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Restoration of metabolic health by decreased consumption of branched-chain amino acids

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Key points

- We recently found that feeding healthy mice a diet with reduced levels of branched-chain amino acids (BCAAs), which are associated with insulin resistance in both humans and rodents, modestly improves glucose tolerance and slows fat mass gain.
- In the present study, we show that a reduced BCAA diet promotes rapid fat mass loss without calorie restriction in obese mice.
- Selective reduction of dietary BCAAs also restores glucose tolerance and insulin sensitivity to obese mice, even as they continue to consume a high-fat, high-sugar diet.
- A low BCAA diet transiently induces FGF21 (fibroblast growth factor 21) and increases energy expenditure.
- We suggest that dietary protein quality (i.e. the precise macronutrient composition of dietary protein) may impact the effectiveness of weight loss diets.

Abstract Obesity and diabetes are increasing problems around the world, and although even moderate weight loss can improve metabolic health, reduced calorie diets are notoriously difficult to sustain. Branched-chain amino acids (BCAAs; leucine, isoleucine and valine) are elevated in the blood of obese, insulin-resistant humans and rodents. We recently demonstrated that specifically reducing dietary levels of BCAAs has beneficial effects on the metabolic health of young, growing mice, improving glucose tolerance and modestly slowing fat mass gain. In the present study, we examine the hypothesis that reducing dietary BCAAs will promote weight loss, reduce adiposity, and improve blood glucose control in diet-induced obese mice with pre-existing metabolic syndrome. We find that specifically reducing dietary BCAAs rapidly reverses diet-induced obesity and improves glucoregulatory control in diet-induced obese mice. Most dramatically, mice eating an otherwise unhealthy high-calorie, high-sugar Western diet with reduced levels of BCAAs lost

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weight and fat mass rapidly until regaining a normal weight. Importantly, this normalization of weight was mediated not by caloric restriction or increased activity, but by increased energy expenditure, and was accompanied by a transient induction of the energy balance regulating hormone FGF21 (fibroblast growth factor 21). Consumption of a Western diet reduced in BCAAs was also accompanied by a dramatic improvement in glucose tolerance and insulin resistance. Our results link dietary BCAAs with the regulation of metabolic health and energy balance in obese animals, and suggest that specifically reducing dietary BCAAs may represent a highly translatable option for the treatment of obesity and insulin resistance.

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Introduction

Over the last four decades, the prevalence of obesity has increased dramatically. In the USA, more than two in three adults are now considered to be overweight or obese (Flegal *et al.* 2012). A similar proportion of males in the European Union are likewise afflicted (Janda *et al.* 2013). Obesity is associated with an increased risk of many diseases, most notably type 2 diabetes, which is also increasing around the world. Although weight loss is a highly effective means of improving metabolic health, reduced calorie diets are notoriously difficult to sustain. Altering the macronutrient composition of the diet while keeping the total number of calories constant is an intriguing alternative that may be more sustainable (Fontana & Partridge, 2015).

Several recent studies have found that high protein consumption is correlated with insulin resistance, diabetes, and increased mortality in both mice and humans (Lagiou *et al.* 2007; Sluijs *et al.* 2010; Solon-Biet *et al.* 2014). Conversely, low protein (LP) diets are associated with metabolic health and increased survival (Levine *et al.* 2014; Solon-Biet *et al.* 2014; Simpson *et al.* 2017), and a recent randomized controlled trial found that a LP diet promotes leanness and decreases fasting blood glucose in humans (Fontana *et al.* 2016). An LP diet also promotes metabolic health in rodents, reducing the accumulation of white adipose tissue (WAT) and increasing glucose tolerance and insulin sensitivity in animals fed a normal diet, as well as improving glucose homeostasis in mice fed a high-fat diet (Laeger *et al.* 2014; Solon-Biet *et al.* 2015; Fontana *et al.* 2016; Maida *et al.* 2016).

We hypothesized that the beneficial effects of a LP diet might be driven by reduced consumption of specific essential amino acids. We focused on the branched-chain amino acids (BCAAs; leucine, isoleucine and valine) as blood levels of BCAAs correlate with insulin-resistant obesity and diabetes in humans and rodents (Felig *et al.* 1969; Newgard *et al.* 2009; Batch *et al.* 2013; Lynch & Adams, 2014; Connelly *et al.* 2017). BCAA levels also correlate well with outcomes in weight loss regimens (Shah *et al.* 2012), and are reduced in both mice and humans consuming LP diets (Solon-Biet *et al.* 2014; Fontana *et al.* 2016). We determined that specifically reducing dietary BCAAs by two-thirds recapitulates many beneficial effects of a LP diet, promoting leanness and glucose tolerance in metabolically normal C57BL/6J mice (Fontana *et al.* 2016). Reducing dietary BCAAs from a young age also slows the accumulation of visceral adipose tissue in Zucker-fatty rats and preserves insulin sensitivity (White *et al.* 2016).

These results led us to hypothesize that specifically reducing dietary BCAAs might not only preserve the metabolic health of young animals, but also might be an effective strategy to restore metabolic health to animals with pre-existing diet-induced metabolic dysfunction. Herein, we test if specifically reducing dietary BCAAs can restore metabolic health to C57BL/6J mice preconditioned with a high-calorie, high-fat, high-sugar Western diet (WD), a well characterized model of diet-induced obesity (DIO) and early type 2 diabetes (Winzell & Ahren, 2004; Newberry *et al.* 2006; Peterson *et al.* 2011; Williams *et al.* 2014). We find that specifically reducing dietary BCAAs is sufficient to promote weight normalization without caloric restriction in less than 4 weeks, primarily as a result of a dramatic reduction in fat mass, and improves metabolic health. Reduction of dietary BCAAs in the context of an otherwise WD transiently induces the energy balance regulating hormone FGF21 (fibroblast growth factor 21) and induces a sustained increase in energy expenditure. Our results suggest that specifically reducing dietary BCAAs, or treatment with pharmaceuticals that mimic this effect, could be an effective and translatable intervention for promoting weight normalization, control of blood glucose, and overall metabolic health.

Methods

Ethical approval and animals

All procedures conformed with institutional guidelines and were approved by the Institutional Animal Care and

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Use Committee of the William S. Middleton Memorial Veterans Hospital (Madison, WI, USA). Animals were killed using methods consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The research complies with the policies of *The Journal of Physiology* (Grundy, 2015).

Animals and diets

Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 5 weeks of age, and preconditioned with WD (TD.88137; Envigo, Madison, WI, USA) starting at 6 weeks of age for 12 weeks; chow control mice were fed Purina 5001 (Purina Mills, Richmond, IN, USA). Mice were then switched to amino acid defined diets or a WD supplemented with additional BCAAs; all diets were obtained from Envigo and diet descriptions, compositions and item numbers are provided in Tables 1–3. Mice were housed in a SPF mouse facility under a 12:12 h light/dark cycle with free access to food and water, except where noted in the procedures below. Animals were group housed in static microisolator cages, except when temporarily housed in a Oxymax/CLAMS metabolic chamber system (Columbus Instruments, Columbus, OH, USA). Group sizes are provided as appropriate. The randomization of obese, WD fed mice was performed at the cage level to ensure that all groups had approximately the same initial starting weight.

Procedures

Glucose tolerance tests were performed by fasting the mice overnight for 16 h and then injecting glucose (1 g kg^{-1}) I.P. (Arriola Apelo *et al.* 2016; Fontana *et al.* 2016). Insulin tolerance tests were performed by fasting mice for 4 h starting at lights on and then injecting insulin (0.75 U kg^{-1}) I.P. Glucose measurements were taken using a Bayer Contour blood glucose meter (Bayer, Leverkusen, Germany) and test strips. Blood for fasting insulin and FGF21 was obtained following an overnight fast; insulin (Crystal Chem, Elk Grove Village, IL, USA) and FGF21 (R&D Systems, Minneapolis, MN, USA) levels were determined by an enzyme-linked immunosorbent assay. Mouse body composition was determined using an EchoMRI 3-in-1 Body Composition Analyser (EchoMRI, Houston, TX, USA). For assay of multiple metabolic parameters [O_2, CO_2, food consumption, respiratory exchange ratio (RER), energy expenditure] and activity tracking, mice were acclimated to housing in a Oxymax/CLAMS metabolic chamber system (Columbus Instruments) for ∼24 h and data from a continuous 24 h period was then recorded and analysed. Triglycerides were measured by Triglyceride Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) in liver collected after fasting for ∼16 h. Other tissues for molecular analysis were flash-frozen in liquid nitrogen or fixed and prepared as described below.

Histology

Samples of brown adipose tissue (BAT), WAT, liver and skin were isolated following death. Adipose was fixed in 4% paraformaldehyde overnight, and then sectioned and haematoxylin and eosin (H&E) stained by the University of Wisconsin Carbone Cancer Center (UWCCC) Experimental Pathology Laboratory. Liver was embedded in OCT, and then cryosectioned and Oil-Red-O stained by the UWCCC Experimental Pathology Laboratory. Liver, BAT and WAT sections were imaged using an EVOS microscope (Thermo Fisher Scientific Inc., Waltham, MA, USA) as described previously (Linnemann *et al.* 2015). Skin was isolated from the belly and back of mice, paraformaldehyde-fixed (4%) overnight, and then paraffin-embedded for evaluation (Kasza *et al.* 2014). Scale bars were inserted automatically or manually by the investigator. For quantification of lipid droplet size, six independent fields were obtained for each tissue from each mouse and quantified using ImageJ (NIH, Bethesda, MD, USA).

Quantitative PCR

Liver or adipose RNA was extracted with Trireagent (Sigma, St Louis, MO, USA). Then, 1 μg of RNA was used to generate cDNA (Superscript III; Invitrogen, Carlsbad, CA, USA). Oligo dT primers and primers for real-time PCR were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA). Reactions were run on an StepOne Plus machine (Applied Biosystems, Foster City, CA, USA) with Sybr Green PCR Master Mix (Invitrogen). Actin was used to normalize the results from gene-specific reactions. The primer sequences used for quantitative PCR are as described previously (Hagiwara *et al.* 2012; Fontana *et al.* 2016) or were designed using the IDT quantitative PCR design tool and were: *Acc1*: F: AAGGCTATGTGAAGGATG, R: CTGTCTGAAGAGGTTAGG; *Acl*: F: GCCAGCGGGAG CACATC, R: CTTTGCAGGTGCCACTTCATC; *Actb*: F: ACCTTCTACAATGAGCTGCG, R: CTGGATGGCTACG TACATGG; *Bmp8*: F: TCAACACAACCCTCCACATCA, R: AGATCGGAGCGTCTGAAGATC; *Dgat1*: F: TGGT GTGTGGTGATGCTGATC, R: GCCAGGCGCTTCTCAA; *Dgat2*: F: AGTGGCAATGCTATCATCATCAT, R: TCTTC TGGACCCATCGGCCCCAGGA; *Fasn*: F: CCCCTCTG TTAATTGGCTCC, R: TTGTGGAAGTGCAGGTTAGG; *Fgf21*: F: CAAATCCTGGGTGTCAAAGC, R: CATGGGC TTCAGACTGGTAC; *Gpat*: F: CAACACCATCCCCGA CATC, R: GTGACCTTCGATTATGCGATCA; *Pparg*: F:

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Table 1. Diet composition of diets used to investigate the effect of reduced levels of dietary BCAAs in the context of a normal calorie diet

Amino acid defined diets	Control AA	ExLow AA	ExLow BCAA	Western (WD)	WD + BCAA
Teklad diet number	TD.140711	TD.140918	TD.150387	TD.88137	TD.150386
Colour	Red	Orange	Blue	Tan	Green
Formula (g kg^{-1})					
L-Alanine	9.38	2.18	12.1566	–	–
L-Arginine	6.3	1.46	6.3	–	–
L-Asparagine	20.58	4.79	22.6388	–	–
L-Aspartic acid	20.58	4.79	24.729	–	–
L-Cysteine	7.2	1.67	7.2	–	–
L-Glutamic acid	28.97	6.74	33.5548	–	–
L-Glutamine	33.77	7.87	36.0672	–	–
Glycine	2.96	0.69	5.2991	–	–
L-Histidine HCl, monohydrate	4.6	1.07	4.6	–	–
L-Isoleucine	7.8	1.81	1.81	–	8.8725
L-Leucine	25.4	5.9	5.9	–	15.6195
L-Lysine HCl	20.38	4.74	20.38	–	–
L-Methionine	6.7	1.56	6.7	–	–
L-Phenylalanine	6.6	1.54	6.6	–	–
L-Proline	7.41	1.72	10.9965	–	–
L-Serine	7.41	1.72	10.6844	–	–
L-Threonine	9.7	2.26	9.7	–	–
L-Tryptophan	3.4	0.79	3.4	–	–
L-Tyrosine	6.9	1.61	6.9	–	–
L-Valine	8.4	1.95	1.95	–	10.725
DL-Methionine	–	–	–	3.0	3.0
Casein	–	–	–	195	195
Sucrose	291.248	291.248	291.248	341.46	341.46
Corn starch	150.0	243.79	153.4368	150	114.683
Maltodextrin	150.0	243.79	153.4368	–	–
Anhydrous milkfat	–	–	–	210	210
Cholesterol	–	–	–	1.5	1.5
Corn oil	52.0	52.0	52.0	–	–
Olive oil	29.0	29.0	29.0	–	–
Cellulose	30.0	30	30	50	50
Mineral mix, AIN-93M-MX (94049)	35.0	35	35	–	–
Mineral mix, AIN-76 (170915)	–	–	–	35	35
Calcium carbonate	–	–	–	4	4
Calcium phosphate $Ca(H_2PO_4)_2 \cdot H_2O$	8.2	8.2	8.2	–	–
Vitamin mix, Teklad (40060)	10.0	10.0	10.0	10.0	10.0
TBHQ, anti-oxidant	0.012	0.012	0.012	–	–
Ethoxyquin, anti-oxidant	–	–	–	0.04	0.04
Food colouring	0.1	0.1	0.1	0.1	0.1
% kcal from:					
Protein (based on N × 6.25)	22	5.1	21.9	15.2	18.3
Carbohydrates	59.4	76.4	59.6	42.7	39.8
vFat	18.6	18.5	18.5	42	41.9
kcal g^{-1}	3.9	3.9	3.9	4.5	4.6

Amino acid defined diets with the specified formulations were obtained from Envigo. These diets were used in the experiment outlined in Fig. 1*A* and used to obtain the results shown in Figs 1–3.

GTACTGCCGTTTTCACAAGTG, R: TCTTTCAGGTCG TGTTCACAG; *Scd1*: F: CTGACCTGAAAGCCGAGAAG, R: AGAAGGTGCTAACGAACAGG; *Srebp1c*: F: GGAGC CATGGATTGCACATT, R: GGCCCGGGAAGTCACTGT.

Immunoblotting

Tissue samples were lysed in cold RIPA buffer supplemented with phosphatase and protease inhibitor cocktail tablets. Tissues were lysed as described previously

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(Baar *et al.* 2016) using a FastPrep 24 (MP Biomedicals, Santa Ana, CA, USA) with bead-beating tubes (13119-500) and ceramic beads (13113-325) from Mo-Bio Laboratories (San Diego, CA, USA) and then centrifuged. Protein concentration was determined by Bradford (Pierce Biotechnology, Rockford, IL, USA). Next, 20 μg of protein was separated by SDS-PAGE on 10% resolving gels (Life Technologies, Grand Island, NY, USA). Antibody for HSP90 (#4874) was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for UCP1 (ab10983) was purchased from Abcam (Cambridge, MA, USA). Imaging was performed using a GE ImageQuant LAS 4000 imaging station (GE Healthcare Chicago, IL, USA). Quantification was determined by densitometry using ImageJ.

Islet isolation and *ex vivo* studies

Islets were isolated and an *ex vivo* glucose stimulated insulin secretion assay was performed as described previously (Neuman *et al.* 2014; Truchan *et al.* 2015; Fontana *et al.* 2016). Briefly, mice were anaesthetized with 240 mg kg^{-1} of freshly prepared avertin, a form of anaesthesia that does not dilate the vasculature or alter blood glucose levels, and the mouse was then killed by exsanguination while the pancreas was inflated with collagenase to isolate the islets. Mitochondrial membrane potential was measured in islets pre-loaded with Rhodamine123 (5 μM, 5 min) (Sigma) and perfused with a standard external solution (135 mM NaCl, 4.8 mM KCl, 5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 20 mM Hepes, pH 7.35) containing 2 or 20 mM glucose, followed by a reference solution containing 20 mM glucose and 5 mM KCN, which was used to normalize the data. Excitation (500/20×) and emission (535/30 m) filters (ET type; Chroma Technology Corporation, Rockingham, VT, USA) were used in combination with an FF444/521/608-Di01 dichroic (Semrock, Rochester, NY, USA) on a Ti-Eclipse microscope (Nikon, Tokyo, Japan); the imaging system has been described previously (Gregg *et al.* 2016). A single region of interest was used to quantify the average response of each islet using Elements (Nikon).

Statistical analysis

Statistical analysis was conducted using Prism, version 7 (GraphPad Software Inc., San Diego, CA, USA). Tests involving repeated measurements were analysed with two-way repeated-measures ANOVA, followed by a Tukey–Kramer or Dunnett's *post hoc* test as specified. All other comparisons of three or more means were analysed by one-way ANOVA followed by a Dunnett's or Tukey–Kramer *post hoc* test as specified where appropriate. Additional comparisons, if any, were corrected for multiple comparisons using the Bonferroni method.

Results

DIO mice switched to normal calorie diets with reduced BCAAs rapidly lose weight and improve glycaemic control

We induced obesity and metabolic dysfunction by feeding C57BL/6J mice a WD for 12 weeks (DIO mice). DIO mice were then switched to one of several different diets of varying amino acid compositions, with an energy density and macronutrient composition typical of rodent chow (Fig. 1*A*). One group of mice was maintained on WD, whereas an additional group of mice was fed WD supplemented with BCAAs. Finally, a parallel group of mice never exposed to a WD was placed on a Control amino acid defined diet. Exact diet formulations are provided in Table 1.

All DIO mice lost weight when switched to a normal calorie diet, whereas mice consuming a WD (with or without supplemental BCAAs) continued to gain weight (Fig. 1*B*). Mice consuming diets in which the BCAAs [extra low (ExLow) BCAA] or all amino acids (ExLow AA) were specifically reduced lost weight very rapidly, shedding ∼25% of their body weight in 2 weeks before eventually stabilizing at a weight lower than mice never exposed to a WD. In contrast, DIO mice switched to a normal calorie Control diet normalized their weight more slowly, over ∼2 additional months. Mice fed the ExLow BCAA or ExLow AA diets lost fat mass, including epididymal WAT, and lean mass, with a net effect of greatly reduced adiposity relative to mice switched to the Control diet as well as to those remaining on a WD (Fig. 1*C* and *D*).

The dramatic weight loss of mice consuming the ExLow BCAA and ExLow AA diets was not the result of decreased food consumption. Indeed, the absolute caloric intake of mice consuming these diets was similar to that of mice switched to the normal calorie Control diet (data not shown) and, relative to their body weight, the caloric intake of mice on either the ExLow BCAA or ExLow AA diets was increased (Fig. 1*E*). Low protein and low amino acid diets promote energy expenditure (Laeger *et al.* 2014; Fontana *et al.* 2016). We assessed activity and energy expenditure via indirect calorimetry once the weights of all groups had stabilized. Although all mice switched to normal calorie diets had similar levels of activity, we observed increased energy expenditure in mice switched to the ExLow AA diet (Fig. 1*F*).

DIO mice switched to any of the normal calorie diets had significantly thinner dermal WAT (dWAT) (Fig. 2*A* and *B*) than mice remaining on a WD. DIO mice fed the ExLow BCAA diet had thinner dWAT than DIO mice switched to the Control diet. DIO mice consuming WD or WD supplemented with BCAAs had evident hepatic steatosis with large fat droplets, whereas DIO

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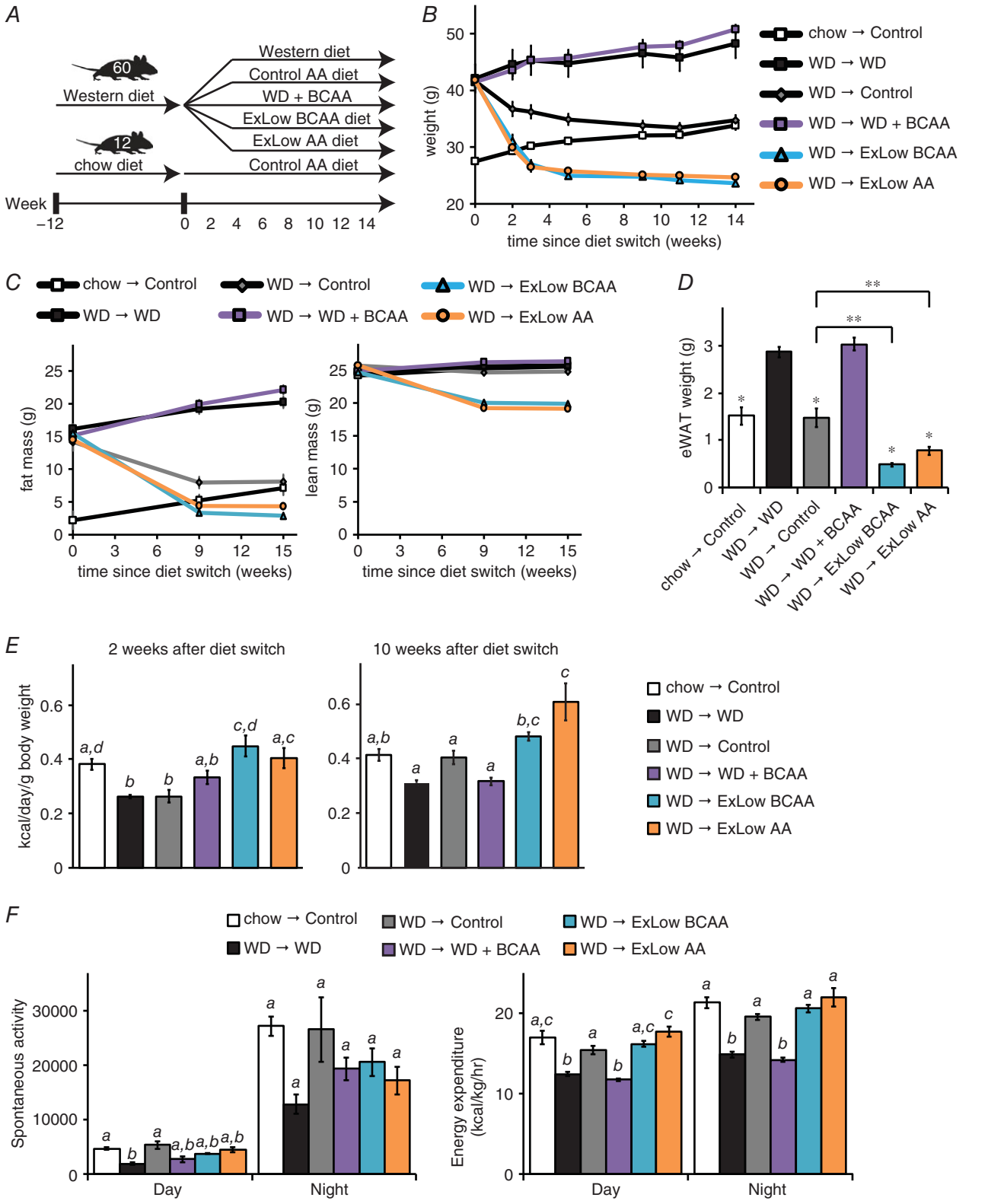


Figure 1. Normal calorie diets with reduced levels of BCAAs promote rapid weight loss
A, schematic representation of the experimental plan; mice were preconditioned with a WD for 12 weeks and then randomized to the five experimental groups shown, whereas chow-fed Control mice were placed on an amino acid defined Control diet. *B*, weight as well as (*C*) adipose and lean mass, of mice in each experimental group, was tracked ($n = 12$ mice per group). *D*, epididymal WAT was collected at necropsy and weighed ($n = 11$–12 mice per

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group; *$P < 0.05$, Dunnett's test following ANOVA, **$P < 0.05$, Bonferroni's test). *E*, 2 and 10 weeks following the start of the specified dietary intervention, food intake was assessed over a 4 day period in home cages, calculated as kcal day g^{-1} body weight ($n = 6$–7 cages per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). *F*, spontaneous activity and energy expenditure were measured using metabolic chambers ∼7–8 weeks after the start of the dietary intervention ($n = 5$–8 mice per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). Error bars represent the SE.

mice switched to either the Control or ExLow BCAA diets had decreased liver droplet size and normal liver histology by the conclusion of the experiment (Fig. 2*C* and *D*). DIO mice switched to the ExLow AA diet had a trend towards reduced lipid droplet size (corrected $P = 0.12$), but hepatic steatosis was still evident. mRNA expression of many lipogenic genes and transcription factors was reduced in the livers of mice switched to Control or ExLow BCAA diets, with similar but less dramatic effects in mice switched to the ExLow AA diet (Fig. 2*E*). Surprisingly, mice fed WD supplemented with BCAAs also had decreased hepatic expression of many lipogenic genes and transcription factors.

Glucose tolerance improved in all mice switched to normal calorie diets; after 3 weeks, mice switched to ExLow BCAA and ExLow AA diets had improved glucose tolerance relative to all other groups, including mice never exposed to a WD (Fig. 3*A*), which was an effect that was sustained throughout the experiment. In contrast, supplementing a WD with BCAAs resulted in worse glucose tolerance *vs.* all other groups after 9 weeks (data not shown). DIO mice that remained on a WD were insulin resistant; all mice placed on normal calorie diets showed improved insulin sensitivity relative to WD mice, with mice switched to an ExLow AA diet showing improved insulin sensitivity relative to all groups (Fig. 3*B*). DIO mice that remained on a WD had fasting hyperglycaemia and hyperinsulinaemia, as well as increased HOMA2-IR (Levy *et al.* 1998; Mather, 2009), relative to mice never exposed to WD (Fig. 3*C*–*E*). In agreement with our tolerance tests, these deficits were corrected in mice switched to any of the normal calorie diets (Fig. 3*C*–*E*).

Specifically reducing dietary BCAAs restores metabolic health to DIO mice continuing to consume a WD

While these results supported our hypothesis that reducing dietary BCAAs would restore metabolic health, the simultaneous alterations in energy density and macronutrient ratios made it difficult to elucidate the precise contribution of the BCAAs. To specifically address this question, we designed a new series of diets based on a novel amino acid-defined WD (WD Control AA) closely matching the macronutrient profile of the naturally sourced diet WD TD.88137. Using this WD Control AA diet as our base, we developed several additional isocaloric WDs with increased or decreased dietary levels of BCAAs (Table 2).

Table 2. Composition of amino acid defined WDs

Diet name	Description
WD Control AA	AA-defined WD – 41% of calories from fat, 38% of calories from carbohydrates (high sucrose), with cholesterol
WD High BCAA	Similar to WD Control AA; with 2× BCAAs
WD Low BCAA	Similar to WD Control AA; with 67% reduction in the BCAAs
WD Low AA	Similar to WD Control AA; with 67% reduction in all AAs
Control AA	AA defined normal calorie diet

The exact formulation of these diets is provided in Table 3.

As shown in Fig. 4*A*, we induced obesity in 48 C57BL/6J mice by feeding them a WD for 12 weeks; these mice were then randomized into four groups of 12 mice each, and each group was placed on either a WD Control AA, WD High BCAA, WD Low BCAA or WD Low AA diet. In parallel, a group of 12 mice never exposed to a WD were switched to the Control amino acid defined diet. Exact diet formulations are provided in Table 3.

DIO mice fed either the WD Control AA or WD High BCAA diets maintained or gained weight, whereas DIO mice switched to the WD Low BCAA or the WD Low AA diets progressively lost weight for 3 weeks (Fig. 4*B*). The weight of mice fed a WD Low BCAA diet then stabilized, matching the weight of Control AA-fed mice never exposed to a WD; mice fed the WD Low AA diet continued to lose weight at a greatly reduced rate. The weight loss of these mice was primarily due to a dramatic decrease in fat mass, whereas lean mass was preserved (Fig. 4*C*), and was not associated with decreased food consumption (Fig. 4*D*). Overall, the mice fed the WD Low BCAA or WD Low AA diets had improved body composition, with decreased adiposity and a resulting increase in the lean fraction (Fig. 4*E*). The dWAT thickness of mice fed the WD Low BCAA or WD Low AA diets was significantly decreased; curiously, mice fed the WD High BCAA diet also had thinner dWAT (Fig. 5*A*).

Mice fed the WD Low BCAA diet had smaller hepatic lipid droplets than mice fed the WD Control AA diet, and cleared much of the hepatic fat deposited by WD feeding (Fig. 5*B*). Intriguingly, there was also qualitative

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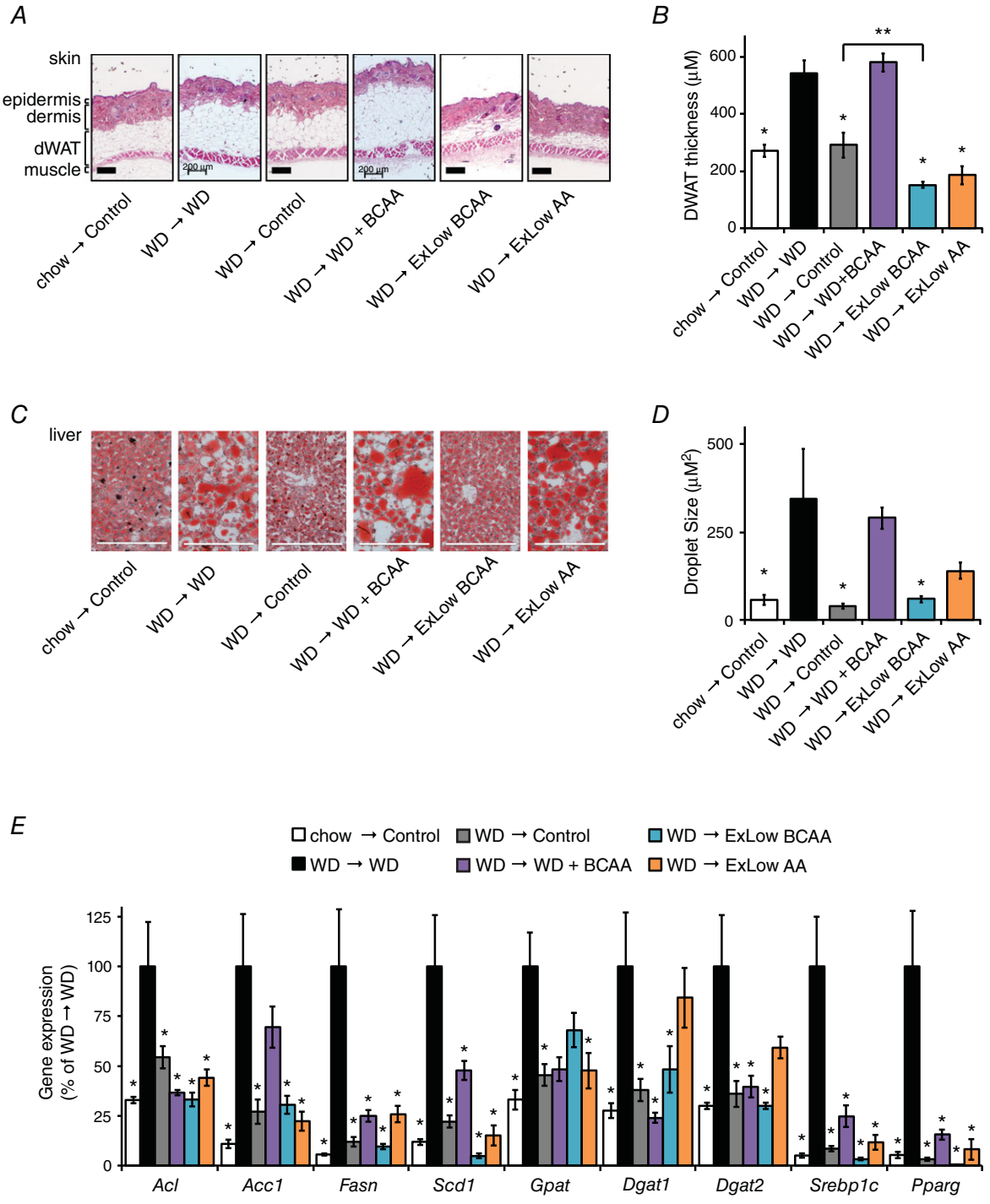


Figure 2. Consumption of an ExLow BCAA diet reduces adipose tissue and reverses diet-induced hepatic steatosis

A, paraffin-embedded skin samples were collected at necropsy ∼15 weeks following the start of the specified dietary intervention, sectioned, and H&E stained, and the thickness of dermal WAT was quantified (*B*) for non-anagen stage skin samples, measuring from muscle to dermis; scale bar = 200 μM (n = 5–7 mice per group, $^{*}P < 0.05$, Dunnett's test following ANOVA, $^{**}P < 0.05$, Bonferroni's test). *C*, liver samples were stained with Oil-Red-O and (*D*) droplet size was quantified; scale bar = 200 μM (n = 3 mice per group, $^{*}P < 0.05$ *vs.* WD, Dunnett's test following ANOVA). *E*, lipogenic gene expression was measured in the livers of fasted mice (n = 5–6 mice per group, $^{*}P < 0.05$ *vs.* WD, Dunnett's test following two-way repeated measures ANOVA). Error bars represent the SE.

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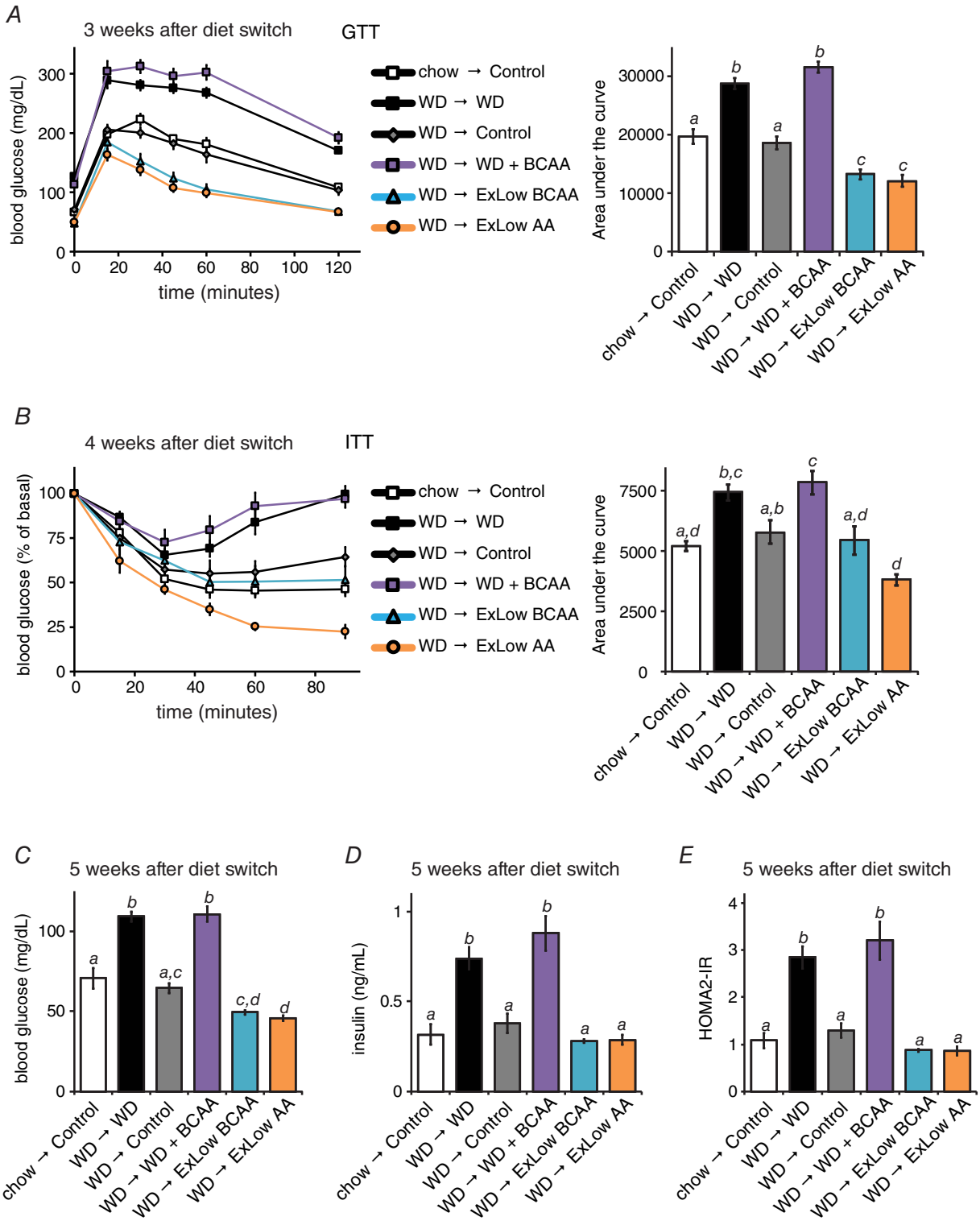


Figure 3. Consumption of BCAAs inversely correlates with glucose tolerance and insulin sensitivity
Glucose (*A*) and (*B*) insulin tolerance tests were conducted at the specified times after the start of the dietary interventions (n = 10–12 per group; for the area under the curve, means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). *C*–*E*, mice were fasted overnight and (*C*) blood glucose and (*D*) insulin were measured and (*E*) the HOMA2-IR was calculated after 5 weeks on the specified diets (n = 5–7 mice per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). Error bars represent the SE.

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Table 3. Diet composition of diets used to investigate the effect of specifically altering dietary BCAAs in the context of a WD

Amino acid defined diets	Control AA	WD Control AA	WD High BCAA	WD Low BCAA	WD Low AA
Teklad diet number	TD.140711	TD.160186	TD.160189	TD.160188	TD.160187
Colour	Red	Aqua	Green	Black	Orange
Formula (g kg^{-1})					
L-Alanine	9.38	9.38	5.5861	11.8183	3.05
L-Arginine	6.3	6.3	6.3	6.3	2.05
L-Asparagine	20.58	20.58	17.7668	22.388	6.7
L-Aspartic acid	20.58	20.58	14.9108	24.2237	6.7
L-Cysteine	7.2	7.2	7.2	7.2	2.34
L-Glutamic acid	28.97	28.97	22.7053	32.9963	9.43
L-Glutamine	33.77	33.77	30.6311	35.7873	11.0
Glycine	2.96	2.96	0.96	5.0141	0.96
L-Histidine HCl, monohydrate	4.6	4.6	4.6	4.6	1.5
L-Isoleucine	7.8	7.8	15.6	2.54	2.54
L-Leucine	25.4	25.4	50.8	8.27	8.27
L-Lysine HCl	20.38	20.38	20.38	20.38	6.64
L-Methionine	6.7	6.7	6.7	6.7	2.18
L-Phenylalanine	6.6	6.6	6.6	6.6	2.15
L-Proline	7.41	7.41	2.5094	10.5596	2.41
L-Serine	7.41	7.41	2.9359	10.2855	2.41
L-Threonine	9.7	9.7	9.7	9.7	3.16
L-Tryptophan	3.4	3.4	3.4	3.4	1.1
L-Tyrosine	6.9	6.9	6.9	6.9	2.25
L-Valine	8.4	8.4	16.8	2.735	2.735
Sucrose	291.248	341.46	341.46	341.46	341.46
Corn starch	150.0	49.63	45.3573	52.6511	132.0625
Maltodextrin	150.0	49.63	45.3573	52.6511	132.0625
Anhydrous milkfat	–	210	210	210	210
Cholesterol	–	1.5	1.5	1.5	1.5
Corn oil	52.0	–	–	–	–
Olive oil	29.0	–	–	–	–
Cellulose	30.0	50	50	50	50
Mineral mix, AIN-93M-MX (94049)	35.0	35	35	35	35
Calcium phosphate $Ca(H_2PO_4)_2{\cdot}H_2O$	8.2	8.2	8.2	8.2	8.2
Vitamin mix, Teklad (40060)	10.0	10.0	10.0	10.0	10.0
TBHQ, anti-oxidant	0.012	0.04	0.04	0.04	0.04
Food colouring	0.1	0.1	0.1	0.1	0.1
% kcal from:					
Protein (based on N × 6.25)	22	20.7	21.4	20.2	6.8
Carbohydrates	59.4	38.5	37.8	39.0	52
Fat	18.6	40.9	40.8	40.9	41.2
kcal g^{-1}	3.9	4.6	4.6	4.6	4.6

Amino acid defined diets with the specified formulations were obtained from Envigo. These diets were used in the experiment outlined in Fig. 4*A* and used to obtain the results shown in Figs 4–10.

histological improvement and a non-significant trend towards reduced lipid droplet size in mice consuming extra BCAAs (WD High BCAA). The livers of mice fed the WD Low AA diet did not have smaller lipid droplets and retained large lipid droplets (Fig. 5*B*). Mice fed the WD Low AA or WD High BCAA diets had increased hepatic triglyceride levels (Fig. 5*C*). We observed numerical decreases in the mRNA expression of two lipogenic genes (*Fasn*, *Scd1*) in the livers of mice fed either the WD Low BCAA diet or the WD High BCAA diet, as well as a statistically significant decrease in the transcription factor *Pparg* in the livers of mice switched to a WD Low BCAA diet (Fig. 5*D*).

We examined glycaemic control by conducting glucose and insulin tolerance tests, as well as by determining fasting glucose and insulin levels. Glucose tolerance was improved in mice eating the WD Low BCAA and WD Low AA diets 3 weeks after the diet switch, a time when these mice still weighed more than Control AA-fed mice never exposed to a WD (Fig. 6*A*). Insulin sensitivity was

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similarly improved in both groups (Fig. 6*B*), even as the mice continued to consume a WD. These improvements were maintained over the course of the study (data not shown). Mice on WD Low BCAA and WD Low AA diets had decreased fasting blood glucose and insulin levels (Fig. 6*C* and *D*), whereas increasing dietary BCAAs resulted in fasting hyperglycaemia and hyperinsulinaemia. HOMA2-IR calculated from these values indicate that insulin sensitivity is inversely correlated with dietary BCAAs (Fig. 6*E*).

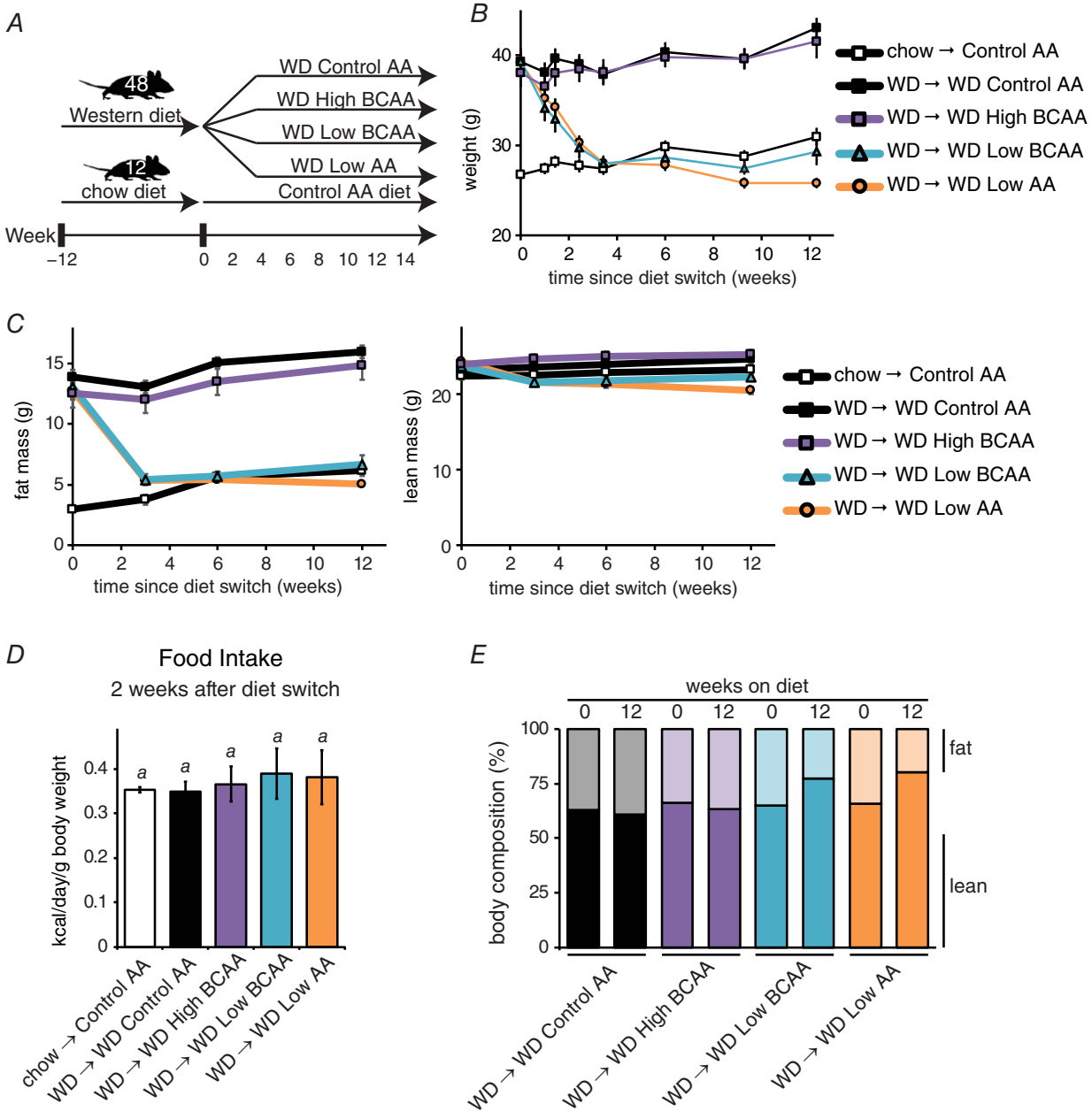


Figure 4. Specifically reducing dietary BCAAs in the context of a WD promotes rapid weight loss and reduced adiposity
A, schematic representation of experimental plan; mice were preconditioned with a WD for 12 weeks and then randomized to the four experimental groups shown, whereas chow-fed Control mice were placed on an amino acid defined Control diet. *B*, weight, as well as (*C*) adipose and lean mass of mice in each experimental group was tracked ($n = 12$ mice per group). *D*, food intake was measured 2 weeks after special diet feeding began ($n = 8$ cages per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). *E*, body composition at diet intervention start and 12 weeks later. Error bars represent the SE.

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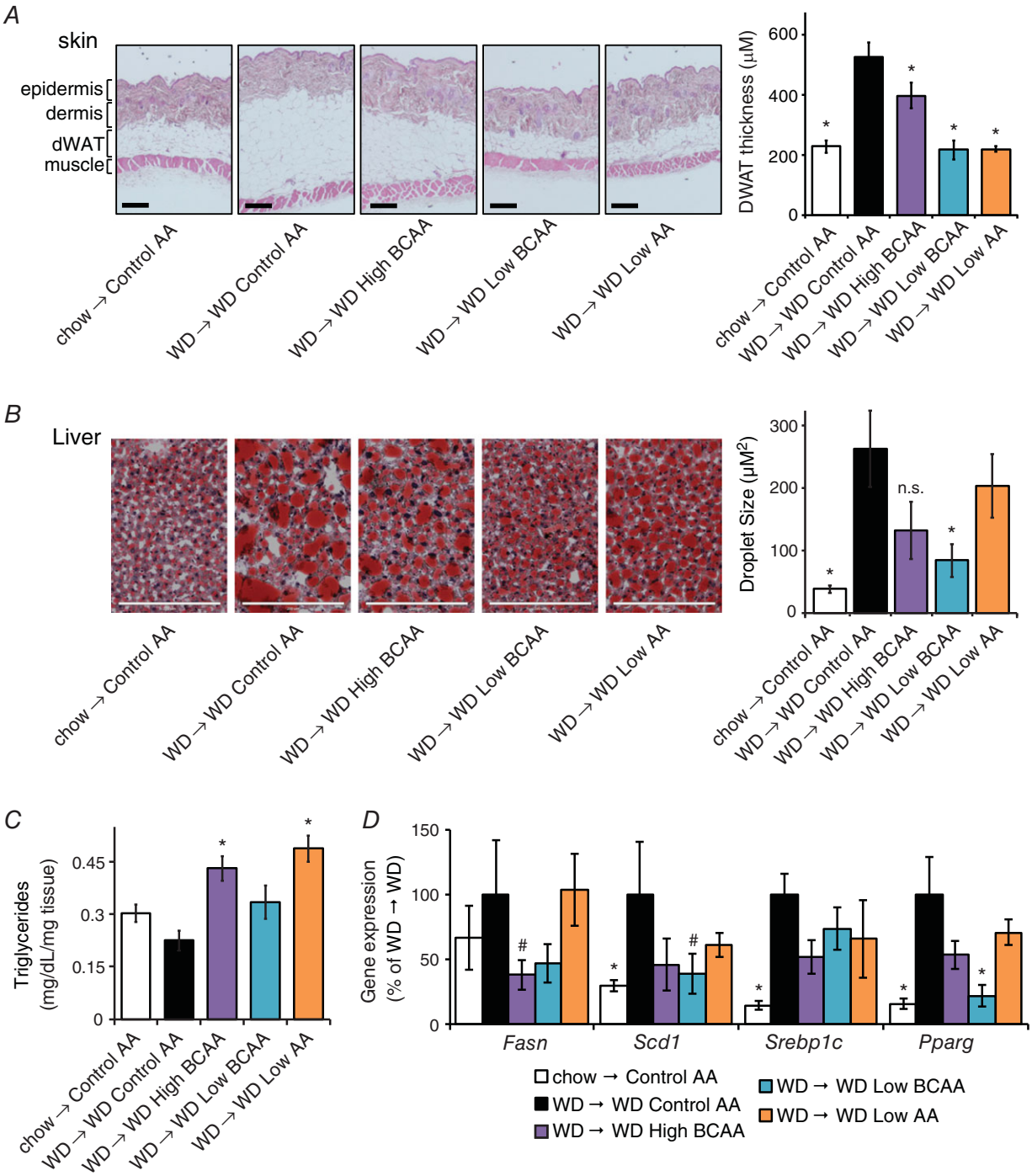


Figure 5. Specifically reducing dietary BCAAs in the context of a WD reduces adipose tissue and reverses diet-induced hepatic steatosis
A, skin samples were collected after feeding mice the indicated diets for ~14 weeks, sectioned, H&E stained and the thickness of dermal WAT was quantified for non-anagen stage skin samples, measuring from muscle to dermis; scale bar = 200 μM ($n = 6$ mice per group, $^*P < 0.05$ *vs*. WD Control AA, Dunnett's test following ANOVA). *B*, OCT-embedded liver samples were cryosectioned and stained with Oil-Red-O, and droplet size was quantified; scale bar = 200 μM ($n = 3$ mice per group, $^*P < 0.05$ *vs*. WD Control AA, Dunnett's test following ANOVA). *C*, triglyceride levels in liver as mg dL^{-1} mg^{-1} of tissue assayed ($n = 6$ mice per group, $^*P < 0.05$ *vs*. WD Control AA, Dunnett's test following ANOVA). *D*, lipogenic gene expression in the livers of mice on the specified diets was measured by quantitative PCR after an overnight fast ($n = 8$–9 mice per group, $^*P < 0.05$, $\#P < 0.1$ *vs*. WD Control AA, Dunnett's test following ANOVA). Error bars represent the SE.

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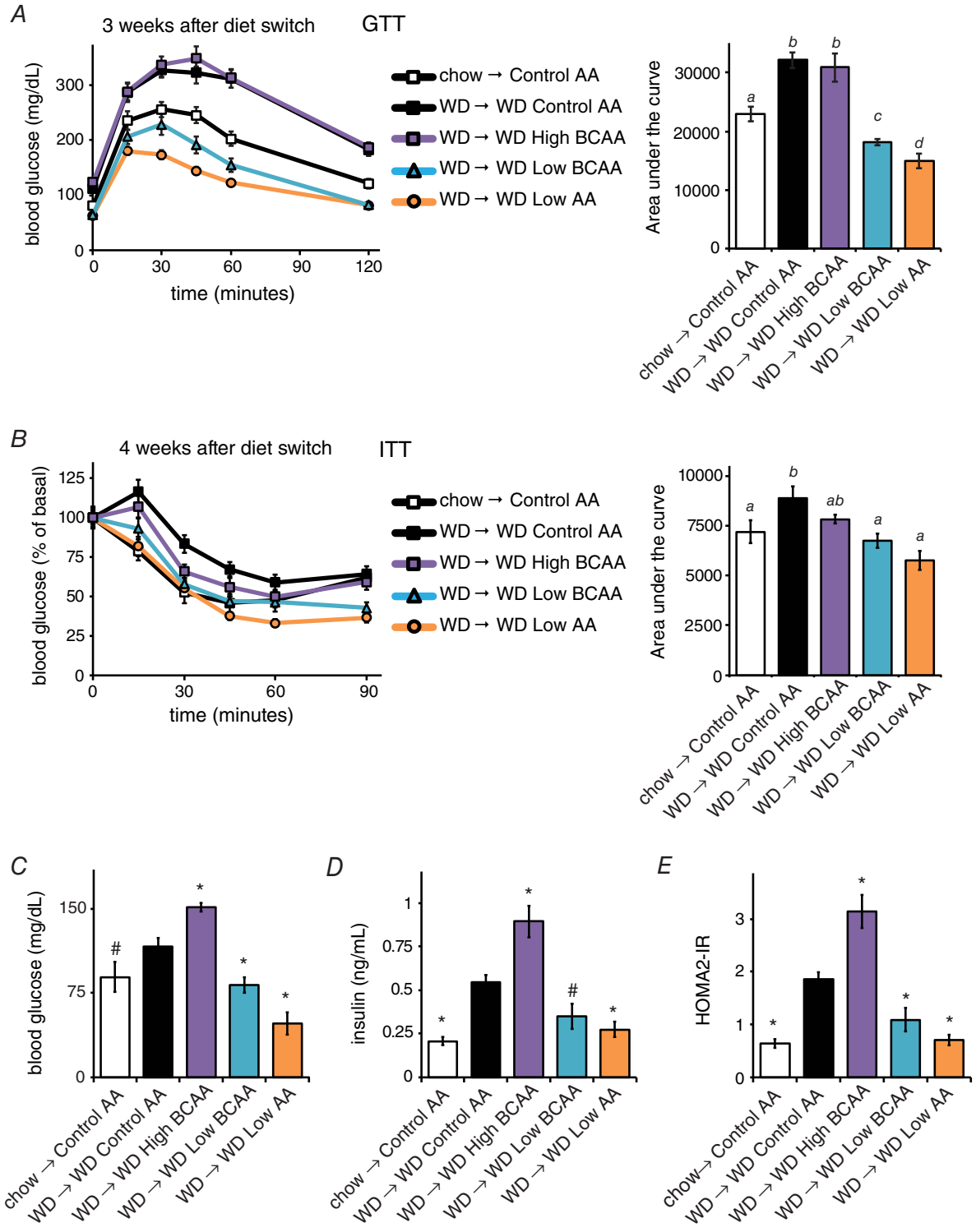


Figure 6. Reduction of dietary BCAAs improves glycaemic control even in mice continuing to consume a high-calorie, high-fat, high-sugar WD
Glucose (*A*) and (*B*) insulin tolerance tests were conducted 3 and 4 weeks, respectively, after the start of the dietary interventions (n = 12–16 per group; for the area under the curve, means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). *C–E*, fasting (*C*) blood glucose and (*D*) insulin were measured and (*E*) the HOMA2-IR was calculated after 5 weeks on the specified diets (n = 3–7 mice per group; $^*P < 0.05$, $\#P < 0.12$ *vs.* WD Control AA, Dunnett's test following ANOVA).

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We examined pancreatic islet function by performing an *ex vivo* glucose-stimulated insulin secretion assay (Fig. 7*A*). Insulin secretion was decreased in mice fed a WD Low AA diet (Fig. 7*A*); however, total islet insulin content was not affected (Fig. 7*A*). To precisely examine β cell metabolic stress, we quantified the mitochondrial membrane potential. This was increased in mice eating a WD Control AA diet, and was reduced and essentially normalized in mice consuming WD Low BCAA and WD Low AA diets (Fig. 7*B*). In every case except WD High BCAA, β cell function was matched with insulin sensitivity, implying that increased BCAA consumption negatively impacts mitochondrial function and insulin secretion.

Chronic consumption of a reduced BCAA WD increases energy expenditure independently of FGF21

To understand how reducing dietary BCAAs promotes leanness without reducing calorie intake, we utilized metabolic chambers to examine food consumption, respiration, activity, and energy expenditure after mice had been on the diets for ∼12 weeks. Mice fed diets with reduced levels of BCAAs or reduced levels of all AAs consumed around twice as many calories than mice fed a WD Control AA diet (data not shown); the increase is even greater when calculated relative to body weight (Fig. 8*A*). As expected, the RER was decreased by WD feeding and increased in mice consuming the high carbohydrate WD Low AA diet (Fig. 8*B*). Intriguingly, the RER of mice fed the WD Low BCAA diet, containing the same level of carbohydrates as the WD Control AA diet, was also increased, and was indistinguishable from the RER of mice consuming a WD Low AA diet.

There was no difference in spontaneous activity between any of the groups (Fig. 8*C*). Mice consuming the WD Low BCAA and WD Low AA diets had greater energy expenditure during both daytime and night-time (Fig. 8*D*). Mice consuming the WD Low AA diet had high

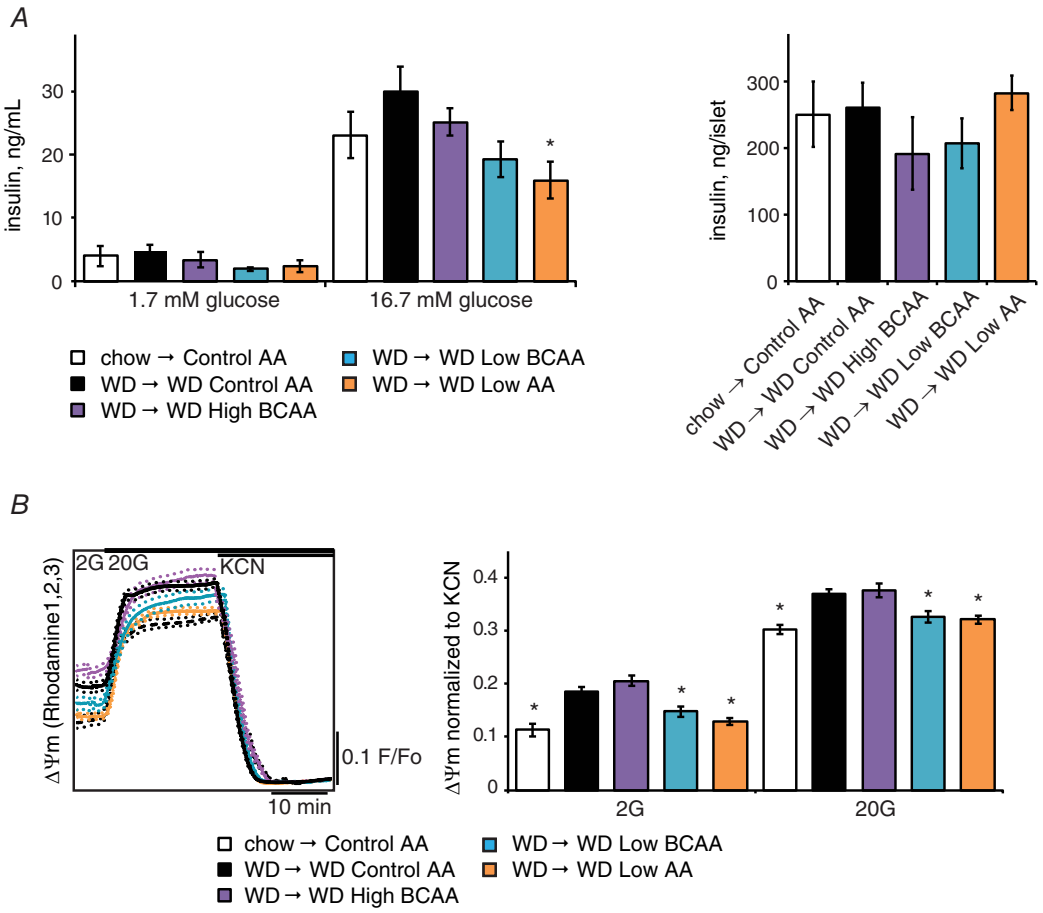


Figure 7. *Ex vivo* analysis of the effect of altering dietary BCAAs on pancreatic beta cells
A, an *ex vivo* insulin secretion assay was performed to assess (left) insulin secretion per islet and (right) islet insulin content in response to low (1.7 mM) and high (16.7 mM) glucose in mice kept on the indicated diets for ∼14 weeks ($n = 6$ mice per group, $^*P < 0.05$ *vs.* WD Control AA, Dunnett's test following ANOVA). *B*, the mitochondrial membrane potential was measured in *ex vivo* isolated pancreatic islets stimulated with low (2 mM) and high (20 mM) glucose levels ($n = 44$–74 islets per group; $^*P < 0.05$ *vs.* WD Control AA, Dunnett's test following ANOVA). Error bars represent the SE.

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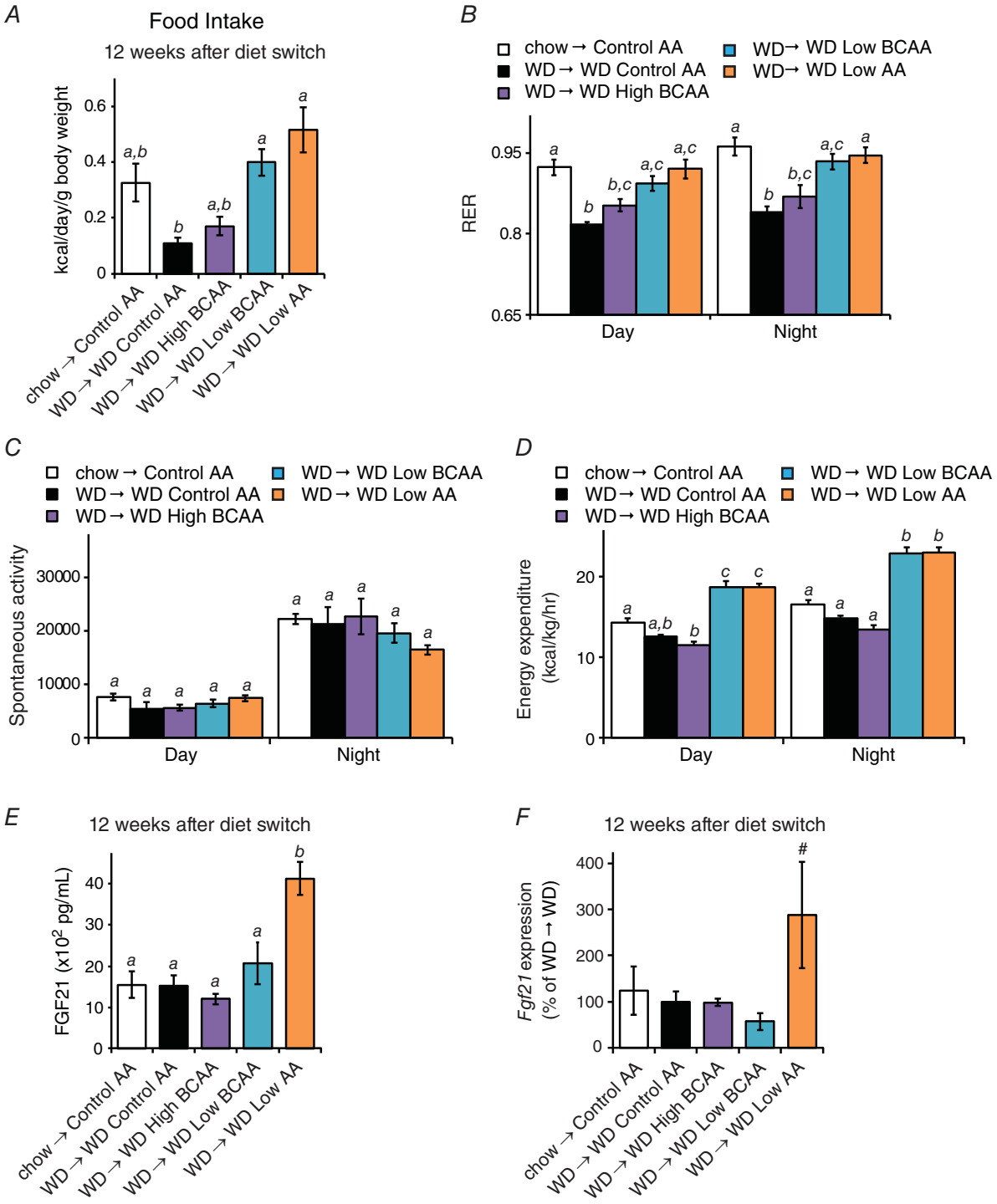


Figure 8. Metabolic impact of sustained consumption of diets with altered dietary BCAAs
A, food intake over a 24 h period (n = 2–5 mice per group; means with the same lowercase letter are not significantly different from each other, Bonferroni test, $P < 0.05$). *B*, RER, (*C*) spontaneous activity and (*D*) energy expenditure were measured ∼12 weeks after the start of the dietary intervention (n = 4–5 mice per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). *E*, FGF21 was measured in the plasma of mice following an overnight fast (n = 4 mice per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). *F*, *Fgf21* gene expression in the liver of mice following an overnight fast was assessed by quantitative PCR (n = 8–9 mice per group; #$P < 0.1$ *vs*. WD Control AA, Dunnett's test following ANOVA). Error bars represent the SE.

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levels of the energy balance regulating hormone FGF21 (Fig. 8*E*); however, there was no increase in FGF21 in the blood of mice consuming the WD Low BCAA diet and no increase in liver *Fgf21* gene expression (Fig. 8*E* and *F*).

Reduction in dietary BCAAs in WD-fed mice is accompanied by a transient increase in FGF21 levels

From the perspective of weight and body composition, the 3 weeks following the diet switch are distinctly different from the time period during which we analysed energy expenditure above. In particular, rapid weight normalization occurs during the first 3 weeks, whereas weights are relatively stable thereafter. We therefore utilized an additional cohort of mice to intensively analyse weight, body composition, activity, and energy expenditure during the 12 days immediately following the diet switch, during which mice fed the WD Low BCAA and WD Low AA diets progressively lost weight and fat mass (Fig. 9*A* and *B*).

In contrast to the increased RER seen at later time points, neither WD Low BCAA or WD Low AA diet fed mice had increased RER 1 week after the diet switch; indeed, WD Low BCAA diet fed mice had a lower RER (Fig. 9*C*). Although there were no significant changes in spontaneous activity between groups, we observed a statistically significant increase in energy expenditure during the daytime and night-time in WD Low AA diet fed mice but, surprisingly, not in WD Low BCAA fed mice (Fig. 9*C*). As weight loss in the absence of a change in energy expenditure or activity was puzzling, we examined energy expenditure more closely over a 24 h period. We determined that mice fed the WD Low BCAA diet have a significant increase in energy expenditure for at least 20% of a 24 h period, with the most pronounced difference at night (Fig. 9*D*). Following completion of this analysis, we determined that FGF21 levels were significantly increased in the blood of both WD Low BCAA and WD Low AA diet fed mice (Fig. 9*E*).

FGF21 promotes browning of WAT and increased activation of BAT (Fisher *et al.* 2012; Owen *et al.* 2014; Douris *et al.* 2015; Hill *et al.* 2017; Wanders *et al.* 2017). We observed decreased adipocyte size in inguinal and gonadal WAT, consistent with the observed loss of adipose mass; however, we did not observe a morphology consistent with increased beiging (Fig. 10*A*) and the expression of UCP1 was not increased in either gonadal or inguinal WAT of WD Low BCAA or WD Low AA diet fed mice (Fig. 10*B* and *C*). We also did not observe increased expression of *Ucp1* in the BAT of mice fed WD Low BCAA or WD Low AA diets (Fig. 10*D*); however, we did observe a significant decrease in lipid droplet size (Fig. 10*E*) and increased expression of *Bmp8* (Fig. 10*F*), which are changes consistent with FGF21-mediated activation of BAT (Whittle *et al.* 2012; Bendayan & Cammisotto, 2016; Quesada-Lopez *et al.* 2016; Wanders *et al.* 2017).

Discussion

Building on recent studies conducted by our laboratory and others showing that reducing dietary BCAAs can promote or preserve metabolic health in young mice and rats (Xiao *et al.* 2014; Fontana *et al.* 2016; White *et al.* 2016), we tested the hypothesis that reducing dietary BCAAs would be a uniquely potent way to intervene in metabolic syndrome. In the present study, we find that specifically reducing dietary BCAAs, without altering energy density, the caloric contribution of amino acids to the diet, or the protein:carbohydrate ratio (Solon-Biet *et al.* 2014, 2015; Simpson *et al.* 2017), is sufficient to robustly restore metabolic health.

These studies represent the first examination of a reduced BCAA diet in a mouse model of pre-existing diet-induced obesity and type 2 diabetes, which are not hyperphagic and have a gradual and reversible onset of metabolic disease (Williams *et al.* 2014). Specifically reducing BCAAs rapidly normalizes the weight of DIO mice without calorie restriction, even in mice continuing to consume a WD, promoting fat mass loss as well as rapid and dramatic improvements in glucose tolerance and insulin sensitivity. While there are likely multiple mechanisms underlying the metabolic benefits of a reduced BCAA diet, the improvements in glucose homeostasis we observe here are likely due in part to weight loss and decreased adiposity resulting from increased energy expenditure.

As summarized in Fig. 11, reducing either all dietary AAs or specifically reducing the BCAAs improves the metabolic health of DIO mice; however, the specific effects of the diets on energy balance differ. During the acute phase of rapid weight loss, mice eating a WD Low AA diet have increased food intake and a substantial increase in energy expenditure, whereas mice eating a WD Low BCAA diet have no change in food intake and a more modest increase in energy expenditure. In contrast, during the chronic phase of relatively stable weight, energy expenditure and caloric intake are identically increased in mice consuming either the WD Low AA diet or WD Low BCAA diet.

A more dramatic difference is observed between mice fed these two diets with regard to the hormone FGF21, which has pleiotropic effects on glucose metabolism and energy expenditure (Berglund *et al.* 2009; Fisher *et al.* 2012; Emanuelli *et al.* 2014; Laeger *et al.* 2014, 2016; Markan *et al.* 2014; Owen *et al.* 2014; Stone *et al.* 2014). FGF21 is induced in both humans and rodents in response to low protein diets, and is proposed to mediate many of the beneficial metabolic effects of these diets (Laeger *et al.* 2014, 2016; Fontana *et al.* 2016; Maida *et al.* 2016). Although our previous work, conducted in the context of a normal calorie

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diet, revealed no effect of BCAA reduction on FGF21, in the present study we find that specifically reducing dietary BCAAs in the context of a WD transiently induces FGF21.

This is intriguing, particularly in light of a recent study which suggests that the increase in FGF21 in mice fed a low protein diet is mediated by the protein:carbohydrate ratio (Solon-Biet *et al.* 2016). Although other groups have previously determined that restricting or eliminating specific essential dietary amino acids, including methionine or leucine (De Sousa-Coelho *et al.* 2012; Lees *et al.* 2014,2017; Wanders *et al.* 2015, 2017), is sufficient to induce FGF21, these studies have utilized diets in which either methionine

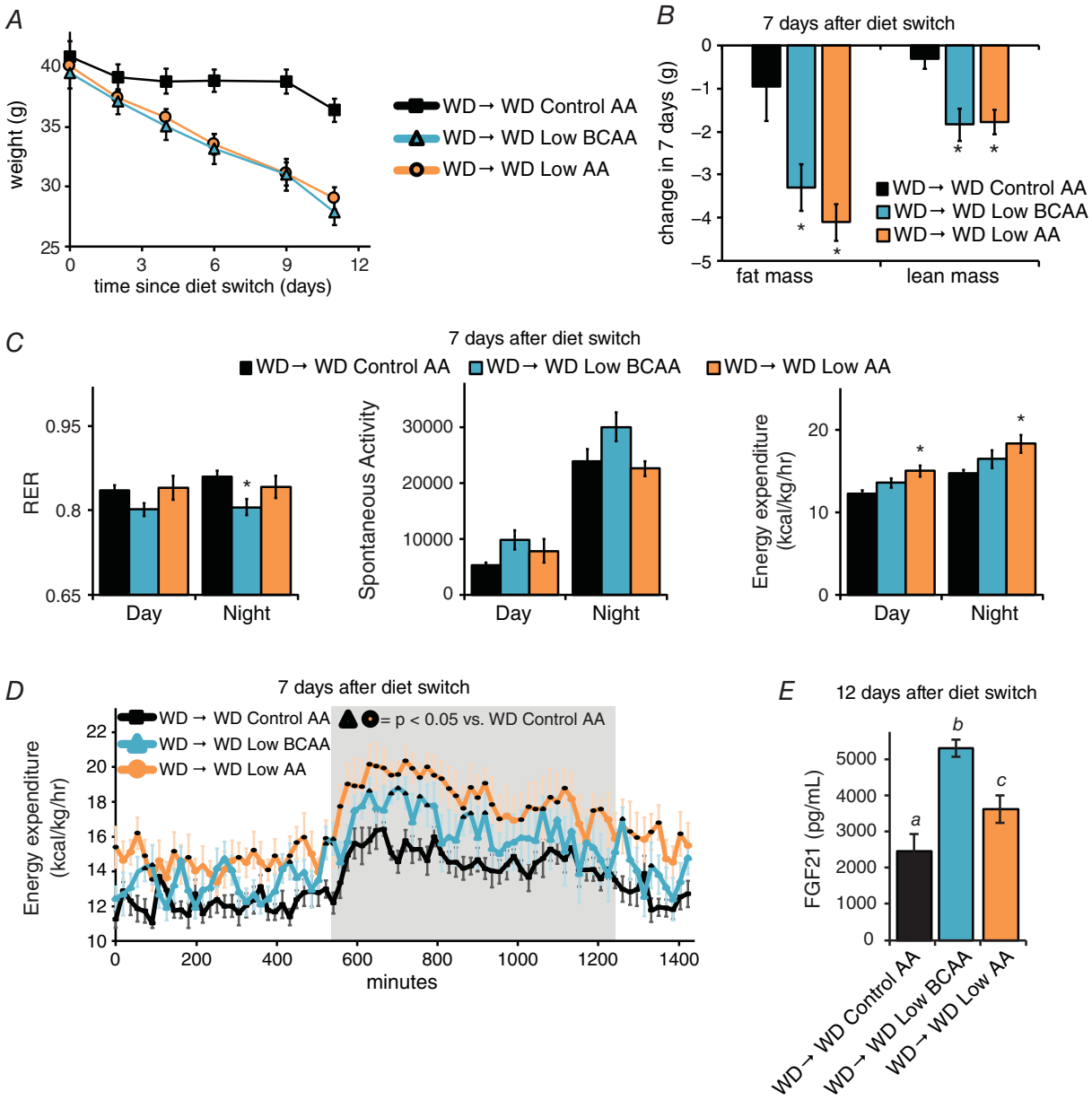


Figure 9. A Western reduced BCAA diet transiently induces FGF21 and increases energy expenditure
A, weight of DIO mice switched to the indicated diet at time 0 ($n = 8$ per group). *B*, change in fat and lean mass of mice placed on each diet for 7 days ($n = 8$ mice per group, $^*P < 0.05$ *vs.* WD Control AA, Dunnett's test following ANOVA). *C*, RER, spontaneous activity and energy expenditure were measured 1 week after the diet switch ($n = 6$–8 mice per group, $^*P < 0.05$ *vs.* WD Control AA, Dunnett's test following ANOVA). *D*, energy expenditure over the course of a 24 h cycle starting at ∼10.00 h ($n = 8$ mice per group; dark outline of symbol indicates $P < 0.05$ *vs.* WD Control AA (Dunnett's test following two-way repeated measures ANOVA). *E*, FGF21 was measured in the plasma of mice following an overnight fast ($n = 6$ mice per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). Error bars represent the SE.

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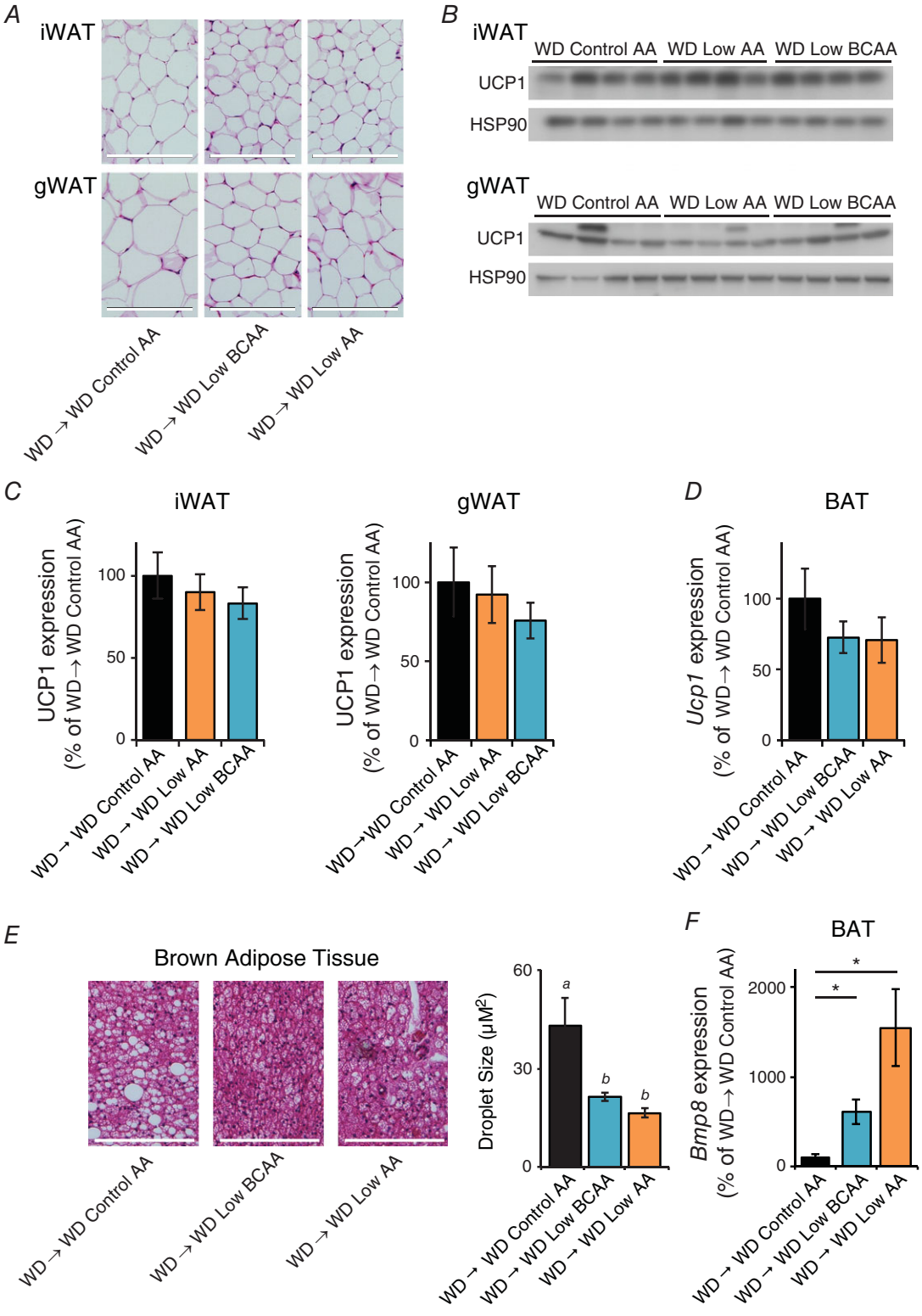


Figure 10. A Western reduced BCAA diet does not beige WAT but activates BAT
Following an overnight fast, tissues were collected from DIO mice switched to the indicated diets for 12 days. *A*, inguinal and gonadal WAT (iWAT and gWAT, respectively) depots were collected, sectioned, and H&E stained; scale bar = 200 μM. *B*, the expression of UCP1 in iWAT and gWAT was determined by Western blotting. *C*, UCP1 expression in iWAT and gWAT was quantified relative to HSP90 ($n = 8$ mice per group, $^*P < 0.05$ *vs*. WD Control AA, Dunnett's test following ANOVA). *D*, *Ucp1* gene expression in BAT was assessed by quantitative PCR

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($n = 6$ mice per group, $^*P < 0.05$ *vs*. WD Control AA, Dunnett's test following ANOVA). *E*, BAT was collected, sectioned, and H&E stained, and lipid droplet size was quantified; scale bar = 200 μM ($n = 6$ mice per group; means with the same lowercase letter are not significantly different from each other, Tukey–Kramer test following ANOVA, $P < 0.05$). *F*, *Bmp8* gene expression in BAT was assessed by quantitative PCR ($n = 6$ mice per group; $^*P < 0.05$ *vs*. WD Control AA, Dunnett's test following ANOVA). Error bars represent the SE.

or leucine levels are restricted by 80% or more. Our finding that FGF21 is responsive to a more physiologically relevant 67% reduction in dietary levels of BCAAs, at least in the context of a WD, suggests that the precise amino acid composition of the dietary protein may also play a role. Further research will be required to define the mechanism by which FGF21 expression is regulated by dietary BCAAs and, indeed, by other AAs such as asparagine (Wilson *et al.* 2015).

FGF21 has been shown to play a key role in the response to cold exposure and dietary interventions such as calorie restriction and methionine restriction, promoting the beiging of WAT (Fisher *et al.* 2012; Shabalina *et al.* 2013; Fabbiano *et al.* 2016; Wanders *et al.* 2017). Dietary protein restriction also promotes the beiging of WAT, and increases energy expenditure via a FGF21 and UCP1-dependent mechanism (Laeger *et al.* 2016; Hill *et al.* 2017). We therefore hypothesized that the increase in FGF21 following the reduction of dietary BCAAs or all AAs promoted energy expenditure and weight loss through the beiging of WAT. However, while mice fed either the WD Low AA or WD Low BCAA diet for ~2 weeks had increased levels of FGF21 and increased energy expenditure, we observed no changes in WAT morphology consistent with beiging, and no increase in the expression of UCP1 in either inguinal or gonadal WAT. *Ucp1* was also not increased in BAT, despite the presence of other changes suggestive of BAT activation.

These results demonstrate that BCAA and AA reduction in the context of a WD does not promote energy expenditure solely by engaging the FGF21-UCP1 axis and promoting WAT beiging. However, as our analysis of WAT and BAT is limited to an early time point, we cannot rule out a role for beiged WAT in the greatly increased energy expenditure we observed in mice consuming a WD Low AA or WD Low BCAA diet for longer periods of time. Determining how UCP1 expression changes in mice fed these diets for longer periods of time will be a key point for further study. FGF21 may also still play a role in the response to reduced dietary BCAAs, as recent research suggests that some of the effects of FGF21 are mechanistically independent of UCP1 (Samms *et al.* 2015). There are also as yet undefined thermogenic mechanisms that act independently of FGF21 and UCP1 (Hill *et al.* 2017; Keipert *et al.* 2017), which could conceivably also play a role in the response to reduced dietary BCAAs. Understanding the molecular mechanisms leading to increased energy expenditure and weight loss, including the role of FGF21 and UCP1, will be key points for further study.

The improvements in the regulation of blood glucose we observe in mice consuming diets with reduced levels of BCAAs are not exclusively the result of weight loss. Notably, while both glucose and insulin tolerance are improved in obese mice switched to diets with reduced levels of the BCAAs, obese mice switched to the WD

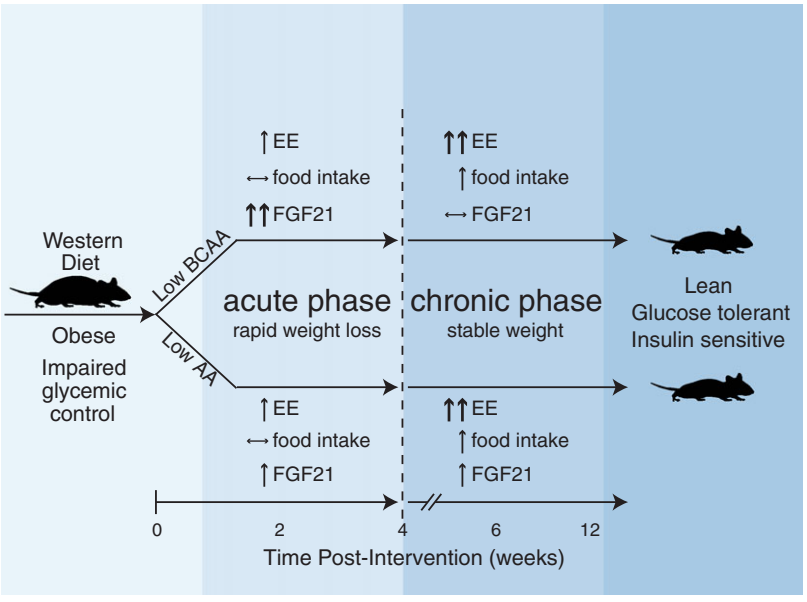


Figure 11. Specifically reducing dietary BCAAs or all AAs restores metabolic health to DIO mice
Altering dietary levels of either the BCAAs or all AAs promotes leanness and restores blood glucose control to DIO mice, but these two dietary interventions have distinct effects on energy expenditure (EE), food intake and blood levels of FGF21. Further, these metabolic effects vary between an acute phase (an ~4 week long period following the diet switch characterized by rapid weight loss), and a chronic phase that persists once mice have reached a stable weight.

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Low BCAA and WD Low AA diets have better glucose tolerance than Control diet-fed mice never exposed to a WD. Based on our previous work (Fontana *et al.* 2016), the decrease in fasting blood glucose we observe in mice switched to BCAA-reduced diets, and the minimal effects of BCAA-reduced diets on islet metabolism, we hypothesize that the improvement in glucose tolerance is most likely due to improved hepatic insulin sensitivity. This effect could be due to alterations in lipid metabolism; increased levels of FGF21, which promotes hepatic insulin sensitivity (Berglund *et al.* 2009); decreased BCAA catabolism, as increased hepatic BCAA catabolism is associated with glucose intolerance in mice (She *et al.* 2007; Ananieva *et al.* 2017); or a combination of these effects. Identifying the physiological and molecular basis for the improvements in blood glucose control we observed here may suggest novel approaches for the treatment of prediabetes and type 2 diabetes.

Intriguingly, increased consumption of BCAAs, at least under the conditions examined here, has minimal impact on weight and body composition. In agreement with previous studies demonstrating that supplementing either a LP diet (Maida *et al.* 2017) or a WD (Newgard *et al.* 2009) with BCAAs impairs glucose homeostasis in wild-type animals, there is an observable negative impact of BCAA supplementation on blood glucose control. Interestingly, while there is both preclinical and clinical data suggesting that BCAA supplementation may be therapeutic for hepatic steatosis (Garcia-Caraballo *et al.* 2013; Barb *et al.* 2016; Honda *et al.* 2017), and we observed decreased expression of several lipogenic genes in mice consuming additional BCAAs, we observed no statistically significant effect of BCAA supplementation on hepatic lipid droplet size. Indeed, we find that BCAA supplementation actually increases liver triglyceride levels. Reconciling our results with data from other groups suggesting a beneficial effect of BCAA supplementation on hepatic steatosis will require additional study.

In contrast to these results, we observed that specifically reducing dietary BCAAs suppresses the expression of numerous lipogenic genes, and also decreases hepatic lipid droplet size. Although the relationship between lipid droplet size and liver health is not clearly understood, smaller lipid droplets may be more metabolically available and better-stabilized by lipid binding proteins (Suzuki *et al.* 2011). Notably, reducing all dietary amino acids, as in a low protein diet, did not reduce lipid droplet size and increased hepatic triglyceride levels. These results suggest that specifically reducing dietary BCAAs may have uniquely beneficial effects on hepatic lipid metabolism not achievable from the consumption of a low protein diet.

Collectively, these results demonstrate that reducing dietary levels of all AAs, or specifically reducing dietary levels of the three BCAAs, leucine, isoleucine, and valine, can rapidly reverse the obesity and metabolic dysfunction resulting from consumption of a high-fat, high-sugar diet without requiring calorie restriction. While many of these metabolic benefits likely result from increased energy expenditure and a resulting decrease in weight, changes in hormones or BCAA catabolism probably also contribute to the improved regulation of blood glucose that we observe in mice fed diets with reduced levels of BCAAs. While the direct applicability of our results to humans remains to be determined, the correlation of blood levels of BCAAs with obesity and insulin resistance in humans is well established (Newgard *et al.* 2009; Lynch & Adams, 2014). If dietary BCAAs have similar effects on energy balance and metabolism in humans, it is implicit in our findings that protein quality (i.e. the precise amino acid composition of dietary protein) may have a significant impact on the efficacy of weight loss diets. Finally, selective reduction of dietary BCAAs through the use of customized diet plans or BCAA-free medical food, or pharmaceuticals that mimic this effect (e.g. by altering BCAA absorption or catabolism), may represent a translatable and sustainable approach to promote metabolic health and treat diabetes and obesity without reducing caloric intake.

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Additional information

Competing interests

The University of Wisconsin-Madison has applied for a patent based in part on the findings reported here, for which NEC and DWL are inventors.

Author contributions

Experiments were performed in the Lamming laboratory, except for the analysis of dWAT, which was performed in the Alexander laboratory, and the analysis of pancreatic islets, which was performed in the Kimple and Merrins laboratories. NEC, MEK, CMA, MJM and DWL conceived the experiments and secured funding. NEC, IK, EMW, ENK, MDS, BAS, CP, DY, SIAA, GG, DSS, SEC, MEB, JAW, RJF and DWL performed the experiments. NEC, IK, EMW, MDS, BAS, CP, KAM, MEK, CMA, MJM and DWL analysed the data. NEC and DWL wrote the manuscript.

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