib fed wild-type and VGF mutant mice in grams.

(F) Activity of wild-type and VGF mutant mice was quantified in Accuscan metabolic cages and is expressed as mean distance traveled per 5 min ± SE (asterisk, $p < 0.05$; $n = 6$).

elevation in food intake per gram of body weight in VGF-deficient (0.24 g of food per gram of body weight) compared with normal (0.16 g of food per gram of body weight) mice. Intake of ad lib fed VGF mutant mice was clearly more similar to that of ad lib fed wild-type mice than to that of refed, 24 hr fasted wild-type mice (wild-type, refed, fasted: 3.1 ± 1.0 g liquid diet/1 hr, 27.6 ± 3.9 g liquid diet/24 hr; −/−, ad lib fed: 0.6 ± 0.2 g liquid diet/1 hr, 20.4 ± 2.1 g liquid diet/24 hr). Comparable hyperphagic responses were noted in refed control and VGF mutant mice following a 24 hr fast. In addition, mutant mice were found to be hyperactive rather than hypoactive (Figure 3F), and the hyperactivity was not associated with elevations in striatal dopamine or 3,4-dihydroxyphenylacetic (DOPAC) levels (data not shown).

Normal mice respond to decreased food intake (i.e., fasting) by lowering serum glucose and leptin levels; the hypothalamus senses these changes, responding with characteristic alterations in the levels of expression of several neuropeptide-encoding genes, including *pro-opiomelanocortin* (*Pomc*), *Npy*, and *Agrp* (*Art*) (Flier and Maratos-Flier, 1998; Woods et al., 1998). In ad lib fed VGF mutant mice, mean serum insulin and glucose levels were 80% and 60%, respectively, of the levels found in normal mice, while the mean serum corticosterone level of VGF mutant mice was 140% normal, all consistent with a fasted state. Hypothalamic levels of NPY and AGRP mRNAs were elevated by 600% and 800%, respectively, while hypothalamic POMC mRNA was reduced by 75% in comparison with hypothalamic mRNA levels in similarly fed littermate controls (Figure 4). Alterations in neuropeptide gene expression comparable to those observed in ad lib fed VGF mutant mice have been noted in normal mice fasted for 48 hr (Mizuno et al., 1998; Mizuno and Mobbs, 1999).

Hypothalamic Structure and Circadian Behavior Are Intact in Mice Lacking VGF

VGF mRNA and polypeptide are expressed at relatively high levels in the rat hypothalamus (van den Pol et al., 1989, 1994; Snyder and Salton, 1998; Snyder et al., 1998b), and previous studies have shown that VGF mRNA levels are regulated in the hamster suprachiasmatic nucleus (SCN) in response to light and the circadian clock (Wisor and Takahashi, 1997). To better evaluate hypothalamic structure and function in VGF mutant mice, we first investigated whether VGF mRNA was similarly distributed in the mouse hypothalamus and comparably regulated in the mouse SCN. By using in situ hybridization, VGF mRNA distribution in the mouse hypothalamus (Figures 5A and 5B) was found to be similar to that reported in the rat (Snyder and Salton, 1998; Snyder et al., 1998b). In addition, VGF mRNA levels were regulated in the mouse SCN by light (Figures 5C–5F) and by the circadian clock in the rostral SCN but not in the caudal SCN (data not shown), as was previously observed in the golden hamster (Wisor and Takahashi, 1997).

We then examined whether targeted deletion of VGF affected specific aspects of hypothalamic anatomy and function. Quantitative neuron counts (see Experimental Procedures) in the hypothalamic paraventricular nuclei of normal and VGF mutant mice were similar (47,700 ± 5800 and 43,560 ± 4600, respectively). The overall distribution of neurons containing gonadotropin-releasing hormone (GnRH), oxytocin, vasopressin, and POMC in specfic hypothalamic regions was also found to be similar in mutant and normal mice (Figure 6A). To assess gross hypothalamic function, circadian behavior of VGF mutant and control mice was compared. Heterozygous and homozygous VGF mutant mice could be entrained to a light:dark cycle, and both maintained a circadian rhythm of wheel-running activity in constant darkness (DD; Figure 6B). The circadian period length of the VGF

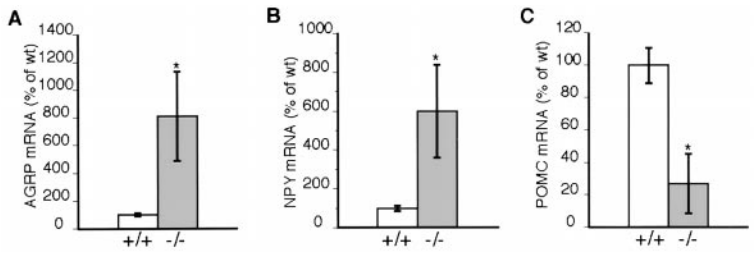

Figure 4. Hypothalamic Neuropeptide Gene Expression in VGF Mutant Mice

Samples of RNA (3.5 μg) isolated from the hypothalami of ad lib fed wild-type or VGF mutant mice were subjected to Northern blot analysis and quantified by densitometric scanning. Amounts are expressed as a percentage of wild-type control (mean ± SE; asterisk, $p < 0.05$; $n = 12$).

(A) AGRP mRNA levels in hypothalamus.

(B) NPY mRNA levels in hypothalamus.

(C) POMC mRNA levels in hypothalamus.

mutant (23.2 ± 0.1 hr) was found to be slightly but significantly shorter than that of normal littermates (23.7 ± 0.1 hr) (Student's t test, $df = 12$, $p < 0.025$).

Expression of VGF mRNA Is Induced in the Hypothalamic Arcuate Nuclei of Normal Mice in Response to Fasting

Since the phenotype of VGF mutant mice indicated that VGF plays a role in the control of metabolism, and in situ hybridization studies demonstrated synthesis of VGF mRNA in the hypothalamus, we examined whether *Vgf* gene expression was regulated in the normal mouse brain by fasting. VGF mRNA levels and distribution were examined by in situ hybridization in the hypothalami of control ad lib fed and 48 hr fasted mice. Robust induction of VGF mRNA in the arcuate nuclei of fasted mice, in comparison to identically processed specimens from ad lib fed mice, was observed (Figure 7).

VGF Mutant Mice Have Reduced Fertility

In the fasted state, falling leptin levels signal the HPG axis to suppress reproduction in order to conserve energy. Since VGF-deficient mice were lean, had a fasting profile of hypothalamic neuropeptide gene expression, and had extremely low levels of circulating leptin, reproductive function was examined in mutant mice. Eighty-five percent of VGF mutant males ($n = 40$) and 90% of mutant females ($n = 40$) aged 8–12 weeks were found to be infertile after repetitive mating for 4 months compared with <10% of heterozygous ($n = 30$) and wild-type ($n = 20$) littermates. Onset of puberty and sexual maturation of VGF-deficient mice were delayed. The VGF mutant females ($n = 30$) were found to undergo vaginal opening between 28 and 35 days of age compared with 22–26 days for wild-type females ($n = 20$). Microscopic examination of vaginal smears revealed a prolonged estrous cycling pattern of up to 12 days in 50% of VGF mutant females, while the remaining 50% failed to cycle. Mating with normal males, on the evening of proestrous, of the fifteen VGF mutant females that had prolonged estrous cycles resulted in copulatory plugs but no pregnancies. Histological examination of ovaries from VGF-deficient females revealed many primary and atretic follicles but no mature follicles or corpus lutea (Figure 8B); ovaries, oviducts, and uteri in −/− females were found to be 18%–30% the weight of those

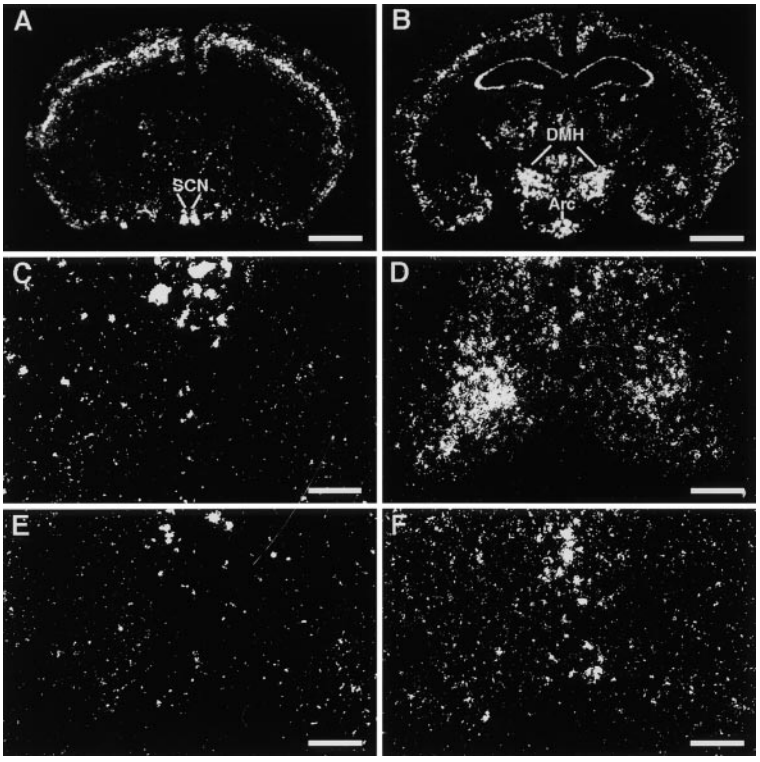

Figure 5. Expression of VGF mRNA in the Normal Mouse Hypothalamus and Its Regulation in the Suprachiasmatic Nucleus by Light

(A and B) In situ hybridization of VGF mRNA in sections through the adult mouse hypothalamus. Abbreviations: SCN, suprachiasmatic nuclei; DMH, dorsomedial hypothalamus; and Arc, arcuate nuclei. Scale bar, 1.5 mm.

(C–F) Photic induction of VGF mRNA in the mouse suprachiasmatic nucleus. Sections were taken from mice exposed to a 30 min light pulse at circadian time (CT) 15 (3 hr after onset of wheel-running activity) that were killed 1 (C) or 6 (D) hr later or from dark control mice killed at the same times (E and F). Scale bar, 100 μm.

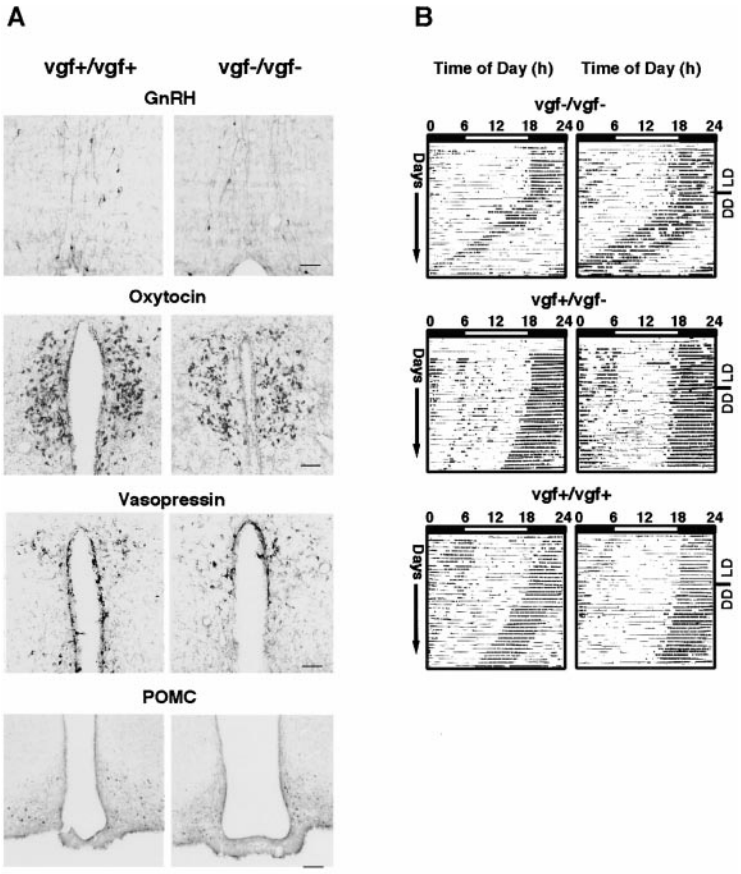

Figure 6. Immunocytochemical Analysis of Hypothalamic Neuropeptides and Examination of Circadian Behavior in VGF Mutant Mice

(A) Representative photomicrographs showing GnRH, oxytocin, vasopressin, and POMC immunocytochemical labeling in wild-type ("vgf+/vgf+") and VGF mutant ("vgf−/vgf−") mice. Immunolabeling of GnRH at the level of the diagonal band/organum vasculosum of the lamina terminalis, oxytocin at the level of the rostral paraventricular nucleus, vasopressin at the level of the paraventricular nucleus, and POMC at the level of the rostral median eminence are shown. Scale bar, 40 μm (GnRH, oxytocin, and vasopressin) and 30 μm (POMC).
(B) Representative wheel-running activity records of homozygous (top) and heterozygous (middle) VGF mutant and wild-type (bottom) mice. Mice were kept in a LD12:12 cycle for the first 14 days of recording. The blackened portion of the horizontal bar at the top of each record indicates the dark part of the cycle. After day 14, indicated by the hatch mark on the right side of each record, mice were maintained in DD for a period of 22 days.

found in wild-type mice. To further investigate the ovarian dysfunction, VGF-deficient and wild-type females were superovulated, and the number of eggs present in the oviducts was determined. Comparable numbers of eggs were produced by superovulated VGF mutant (22 ± 10 [mean ± SE], n = 6) and wild-type (23 ± 10 [mean ± SE], n = 6) females. Ovaries transplanted from VGF mutant females into ovariectomized normal females restored fertility, suggesting that the reproductive deficits of VGF-deficient females were not the result of primary ovarian pathology but rather were due to failed stimulation by the hypothalamic pituitary axis.

None of the pups born to the four homozygous mutant females lived more than 48 hr postpartum. VGF-deficient and +/− pups failed to survive if they were nursed by their VGF mutant mothers but did survive if they were fostered by a wild-type mother. Immediately following birth, pups born to VGF mutant females were replaced with P3 wild-type pups, which were observed to suckle but did not gain weight (Figure 8E). Histological examination of the mammary glands of postpartum females revealed relatively immature, milk-engorged ducts in the VGF-deficient mice (Figure 8D) in comparison with control females (Figure 8C). Daily postpartum injections of oxytocin into the nursing VGF mutant mother had no effect on milk letdown or infant mortality (data not shown).

Sexual maturation of VGF-deficient males was delayed, adult sexual behavior was abnormal, and fertility was reduced; in addition, adult VGF mutant males failed to attack intruders (data not shown). The presence of mobile spermatozoa in the lumen of the testis and epididymis of VGF-deficient males was confirmed by microscopic examination. Gross testicular morphology appeared to be normal in mutant males, but the testis and seminal vesicles were, respectively, 70% and 35% the weight of these organs in wild-type males (289 ± 28 mg versus 197 ± 53 mg and 251 ± 64 mg versus 83 ± 44 mg [mean ± SE]).

Mice Lacking VGF Have Reduced Pituitary Gonadotropin Content

Since the ovaries of VGF mutant females were abnormal, but the function could be rescued by transplantation, we examined whether pituitary function was normal in VGF-deficient animals. VGF is found in the developing (Snyder et al., 1998b) and adult (Ferri et al., 1995) pituitary, and its expression is regulated in gonadotropes in response to ovariectomy and the reproductive cycle (Ferri et al., 1995). Pituitary luteinizing hormone (LHβ) and follicle-stimulating hormone (FSHβ) mRNA levels (data not shown) and protein content were found to be reduced in male and female VGF-deficient mice (Figure 9A); hypothalamic GnRH mRNA levels (data not shown) and immunocytochemistry (see Figure 6) were found, however, to be within normal limits. For comparison, prolactin (Prl) and growth hormone (GH) mRNA (data not shown) and polypeptide content in mutant and wild-type pituitaries were not significantly different (Figure 9A), while TSH content was slightly lower in VGF mutant

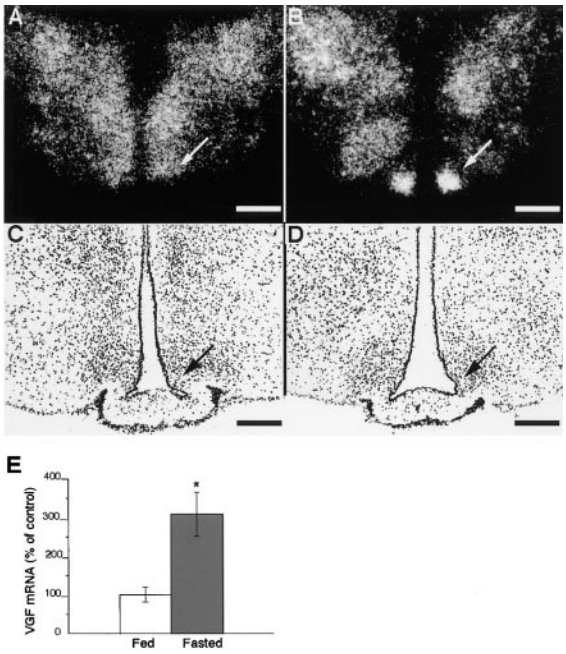

Figure 7. VGF mRNA Levels Are Regulated by Fasting in the Arcuate Nucleus of the Hypothalamus

VGF expression was examined in the hypothalami of ad lib fed and 48 hr fasted mice. Brains were removed and in situ hybridization was carried out on coronal sections from fed (n = 7) and fasted (n = 4) animals as previously described (Mizuno et al., 1998) using a mouse VGF antisense probe. Similar results were found in two additional studies.

(A and B) VGF mRNA expression in the hypothalami of ad lib fed and 48 hr fasted mice, respectively. Arrows are positioned to indicate the arcuate nuclei in the corresponding film autoradiograms and photomicrographs. Scale bars, 100 μm.

(C and D) Nissel-stained sections of (A) and (B), respectively. Scale bars, 100 μm.

(E) Quantitative analysis (mean ± SE; asterisk, $p < 0.05$) of VGF expression in the arcuate nuclei of 48 hr fasted and fed mice. VGF expression, quantified by densitometry of film autoradiograms, is expressed as a percentage of fed wild-type controls.

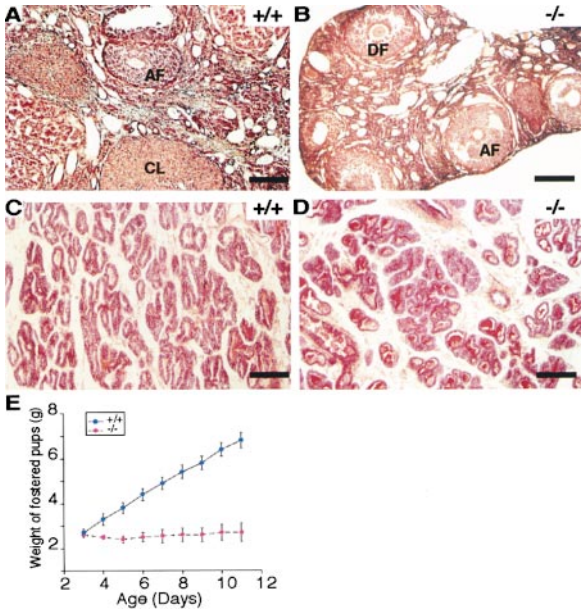

Figure 8. Histological (H and E) Examination of Ovaries and Mammary Glands from VGF Mutant and Wild-Type Females

(A) Ovary of 4-month-old wild-type female. Abbreviations: AF, atretic follicle, and CL, corpus luteum. Scale bar, 150 μm.

(B) Ovary of 4-month-old homozygous mutant female. Abbreviations: AF, atretic follicle, and DF, developing follicle. Scale bar, 150 μm.

(C) Inguinal mammary gland of 8 day postpartum wild-type female. Scale bar, 150 μm.

(D) Inguinal mammary gland of 8 day postpartum homozygous mutant female. Note that alveoli containing milk stain with eosin. Scale bar, 150 μm.

(E) Mean weight (g) of P3–P11 wild-type pups fostered either with postpartum wild-type (+/+) or VGF mutant (−/−) females.

mice (Figure 9A). Histological and immunocytochemical analyses of pituitary glands from VGF mutant mice revealed no gross anatomical defects and the normal complement of gonadotropes, somatotropes, thyrotropes, and lactotropes (Figure 9B).

Discussion

The results of classical brain lesion experiments have helped to establish that the hypothalamus is the central integrator of energy homeostasis in the brain, balancing energy intake (feeding) with energy expenditure (activity and metabolic rate). Molecular genetic studies in mice have further increased our understanding of the contributions that various hypothalamic neuropeptides make to the central control of energy balance. For example, ectopic, constitutive expression of agouti, the endogenous antagonist of the melanocortin receptor, results in obesity, as does deficiency of the MC4 receptor or overexpression of the agouti-related protein (AGRP or ART) (Huszar et al., 1997; Ollmann et al., 1997; Shutter et al., 1997). Analysis of additional obese mouse mutants, including *Lep*^{ob}/*Lep*^{ob} and *Lepr*^{db}/*Lepr*^{db}, indicate that the disruption of pathways that limit food intake is poorly compensated for (Elmquist et al., 1998; Friedman and Halaas, 1998). Indeed, until the recent description of lean mice with a targeted deletion of MCH (Shimada et al., 1998), the redundancy of hypothalamic orexigenic neuropeptides that promote feeding was felt to be responsible for the lack of effect that the genetic ablation of genes such as *Npy* had on feeding and body weight in mice (Erickson et al., 1996). MCH-deficient mice, however, have reduced body weight, are hypophagic, and have an inappropriate increase in metabolic rate, despite reduced peripheral leptin levels and decreased arcuate POMC mRNA. Interestingly, the phenotype we have described here for VGF-deficient mice is in general more severe and is associated with greater reductions in weight, peripheral fat, and leptin levels; with decreased fertility; and with a greater elevation in basal metabolic rate, which is associated with hyperactivity.

Our studies suggest that VGF plays a critical role in the central regulation of energy expenditure and in the normal animal may control energy storage, perhaps by damping metabolic rate and activity. Intriguingly, ad lib fed VGF mutants have not only decreased levels of hypothalamic POMC mRNA but also dramatically increased

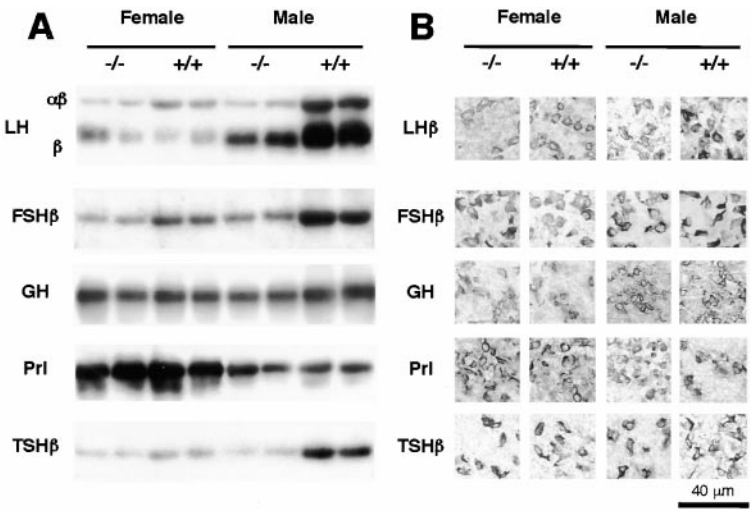

Figure 9. Analysis of Pituitary Hormone Expression in VGF Mutant and Wild-Type Mice

(A) Immunoblot analysis of LH, FSH, GH, Prl, and TSH content in pituitary protein samples (15 μg for each except for PRL analysis, for which 1.5 μg samples were used) from female and male VGF mutant (−/−) and wild-type (+/+) mice.

(B) Immunocytochemical analysis of pituitary hormone expression.

Representative photomicrographs taken from coronal sections of the anterior pituitary show LHβ, FSHβ, GH, Prl, and TSHβ immunocytochemical labeling in female and male VGF mutant (−/−) and wild-type (+/+) mice. Scale bar, 40 μm.

levels of hypothalamic NPY and AGRP mRNAs, a profile of gene expression that is seen in normal mice fasted for 48 hr (Mizuno et al., 1998; Mizuno and Mobbs, 1999). Since food intake of VGF-deficient mice is comparable to that of ad lib fed normal mice, not that of refed fasted mice, a relative insensitivity of VGF mutant mice to decreased hypothalamic POMC and serum leptin, and to increased hypothalamic NPY and AGRP levels, is suggested. These data imply that VGF might also function to stimulate food intake, but like MCH (Shimada et al., 1998), VGF does not appear to be required to mount a hyperphagic response, since fasted VGF mutant mice are hungry, and intake on refeeding is similar to that of control mice. Our results indicate either that VGF is a critical downstream effector in the leptin, POMC, NPY, and AGRP pathways or, alternatively, that even appropriate regulatory responses of these hypothalamic genes cannot compensate for the VGF mutant's hyperactive, hypermetabolic state. These hypotheses may be more directly tested by performing genetic crosses between VGF mutant mice and obese models such as agouti or *Lep*^{ob}/*Lep*^{ob}.

Animals regulate body weight by balancing daily energy expenditure with food intake at a particular body weight (or set point). The body weight set point of the VGF knockout mice is reduced and is resistant to change either on a hypercaloric high-fat diet or following gold thioglucose lesioning of the hypothalamus, both of which normally lead to obesity (S. H. et al., unpublished data). Suppression of diet-induced obesity in the VGF mutant mouse should encourage the search for therapeutic inhibitors of VGF actions. In support of a significant role for VGF in the regulation of energy homeostasis, hypothalamic VGF mRNA expression is induced, as are NPY mRNA levels, in the arcuate nuclei of fasted mice. In addition, VGF mRNA levels are also rapidly induced following cysteamine treatment, prior to the formation of duodenal ulcers but perhaps as a result of increased neuronal activity in regions of the nucleus tractus solitarius and dorsal motor nucleus of the vagus that project to the stomach (Kanemasa et al., 1995). Activation of neuronal pathways after feeding, projecting from the digestive tract to the brainstem, might therefore directly influence VGF synthesis in the medulla oblongata, suggesting that VGF expression is rapidly regulated in different regions of the brain in response to feeding and fasting. Finally, our studies indicate that VGF mRNA levels in the mouse are regulated by circadian behavior and light in the SCN, as they are in the hamster (Wisor and Takahashi, 1997), providing a potential molecular link between the circadian rhythm and the diurnal regulation of feeding, activity, and metabolic rate.

Serum leptin and adipose leptin mRNA levels are reduced in VGF mutant mice and, like mouse strains that are defective in leptin signaling (*Lep*^{ob}/*Lep*^{ob} or *Lepr*^{db}/*Lepr*^{db}), VGF-deficient mice have significant reproductive abnormalities. Delays in sexual maturation, growth retardation, defective mammary gland development, and amenorrhea are seen in leptin-deficient humans (Clement et al., 1998; Strobel et al., 1998) and rodents (Campfield et al., 1998) and in VGF mutant mice. The reproductive deficits and alterations in pituitary gonadotropin content found in VGF-deficient mice may therefore be a consequence of the effect that low-circulating leptin levels have on the HPG axis. Since VGF is expressed and processed in the pituitary (Ferri et al., 1995), primarily in gonadotropes in the female, and VGF levels are regulated throughout the estrous cycle and in response to ovariectomy (Ferri et al., 1995), it is also possible that VGF or VGF-derived peptides may play a more direct role in HPG axis function.

The VGF polypeptide is synthesized in neurons, stored in dense core secretory vesicles, and cleaved in a cell type–specific pattern (Trani et al., 1995). The carboxy-terminal 30 amino acid peptide has been isolated from bovine posterior pituitary (Liu et al., 1994), suggesting that the dibasic amino acid–rich VGF protein may be transported from the hypothalamus and processed, possibly into bioactive peptides. Other vesicular components, the secretogranins and chromogranins, have been postulated to function as neuromodulators. In fact, secretoneurin, a peptide derived from secretogranin II, has been found to modulate dopamine release from rat striatal slices (Saria et al., 1993). As a protein that is secreted from dense core vesicles (Possenti et al., 1989),

VGF might be anticipated to be targeted to the regulated release pathway by carboxypeptidase E (CPE) (Cool et al., 1997). Subsequent misrouting and disruption of the regulated release of VGF might be expected in the CPE-deficient mouse model (*Cpe*^{fat}) (Naggert et al., 1995), which has multiple endocrine abnormalities, including hyperproinsulinemia, infertility, and obesity, and a possible overlap of the phenotypes of *Cpe*^{fat} and VGF mutant mice might be anticipated on this basis. VGF, however, does not appear to be sorted into the regulated release pathway by the CPE receptor (E. Normant, Y. P Loh, and S. R. J. S., unpublished data), and this may in part be responsible for the lack of phenotypic similarities between *Cpe*^{fat} and VGF-deficient mice.

Could the metabolic dysfunction that we detected in adult VGF mutant mice have arisen as a consequence of developmental abnormalities? Detailed investigation of hypothalamic and pituitary structure did not demonstrate major differences in relative cell type or number, nor were gross functional defects observed, but our studies may not have detected subtle but potentially critical alterations in neuronal connectivity, providing an impetus to develop models in which VGF synthesis is eliminated only in the adult or, alternatively, to introduce recombinant VGF or VGF peptides into adult VGF mutant mice. Although no developmental defects are apparent at birth, as they age, VGF-deficient pups become somewhat runted, most likely secondary to increased metabolic rate, reduced peripheral fat stores, and low-circulating leptin, insulin, and T4 levels. Do other small, runted mutant mice have alterations in energy metabolism similar to those we have detected in VGF-deficient mice? A number of immunologically compromised mice are runted and most likely chronically ill, with decreased fat and lean body mass resulting in part from reduced food consumption. We are not aware of reports, however, that any of these knockout mice are hypermetabolic and hyperactive, nor conversely did our analysis of VGF mutants demonstrate any significant abnormalities in immune function in comparison with littermate controls. Mutation of genes involved in osteogenesis (e.g., fibrobast growth factor receptor 3 in achondroplasia; Rousseau et al., 1994) and pituitary development (e.g., *Pit-1*; Li et al., 1990) can lead to dwarfism, with a constellation of neuroendocrine problems in the latter that are shared in part with VGF mutant mice, but to our knowledge none of these mice have been tested for defects in energy homeostasis. Finally, insulin-like growth factor II heterozygotes (DeChiara et al., 1990) and thyrotropin-releasing hormone (Yamada et al., 1997), GH receptor binding protein (Zhou et al., 1997), and Otx-1 (Acampora et al., 1998) null homozygotes are all runted to varying degrees but are fully fertile, suggesting that despite their small size, these mice are unlikely to have extreme alterations in peripheral fat stores, leptin levels, and energy balance.

In this study, we have reported the generation and characterization of mice resulting from the targeted deletion of *Vgf*. Homozygous mutant mice produce no VGF polypeptide and, although appearing normal at birth, by the third or forth postnatal day manifest reduced body weight. Adult VGF-deficient mice are hypermetabolic and hyperactive, have dramatically reduced peripheral fat stores, and display a profile of hypothalamic gene expression that is comparable to that found in a fasted animal. VGF and/or VGF-derived peptides may therefore be required to appropriately regulate energy balance, since the expected behavioral responses of a normal mouse to fasting include decreased activity, decreased energy output, and hyperphagia. Investigation of the central mechanisms and hypothalamic circuits that are involved in obesity and wasting may consequently be facilitated through the study of lean VGF mutant mice. Further experimentation will be necessary to identify the biologically active portions of the VGF polypeptide and the mechanism and sites of VGF action. Pharmacologic antagonism of VGF actions through the use of small molecule inhibitors might therefore represent a novel approach to the treatment of human obesity.

Experimental Procedures

Cloning, Targeted Deletion, and Chromosomal Localization of the Murine *Vgf* Gene

The mouse *Vgf* gene was cloned from a strain 129/SvJ genomic DNA library. The chromosomal location of the mouse *Vgf* gene was determined by probing Southern blots with the rat VGF cDNA (coding sequence), employing methods described previously (Kozak et al., 1990). The targeting construct was generated by replacing the entire VGF coding sequence with a PGK-*neo* cassette. After linearization, the resultant plasmid was electroporated into R1 ES cells, and neomycin-resistant clones were selected in G418-containing media. Of 750 G418-resistant clones selected, Southern analysis using a 1 kb KpnI–SalI fragment 3′ to the targeted sequence as a probe indicated disruption of the *Vgf* locus in two clones. Both clones were expanded and injected into C57BL/6 blastocysts to generate chimeric mice. Chimeric males were crossed to either 129/SvJ or C57BL/6 females, and germline transmission of the mutant allele was determined by Southern analysis. To verify that the deletion of *Vgf* coding sequences resulted in a null mutation, total RNA (Salton, 1991) and protein (Laemmli, 1970) were prepared from the brains of 4-week-old homozygous, heterozygous, and control mice. Northern and Western blot analyses employing VGF cDNA probes (Salton, 1991) and rabbit anti-VGF (rat) antisera (Salton et al., 1995), respectively, were carried out as previously described. Chimeric males were directly crossed to 129/SvJ or repetitively backcrossed to C57BL/6 strains for seven generations; homozygous VGF-deficient offspring of F7 and F1 heterozygotes on either background were phenotypically indistinguishable.

Tissue Preparation and Histology

Mice were sacrificed by an overdose of chloral hydrate (800 mg/kg body weight) and perfused transcardially with 5 ml of 0.1 M sodium phosphate containing 50 mM NaCl (PBS [pH 7.2]) followed by 50 ml of fixative (4% paraformaldehyde in PBS). Following perfusion, brains and pituitaries were removed and postfixed in the same fixative for 6 hr at 4°C. Subsequently, the tissues were equilibrated in 30% sucrose overnight by a stepwise increase in sucrose concentration (10%, 20%, and 30%, respectively), embedded in OCT compound (Miles, Elkhart, IN), and frozen in 2-methyl-butane on dry ice. Frozen sections (25 μm) were cut on a Bright-Hacker cryostat (Huntingdon, UK), thaw mounted onto Superfrost/Plus slides (Fisher Scientific, Fairlawn, NJ), and stained with cresyl violet or processed for immunocytochemistry. The sections were stored at −20°C until use. After removal, ovaries, testes, and mammary glands were stored in Bouin's fixative overnight and embedded in paraffin. Sections (8–10 μm) were cut at room temperature with a sliding microtome. Subsequently, these sections were deparafinized with decreasing alcohol prior to staining with hematoxylin and eosin (H and E). After staining, the sections were dehyhydrated in graded alcohol, cleared in xylene, and coverslipped.

Immunocytochemical Analysis

Immunocytochemistry was carried out as previously described (Wu et al., 1992). Briefly, sections were treated with 0.5% H_2O_2 to remove

endogenous activity followed by several PBS washes that contained 0.1% Triton X-100 and 5% normal serum. All sections were incubated for 48 hr at room temperature with antiserum diluted in PBS containing 1% normal serum, 0.02% sodium azide, and 0.1% Triton X-100. This was followed by 4 hr incubations in biotinylated antiprimary antibody immunoglobulin G (1:400; Vector Laboratories, Burlingame, CA) and an avidin–biotin–horseradish peroxidase complex (1:500; Vector Laboratories, Burlingame, CA). Sections were extensively washed with PBS, and immunoreactivity was visualized by incubating the sections at room temperature with 3,3′-diaminobenzidine tetrahydrochloride (50 mg/100 ml in PBS; Sigma) with H_2O_2 (6 μl/100 ml) as the catalyzing agent. Sections were then dehydrated in graded alcohol, cleared in xylene, and coverslipped.

Neuron Counts

Quantitative analyses were performed on a computer-assisted image analysis system consisting of a Zeiss Axiophot photomicroscope equipped with a Zeiss MSP65 computer-controlled motorized stage, a Zeiss ZVS-47E video camera, a Macintosh 84-AV microcomputer, and Neurozoom, a custom designed morphology and stereology software (Bloom et al., 1997; Howard and Reed, 1998).

RNA and Protein Analysis

Rabbit anti-rat VGF antisera (Salton et al., 1995), rabbit anti-POMC (Dr. R. Allen, Oregon Health Sciences University), mouse anti-vasopressin and anti-oxytocin (Dr. A. J. Silverman, Columbia University), mouse anti-GnRH (Sternberger Monoclonals, Baltimore, MD), or the following primary antisera obtained from the National Hormone and Pituitary Program, rabbit anti-rat LHβ (lot AFP5733093), rabbit anti-human FSHβ (lot AFP005), rabbit anti-rat TSHβ, monkey anti-rat GH (lot AFP411S), and rabbit anti-rat Prl (lot AFP4251091), were employed for Western and immunohistochemical analyses. SDS polyacrylamide and Western analyses were carried out as previously described (Salton et al., 1995). Total RNA was isolated with Trizol (Life Technologies), and Northern analysis was carried out as described (Mizuno et al., 1996) using probes to NPY (Mizuno et al., 1996), POMC (Mizuno et al., 1998), and AGRP (Mizuno and Mobbs, 1999). Qualitative in situ hybridization was carried out essentially as previously described (Zheng and Pintar, 1995) using [^{35}S]-labeled antisense mouse VGF RNA probes. To investigate regulation of VGF mRNA levels by starvation, freshly dissected brains from 48 hr fasted and ad lib fed C57BL/6 mice were rapidly frozen, matching sections through the hypothalami were processed for in situ hybridization, and [^{32}P]-labeled single stranded mouse VGF cDNA probes were employed as previously described (Mizuno et al., 1998). Relative VGF mRNA expression was determined by densitometric analysis of film autoradiograms.

Tissue Neurotransmitter Content

Catecholamine levels in the striatum and adrenal gland were determined by previously described methods (Matson et al., 1987). Tissues were thawed, weighed, and homogenized in ten volumes of 0.4 N perchloric acid containing N-methyl dopamine as an internal standard. The homogenate was centrifuged at 15,000 × g for 15 min at 4°C, and the pellet was retained for protein determination. The supernatant was injected into an ESA CEAS 5500 high-performance liquid chromatograph (ESA, Bedford, MA) with a flow rate of 0.6 mL/min and a mobile phase of 75 mM PO_4, 1.7 mM octanesulfonic acid, 100 μL/L triethylamine, 25 μM EDTA, and 10% acetonitrile (pH 3.00). The concentrations of striatal dopamine, DOPAC, and serotonin, and adrenal norepinephrine and epinephrine, were calculated after comparison with standards, and the yield was calculated by normalization to the internal standard.

Blood and Serum Analysis

Mice were anesthetized with avertin, and blood samples were collected by cardiac puncture. Blood glucose levels were determined with a one touch profile meter (Lifescan). Serum corticosterone, insulin, T4, and leptin levels were determined by radioimmunoassay (ICN Biomedicals).

Energy Balance

Daily food consumption was measured by using either a liquid diet or a weighable solid pellet delivery system (Bio-Serv, Frenchtown, NJ). Total cumulative food consumption was measured over 5 consecutive days and average daily consumption determined. Oxygen consumption and ambulatory activity of individual mice were measured with an Oxyscan (O_2/CO_2) metabolic system and Digiscan activity monitor (AccuScan Instruments, Columbus, Ohio). Individual mice were placed into metabolic chambers (21 cm × 21 cm × 16 cm) for 24 hr. The basal rate of oxygen consumption was determined during the circadian light phase, which corresponded to a period of relative inactivity, and the ambulatory activity of the individual mice was determined by the number of infrared beam breaks (distance between beams, 2.54 cm). Total distance traveled (cm) by the mice was recorded every 5 min for a period of 24 hr.

Lean Body Mass

Mice were killed by CO_2 narcosis. After removing undigested food and fecal material from the digestive tract, initial body weight was recorded, and total body water, carcass lipid, and carcass protein were determined as previously described (Chung et al., 1998).

Superovulation and Ovary Transplantation

To determine estrous cycle length, epithelial cells lining the vaginal wall were suspended in saline, transferred into 96-well tissue culture plates, and viewed by light microscopy.

Female mice (12–16 weeks of age) to be superovulated were injected subcutaneously with five units of pregnant mare serum, and 48 hr later with five units of human chorionic gonadotropin. After 24 hr, superovulated females were anesthetized, ovaries and oviducts were removed, and oocytes were flushed from the oviduct with PBS and counted under a dissecting microscope.

To transplant ovaries from VGF mutant females into C57BL/6 × 129/SvJ host females (Jackson Lab), mice were anesthetized, and *Vgf*$^{-/-}$ ovaries were removed and stored in PBS. Host females were bilaterally ovariectomized, and a *Vgf*$^{-/-}$ ovary was sutured into the bursa that had surrounded the left ovary. Ovary and periovarian fat were returned to the abdominal cavity, and the incision was closed. Mice were mated after 2 weeks of postoperative recovery.

Behavioral Analysis

Male Sexual Behavior

Sixteen 6-month-old female mice (C57BL6) underwent bilateral ovariectomy (OVX) under avertin anesthesia, after which mice were implanted with subcutaneous Silastic capsules (Dow Corning, Midland, MI) containing 1.0 μg 17β-estradiol (E2) in sesame oil. After recovering for 2 weeks, female mice were sequentially treated with estradiol benzoate and progesterone as previously described (Wu et al., 1992). The male mice were scored for sexual behavior based on their response to the primed OVX females. On the morning of the experiment, males were housed individually in freshly prepared clear polyethylene cages. All OVX-primed females were introduced to males at 1900 hr and observed for sexual behavior. Responses of the males were categorized as number of mounts and intromissions (Wu et al., 1992).

Aggression

Intermale aggression testing was performed as described (Everts et al., 1997) with minor modification. Male mice (4–6 months old) were individually caged and separated into three groups ($n = 5$). Wild-type and VGF mutant mice (groups I and II, respectively) were treated as resident males, and naive males (group III) were introduced as intruders subject to attack. The encounter, initiated by placing the naive male into the resident male's cage, was monitored for 10 min, and the latency to attack was scored. All experiments were carried out 1 hr before lights out.

Circadian Behavior

Activity was monitored continuously with an Esterline-Angus mechanical recorder (Angus Electronics, Indianapolis, IN) and stored on polygraphic paper with 10 min gradations. Animals were kept in LD12:12 (12 hr of light and 12 hr of dark) conditions for 2 weeks. Following entrainment, the animals were kept in DD conditions (with the exception of light pulses) for the remainder of the experiment. The free-running period was determined from the first 10 days in

DD and second 10 days in DD separately by measurement of the angle of a line eye fitted to the daily activity onsets.

Olfaction

Male wild-type and VGF mutant mice were fasted for 24 hr, and the time taken to locate a cookie buried under 10 cm, 8 cm, or 6 cm of fresh bedding was measured essentially as described (Beard and Mackay-Sim, 1987).

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