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Article

Disruption of BCATm in Mice Leads to Increased Energy Expenditure Associated with the Activation of a Futile Protein Turnover Cycle

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SUMMARY

Leucine is recognized as a nutrient signal; however, the long-term in vivo consequences of leucine signaling and the role of branched-chain amino acid (BCAA) metabolism in this signaling remain unclear. To investigate these questions, we disrupted the *BCATm* gene, which encodes the enzyme catalyzing the first step in peripheral BCAA metabolism. *BCATm*$^{-/-}$ mice exhibited elevated plasma BCAAs and decreased adiposity and body weight, despite eating more food, along with increased energy expenditure, remarkable improvements in glucose and insulin tolerance, and protection from diet-induced obesity. The increased energy expenditure did not seem to be due to altered locomotor activity, uncoupling proteins, sympathetic activity, or thyroid hormones but was strongly associated with food consumption and an active futile cycle of increased protein degradation and synthesis. These observations suggest that elevated BCAAs and/or loss of BCAA catabolism in peripheral tissues play an important role in regulating insulin sensitivity and energy expenditure.

INTRODUCTION

Abundant food supplies and sedentary lifestyle contribute to the current epidemic of obesity in Western nations. Obesity results from a positive balance of energy intake versus expenditure—i.e., energy intake exceeds energy expenditure. Total energy expenditure consists of obligatory energy expenditure, physical activity, and adaptive thermogenesis (Lowell and Spiegelman, 2000). Adaptive thermogenesis is particularly influenced by environmental temperature and diet; the latter type is termed diet-induced thermogenesis (DIT). Despite extensive research, the pathogenesis of human obesity has not been fully elucidated, and the prevention and treatment of human obesity have proved difficult. Yet recent studies in humans suggest that increasing dietary protein intake may improve body weight control by poorly defined mechanisms that appear to involve both satiety and energy expenditure (Halton and Hu, 2004; Westerterp, 2004). In both short-term and relatively long-term studies, high-protein and low-fat diets have been shown to increase energy expenditure, while short-term protein intake induces satiety (Johnston et al., 2002; Leidy et al., 2007; Lejeune et al., 2006). After a fast in humans, whole-body nitrogen turnover and the thermic response to protein diet feeding were found to be significantly greater when compared with a high-carbohydrate meal (Robinson et al., 1990). This may be due to the fact that both protein synthesis and proteolysis are energy-demanding processes (Reeds et al., 1985). Thus, protein intake and metabolism positively affect energy expenditure.

The effects of dietary protein are thought to be mediated at least in part by the essential amino acid leucine (Leu), and perhaps by the other branched-chain amino acids (BCAAs). Leu is recognized as a nutrient signal and is an efficacious regulator of protein turnover through stimulating protein synthesis and inhibiting protein degradation (Buse and Reid, 1975; Fulks et al., 1975). Its stimulation of protein synthesis is linked to activation of a cell signaling pathway involving the mammalian target of rapamycin complex 1 (mTORC1) (Kimball and Jefferson, 2006). An in vitro study has shown that one or more metabolites of Leu catabolism inhibit proteolysis, while intracellular Leu, not Leu metabolites, regulates protein synthesis (Tischler et al., 1982). Like dietary protein, Leu has been linked to satiety, body weight control, and whole-body energy expenditure. For example, Leu has been reported to directly stimulate mTOR signaling in the hypothalamus, leading to decreased food intake (Cota et al., 2006). In addition, Leu may influence satiety by stimulating leptin secretion (Lynch et al., 2006). Dietary supplements of Leu or BCAAs have been shown to decrease fat mass and body weight

and to improve glucose metabolism in some cases (Bianchi et al., 2005; Donato et al., 2006; Gordon-Elliott and Margolese, 2006; Layman and Walker, 2006; Mourier et al., 1997; Zhang et al., 2007). These findings suggest that BCAA supplements may be beneficial in controlling obesity.

Paradoxically, other findings are not consistent with an antiobesity role of dietary Leu and Leu signaling. For example, hyperactivation of the TORC1 signaling pathway resulting from overnutrition, which includes excessive Leu intake, appears to worsen insulin resistance in obesity (Khamzina et al., 2005; Um et al., 2004, 2006). In addition, plasma BCAA concentrations are elevated in humans and animal models of obesity (Felig et al., 1969; Rafecas et al., 1991; Wijekoon et al., 2004). Thus, further research is needed to clarify the physiological role of Leu and its potential for protecting or worsening obesity.

In order to examine the effects of persistently elevated plasma Leu resulting from blockage of BCAA metabolism, we have generated and characterized mice in which the gene encoding the mitochondrial branched-chain aminotransferase isozyme (*BCAT2*) has been disrupted. This enzyme catalyzes the first step in BCAA metabolism, which is transfer of the α-amino group of a BCAA to α-ketoglutarate to form glutamate and the three respective branched-chain α-keto acids. BCATm is expressed in most nonneuronal tissues except liver, while the cytosolic isozyme (BCATc) is expressed in the central nervous system (CNS) and in peripheral nerves (Hutson et al., 1992; Suryawan et al., 1998; Sweatt et al., 2004). The expression pattern of BCAA catabolic enzymes in body tissues serves to regulate Leu signaling (Lynch et al., 2003) and to promote interorgan exchange of BCAA metabolites (Suryawan et al., 1998). Because peripheral BCAA catabolism is blocked in *BCATm*$^{-/-}$ mice, plasma BCAA concentrations are chronically elevated. These animals consume more food, exhibit increased DIT, and are lean when compared with wild-type mice. In addition, their protein turnover rate is elevated. We propose that increased protein synthesis and degradation contribute directly to increased energy expenditure in mice lacking peripheral BCAA metabolism.

RESULTS

Growth Curve, Food Intake, and Plasma Concentrations of Hormones, Amino Acids, and Other Metabolites

Targeting of the *BCATm* gene and generation of the conditional and total null alleles using the Cre-*loxP* system (Figures S1A–S1D) are described in the Supplemental Data available with this article online. As expected, BCATm protein was not detectable in skeletal muscle, kidney, heart, pancreas, brain, or adipose tissue of *BCATm*$^{-/-}$ mice (Figure 1A). Heterozygotes had approximately half as much BCATm protein in measured tissues (data not shown). Liver, which does not express BCATm, showed no detectable bands in either *BCATm*$^{+/+}$ or *BCATm*$^{-/-}$ mice (Figure 1A). BCATm activity was 628 ± 25, 291 ± 18, and −0.2 ± 1.5 mU/g tissue (n = 2) in gastrocnemius muscle of *BCATm*$^{+/+}$, *BCATm*$^{+/-}$, and *BCATm*$^{-/-}$ mice, respectively. Consistent with expression of BCATc exclusively in neurons (Sweatt et al., 2004), BCATc protein amount in brain was unaltered in *BCATm*$^{-/-}$ mice (Figure 1A), and immunohistochemistry showed no alteration in the pattern of BCATc expression in brain (data not shown).

BCATm$^{-/-}$ mice grew at the same rate as their littermate controls until ~6 weeks of age, when the growth curves diverged and the male *BCATm*$^{-/-}$ mice began to exhibit a 10%–15% lower body weight than control animals (Figure 1B). Because the extreme elevations in plasma BCAAs and their α-keto acids that are observed in maple syrup urine disease have severe neurological consequences (Chuang and Shih, 2001), we took advantage of the rodent's ability to discriminate between diets of differing amino acid composition (Harper and Peters, 1989) to prevent a toxic accumulation of BCAAs. Both the *BCATm*$^{-/-}$ and wild-type mice were provided access to normal chow (NC, Harlan 2018) diet and a purified amino acid BCAA-free diet (−BCAA, Dyets 510081). Male *BCATm*$^{-/-}$ mice preferred the −BCAA diet and consumed 76% of this diet and 24% of the NC diet, whereas the wild-type mice consumed 45% and 55%, respectively, of the −BCAA and NC diets (Figure 1C). Total food intake (Figure 1C) and calculated caloric intake (data not shown) did not differ, but when adjusted for body weight, food intake was greater in the *BCATm*$^{-/-}$ mice (Figure 1C). Male *BCATm*$^{-/-}$ mice had 55% lower epididymal fat pad weight (Figures 1D), and fat cell size was accordingly decreased compared to *BCATm*$^{+/+}$ animals (Figure 1E). Body composition determined by EchoMRI 3-in-1 showed that fat mass expressed as a percent of body weight was lower in the *BCATm*$^{-/-}$ mice (11.9% ± 0.3%) compared to the wild-type mice (18.3% ± 2.1%; $p < 0.01$, $n = 7$), whereas the percent of lean body mass was higher in the *BCATm*$^{-/-}$ mice (83.5% ± 0.3%) than in the wild-type mice (78.6% ± 2.1%; $p < 0.05$, $n = 7$ males). While the male *BCATm*$^{-/-}$ mice had somewhat enlarged kidneys, other tissues were normal for their body weight (Figure 1D). The female *BCATm*$^{-/-}$ mice were also lighter, but to a lesser extent than observed in the males (data not shown). Thus, the animals appeared healthy and lean.

Even though *BCATm*$^{-/-}$ mice consumed far less BCAAs, their fed plasma Leu, Ile, and Val levels were increased 14-, 21-, and 31-fold, respectively, in the male mice (Table 1) and 25-, 33-, and 37-fold, respectively, in the female mice (data not shown), consistent with disruption of BCATm, the predominant BCAT isozyme in tissues outside the CNS (Suryawan et al., 1998). Asp and Ala were decreased and Thr, Cit, and Arg were elevated in the null mice of both genders (Table 1 and data not shown). We also measured plasma BCAA transamination products, the branched-chain α-keto acids KIC, KMV, and KIV, for Leu, Ile, and Val, respectively. KIC did not differ (data not shown); KMV and KIV concentrations were lower in the female *BCATm*$^{-/-}$ mice (10.6 ± 0.9 μM for KMV; 6.1 ± 0.7 μM for KIV) than in the wild-type mice (17.6 ± 1.2 μM for KMV; 11.8 ± 0.9 μM for KIV; $p < 0.01$, $n = 7$). Plasma

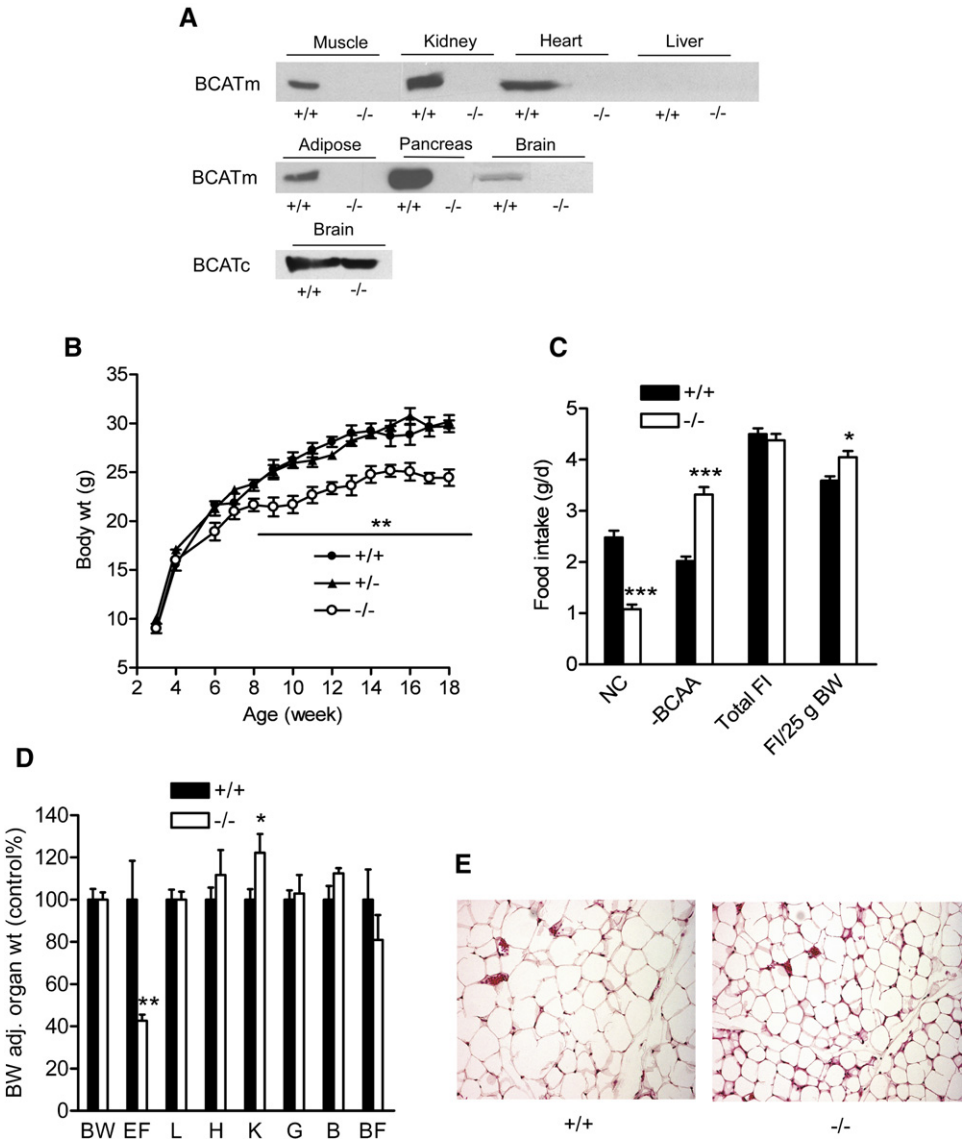

Figure 1. Growth Curve, Food Intake, Organ Weight, and Fat Cell Size

(A) Immunoblots of BCATm and BCATc in selected organs from wild-type and *BCATm* null mice. Equal amounts of protein (20 μg) were loaded in each lane of tissue samples examined.

(B) Growth curves of *BCATm* wild-type, heterozygous, and homozygous null mice. Male mice were weighed weekly. **$p < 0.01$; $n = 6–12$. In this and all other figures, error bars represent ± SEM.

(C) Food intake in $BCATm^{-/-}$ and wild-type mice. Mice were fed a choice of normal chow (NC) and purified amino acid BCAA-free (−BCAA) diets at weaning. Food intake (FI) was measured for 3 weeks at ~10 weeks of age and calculated as average daily values. It was also adjusted for body weight (FI/25 g BW). *$p < 0.05$, ***$p < 0.001$; $n = 8–10$.

(D) Relative organ weights in *BCATm* null and wild-type mice. Values are expressed as a percent of $BCATm^{+/+}$ mice and adjusted for body weight (BW). EF, epididymal fat; L, liver; H, heart; K, kidney; G, gastrocnemius muscle; B, brain; BF, brown fat. *$p < 0.05$, **$p < 0.001$; $n = 6$ male mice at ~16 weeks of age.

(E) H&E staining of paraformaldehyde-fixed sections of epididymal fat.

KMV and KIV also tended to be lower in the male $BCATm^{-/-}$ mice. The lower levels of branched-chain α-keto acids in the $BCATm^{-/-}$ mice are consistent with disrupted BCAA metabolism at the BCATm-catalyzed step.

Table 1 also shows plasma concentrations of relevant hormones and metabolites. Plasma leptin, adiponectin, and resistin were decreased 88%, 55%, and 34%, respectively, in the male $BCATm^{-/-}$ mice, whereas IGF-1 and PAI-1 were unaltered compared to the wild-type mice. Plasma adiponectin was also decreased by half in the female mice (data not shown). The lowered adiponectin is unexpected considering the lower adiposity of the $BCATm^{-/-}$ mice. Although fed glucose was unaltered, fasting blood glucose and plasma insulin were 31% and 65% lower,

Table 1. Plasma Concentrations of Hormones, Amino Acids, and Other Metabolites

	Nutritional State	+/+	−/−
Hormones			
Leptin (pg/ml)	6 hr fast	2309 ± 475	277 ± 62***
PAI-1 (pg/ml)	6 hr fast	805 ± 163	597 ± 150
Resistin (pg/ml)	6 hr fast	1970 ± 186	1247 ± 170*
Insulin (ng/ml)	6 hr fast	0.75 ± 0.17	0.26 ± 0.04*
Adiponectin (ng/ml)	overnight fast	10050 ± 1769	4500 ± 288**
Thyroxine (μg/ml)	fed	6.02 ± 0.40	6.25 ± 0.21
IGF-1 (ng/ml)	fed	429 ± 32	398 ± 53
Norepinephrine[a] (pg/ml)	fed	245 ± 52	121 ± 17*
Amino acids[b] (μM)			
Leu	fed	115.4 ± 9.0	1621 ± 361***
Ile	fed	57.1 ± 4.9	1236 ± 301***
Val	fed	139.0 ± 7.7	4243 ± 700***
Asp	fed	9.6 ± 1.3	5.7 ± 0.7*
Gly	fed	347.5 ± 55.6	522.9 ± 53.1*
Thr	fed	189.0 ± 26.0	328.4 ± 42.7**
Cit	fed	44.6 ± 1.9	74.7 ± 7.1**
Arg	fed	118.5 ± 12.4	244.6 ± 36.1**
β-Ala	fed	6.3 ± 0.5	3.5 ± 0.5***
Ala	fed	436.5 ± 44.7	245.4 ± 25.9**
Other metabolites			
Triglycerides (mg/dl)	6 hr fast	50 ± 5.5	41.7 ± 2.0
Cholesterol (mg/dl)	6 hr fast	116.0 ± 10.4	95.8 ± 10.0
Free fatty acids (μM)	overnight fast	1361 ± 152	814 ± 74**
β-hydroxybutyrate (mg/dl)	overnight fast	13.4 ± 1.8	6.9 ± 1.2**
Albumin (g/dl)	6 hr fast	2.4 ± 0.0	2.5 ± 0.1
Blood urea nitrogen (mg/dl)	fed	19.1 ± 1.1	16.8 ± 1.8
Creatinine (μM)	fed	118 ± 20	138 ± 25
Lactate (mM)	fed	13.9 ± 2.2	14.9 ± 1.6
Glucose (mg/dl)	fed	202 ± 10.8	195 ± 12.5
Glucose (mg/dl)	overnight fast	151.1 ± 17.8	104.1 ± 10.8

Male *BCATm*$^{-/-}$ and wild-type mice were fed a choice of normal chow and defined amino acid BCAA-free diets. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 6–10.
[a] Mice fed a choice of defined amino acid BCAA-containing and BCAA-free diets.
[b] Data for all other amino acids that were unaltered are not shown.

respectively, in the null mice. Fasting plasma concentrations of free fatty acids (FFAs) and β-hydroxybutyrate were 40% and 50% lower, respectively, in the *BCATm*$^{-/-}$ than in the *BCATm*$^{+/+}$ mice. Plasma concentrations of triglyceride, cholesterol, albumin, creatinine, urea nitrogen, and lactate were unaffected by the loss of *BCATm*.

Plasma hormones and metabolites measured in Table 1 were unaltered in *BCATm*$^{+/-}$ mice (data not shown). In addition, body weight (Figure 1B), food intake, body composition, and organ weights (data not shown) as well as glucose tolerance and insulin sensitivity (Figure 2) did not differ between the heterozygotes and the wild-types. Thus, a loss of ~50% of the BCATm in heterozygotes is insufficient to cause apparent metabolic alterations.

Improved Insulin Sensitivity, Glucose Tolerance, and Resistance to High-Fat-Diet-Induced Obesity in *BCATm* Null Mice

The leanness and lower fasted plasma glucose and insulin concentrations of the *BCATm*$^{-/-}$ mice prompted us to

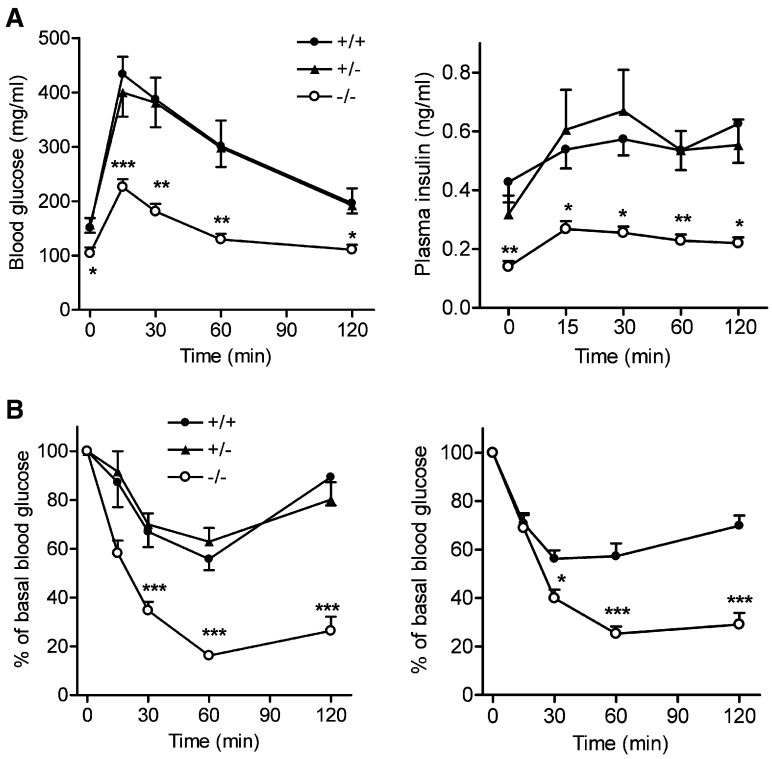

Figure 2. Glucose and Insulin Tolerance Tests in *BCATm* Null and Wild-Type Mice

(A) Blood glucose (left panel) and plasma insulin concentrations (right panel) during glucose tolerance test. *p < 0.05, **p < 0.01, ***p < 0.001; n = 7–8.

(B) Blood glucose expressed as a percent of basal level during insulin tolerance test. Left panel: ITT in male mice fed a choice of normal chow and −BCAA diets as described in Figure 1. ***p < 0.001; n = 7–9 for each group. Right panel: ITT in male mice fed a 60% fat-containing diet for 10 weeks as described in Figure 3. *p < 0.05, ***p < 0.001; n = 7–8.

examine glucose metabolism in these animals. After an overnight fast, blood glucose and plasma insulin were again decreased by 33% and 67%, respectively, compared to controls (Figure 2A, left and right panels). Calculated homeostasis model assessment of insulin resistance (HOMA-IR) index was lower in the null mice (0.95 ± 0.0.12) than in the wild-type mice (3.95 ± 0.38; p < 0.001, n = 8). During glucose tolerance test (GTT), blood glucose concentrations remained significantly lower (Figure 2A, left panel), and area under the curve was 51% less in the *BCATm*$^{-/-}$ mice compared to wild-type mice, suggesting markedly improved glucose tolerance. Their insulin response to glucose was much lower (Figure 2A, right panel), suggesting improved insulin sensitivity. Indeed, an insulin tolerance test (ITT) showed much greater decreases in blood glucose in response to insulin in both male (Figure 2B, left panel) and female *BCATm*$^{-/-}$ mice (data not shown) compared to wild-type mice.

We examined the effects of the knockout on diet-induced obesity (DIO) by feeding the mice a 60% fat diet for 15 weeks starting at 6–7 weeks of age (Figure 3). Whereas wild-type and *BCATm*$^{+/-}$ mice became obese on the high-fat diet (HFD), the *BCATm*$^{-/-}$ mice were totally protected from HFD-induced obesity (Figure 3A). MRI showed that both abdominal and subcutaneous fat deposition was much less in the null mice compared to the wild-type mice (Figure 3C). Importantly, food intake in the null mice was not decreased and was actually 30% greater when normalized for body weight (Figure 3B). ITT showed that null mice were protected from worsening of insulin sensitivity caused by HFD feeding (Figure 2B, right panel).

Enhanced Energy Expenditure Associated with Diet-Induced Thermogenesis in *BCATm* Null Mice

To determine the mechanism by which *BCATm*$^{-/-}$ mice are lean and resistant to DIO, we measured energy expenditure using indirect calorimetry (Figure 4). Compared to the wild-type mice, VO_2 was increased by 18.5% in the *BCATm*$^{-/-}$ mice fed the NC/−BCAA choice of diets (data not shown). To eliminate the possibility that the differences in diet composition and amino acid source (protein versus free amino acids) were influencing food choice, food intake, and VO_2, 12-week-old mice were switched from the NC/−BCAA choice to a choice between a BCAA-containing defined amino acid diet (+BCAA, Dyets 510090) and the −BCAA diet for 5 weeks. Unlike the NC/−BCAA choice, the +BCAA/−BCAA diets were isonitrogenous and isocaloric. Differences in VO_2 measured with mice fed the +BCAA/−BCAA choice of diets were greater than in animals fed the original NC/−BCAA choice of diets. VO_2 was 32% higher in the *BCATm*$^{-/-}$ mice compared to controls based on body weight (Figure 4A, left panel). Commercial calorimeters frequently normalize data to body weight, but it is unclear whether this is valid. However, even VO_2 per mouse was 20% higher in the null mice (114.1 ± 2.1 versus 94.9 ± 1.5 ml/mouse/hr in controls; n = 8, p < 0.001). Furthermore, when absolute energy expenditure was plotted against fat-free mass, different slopes emerged between the genotypes (Figure S2). Taken together, these results provide strong evidence for an effect of BCATm disruption on energy expenditure.

Respiratory quotient (RQ) during the light phase was elevated in the null mice, suggesting that the *BCATm*$^{-/-}$

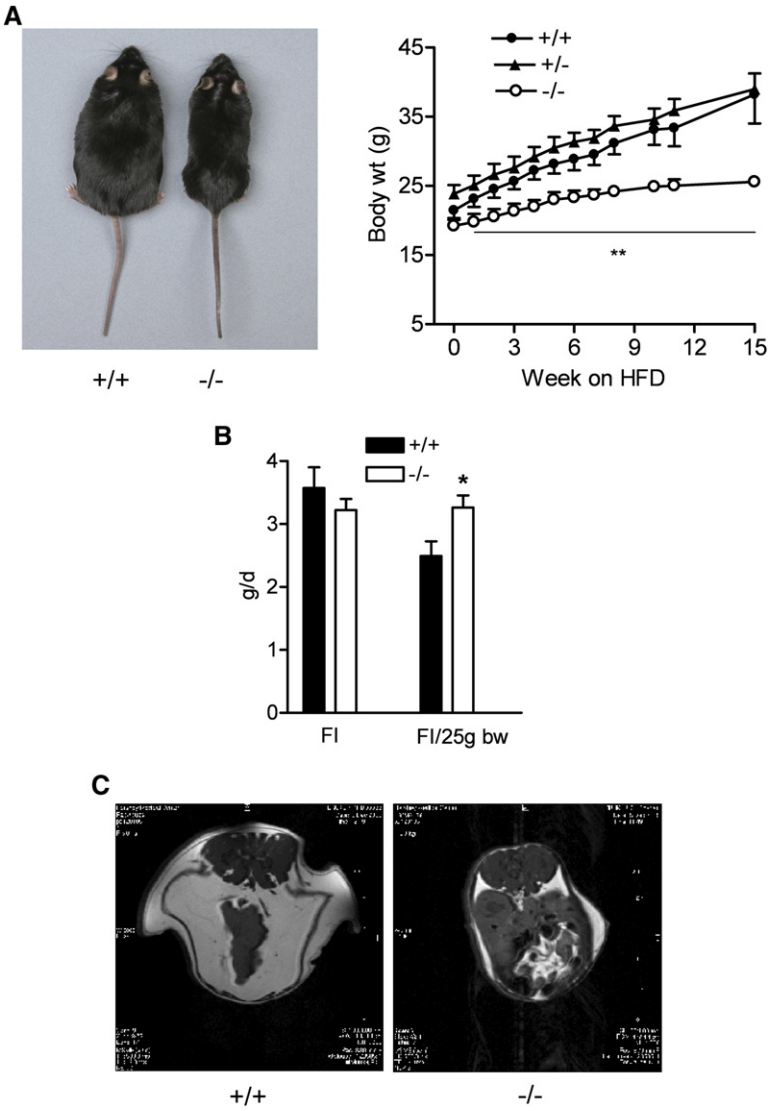

Figure 3. *BCATm* Null Mice Are Protected from High-Fat-Diet-Induced Obesity

(A) Growth curve (right panel) and representative picture of $BCATm^{+/+}$ and $BCATm^{-/-}$ mice after high-fat-diet feeding (left panel). **$p < 0.01$ for −/− versus +/+ males at each time point; n = 7–8 for each group.

(B) Food intake (FI) measured during high-fat-diet feeding. Food consumption was measured for 1 week, and average food intake was calculated and also normalized to body weight. *$p < 0.05$; n = 7–9.

(C) Representative MRI of mice after high-fat-diet feeding. Transverse images set at the same distance from the anus were taken for both mice. Abdominal and subcutaneous fat (shown as white) were separated by the peritoneal membrane.

mice used more carbohydrate as a fuel (Figure 4A, middle panel). As observed with the NC/−BCAA diet choice, $BCATm^{-/-}$ mice fed the +BCAA/−BCAA diet choice still preferred the −BCAA diet, while the wild-type mice mainly consumed the +BCAA diet (data not shown). Total food intake and body-weight-adjusted food intake were 12% and 22% greater in the $BCATm^{-/-}$ than in the $BCATm^{+/+}$ mice (Figure 4A, right panel). The body weight difference between the two groups of mice was 9.1% after 5 weeks on the +BCAA/−BCAA choice of diets. Epididymal fat pad weight was 48% less in the null mice (0.32 ± 0.03 versus 0.61 ± 0.05 g in wild-type mice; $p < 0.001$, n = 8). Thus, even when fed the choice of defined amino acid diets, the *BCATm* null mice still consumed more food, expended more energy, and were leaner than wild-type controls.

To further investigate the association between food intake and VO_2, we measured VO_2 during fasting and refeeding (Figure 4B, left panel). While VO_2 in the $BCATm^{-/-}$ mice during initial fasting (light phase) was 11% greater than that in the wild-type mice, it normalized during longer fasting (dark phase). A 3 hr refeeding led to a 31% elevation of VO_2 from the dark-phase level in the null mice, but only a 13% elevation in the wild-type mice. RQ during the light phase was lower in the $BCATm^{-/-}$ mice (Figure 4B, right panel), suggesting that the *BCATm* null can readily use fat as a fuel. These results suggest that increased energy expenditure in the $BCATm^{-/-}$ mice is strongly associated with food consumption.

No Major Alterations in Common Factors Regulating Bioenergetics in *BCATm* Null Mice

We sought to determine how energy expenditure is elevated in these null mice. Locomotor activities measured with the Opto-M3 activity meter were unaltered under most conditions in $BCATm^{-/-}$ mice (Figure 5A) and were even lower during refeeding (data not shown). At 2 p.m., rectal core temperature was unaltered, but it was 0.7°C higher in the $BCATm^{-/-}$ mice when measured at 9 p.m.

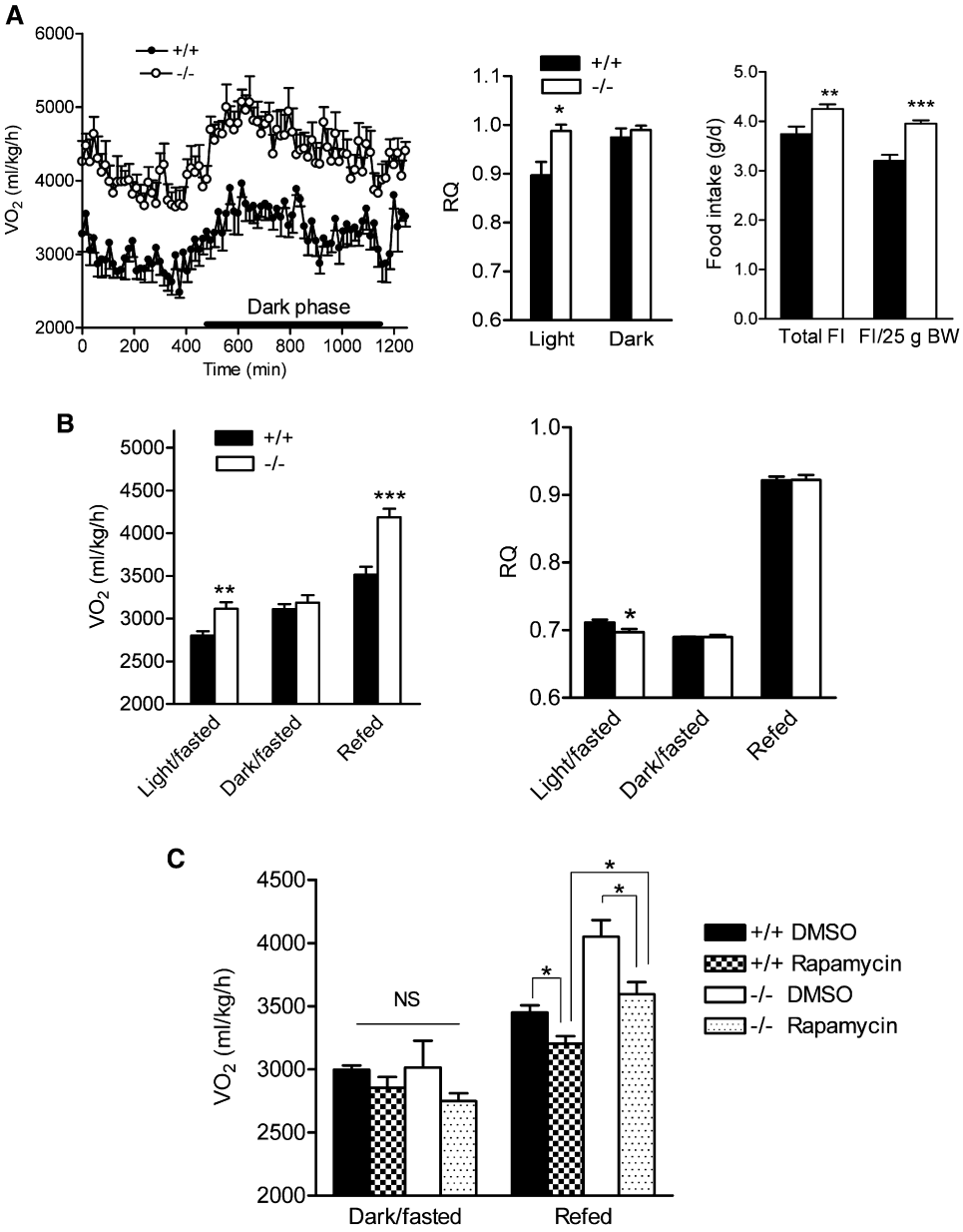

Figure 4. Elevated Energy Expenditure in *BCATm* Null Mice Is Associated with Food Consumption and Is Partially Blunted by Rapamycin

(A) Oxygen consumption (VO_2, left panel), respiratory quotient (RQ, middle panel), and food intake (FI, right panel). Male mice fed a choice of purified amino acid BCAA-containing (+BCAA) and BCAA-free diets for 4 weeks were placed in indirect calorimetry chambers at ~16 weeks of age. Food intake was measured for 3 weeks and calculated as average daily values. It was also adjusted for body weight (FI/25 g BW). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 8$.

(B) VO_2 (left panel) and RQ (right panel) during fasting and refeeding. Male mice fed a mix of NC and −BCAA diets were fasted for 21 hr and refed with a choice of NC and −BCAA diets. VO_2 and RQ were measured during fasting (light and dark phases) and a 3 hr refeeding period. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 6$.

(C) VO_2 after treatment with rapamycin during fasting and refeeding. Twelve- to fifteen-week-old mice were intraperitoneally injected with 0.75 mg/kg of rapamycin at 11 a.m., fasted for 21 hr, and injected again with the same dose of rapamycin. Food was provided 1 hr after the second injection for 3 hr. VO_2 was measured during the dark phase and refeeding. One-way ANOVA was used to compare VO_2 between groups under each nutritional condition. No difference was found between groups during fasting. *$p < 0.05$ between groups during refeeding; $n = 8–11$.

postprandially (Figure 5B), consistent with increased DIT in the null mice. Plasma thyroxin (total T4) did not differ between $BCATm^{-/-}$ and $BCATm^{+/+}$ mice (Table 1).

We measured *UCP* (uncoupling protein) mRNA and protein levels in various tissues (Figure 5C; Figure S3; data not shown). UCP1 is mainly expressed in brown

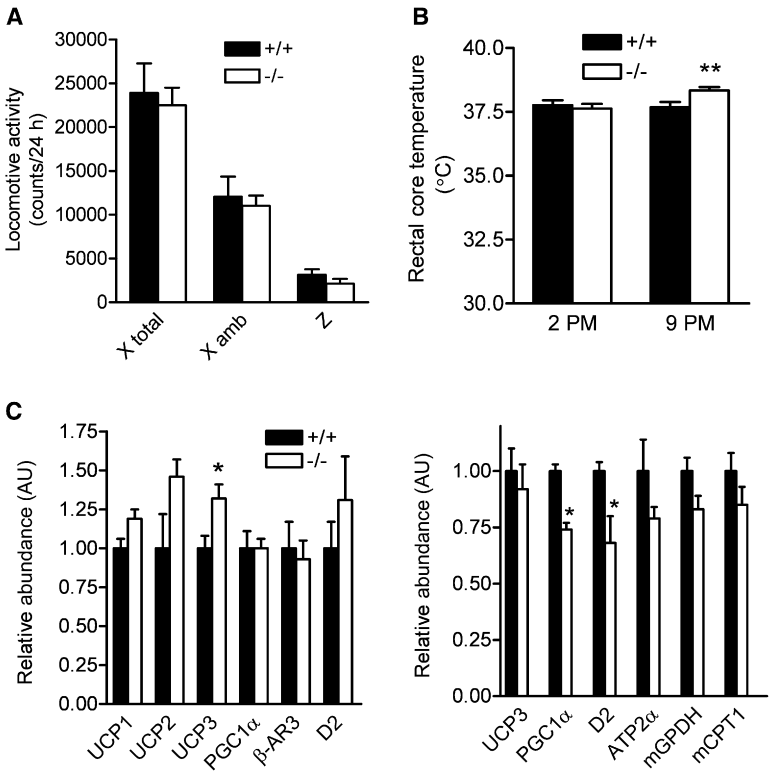

Figure 5. Common Factors Associated with Thermogenesis

(A and B) Locomotor activity measured in indirect calorimetry (A) and rectal core temperature (B) in *BCATm* null and wild-type mice fed a choice of NC and −BCAA diets. (A) $p > 0.05$, n = 8–10; (B) $^{**}p < 0.01$, n = 8.

(C) mRNA expression in arbitrary units (AU) of selected genes in brown fat (left panel) and gastrocnemius muscle (right panel) in mice fed a choice of +BCAA and −BCAA diets. $^{*}p < 0.05$, n = 8.

fat, and both *UCP1* mRNA and protein amounts were unaltered in this tissue of *BCATm*$^{-/-}$ mice. *UCP1* mRNA abundance in gastrocnemius was 1.00 ± 0.73 versus 15.83 ± 8.03 arbitrary units ($p > 0.05$, n = 8) for *BCATm* wild-type and null mice, respectively. Although *UCP1* mRNA in gastrocnemius muscle of some null mice was increased, it did not correlate with VO_2 (data not shown). Moreover, we could not detect UCP1 protein in gastrocnemius and white fat, suggesting very low levels of this protein in these tissues. UCP2 is ubiquitously expressed, and its protein expression was unaltered in gastrocnemius, epididymal fat, kidney, and liver of *BCATm*$^{-/-}$ mice (Figure S2). UCP3 is mainly expressed in skeletal muscle, and its mRNA and protein levels were unaltered in this tissue of the null mice. Since UCP2 and UCP3 are thought not to promote gross thermogenesis or energetic inefficiency (Brand and Esteves, 2005), a small increase in *UCP3* mRNA in brown fat is unlikely to be the major contributor to elevated energy expenditure in the *BCATm*$^{-/-}$ mice.

We also measured mRNA for other selective genes involved in the regulation of thermogenesis and mitochondrial biogenesis in *BCATm*$^{-/-}$ mice compared to wild-type mice (Figure 5C). *PGC-1α* (PPARγ coactivator 1α) mRNA was unaltered in brown fat and was actually 26% lower in gastrocnemius muscle of *BCATm*$^{-/-}$ mice. *β-AR3* (β3-adrenergic receptor, also *B3AR*) mRNA was unaltered in brown fat, and plasma norepinephrine concentrations were 51% lower (Table 1). *D2* (type 2 iodothyronine deiodinase) mRNA was unaltered in brown fat and was decreased by 32% in gastrocnemius muscle, suggesting that the conversion of T4 to active T3 is not upregulated in the null mice. mRNA levels of *mGPDH* (mitochondrial glycerol-3-phosphate dehydrogenase) (Lee and Lardy, 1965) and *mCPT-1* (mitochondrial carnitine palmitoyltransferase-1) were unaltered in white fat and skeletal muscle. *ATP2α1* (also called *SERCA1*) (Simonides et al., 2001) mRNA was also unaltered in skeletal muscle. Thus, physical activity, thyroid hormone, uncoupling proteins, adrenergic outflow, and futile cycling involving the glycerol phosphate shuttle or calcium release and reuptake do not appear to contribute to the elevated energy expenditure in the *BCATm*$^{-/-}$ mice.

Elevated Protein Turnover and mTOR Signaling in *BCATm* Null Mice

Leu is known to stimulate protein synthesis through rapamycin-sensitive and -insensitive mechanisms (Anthony et al., 2000). If protein synthesis were elevated in the *BCATm*$^{-/-}$ mice due to chronically high levels of plasma Leu with a concomitant increase in protein degradation (organ weights showed little change; Figure 2C), the increased energy expenditure observed in the *BCATm*$^{-/-}$ mice might be related to the energy demand associated with increased protein turnover (protein synthesis and degradation). Compared to wild-type mice, in vivo protein synthesis rates measured in *BCATm*$^{-/-}$ mice fed a choice of NC or −BCAA diets were elevated by 40%, 39%, 74%, and 40% in heart, skeletal muscle, epididymal fat, and kidney, respectively, and showed a trend of increase (22%) in liver (Figure 6A). To assess protein degradation, we measured urinary creatinine and 3-methylhistidine, an index of breakdown of myofibrillar

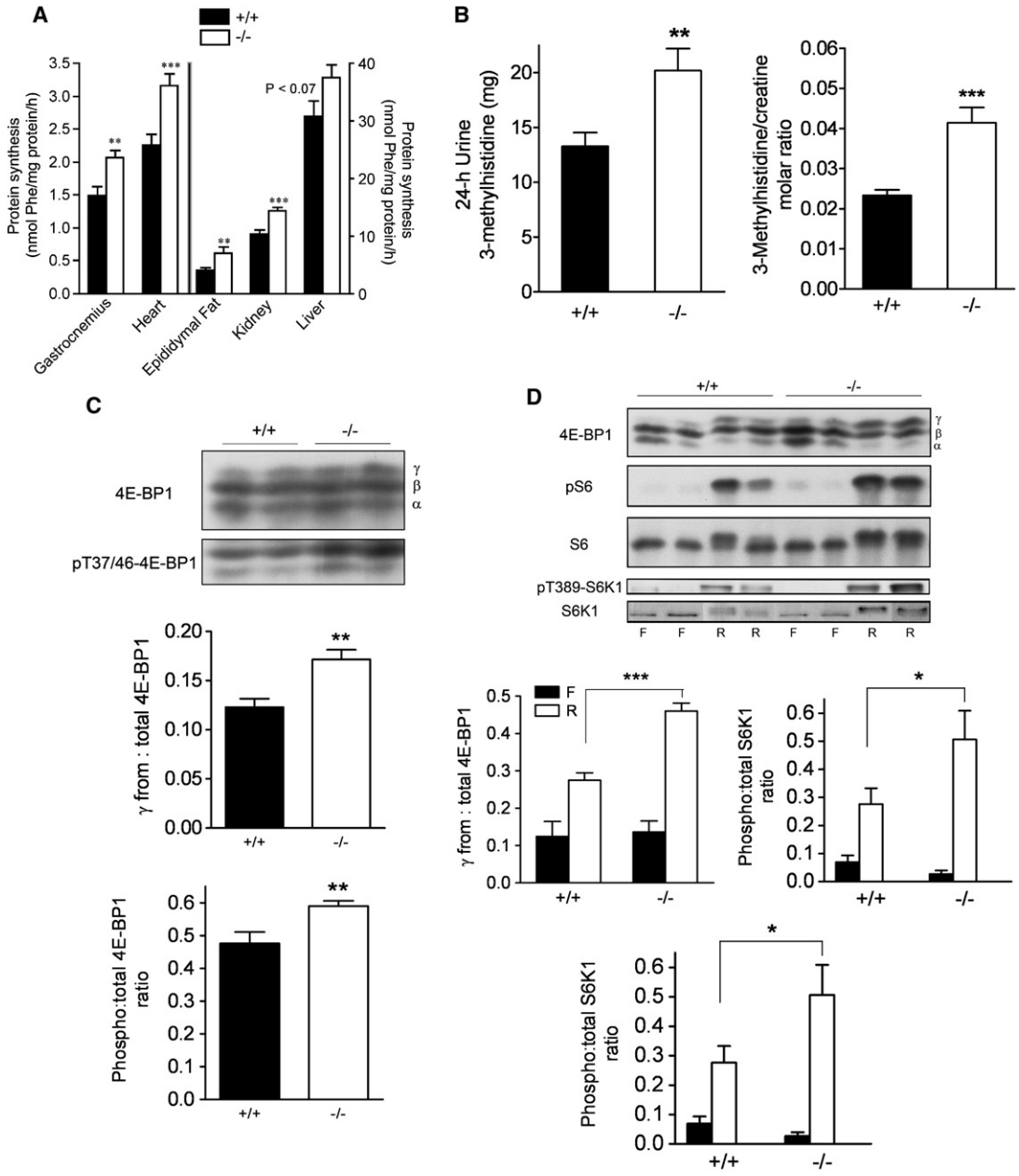

Figure 6. Elevated Protein Turnover In Vivo and mTOR Signaling in *BCATm* Null Mice

(A) In vivo protein synthesis rates measured by flooding does of L-[^{3}H]phenylalanine. **p < 0.01; n = 10–13. ***p < 0.001.

(B) Amount of 24 hr urinary 3-methylhistidine (left panel) and molar ratio of urine 3-methylhistidine to creatinine (right panel). Eight male mice from each genotype were placed in individual metabolic cages (Nalgene) for 2 days. Daily urine was collected for analysis of 3-methylhistidine and creatinine. **p < 0.01, ***p < 0.001; n = 16.

(C) Western blot analysis for 4E-BP1 and pT37/46 4E-BP1 in gastrocnemius of *BCATm*$^{-/-}$ mice fed a choice of NC and −BCAA diets. **p < 0.01; n = 8.

(D) Western blot analysis for 4E-BP1, pS235/236 S6, S6, pT389 S6K1, and S6K1 in gastrocnemius of fasted (F) and fasted-refed (R) *BCATm*$^{-/-}$ mice. *p < 0.05, **p < 0.01, ***p < 0.001; n = 4 for fasted, n = 8 for fasted-refed.

proteins (Young and Munro, 1978). While 24 hr urine volume was increased by 33% in the null mice, total urinary creatinine output was unaltered (Table S2). The 24 hr 3-methyhistidine level and urinary 3-methylhistidine/creatinine ratio were increased by 52% and 78%, respectively, in the *BCATm*$^{-/-}$ mice (Figure 6B), suggesting increased protein breakdown in muscle tissues. Unaltered skeletal muscle and heart weights as well as plasma and urinary creatinine excretion, indices of muscle mass, agree with the observation that protein synthesis and degradation are simultaneously elevated in mice lacking peripheral BCAA catabolism.

To investigate the mechanism by which protein synthesis is elevated in *BCATm*$^{-/-}$ mice, we examined mTOR signaling and factors involved in protein synthesis. In randomly fed *BCATm*$^{-/-}$ mice, total S6K1 protein levels were unaltered, and pT389 S6K1 was highly variable as measured in gastrocnemius and liver. Both the percent of 4E-BP1 in the γ form and the pT37/46 4E-BP1 to total 4E-BP1 ratio were significantly elevated in null gastrocnemius compared to wild-type gastrocnemius (Figure 6C). No significant changes were observed in the concentration of eIF2Bε, eIF4G, eIf4B, or eEF2 in gastrocnemius (data not shown).

In response to refeeding after a 21 hr fast, the ratios of pT389 S6K1 to total S6K1 and pS235/236 S6 to total S6 (a target of S6K1) as well as 4E-BP1 in the γ form were greater in the *BCATm*$^{-/-}$ mice compared to the wild-type mice (Figure 6D), suggesting greater mTOR activation during refeeding by BCATm disruption. However, no differences were observed in eIF2α phosphorylation, eIF2Bε levels and phosphorylation, or mTOR protein in muscle, heart, and liver between the null mice and control mice in the ad libitum fed or fasted-refed states (data not shown). Thus, as expected, increased TORC1 activity appears to be associated with elevated protein synthesis observed in the *BCATm*$^{-/-}$ mice.

Finally, to evaluate the role of protein synthesis in energy expenditure, we measured VO_2 in fasted-refed mice treated with an mTOR inhibitor, rapamycin, at a dose reported to largely abolish mTOR signaling (Anthony et al., 2000; Lynch et al., 2006) (Figure 4C). While VO_2 did not differ among the groups during longer fasting, it was partially blunted by rapamycin in both wild-type and *BCATm*$^{-/-}$ mice compared with vehicle-treated mice in response to refeeding, suggesting that elevated protein synthesis does contribute to thermogenesis during feeding. However, VO_2 during refeeding was still higher in rapamycin-treated *BCATm*$^{-/-}$ mice than wild-type mice, suggesting that factors other than mTOR may also lead to elevated energy expenditure in the *BCATm*$^{-/-}$ mice.

DISCUSSION

We have demonstrated in the present study that mice lacking BCATm-catalyzed BCAA metabolism exhibit high levels of plasma BCAAs without elevated branched-chain α-keto acids, resulting in a phenotype that includes low body fat and increased energy expenditure that is associated with increased food intake, glucose tolerance, insulin sensitivity, and protein turnover. Importantly, we found that VO_2 was strongly associated with food consumption in the *BCATm*$^{-/-}$ mice. During longer fasting, VO_2 differences between the null and wild-type mice disappeared, but they reappeared during refeeding. We posit that these feeding-related changes in VO_2 are partially related to elevated protein turnover in these animals. Indeed, fasting and feeding are well known to regulate both protein synthesis and energy utilization. Fasting inhibits protein synthesis and enhances protein degradation, whereas refeeding immediately stimulates protein synthesis due to elevated insulin and availability of amino acids (Yoshizawa et al., 1997). Feeding has short-term (i.e., after a meal) in addition to long-term (adaptive changes from overeating or underfeeding) effects on metabolic rates (Rolfe and Brown, 1997). Theoretical stoichiomteric calculation and in vitro experiments have suggested that the energy cost of pathways of nutrient metabolism greatly varies; and protein synthesis is most sensitive to energy supply (Buttgereit and Brand, 1995). Indeed, protein synthesis accounts for a minimum of 20% of calculated total daily heat production, while fatty-acid synthesis accounts for only 1% of total heat production in young growing animals (Reeds et al., 1982b). The association between increased energy expenditure and feeding in the *BCATm*$^{-/-}$ mice and the lack of increased locomotion suggest that the elevated VO_2 is associated with DIT, which includes protein turnover. Their increased food intake no doubt fuels this increased DIT. It is also conceivable that DIT associated with protein turnover may contribute to the decreased energy expenditure in leptin-deficient ob/ob mice and Zucker fatty rats in which protein synthesis, at least in muscle, is diminished (Reeds et al., 1982a).

On the other hand, we did not observe significant increases in factors frequently associated with altered energy expenditure, such as UCPs, PGC-1α, β-AR3, SERCA1, thyroid hormone, plasma norepinephrine, and locomotor activity, in the *BCATm*$^{-/-}$ mice. Moreover, leptin and adiponectin, two important fat-derived hormones known to significantly enhance energy expenditure, were greatly decreased in the *BCATm*$^{-/-}$ mice. PGC-1α is known to be the master regulator of glucose and lipid metabolism as well as mitochondrial function at the transcriptional and posttranslational levels (Handschin and Spiegelman, 2006). Moreover, cold exposure causes marked and rapid induction of PGC-1α expression in brown fat and skeletal muscle, thereby upregulating UCP1 and enhancing thermogenesis in these tissues (Lowell and Spiegelman, 2000). The role of UCP1 in maintaining normal body temperature has been demonstrated in *UCP1*$^{-/-}$ mice; however, they do not develop obesity and are paradoxically resistant to DIO (Enerback et al., 1997; Liu et al., 2003). Zhang et al. (2007) have reported that Leu supplementation increases energy expenditure and resistance to DIO due to upregulation of UCP3. However, in another recent study, overexpression of UCP3 did not increase energy expenditure in mice (Bezaire et al., 2005). We did not observe an increase in muscle UCP3 in our mice and have been unable to reproduce the Zhang et al. findings on DIO, energy expenditure, and insulin tolerance, even using a slightly higher concentration of Leu in the animals' drinking water (unpublished data). While PGC-1α and uncoupling proteins are important in regulating energy expenditure and weight control, alternative thermogenic mechanisms also exist (Lowell and Spiegelman, 2000; Rolfe and Brown, 1997), especially because little brown fat is present in large adult animals and humans living in a thermoneutral environment. Thus, it is highly likely that the elevated protein turnover directly contributes to

enhanced energy expenditure in mice lacking BCAA metabolism.

Others have proposed that sympathetic nerve activity through β-adrenergic receptors plays a major role in DIT, as demonstrated by β-less mice (lacking all three β-adrenergic receptors), which are prone to DIO (Lowell and Bachman, 2003). However, we found no difference in β-*AR3* mRNA expression in brown fat, and plasma norepinephrine was 50% lower in *BCATm*$^{-/-}$ mice. Moreover, we found that brain tyrosine was decreased by 87% in male and 66% in female *BCATm* null mice (unpublished data). Decreases in brain tyrosine could lead to decreased catecholamine concentrations in the nervous system and throughout the body. Mice lacking the ability to synthesize epinephrine and norepinephrine also have elevated energy expenditure and food intake and decreased body weight (Thomas and Palmiter, 1997).

The mechanisms for diet selectivity and elevated food intake in the *BCATm* null mice are unknown. Seeley and coworkers (Cota et al., 2006) have shown that direct injection of high concentrations of Leu into the feeding center of the hypothalamus results in cessation of feeding. In the *BCATm*$^{-/-}$ mice, chronically high levels of BCAAs do not impair food intake. The lowered plasma leptin in the null animals could contribute to their increased food intake; however, it remains to be determined whether neurotransmitter pathways affect food intake and energy expenditure in these mice. On the other hand, the lack of apparent neurological consequences of pathologic levels of plasma BCAAs in the *BCATm*$^{-/-}$ mice agree with studies suggesting that branched-chain α-keto acids, rather than BCAAs, are the toxic metabolites in maple syrup urine disease (Jouvet et al., 2000). Because elevations in brain BCAA concentrations were modest (data not shown), the results suggest that BCATc can handle the increased BCAA supply in the CNS of these mice.

Our finding of elevated protein turnover in mice lacking BCAA catabolism raises important questions. What are the mechanisms for elevated protein synthesis and degradation in these mice? We found that mTOR signaling (i.e., 4E-BP1 and S6K1 activation) was elevated in vivo in randomly fed *BCATm*$^{-/-}$ mice and/or during fasting and refeeding. eIF4E dissociated from hyperphosphorylated 4E-BP1 binds to eIF4G and hence forms an eIF4F complex, thereby promoting protein synthesis through a cap-dependent translation initiation mechanism. Other unidentified mechanisms that increase global protein synthesis in these mice could also exist. The mechanisms regulating global protein degradation as occurs in catabolic diseases are not as well understood as protein synthesis. Thus, *BCATm*$^{-/-}$ mice may provide a useful model to explore such regulation. We hypothesize that lack of BCAA catabolism elevates intracellular Leu concentrations, thereby driving the increase in protein synthesis, while a deficiency of certain metabolites of BCAA catabolism leads to elevated protein degradation in mice lacking BCATm. This is in agreement with a study showing that KIC, but not Leu, infusions significantly lowered negative nitrogen balance and 3-methylhistidine excretion in postoperative patients (Sapir et al., 1983). Similarly, it has been reported that KIC, but not Leu, decreases the nitrogen wasting of starvation (Mitch et al., 1981).

While the mechanism or mechanisms underlying the markedly improved insulin sensitivity and glucose tolerance remain undetermined, increased insulin sensitivity can contribute to elevated protein synthesis in these mice. Because enhanced protein turnover consumes a large amount of ATP, it is conceivable that ATP production from substrate oxidation in mitochondria could be elevated. Indeed, we have found that the mitochondrial membrane potential is significantly increased in cultured primary fibroblasts from *BCATm* null neonates (unpublished data). This is also inconsistent with rises in UCP levels and mitochondrial uncoupling. Furthermore, enhanced insulin sensitivity in these mice could lead to increased mitochondrial oxidative capacity. It has been reported that insulin stimulates mitochondrial oxidative phosphorylation in skeletal muscle associated with synthesis of mitochondrial gene transcripts and protein in human subjects (Stump et al., 2003). While it seems counterintuitive that elevated mTOR signaling would be associated with improved insulin signaling, we have found that Leu- and KIC-stimulated, but not insulin-stimulated, phosphorylation of S6K1 is largely abolished in isolated fat cells, cultured primary fibroblasts, and perfused hearts lacking BCATm (unpublished data). While further studies are needed to determine the mechanism of these changes, the increased insulin sensitivity in *BCATm*$^{-/-}$ mice is consistent with findings in *S6K1*$^{-/-}$ mice (Um et al., 2004).

In summary, we have clearly demonstrated that deletion of *BCATm* knockout leads to activation of a futile protein turnover cycle that is associated with elevated energy expenditure and improved insulin sensitivity. Since BCAA metabolism is blocked in *BCATm*$^{-/-}$ mice, the effects of *BCATm* gene disruption may not be the same as those of high-protein diets and dietary BCAA supplements. Nevertheless, given that humans and animals can tolerate much higher doses of BCAA supplements (Baker, 2005; Fernstrom, 2005), our study suggests that BCATm may be a suitable peripheral therapeutic target for obesity.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine. Animals were given free access to water and offered a choice of standard rodent chow (Harlan Teklad 2018) with protein as 18% percent of total weight and a defined amino acid BCAA-free diet (Dyets Inc.) with amino acids as 17% percent of total weight. Subsequently, the rodent chow was replaced with a choice of a defined amino acid diet with 17% amino acids including BCAA and a defined amino acid BCAA-free diet. The BCAA concentration of the defined diet was 43%, 4%, and 14% less for Leu, Ile, and Val, respectively, compared to the standard chow diet. Extra glutamate was added to the −BCAA diet to make it isonitrogenous to the control defined amino acid BCAA-containing diet. These defined amino acid diets contained amounts of carbohydrate, fat, vitamins, and minerals similar to standard mouse chow. For DIO, ~5-week-old wild-type

and *BCATm* knockout mice were fed a 60% high-fat diet (Research Diets D12492) for 15 weeks.

Insulin Sensitivity and Glucose Tolerance Tests

ITT and GTT were performed in 6 hr and overnight food-deprived mice, respectively. Mice were injected intraperitoneally with insulin (human insulin, Eli Lilly) at 0.75 mU/g body weight or 20% glucose at 2.0 mg/g body weight, and blood glucose was measured at 15, 30, 60, and 120 min after injection. Approximately 20 μl of blood was collected from the tail at each time point during GTT for measuring plasma insulin.

Energy Expenditure, Activity, and Core Temperature

Energy expenditure was assessed using indirect calorimetry (Oxymax, Columbus Instruments). Constant airflow (0.6 l/min) was drawn through the chamber and monitored by a mass-sensitive flow meter. The concentrations of oxygen and carbon dioxide were monitored at the inlet and outlet of the sealed chambers to calculate oxygen consumption and RQ. Each chamber was measured for 1 min at 15 min intervals. Physical activity was measured using infrared technology (OPT-M3, Columbus Instruments). The counts of three-dimensional beam breaking (X total, X ambulatory, and Z) were measured. Rectal core temperature was measured using a Fluke 51II thermometer with a mouse thermocouple probe (Harvard Apparatus).

Body Composition

MRI scans were taken starting at the lungs and ending at the hips of mice using a 7T (300 MHz) MRI magnet with a 20 cm bore (BioSpec 70/20as, Bruker Instruments). The pulse sequence used had a TR/TE = 500/12, 2 averages, 1 echo, 256 × 256 matrix, 4.3 cm^2 FOV, 1 mm slice thickness, and 1 mm slice distance, with a total scan time of 4 min for each mouse. Body fat and lean body mass were also measured using a qNMR system (Echo Medical Systems).

Protein Synthesis and Degradation

Rates of protein synthesis in ad libitum-fed mice were measured using the flooding-dose method to measure the incorporation of radioactive phenylalanine into protein as previously described (Lynch et al., 2002). Briefly, mice were injected intraperitoneally with L-[^{3}H]phenylalanine (150 mM, 30 μCi/ml, 1 ml/100 g body weight). Fifteen minutes after injection of the radioisotope, mice were decapitated, and blood and tissue samples were collected. Plasma phenylalanine concentrations were determined by HPLC analysis of supernatants from TCA extracts of plasma. The radioactivity in the phenylalanine peak was measured to calculate plasma specific activity of [^{3}H]phenylalanine. Frozen powdered tissue was homogenized in ice-cold 3.6% PCA and centrifuged. The supernatant was decanted, and the pellets were dissolved in 0.1 M NaOH after washing with 3.6% PCA, acetone, a mixture of chloroform-methanol, and water. Aliquots were used for assays of protein and radioactivity. Urine 3-methylhistidine was measured by Scientific Research Consortium, Inc. (St. Paul, MN, USA) using the method of Moore et al. (1958), which employs postcolumn derivatization by ninhydrin.

Real-Time Quantitative RT-PCR

Tissue total RNA was isolated using combined reagents of TRIzol (Invitrogen) and an RNeasy kit (QIAGEN). First-strand cDNA was synthesized from 1.0 μg of total RNA using the SuperScript III reverse transcription kit (Invitrogen). Quantitative RT-PCR was performed on an ABI 7900HT Sequence Detection System using the appropriate primers and probes and TaqMan Universal PCR Master Mix (Applied Biosystems). The primers for individual genes were ordered from Applied Biosystems. ABI SDS 2.2.2 software and the $2^{\Delta\Delta Ct}$ analysis method were used to quantify relative amounts of product using β-actin as an endogenous control.

Western Blot Analysis

Standard procedures were used as described previously (Lynch et al., 2002). Briefly, aliquots of frozen powdered tissues were homogenized on ice in 7 or 3 (adipose tissue) volumes of a phosphopreserving homogenization buffer. Equal amounts of protein were loaded for electrophoresis and transferred to PVDF membranes. The membranes were then probed with antibodies against S6K1, 4E-BP1 (Bethyl Laboratories, Inc.), pT389 S6K1, S6, pS235/236 S6, or pT37/46 4E-BP1 (Cell Signaling). For detection of BCATm, affinity-purified BCATm antibodies were used as described (Suryawan et al., 1998).

Analytical Procedures

Plasma concentrations of glucose, triglyceride, cholesterol, urea, albumin, creatinine, and lactate were measured using a Vitros Chemistry Analyzer (Ortho-Clinical Diagnosis). Plasma insulin (Linco Research, Inc.) and thyroxine (Alpha Diagnostic International) were measured using ELISA kits. Plasma adiponectin was measured using an RIA kit (Linco Research, Inc.). Plasma concentrations of FFA (Wako Pure Chemical Industries) and β-hydroxybutyrate (Stanbio Laboratory) were measured using commercial kits. Plasma leptin, PAI-1, and resistin were measured using a LINCOplex panel (Linco Research, Inc.). Plasma norepinephrine was measured by HPLC with electrochemical detection (CoulArray system, ESA). A one-step ultrafiltration method was used as described previously (Ueyama et al., 2003). Samples (10 μl) were injected into a 15 cm column with a 3 mm bore and 3 μm C-18 packing (ESA MD-150). Plasma amino acids and branched-chain α-keto acids were measured using fluorometric HPLC methods. Separation of the o-phthaldialdehyde amino acid derivatives was performed by gradient elution from a SUPELCOSIL LC-18 column (15cm × 4.6 mm, 3μm; Sigma) (Wu and Knabe, 1994). Plasma α-keto acids were derivatized with o-phenylenediamine, and separation was performed by gradient elution from a Spherisorb ODS2 column (250 mm × 4.6 mm, 5μm; Waters) (Pailla et al., 2000). Total plasma BCAA concentrations were also measured by an enzymatic method (Beckett, 2000). BCAT activity was measured as described previously (Hutson et al., 1988).

Statistical Analysis

A two-tailed nonpaired t test was used to assess differences between *BCATm*$^{-/-}$ and wild-type mice. Values are presented as means ± SEM; $p < 0.05$ was considered significantly different.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three figures, and two tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/6/3/181/DC1/.

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