Germline ablation of VGF increases lipolysis in white adipose tissue

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Abstract

Targeted deletion of VGF, a neuronal and endocrine secreted protein and neuropeptide precursor, produces a lean, hypermetabolic mouse that is resistant to diet-, lesion-, and genetically induced obesity and diabetes. We hypothesized that increased sympathetic nervous system activity in *Vgf*^{−/−} mice is responsible for increased energy expenditure and decreased fat storage and that increased β -adrenergic receptor stimulation induces lipolysis in white adipose tissue (WAT) of *Vgf*^{−/−} mice. We found that fat mass was markedly reduced in *Vgf*^{−/−} mice. Within knockout WAT, phosphorylation of protein kinase A substrate increased in males and females, phosphorylation of hormone-sensitive lipase (HSL) (ser563) increased in females, and levels of adipose triglyceride lipase, comparative gene identification-58, and phosphoperilipin were higher in male *Vgf*^{−/−} WAT compared

with wild-type, consistent with increased lipolysis. The phosphorylation of AMP-activated protein kinase (AMPK) (Thr172) and levels of the AMPK kinase, transforming growth factor β -activated kinase 1, were decreased. This was associated with a decrease in HSL ser565 phosphorylation, the site phosphorylated by AMPK, in both male and female *Vgf*^{−/−} WAT. No significant differences in phosphorylation of CREB or the p42/44 MAPK were noted. Despite this evidence supporting increased cAMP signaling and lipolysis, lipogenesis as assessed by fatty acid synthase protein expression and phosphorylated acetyl-CoA carboxylase was not decreased. Our data suggest that the VGF precursor or selected VGF-derived peptides dampen sympathetic outflow pathway activity to WAT to regulate fat storage and lipolysis.

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Introduction

The sympathetic nervous system (SNS) is an important regulator of glucose and fat metabolism, and its dysfunction can predispose to obesity and type 2 diabetes mellitus. The melanocortin pathway projects from the hypothalamic paraventricular nucleus, innervating parasympathetic and sympathetic preganglionic cells in the brainstem and spinal cord, to form sympathetic circuits that innervate brown adipose tissue (BAT), white adipose tissue (WAT), liver, and pancreas (Giordano *et al.* 2005, Song *et al.* 2005, Penn *et al.* 2006, Voss-Andreae *et al.* 2007, Foster *et al.* 2010). This pathway, through its modulation of autonomic outflow, has been proposed to regulate food intake, energy expenditure, and insulin secretion (Bray & York 1998, Friedman & Halaas 1998, Fan *et al.* 2000, Li *et al.* 2003). Norepinephrine, released from sympathetic nerve endings, stimulates lipolysis in WAT and increases thermogenesis in BAT via activation of β -adrenergic receptors (Collins *et al.* 2004), and mice that

lack all three β -adrenergic receptor subtypes (betaless) develop massive diet-induced obesity (Bachman *et al.* 2002).

VGF is a secreted protein and peptide precursor, a member of the chromogranin/secretogranin family (Bartolomucci *et al.* 2011), which is expressed in neurons throughout the brain and in several neuroendocrine and endocrine tissues (Salton *et al.* 2000, Levi *et al.* 2004). Homozygous germline *Vgf* knockout mice are lean and hypermetabolic and resist developing obesity and diabetes when fed a high-fat diet (Hahm *et al.* 1999), suggesting that VGF regulates energy balance by modulating sympathetic outflow. Consistent with this hypothesis, neonatal treatment of *Vgf*^{−/−} mice with either monosodium glutamate, which damages the hypothalamus and the hypothalamic projections to the autonomic nervous system (Bergen *et al.* 1998, Morris *et al.* 1998, Tsukahara *et al.* 1998), or guanethidine, which results in a peripheral sympathectomy (Watson *et al.* 2009), blocks development of the lean phenotype (Hahm *et al.* 2002, Watson *et al.* 2005). Moreover, *Vgf* knockout mice have also

increased serum-free fatty acid (FFA) levels, suggesting increased fat mobilization in WAT (Watson *et al.* 2005). Targeted deletion of *Vgf* also suppresses obesity, hyperinsulinemia, and hyperglycemia in *A^y/a* agouti and melanocortin 4 receptor-deficient (*Mc4r*[−]/*Mc4r*[−]) mice (Hahm *et al.* 2002, Watson *et al.* 2005), supporting a role for VGF in the melanocortin pathway and its projections. Here, we examined WAT to determine the effect that targeted germline ablation of VGF has on the activation state and the protein expression of key lipolytic and lipogenic enzymes. We noted alterations in the level and/or phosphorylation of proteins that control fat breakdown, consistent with increased SNS activity. Our studies support the hypothesis that VGF and/or one or more VGF peptides modulate sympathetic outflow pathway activity to control fat storage and energy expenditure.

Materials and Methods

Mouse strains and diets

The VGF-deficient line used here was generated by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY, USA) as described previously (Valenzuela *et al.* 2003) using F1H4 ES cells (a 129B6/F1-derived cell line) and a BAC-based targeting vector with deletion of the entire *Vgf* coding sequence and insertion of an in-frame *lacZ* reporter gene and neomycin selection cassette; chimeric mice resulted from the injection of two independent *Vgf*[−]/*Vgf*[−] embryonic stem cell clones into C57BL6/J blastocysts. Male chimeras were mated with C57BL6/J females to produce F1 breeders and experiments were performed on N2F1 mice (>83% C57BL6 background). As described previously (Watson *et al.* 2009), the phenotype of the Regeneron VGF-deficient line is extremely similar to an earlier line generated using R1 ES cells (Hahm *et al.* 1999). Mice were housed at room temperature in a 12 h light:12 h darkness cycle with chow and water available *ad libitum* unless otherwise specified. Mice fed standard chow received a 4.5% fat, 55% carbohydrate, 20% protein, and 4.7% fiber diet (Purina PicoLab Rodent Diet 20-5053; 4 kcal/g; Purina, St Louis, MO, USA). All animal studies were conducted in accordance with the Guide for Care and Use of Experimental Animals, using protocols approved by Institutional Animal Care and Use Committees at Mount Sinai School of Medicine.

Western blot analysis

Gonadal WAT from wild-type (*Vgf*⁺/*Vgf*⁺), heterozygous knockout (*Vgf*⁺/*Vgf*[−]), and homozygous knockout (*Vgf*[−]/*Vgf*[−]) (*n* = 4–17) mice was homogenized in ice-cold lysis buffer. Two different buffers were used with comparable results: i) 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, and 1% NP40 supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics; Thermo Scientific, Waltham, MA, USA;

Watson *et al.* 2009) and ii) 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 0.5% NP-40, and complete protease inhibitor cocktail (Roche; Scherer *et al.* 2011). Lysates were cleared by centrifugation (16 800 *g*, 10 min at 4 °C), and the protein concentration of the supernatant was determined by the BCA protein method (Thermo Scientific). Protein samples (25 μg) were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% (w/v) BSA in PBS or with Odyssey LI-COR Blocking Buffer (LI-COR, Lincoln, NE, USA) 1:1 in TBS.

Membranes were incubated overnight at 4 °C with the following rabbit antisera (Cell Signaling, Boston, MA, USA), unless otherwise indicated, diluted in blocking buffer: phospho-AMP-activated protein kinase (AMPKα) (Thr172) (1:1000 (v/v)), total AMPKα (1:1000 (v/v)); TAK1 (1:1000 (v/v)), mouse monoclonal anti-GAPDH (1:1000 (v/v)), phospho-TAK1 (Thr187) (1:1000 (v/v)), transducer of regulated CREB activity 3 (TORC3) (1:1000 (v/v)), phospho-hormone-sensitive lipase (HSL) ser563 (1:1000 (v/v)), phospho-HSL ser565 (1:1000 (v/v)), phospho-HSL ser660 (1:1000 (v/v)), HSL total (1:2000 (v/v)), ATGL (1:1000 (v/v)), phospho-protein kinase A (PKA) substrate (1:1000 (v/v)), phospho-acetyl CoA carboxylase (ACC) (1:1000 (v/v)), ACC (1:1000 (v/v)), p42/44 MAPK (1:1000 (v/v)), phospho-p42/44 MAPK (1:1000 (v/v)), GLUT4 (1:1000 (v/v)), and phospho-CREB (1:1000 (v/v)). Other antisera included FAS (1:1000 (v/v)) (BD Bioscience, San Jose, CA, USA), insulin receptor β (1:500 (v/v)) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:10 000 (v/v)) (Abcam, Cambridge, MA, USA), GAPDH (1:5000 (v/v)) (Abcam), perilipin (1:2000 (v/v)) (Souza *et al.* 2002) (gift from Dr Andrew Greenberg, Tufts University, MA), and CGI-58 (1:2000 (v/v)) (Subramanian *et al.* 2004) (gift from Dawn Brasaemle, Rutgers University, NJ, USA).

After three consecutive 5 min washes in PBS-T or TBS-T (0.1% Tween 20 in PBS or TBS respectively), membranes were incubated for 1 h at room temperature with either HRP-conjugated secondary antibody (1:2000 (v/v) in PBS-T; GE Healthcare, Piscataway, NJ, USA) containing 5% nonfat dry milk or with Dylight 680-conjugated goat anti-rabbit IgG and Dylight 800-conjugated goat anti-mouse IgG (both Thermo Scientific) in blocking buffer containing 0.1% TBS-T and 0.1% SDS. After three washes in PBS-T or TBS-T, bound antibodies were detected using either ECL (Thermo Scientific), exposure to HyBlot CL (Denville Scientific, Metuchen, NJ, USA), and densitometric quantification using NIH ImageJ or were scanned with the LI-COR Odyssey (LI-COR) and quantified with Odyssey 3.0 software based on direct fluorescence measurement.

Data and statistical analysis

Data are expressed as mean ± S.E.M. The statistical significance of differences among *Vgf*[−]/*Vgf*[−], *Vgf*[−]/*Vgf*⁺, and

Vgf⁺/*Vgf*⁺ groups was subjected to a one-way ANOVA and Tukey's multiple-range test; comparisons were performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). *P* values <0.05 were considered significant. For western blot assays, values ($\pm$ S.E.M.) from *Vgf*[−]/*Vgf*[−] and *Vgf*⁺/*Vgf*⁺ adipose samples were compared by two-tailed Student's *t*-test.

Results

Vgf[−]/*Vgf*[−] knockout mice showed significantly reduced body and gonadal fat pad weights compared with *Vgf*⁺/*Vgf*[−] and *Vgf*⁺/*Vgf*⁺ mice

Quantification of *Vgf*[−]/*Vgf*[−], *Vgf*⁺/*Vgf*[−], and *Vgf*⁺/*Vgf*⁺ body and adipose tissue weights was initially performed. Because fat mass is often dramatically reduced in homozygous *Vgf* knockout mice, only gonadal WAT could be reliably isolated and quantified from all mice. *Vgf*[−]/*Vgf*[−] male and female mice are lean with 42 and 31% less body weight respectively than *Vgf*⁺/*Vgf*⁺ mice (Fig. 1A and B), which was accompanied by a significant eightfold decrease in gonadal fat pad mass (Fig. 1F, ****P*<0.0001, ANOVA), which was also significantly decreased when fat pad mass was examined as a percentage of body mass (Fig. 1I, ****P*<0.0001, ANOVA). In addition, similar differences between *Vgf*[−]/*Vgf*⁺ heterozygous and *Vgf*[−]/*Vgf*[−] mice were noted (Fig. 1C, ****P*<0.0001, ANOVA). Neither body weight nor fat pad weight differed significantly between *Vgf*[−]/*Vgf*⁺ and *Vgf*⁺/*Vgf*⁺ mice. Photographs of representative 8-week-old female mice of each genotype, and adipose depots dissected from each, are shown in Fig. 1J, K, L, M, N, O, P, Q and R.

Altered protein expression in Vgf[−]/*Vgf*[−] WAT is consistent with increased lipolysis

Western analysis of adipose tissues from *Vgf*[−]/*Vgf*[−], *Vgf*⁺/*Vgf*[−], and *Vgf*⁺/*Vgf*⁺ mice was performed to investigate potential mechanisms underlying decreased adiposity in *Vgf* knockout mice. Expression of proteins involved in glucose uptake or lipogenesis was unchanged: GLUT4, the main transporter involved in glucose uptake into WAT, fatty acid synthase (FAS), and additional enzymes important in adipose tissue triglyceride accumulation, including total and phosphorylated ACC, were unchanged in *Vgf* knockout WAT compared with wild-type (Table 1).

Next, we analyzed whether reduced gonadal fat pad weight might be due to increased lipolysis. Phosphorylation of HSL, an enzyme responsible for the mobilization of FFAs from adipose tissue, on serine 563 was significantly increased in female *Vgf*[−]/*Vgf*[−] WAT (Fig. 2A), although phosphorylation of HSL at serine 660 was unchanged (Fig. 2B). We found that levels of adipose triglyceride lipase (ATGL), the main lipase responsible for the first step in intracellular

triglyceride hydrolysis, were significantly upregulated in male *Vgf*[−]/*Vgf*[−] WAT (Fig. 2C). This was consistent with increased expression of comparative gene identification-58 (CGI-58, also known as a/b hydrolase domain containing protein 5; Fig. 2D) and increased levels of phospho-perilipin (Fig. 2E), both important activators of ATGL-mediated lipolysis, in male *Vgf* knockout WAT.

AMPK, an enzyme that is regulated by phosphorylation, has been implicated in the regulation of HSL. Phosphorylation of AMPK α , the catalytic subunit of AMPK, was significantly reduced in *Vgf*[−]/*Vgf*[−] relative to *Vgf*⁺/*Vgf*⁺ WAT (Fig. 3A; ****P*=0.0001). In support of these findings, previous studies noted that activation of AMPK decreased HSL phosphorylation of ser563, while dominant negative inhibitors or targeted ablation of AMPK increased phospho-HSL (563) levels in adipocytes (Daval *et al.* 2005). Levels of transforming growth factor β -activated kinase 1 (TAK1), a MAPK kinase kinase, and AMPK kinase (Momcilovic *et al.* 2006, Xie *et al.* 2006), decreased in *Vgf*[−]/*Vgf*[−] WAT (Fig. 3B; **P*=0.0207). Although we were unable to detect TAK1 (Thr187) phosphorylation in our WAT samples, phosphorylation of HSL at serine 565, a downstream target of AMPK (Anthonson *et al.* 1998), was found to be significantly decreased in *Vgf*[−]/*Vgf*[−] WAT (Fig. 3C; ***P*=0.0039), suggesting reduced activation of AMPK in male and female *Vgf* knockout WAT. In addition, the overall pattern of phosphorylation of PKA substrate (Fig. 3D) was significantly increased in male and female *Vgf*[−]/*Vgf*[−] WAT, consistent with increased PKA activity.

No changes in the expression of p42/44 MAPK were detected (Table 1), which is consistent with our finding that phosphorylation of HSL at serine 660 was not altered; phosphorylation at serine 660 by p42/44 MAPK enhances the enzymatic activity of HSL (Greenberg *et al.* 2001). Levels of perilipin, a protein associated with the lipid droplet, were not changed (Table 1). Lastly, no significant differences in the levels of phosphorylated CREB (pCREB), its coactivator TORC3, or the insulin receptor protein IR β were noted between *Vgf*[−]/*Vgf*[−] and *Vgf*⁺/*Vgf*⁺ WAT (Table 1).

Discussion

We examined the effect that targeted germline *Vgf* gene ablation has on fat pad weight and WAT lipolysis and lipogenesis. Characterization of *Vgf* knockout mice previously revealed increased circulating FFA levels, consistent with increased lipolysis (Watson *et al.* 2005). Here, we found that *Vgf* knockout mice have reduced body weight, decreased gonadal fat pad weight, and alterations in a number of key lipolytic proteins by western blot analysis compared with wild-type mice. Phosphorylation of HSL on serine 563, which activates the enzyme, was increased in *Vgf* knockout mice in comparison with wild-type mice, significantly in females. This site is generally phosphorylated by PKA (Anthonson *et al.* 1998), and consistent with increased PKA

Table 1 Expression of proteins that regulate lipolysis or lipogenesis: comparison of *Vgf* knockout and wild-type gonadal WAT. Protein levels were determined in gonadal fat pads from 9–10-week-old male and female mice using western blot analysis as described in the Materials and Methods section. All data are normalized to the loading control β -actin and are expressed in arbitrary units (mean $\pm$ S.E.M.); statistical significance was determined using the two-tailed Student's *t*-test with $P < 0.05$ considered significant (male, $n = 3$ –6 (*Vgf*+/*Vgf*+), $n = 3$ –5 (*Vgf*-/*Vgf*-); female, $n = 3$ –4 (*Vgf*+/*Vgf*+), $n = 3$ –5 (*Vgf*-/*Vgf*-)). No significant differences in the levels of these proteins were found between *Vgf*-/*Vgf*- and *Vgf*+/*Vgf*+ WAT

Protein quantified in male WAT	Protein expression (arbitrary units) mean $\pm$ S.E.M.	P value	Protein quantified in female WAT	Protein expression (arbitrary units) mean $\pm$ S.E.M.	P value
FAS	<i>Vgf</i> +/ <i>Vgf</i> + = 0.19 $\pm$ 0.21 <i>Vgf</i> -/ <i>Vgf</i> - = 0.17 $\pm$ 0.06	0.93	FAS	<i>Vgf</i> +/ <i>Vgf</i> + = 0.27 $\pm$ 0.04 <i>Vgf</i> -/ <i>Vgf</i> - = 0.37 $\pm$ 0.11	0.45
Perilipin	<i>Vgf</i> +/ <i>Vgf</i> + = 0.46 $\pm$ 0.12 <i>Vgf</i> -/ <i>Vgf</i> - = 0.55 $\pm$ 0.05	0.62	Perilipin	<i>Vgf</i> +/ <i>Vgf</i> + = 1.14 $\pm$ 0.22 <i>Vgf</i> -/ <i>Vgf</i> - = 1.01 $\pm$ 0.32	0.79
TORC3	<i>Vgf</i> +/ <i>Vgf</i> + = 0.015 $\pm$ 0.003 <i>Vgf</i> -/ <i>Vgf</i> - = 0.017 $\pm$ 0.002	0.55	TORC3	<i>Vgf</i> +/ <i>Vgf</i> + = 0.03 $\pm$ 0.01 <i>Vgf</i> -/ <i>Vgf</i> - = 0.04 $\pm$ 0.01	0.58
pCREB	<i>Vgf</i> +/ <i>Vgf</i> + = 0.006 $\pm$ 0.002 <i>Vgf</i> -/ <i>Vgf</i> - = 0.002 $\pm$ 0.001	0.25	pCREB	<i>Vgf</i> +/ <i>Vgf</i> + = 0.01 $\pm$ 0.00 <i>Vgf</i> -/ <i>Vgf</i> - = 0.01 $\pm$ 0.00	0.30
IR β	<i>Vgf</i> +/ <i>Vgf</i> + = 0.06 $\pm$ 0.003 <i>Vgf</i> -/ <i>Vgf</i> - = 0.08 $\pm$ 0.02	0.55	IR β	<i>Vgf</i> +/ <i>Vgf</i> + = 0.05 $\pm$ 0.01 <i>Vgf</i> -/ <i>Vgf</i> - = 0.07 $\pm$ 0.02	0.57
ACC	<i>Vgf</i> +/ <i>Vgf</i> + = 0.008 $\pm$ 0.002 <i>Vgf</i> -/ <i>Vgf</i> - = 0.074 $\pm$ 0.046	0.23	ACC	<i>Vgf</i> +/ <i>Vgf</i> + = 0.114 $\pm$ 0.06 <i>Vgf</i> -/ <i>Vgf</i> - = 0.077 $\pm$ 0.02	0.63
pACC	<i>Vgf</i> +/ <i>Vgf</i> + = 0.15 $\pm$ 0.061 <i>Vgf</i> -/ <i>Vgf</i> - = 0.42 $\pm$ 0.146	0.16	pACC	<i>Vgf</i> +/ <i>Vgf</i> + = 0.39 $\pm$ 0.104 <i>Vgf</i> -/ <i>Vgf</i> - = 0.22 $\pm$ 0.045	0.19
p42/44 MAPK	<i>Vgf</i> +/ <i>Vgf</i> + = 0.41 $\pm$ 0.053 <i>Vgf</i> -/ <i>Vgf</i> - = 0.56 $\pm$ 0.044	0.11	p42/44 MAPK	<i>Vgf</i> +/ <i>Vgf</i> + = 0.42 $\pm$ 0.04 <i>Vgf</i> -/ <i>Vgf</i> - = 0.41 $\pm$ 0.015	0.99
Phospho-p42/44 MAPK	<i>Vgf</i> +/ <i>Vgf</i> + = 0.04 $\pm$ 0.002 <i>Vgf</i> -/ <i>Vgf</i> - = 0.25 $\pm$ 0.083	0.06	Phospho-p42/44 MAPK	<i>Vgf</i> +/ <i>Vgf</i> + = 0.06 $\pm$ 0.008 <i>Vgf</i> -/ <i>Vgf</i> - = 0.17 $\pm$ 0.065	0.18
GLUT4	<i>Vgf</i> +/ <i>Vgf</i> + = 0.03 $\pm$ 0.006 <i>Vgf</i> -/ <i>Vgf</i> - = 0.04 $\pm$ 0.021	0.58	GLUT4	<i>Vgf</i> +/ <i>Vgf</i> + = 0.038 $\pm$ 0.009 <i>Vgf</i> -/ <i>Vgf</i> - = 0.057 $\pm$ 0.008	0.20
HSL total	<i>Vgf</i> +/ <i>Vgf</i> + = 0.16 $\pm$ 0.017 <i>Vgf</i> -/ <i>Vgf</i> - = 0.14 $\pm$ 0.060	0.80	HSL total	<i>Vgf</i> +/ <i>Vgf</i> + = 0.15 $\pm$ 0.061 <i>Vgf</i> -/ <i>Vgf</i> - = 0.076 $\pm$ 0.024	0.32

activity, significant changes were noted in PKA substrate phosphorylation (Fig. 3D) in both male and female knockout WAT. In addition, we showed that the lipase ATGL, and its activators CGI-58 and phospho-perilipin, were significantly increased in WAT from male *Vgf*-/*Vgf*- mice. Recent studies have demonstrated that interaction of CGI-58 with ATGL enhances its activity up to 20-fold (Lass *et al.* 2006, Gruber *et al.* 2010), so taken together, our western blot results are consistent with increased lipolysis in *Vgf* knockout adipose tissue, although there may be some sex-specific differences that need further exploration.

The role that AMPK plays in regulating lipid storage in the adipocyte is incompletely understood. Phosphorylation of

HSL at serine 565 by AMPK reduces HSL phosphorylation at serine 563 by PKA, inhibiting HSL activity (Anthonsen *et al.* 1998), while a number of other studies have shown an inhibitory effect of AMPK on adipocyte lipolysis (Daval *et al.* 2005, Anthony *et al.* 2009, Gaidhu *et al.* 2009). Consistent with the former, we noted decreased pAMPK, decreased pHSL (ser565), and increased pHSL (ser563) in *Vgf* knockout WAT. The anti-lipolytic role of AMPK was demonstrated using adipocytes from *Ampk α 1*-knockout mice, treatment of adipocytes with AMPK inhibitors, and transfection of dominant negative and constitutively active AMPK constructs into adipocytes (Daval *et al.* 2005). On the other hand, treatment of rodents with AMPK agonists is associated with

Figure 1 Body weight (BW) and gonadal WAT are reduced in *Vgf* knockout mice. BWs (g) of male and female *Vgf*-/*Vgf*- (KO), *Vgf*+/*Vgf*- (HET), and *Vgf*+/*Vgf*+ (WT) mice were measured at 9 weeks of age (A, B, and C); male and female *Vgf*-/*Vgf*- mice had significantly lower BW compared with *Vgf*+/*Vgf*- and *Vgf*+/*Vgf*+ mice. In D, gonadal fat pad weights (g) were significantly reduced in *Vgf*-/*Vgf*- ($n = 5$ males, 4 females), compared with *Vgf*+/*Vgf*- ($n = 6$ males, 7 females), and *Vgf*+/*Vgf*+ ($n = 9$ male, 7 females). No significant differences in BW, fat pad weight, or the ratio of fat pad weight to BW, between *Vgf*+/*Vgf*- and *Vgf*+/*Vgf*+ mice, were noted (A, B, C, D, E, F, G, H and I). BWs and epididymal and ovarian fat pad weights were measured for each genotype at 9–10 weeks of age; values are mean $\pm$ S.E.M., * $P = 0.0430$, ** $P = 0.0031$, *** $P < 0.0001$ compared with wild-type (WT) (ANOVA and Tukey's multiples comparison). Eight-week-old female mice (*Vgf*+/*Vgf*+ WT (24.6 g body weight), *Vgf*+/*Vgf*- HET (20.5 g), and *Vgf*-/*Vgf*- KO (13.6 g)) (P, Q and R respectively) were transcardially perfused with 4% paraformaldehyde in PBS, intraperitoneal fat depots were exposed (J indicated by arrows in, K and L), and the subcutaneous and visceral depots were dissected and placed on a mouse template to show their location in the body (M, N and O). Fat depots are labeled with lower case letters: a (subcutaneous anterior: deep and superficial cervical, interscapular, subscapular, axillo-thoracic); b (interscapular BAT); c (mesenteric); d (perirenal, periovarian, parametrial, perivesical); e (subcutaneous posterior: dorsolumbar, inguinal, gluteal) (Murano *et al.* 2009).

Figure 2 Altered expression of proteins involved in lipolysis in *Vgf* knockout (KO) compared with wild-type (WT) WAT. WAT tissues were collected from *Vgf*^{−/−} knockout mice ($n=11$ –13) and *Vgf*^{+/+} wild-type littermate controls ($n=9$ –11), and protein expression was determined using western blot analysis and Image J as described in the Materials and Methods section. Values are expressed as fold change in knockout relative to wild-type WAT, normalized to β -actin as a loading control, and statistical significance was determined using the two-tailed Student's *t*-test (* $P<0.05$). Levels of phospho-HSL (ser563) were significantly increased in *Vgf* KO WAT (A, ** $P=0.0030$), while phospho-HSL (ser660) levels were unchanged (B). Total HSL levels were also unchanged (see Table 1). Expression of ATGL (C, * $P=0.0312$), CGI-58 (D, ** $P=0.0074$), and phospho-perilipin (E, * $P=0.0317$) was significantly increased in *Vgf* knockout WAT.

leanness (Narkar *et al.* 2008), most likely through the inhibition of ACC2 activity, which leads to reduced CPT1 activity, increased long-chain fatty acid entry into mitochondria, and increased fatty acid oxidation, although the mechanism remains uncertain and likely differs with chronic and acute treatment (Hoehn *et al.* 2010). Targeted ablation of

ACC2 in mice has resulted in a marked reduction in whole body adiposity (Abu-Elheiga *et al.* 2001), increased fatty acid oxidation coupled with elevated energy expenditure (Choi *et al.* 2007), or no net effect on energy balance or adiposity (Hoehn *et al.* 2010), depending on the line analyzed. The latter study (Hoehn *et al.* 2010) suggests that chronically

Hsl (*Lipe*) and *Fas* mRNA levels and decreased *Acc* mRNA levels in knockout WAT (Watson *et al.* 2009) were not predictive of similar changes in protein levels (see Table 1). We previously found that increased mitochondrial number and UCP1 protein levels in *Vgf* knockout BAT were associated with decreased rather than increased *Ucp1* mRNA levels compared with wild-type (Watson *et al.* 2009). So although RNA levels often correlate with protein levels, greater understanding of the roles that specific gene products play in the regulation of metabolic flux ultimately relies on the determination of protein levels and activation state.

The VGF-derived peptide TLQP21 (VGF_{556–576}) robustly potentiates β -adrenergic receptor-induced lipolysis, increases sympathetic tone, increases energy expenditure, and prevents diet-induced obesity in mice (Bartolomucci *et al.* 2006, Possenti *et al.* 2012). This is somewhat paradoxical given the lean, hypermetabolic phenotype of germline *Vgf* knockout mice and our current and previous findings (Watson *et al.* 2005, 2009), which suggest increased sympathetic tone and lipolysis in these mice. Morphological alterations in WAT and BAT from *Vgf*-deficient mice, including decreased lipid accumulation in WAT, smaller interscapular WAT depots that are associated with BAT, and regions of fat accretion in BAT, and increased fatty acid oxidation, increased UCP1 and UCP2 protein levels, and increased mitochondrial number and cristae density in BAT are consistent with increased SNS activity (Watson *et al.* 2005, 2009). Individual VGF-derived peptides may therefore have opposing activities, much as has been shown previously for the pro-opiomelanocortin (POMC)-derived peptides, α -melanocyte stimulating hormone (α -MSH) and β -endorphin, which reduce and increase feeding respectively (Raffin-Sanson *et al.* 2003), while germline ablation of the *Pomc* gene in mice and humans leads to profound obesity (Yaswen *et al.* 1999). Moreover, increased feeding was found following intracranial administration of β -endorphin (Grandison & Guidotti 1977), and also following selective ablation of the β -endorphin coding sequence from the mouse *Pomc* gene (Appleyard *et al.* 2003), a similarly paradoxical situation to the increased lipolysis noted in TLQP21-treated and *Vgf* knockout mice. Ablation of VGF-derived peptides other than TLQP21, including the neuroendocrine regulatory peptide-2 that regulates feeding and energy expenditure via an orexin-dependent mechanism, could therefore be responsible for the observed germline *Vgf* knockout phenotype (Toshinai *et al.* 2010). Alternatively, germline ablation of VGF could result in developmental abnormalities in hypothalamic/sympathetic outflow pathways, or lack of this chromogranin- and secretogranin-like protein could impact dense core secretory vesicles structure and function, possibly altering catecholamine release from sympathetic terminals that innervate WAT, leading to chronic changes in β -adrenergic signaling. Additional experimentation using conditional knockout mice should allow these different hypotheses to be further tested.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S R S and C B designed the study; M S generated and genotyped the mice and assisted S F with sample preparation; S F, T S, and A C S carried out the protein analysis; S F, T S, C B, and S R S wrote the manuscript; all authors approved the final version of the manuscript.

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