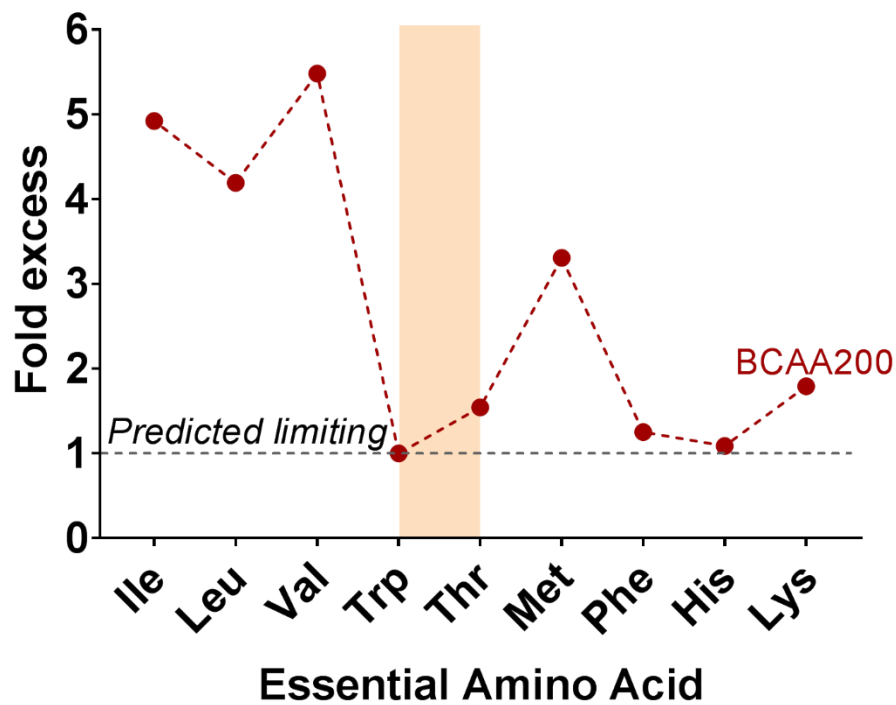


In the format provided by the authors and unedited.

Branched-chain amino acids impact health and lifespan indirectly via amino acid balance and appetite control

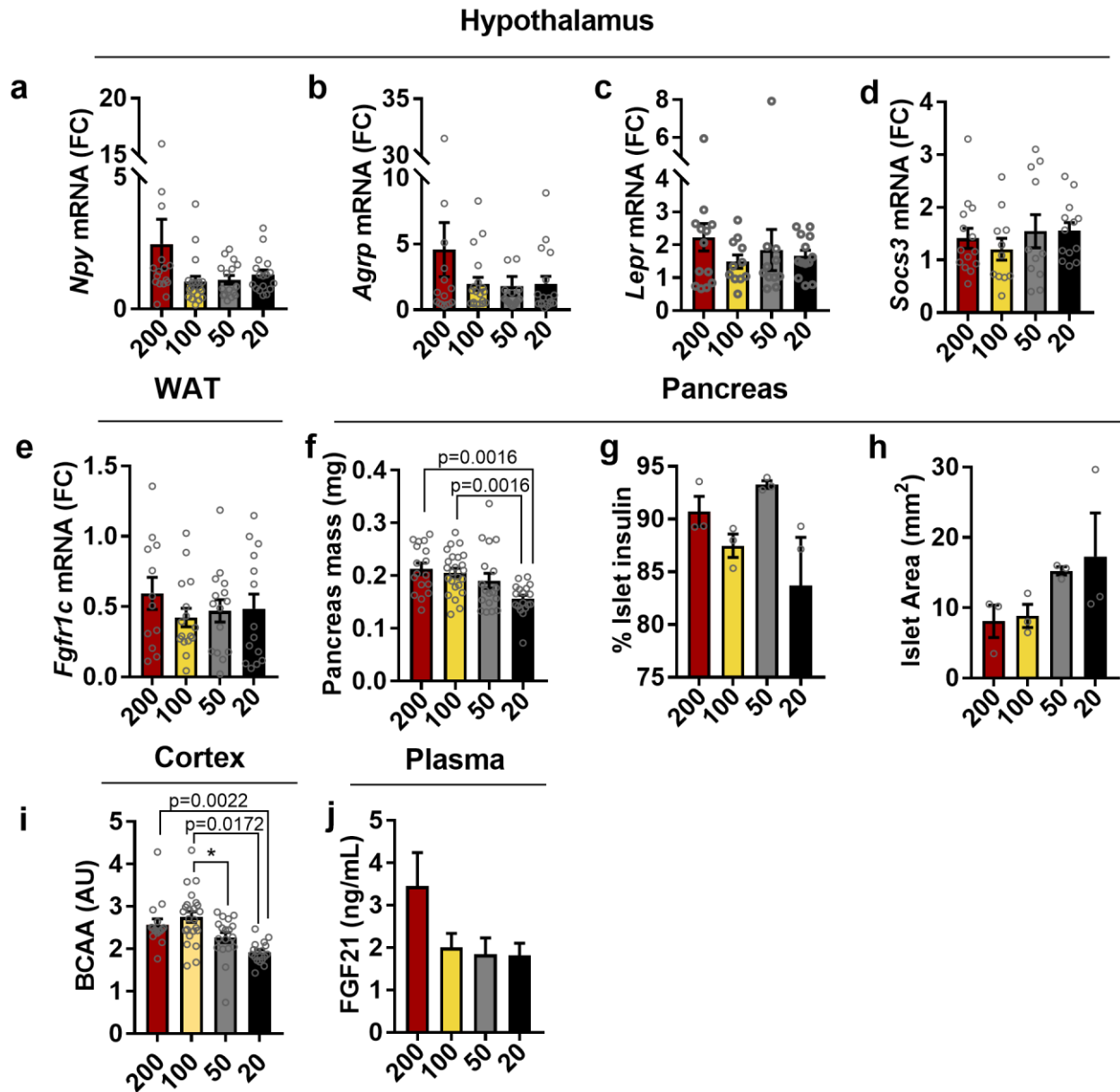
Samantha M. Solon-Biet^{1,2*}, Victoria C. Cogger^{1,3,4,5}, Tamara Pulpitel^{1,3}, Devin Wahl^{1,3,4}, Ximonie Clark^{1,2}, Elena E. Bagley^{1,6}, Gabrielle C. Gregoriou^{1,6}, Alistair M. Senior^{1,2}, Qiao-Ping Wang^{1,2,7}, Amanda E. Brandon^{1,3}, Ruth Perks¹, John O'Sullivan^{1,8}, Yen Chin Koay^{1,8}, Kim Bell-Anderson^{1,2}, Melkam Kebede^{1,2}, Belinda Yau^{1,2}, Clare Atkinson^{1,2}, Gunbjorg Svineng⁹, Tim Dodgson^{1,2}, Jibran A. Wali^{1,2}, Matthew D. W. Piper¹⁰, Paula Juricic¹¹, Linda Partridge¹¹, Adam J. Rose¹², David Raubenheimer^{1,2}, Gregory J. Cooney^{1,3}, David G. Le Couteur^{1,3,4,5} and Stephen J. Simpson^{1,2*}

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Supplementary Fig. 1

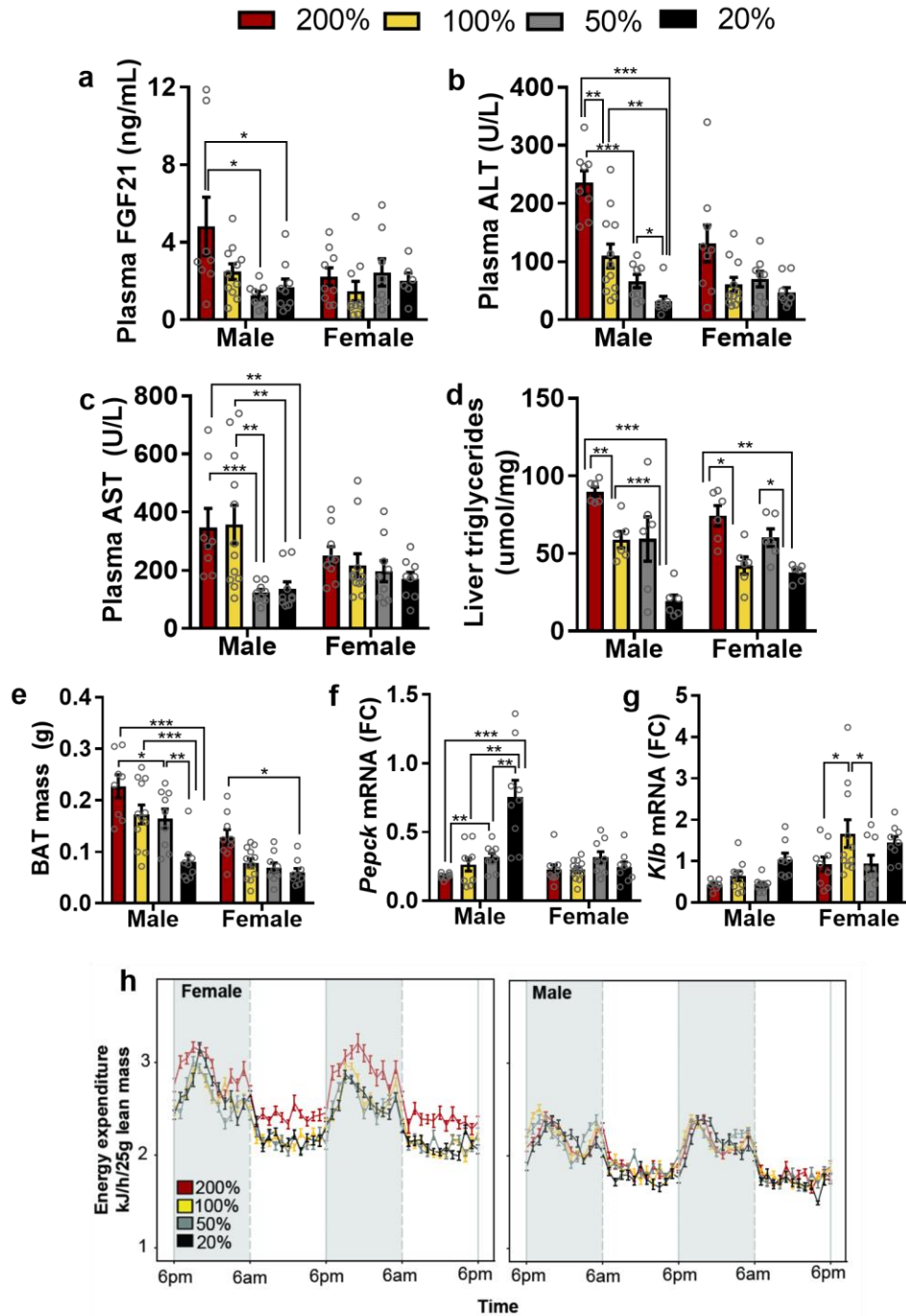
Prediction of amino acid limitation based on exome-matching. The relative proportional representation of each essential amino acid in the BCAA200 diet is plotted as a function of its value in the organism (x-axis) and food (y-axis) based on exome matching. The amino acids with lowest abundance in food relative to the predicted needs of the organism fall lowest (equal to 1). Thr and Trp are highlighted in the orange box.



Supplementary Fig. 2

Appetite signaling and metabolic outcomes. (a-d) Hypothalamic mRNA expression of key appetite markers measured by qRT-PCR (Fold change; FC). For (a,b) 200%, n=16; 100%, n=19; 50%, n=17; 20%, n=17 biologically independent mice. For (c,d) 200%, n=14; 100% and 50%, n=11, 20%, n=13 biologically independence mice. (e) mRNA expression of FGF21 co-receptor *Fgfr1c* quantified in gonadal white adipose tissue (200%, n=12; 100%, n=16; 50%, n=15; 20%, n=14 biologically independent mice). (f) Pancreas mass (mg) (200% and 50%, n=17; 100%, n=24; 20%, n=18 biologically independent mice), (g) % of insulin/islet and (h) the average islet area per mouse (n=3 biologically independent mice for all groups). (i) Brain BCAA levels measured by

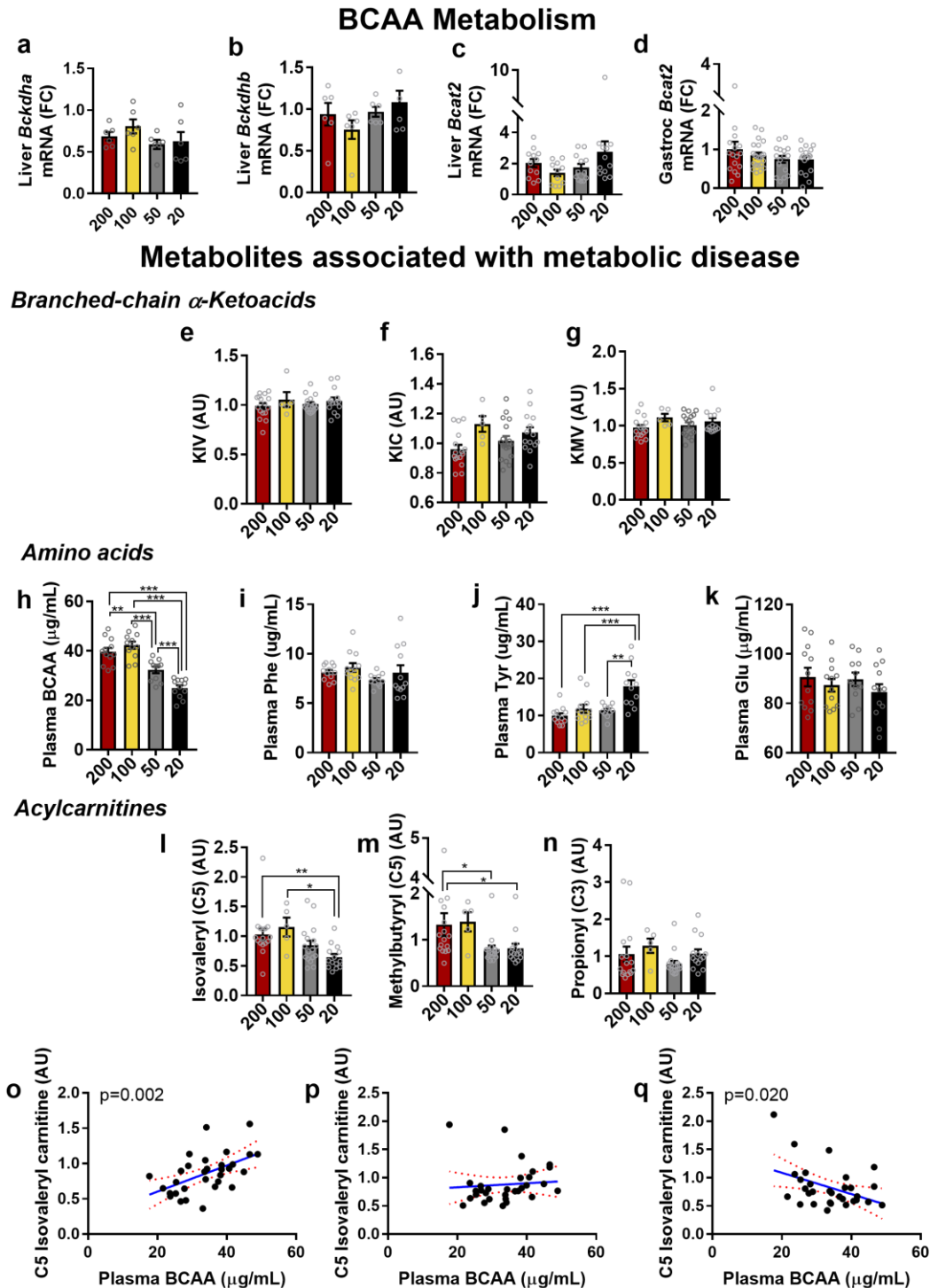
LC-QQQ-MS (200%, n=16; 100%, n=24, 50%, n=50; 20%, n=17 biologically independent mice).
(j) plasma FGF21 levels (200%, n=17; 100%, n=21; 50%, n=16; 20%, n=15 biologically independent mice). Data are from 15 month old male and female mice. ANOVA was used for normal and log-normal data, and Kruskal Wallis for non-normal data. Pairwise comparisons amongst diets for normal and log-normal data were made using t-tests (two-sided). For non-normal data, pairwise comparisons amongst diets were made using Kruskal Wallis test. All bars indicate means $\pm$ SEM. In (i), * indicates $p=1.2 \times 10^{-5}$.



Supplementary Fig. 3

The effect of diet and sex on response variables. Response variables showing a significant sex effect include (a) FGF21 levels (200%, n=17; 100%, n=21; 50%, n=16; 20%, n=15 biologically independent mice), (b) ALT (200%, n=17; 100%, n=23; 50%, n=17; 20%, n=15 biologically independent mice) and (c) AST (200%, n=17; 100%, n=14; 50%, n=18; 20%, n=18 biologically independent mice). (d) Hepatic triglyceride content (n=12 biologically independent mice for all groups), (e) BAT mass (n=12 biologically independent mice for all groups), (f) hepatic *Pepck*

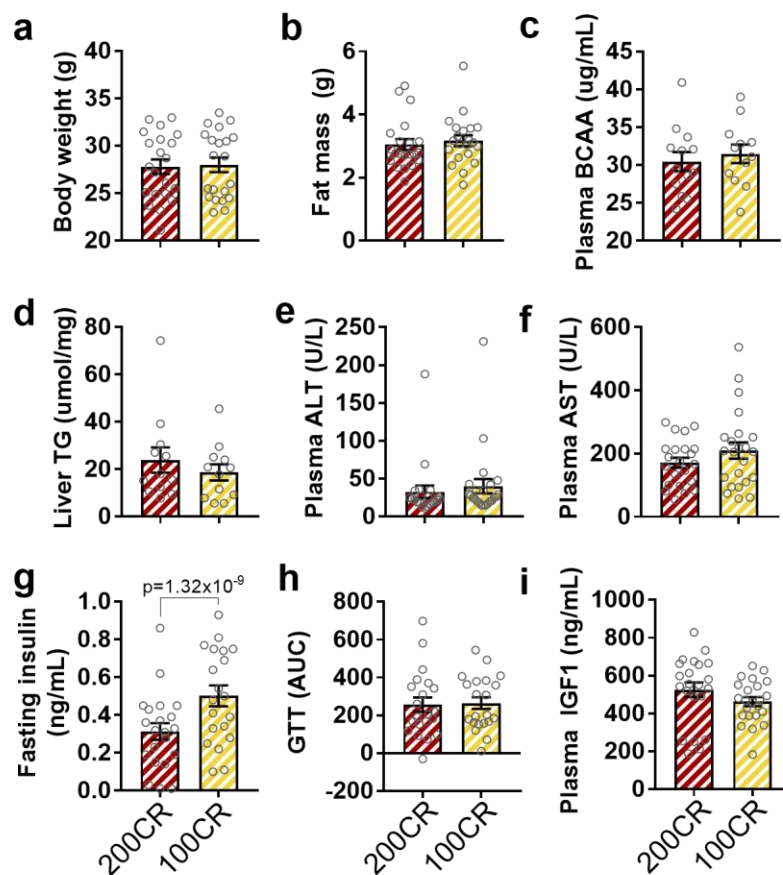
mRNA levels (200%, n=17; 100%, n=21; 50% and 20%, n=18 biologically independent mice) and (g) *Klb* mRNA expression in gonadal white adipose tissue (200%, n=12; 100%, n=16; 50%, n=15; 20%, n=14 biologically independent mice). (h) Energy expenditure (200%, 50% and 20%, n=12; 100%, n=16 biologically independent mice). Data were parsed by sex only if analysis revealed a statistically significant main effect of Sex or a significant Diet*Sex interaction effect. Sexes were then analyzed separately by ANOVA. All data are from mice at 15 months of age. All bars indicate means $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ based on posthoc analysis following correction for multiple testing.



Supplementary Fig. 4

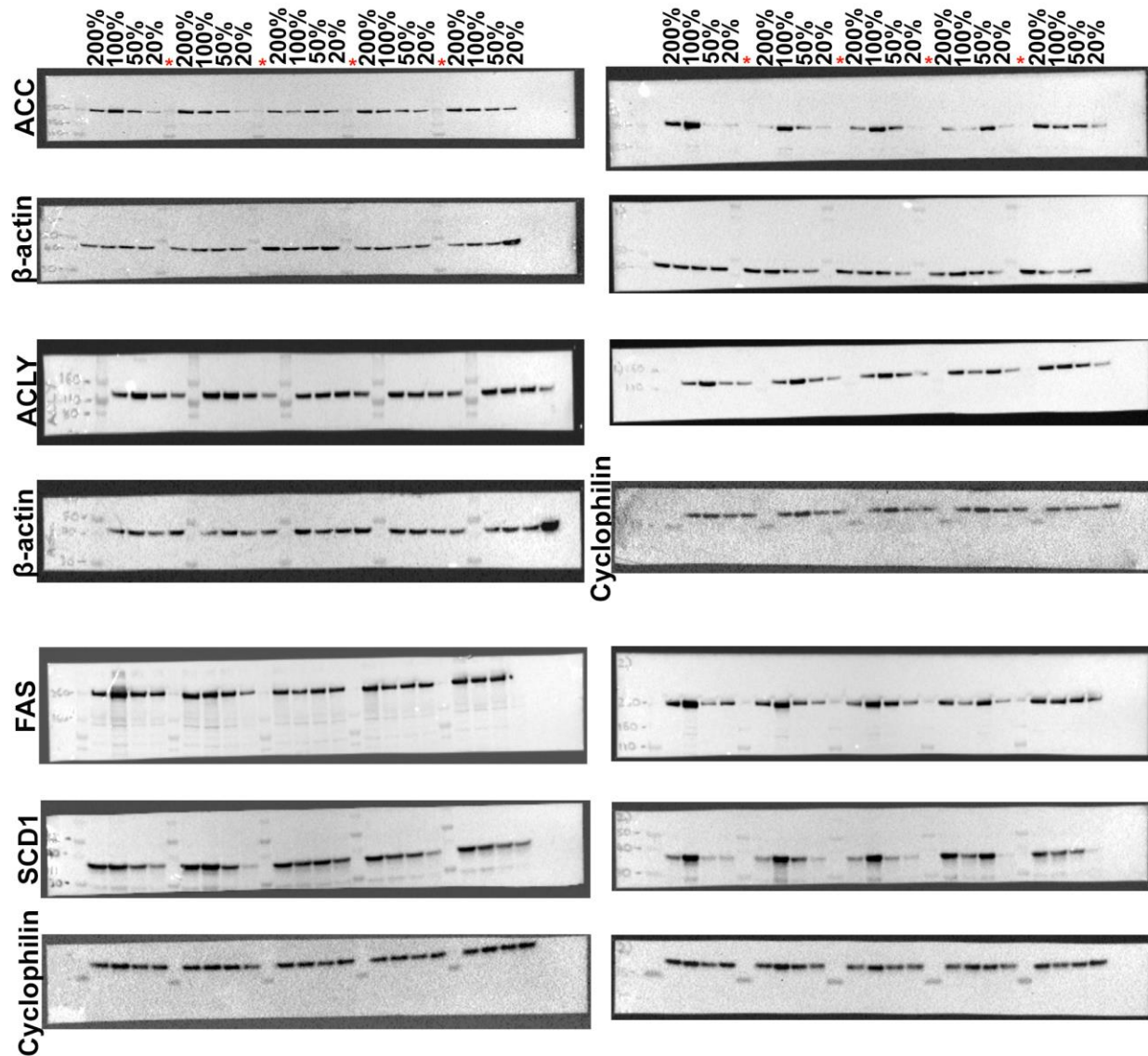
Markers of BCAA metabolism in 15 month old mice. mRNA expression of hepatic (a) *Bckdha* (b) *Bckdhb* ($n=6$ biologically independent mice) (c) Liver *Bcat2* (200%, 50%, 20%, $n=12$; 100%, $n=10$ biologically independent mice) and (d) *Bcat2* (200%, 100%, 50%, $n=16$; 20%, $n=17$ biologically independent mice) in gastrocnemius muscle. (e-g) Plasma branched-chain α -ketoacids

(200%, n=16; 100%, n=5; 50%, n=17; 20%, n=14 biologically independent mice). α -ketoisovalerate (KIV), α -ketoisocaproate (KIC) and α -keto-methylvalerate (KMV). Plasma concentrations of metabolites associated with metabolic dysfunction including the amino acids. (h) BCAA, (i) phenylalanine (Phe), tyrosine (Tyr) and glutamine (Glu) and the acylcarnitines (200%, 100% and 20%, n=12; 50%, n=11 biologically independent mice). (l) C5 isovaleryl carnitine, (m) C5 methylbutyryl carnitine and (n) C3 propionyl carnitine (200%, n=15; 100%, n=5; 50%, n=16; 20%, n=14 biologically independent mice). (o-q) The relationship between plasma acylcarnitines and BCAAs analyzed by Pearson correlation. $r=0.533$, 0.083 and -0.415 , for (o-q), respectively (n=32 independent data points). Red lines show the 95% confidence interval. Data is from males and females combined. All bars indicate means $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ based on posthoc analysis following correction for multiple testing.



Supplementary Fig. 5

Metabolic outcomes of mice fed a BCAA200 or BCAA100 diet under 20%CR. (a) body weight and (b) fat mass did not differ between groups (n=21 biologically independent mice for all groups). (c) Plasma BCAAs remained identical, despite increased dietary BCAAs in the BCAA200 diet (200CR, n=13; 100CR n=12 biologically independent mice). (d-f) liver lipids (n=12 biologically independent mice) and liver function did not differ (200CR, n=21; 100CR, n=23 biologically independent mice). Among the responses measured, only (g) fasting insulin showed significant differences (p=0.012; 200CR, n=21; 100CR, n=20) with no change in (h) glucose tolerance (GTT) (n=21 biologically independent mice) or (i) IGF1 levels (200CR, n=22; 100CR, n=23). Data are from males and females combined, collected at 15 months of age and analyzed using t-test (two-sided). Error bars indicate mean ± SEM.



Supplementary Fig. 6

Markers of *de novo* lipogenesis in liver quantified using western blot. Data are from mice culled at 15 months of age. ACLY: ATP-citrate lyase; SCD1: stearoyl-coA desaturase-1; FAS: fatty acid synthase; ACC: acetyl-coA carboxylase. Data are from males and females combined, collected at 15 months of age. Uncropped images of western blot membranes are shown as composite images of the chemiluminescent image of the proteins of interest and the optical image of the molecular weight marker (*, in red). For all blots, n=10 biologically independent mice. Please see the Reporting Summary and methods for details on all antibodies.

Supplementary Table 1

Composition of experimental diets. The % of protein, carbohydrate and fat remained constant. Total protein (casein, amino acids), carbohydrate (sucrose, wheatstarch, dextrinized starch) and fat (soy oil) were fixed across all diets. Trace minerals and vitamins were kept constant at 1.4 g/kg and 10 g/kg, respectively. Manipulation of amino acid content was achieved by first adjusting the proportion of casein, then supplemented with free amino acids as indicated by +. NME of amino acids from casein was calculated at 13.3 kJ/g and 18 kJ/g for free amino acids. NME of wheatstarch, sucrose and dextrinized starch was calculated at 14.13, 14.92 and 14.6 kJ/g, respectively. NME of fat was calculated at 37 kJ/g.

Diet	BCAA200			BCAA100			BCAA50			BCAA20		
NME (kJ/g)	14.3			14.3			14.3			14.3		
% Protein energy	18			18			18			18		
% Carbohydrate energy	64			64			64			64		
% Fat energy	18			18			18			18		
Amino acid (g/kg)	From casein	+	Final	From casein	+	Final	From casein	+	Final	From casein	+	Final
Valine	6.6	18.6	25.2	12.5		12.5	6.3		6.3	2.5		2.5
Leucine	9.0	26.6	35.6	17.1		17.1	8.6		8.6	3.4		3.4
Isoleucine	5.0	12.9	17.9	9.4		9.4	4.7		4.7	1.9		1.9
Threonine	4.1	2.1	6.2	7.9		7.9	3.9	2.3	6.2	1.6	5.1	6.7
Methionine	2.7	5.9	8.6	5.3	3.0	8.3	2.6	6.9	9.5	1.0	8.8	9.8
Tryptophan	0.9	0.6	1.5	1.7		1.7	0.9	1.2	2.1	0.3	2.2	2.5
Cysteine	0.4		0.4	0.8		0.8	0.4		0.4	0.2		0.2
Lysine	7.5	1.8	9.3	14.2		14.2	7.1	9.4	16.5	2.8	13.9	16.7
Phenylalanine	4.9		4.9	9.3		9.3	4.7	9.8	14.5	1.9	15.9	17.8
Tyrosine	5.2		5.2	9.9		9.9	5.0		5.0	2.0		2.0
Alanine	2.9		2.9	5.5		5.5	2.8	2.2	5.0	1.1	3.6	4.7
Aspartic Acid	3.3		3.3	6.3		6.3	3.2	4.2	7.4	1.3	5.9	7.2
Asparagine	3.2		3.2	6.0		6.0	3.0		3.0	1.2		1.2
Glutamic Acid	11.2		11.2	21.4		21.4	10.7	20.6	31.3	4.3	33.4	37.7
Glutamine	9.5		9.5	18.0		18.0	9.0		9.0	3.6		3.6
Proline	10.3		10.3	19.6		19.6	9.8	7.5	17.3	3.9	12.6	16.5
Arginine	3.7	0.8	4.5	7.1		7.1	3.6	3.3	6.9	1.4	5.3	6.7
Glycine	1.8		1.8	3.5		3.5	1.7	1.7	3.4	0.7	2.3	3.0
Histidine	2.7		2.7	5.2		5.2	2.6	3.0	5.6	1.0	4.8	5.8
Serine	5.6		5.6	10.6		10.6	5.3		5.3	2.1		2.1
Total protein (g/kg)	169.7			194.4			167.7			152.0		
Other (g/kg)												
Soy oil	70			70			70			70		
Wheatstarch	405			404			405			405		
Sucrose	100			100			100			100		
Dextrinized starch	132			132			132			132		
Cellulose	78			50			80			96		
AA energy (kJ/g)												
BCAA	1.32			0.52			0.26			0.1		
non-BCAA	1.26			2.08			2.31			2.45		
Ratio BCAA: non-BCAA	1.05			0.25			0.11			0.04		

Supplementary Table 2

Macronutrient and amino acid composition of experimental diets supplemented with either Met, Thr or Trp. The % of protein, carbohydrate and fat (as a % of total net metabolizable energy) remained constant. The BCAA content of each diet is matched to the BCAA200 diet (Table S1). Total protein (casein, amino acids), carbohydrate (sucrose, wheatstarch, dextrinized starch) and fat (soy oil) were fixed across all diets. Trace minerals and vitamins were kept constant at 1.4 g/kg and 10 g/kg, respectively. Manipulation of amino acid content was achieved by first adjusting the proportion of casein, followed by the addition of individual amino acids as indicated by +. NME of amino acids from casein was calculated at 13.3 kJ/g and 18 kJ/g for free amino acids. NME of wheatstarch, sucrose and dextrinized starch was calculated at 14.13, 14.92 and 14.6 kJ/g, respectively. NME of fat was calculated at 37 kJ/g.

Diet	BCAA200 + Met			BCAA200 + Thr			BCAA200 + Trp		
NME (kJ/g)	14.3			14.3			14.3		
% Protein	18			18			18		
% Carbohydrate	64			64			64		
% Fat	18			18			18		
Amino acid (g/kg)	From casein	+	Final	From casein	+	Final	From casein	+	Final
Valine	5.9	19.3	25.2	6.3	18.9	25.2	6.3	18.9	25.2
Leucine	8.1	27.5	35.6	8.6	27.0	35.6	8.6	27.0	35.6
Isoleucine	4.5	13.4	17.9	4.8	13.1	17.9	4.8	13.1	17.9
Threonine	3.7	1.9	5.6	4.0	5.3	9.3	4.0	1.9	5.9
Methionine	2.5	10.4	12.9	2.7	4.4	7.1	2.7	5.6	8.3
Tryptophan	0.8	0.5	1.3	0.6	0.8	1.4	0.9	1.4	2.3
Cysteine	0.4		0.4	0.4		0.4	0.4		0.4
Lysine	6.7	1.6	8.3	7.2	1.8	9.0	7.2	1.8	9.0
Phenylalanine	4.4		4.4	4.7		4.7	4.7		4.7
Tyrosine	4.7		4.7	5.0		5.0	5.0		5.0
Alanine	2.6	1.4	4.0	2.8	0.0	2.8	2.8		2.8
Aspartic Acid	2.8	0.1	2.9	3.0	0.2	3.2	3.0	0.2	3.2
Asparagine	3.0		3.0	3.2		3.2	3.2		3.2
Glutamic Acid	10.1		10.1	10.8		10.8	10.8		10.8
Glutamine	8.5		8.5	9.1		9.1	9.1		9.1
Proline	9.2		9.2	9.9		9.9	9.9		9.9
Arginine	3.4	0.6	4.0	3.6	0.4	4.0	3.6	0.4	4.0
Glycine	1.6		1.6	1.7		1.7	1.7		1.7
Histidine	2.5		2.5	2.6		2.6	2.6		2.6
Serine	5.0		5.0	5.3	0.1	5.4	5.3	0.1	5.4
Total protein	167.1			168.3			167.0		
Other (g/kg)									
Casein	95			101			101		
Soy oil	70			70			70		
Wheatstarch	404			404			406		
Sucrose	100			100			100		
Dextrinized starch	132			132			132		
Cellulose	82			80			80		

Supplementary Table 3

Plasma metabolites and associated KEGG pathways correlated with BCAA intake. Metabolites with a Pearson correlation coefficient (r) >0.1 were analyzed in Metaboanalyst using the KEGG (*Mus musculus*) pathway library. Both positively and negatively correlated metabolite sets were analyzed using the integrated pathway analysis and pathways with significant Holm-adjusted p-values are shown.

Metabolite	Pathway	KEGG	Pearson
L-3-Hydroxykynurenine	Tryptophan metabolism	C03227	-0.466
Pyruvic acid	TCA cycle	C00022	-0.419
2-Aminobenzoic acid	Tryptophan metabolism	C00108	-0.377
5-Hydroxyindoleacetic acid	Tryptophan metabolism	C05635	-0.342
Oxalacetic acid	TCA cycle	C00036	-0.331
Fumaric acid	TCA cycle	C00122	-0.272
L-Malic acid	TCA cycle	C00149	-0.263
L-Kynurenine	Tryptophan metabolism	C00328	-0.211
Serotonin	Tryptophan metabolism	C00780	-0.194
L-Tryptophan	Tryptophan metabolism	C00078	-0.141
cis-Aconitic acid	TCA cycle	C00417	-0.114
NADP	Glutathione metabolism	C00006	0.105
L-Valine	Aminoacyl-tRNA biosynthesis	C00183	0.108
L-Asparagine	Aminoacyl-tRNA biosynthesis	C00152	0.223
L-Glutamic acid	Glutathione metabolism; aminoacyl t-RNA biosynthesis	C00025	0.262
Pyroglutamic acid	Glutathione metabolism	C01879	0.272
L-Lysine	Aminoacyl-tRNA biosynthesis	C00047	0.284
Glycine	Glutathione metabolism	C00037	0.298
L-Isoleucine	Aminoacyl-tRNA biosynthesis	C00407	0.298
Ornithine	Glutathione metabolism	C00077	0.305
L-Phenylalanine	Aminoacyl-tRNA biosynthesis	C00079	0.363
L-Glutamine	Aminoacyl-tRNA biosynthesis	C00064	0.374
L-Leucine	Aminoacyl-tRNA biosynthesis	C00123	0.375
Spermine	Glutathione metabolism	C00750	0.433
Pathway name	Total compounds in pathway/Actual hits	Raw p	Holm adj. p
Tryptophan metabolism	40/7	2.28E-04	0.019
Citrate cycle (TCA cycle)	20/5	3.45E-04	0.028
Glutathione metabolism	26/6	2.39E-04	0.020
Aminoacyl-tRNA biosynthesis	69/9	6.23E-04	0.050

Supplementary Table 4

KEGG pathways associated with hypothalamic gene expression. 949 genes were positively correlated with BCAA intake and 989 genes were negatively correlated with BCAA intake ($r>0.3$) as analysed by Pearson's correlation. Counts indicate the number of genes in the corresponding pathway and only pathways with a minimum of 6 genes are shown. EASE Score indicates the raw, non-adjusted p-value (modified one-sided Fisher Exact Test from analysis in DAVID). Fold enrichment indicates the magnitude of enrichment and ranking enriched terms should be done along with the EASE score. Benjamini is the adjusted p-value, corrected for multiple testing. Statistical significant indicates adjusted p-values <0.05 .

KEGG Pathway	Count	EASE Score	Fold Enrichment	Benjamini
Positively correlated				
MicroRNAs in cancer	16	0.029	1.8	0.620
Pathways in cancer	21	0.025	1.7	0.630
Rap1 signaling pathway	14	0.019	2.1	0.660
Transcriptional misregulation in cancer	12	0.016	2.3	0.700
Thyroid hormone signaling pathway	12	0.001	3.3	0.190
NF-kappa B signaling pathway	11	0.001	3.6	0.100
Notch signaling pathway	6	0.019	3.8	0.580
Negatively correlated				
p53 signaling pathway	9	0.001	4.1	0.270
Apoptosis	6	0.043	3.1	0.830
Peroxisome	8	0.017	3	0.750
Pyrimidine metabolism	8	0.042	2.5	0.870
Cell cycle	9	0.047	2.2	0.760
Metabolic pathways	55	0.017	1.3	0.860
Biosynthesis of antibiotics	13	0.044	1.9	0.780

Supplementary Table 5 is supplied as a separate supplementary information file.

Supplementary Table 6

Macronutrient and amino acid composition of exome matched diets.

Diet	P23	P6	P6+BCAAs
NME (kJ/g)	16.7	16.6	16.6
% Protein	23	6	6
% Carbohydrate	57	74	74
% Fat	20	20	20
Amino acid (g/kg)	Final	Final	Final
Valine	13.85	3.67	4.98
Leucine	26.05	6.91	9.52
Isoleucine	10.82	2.87	4.21
Threonine	12.54	3.33	3.09
Methionine	6.54	1.73	1.61
Tryptophan	4.61	1.22	1.14
Cysteine	5.06	1.34	1.25
Lysine	20.59	5.46	5.08
Phenylalanine	12.34	3.27	3.05
Tyrosine	10.74	2.85	2.66
Alanine	11.54	3.09	2.85
Aspartic Acid	12.31	3.27	3.04
Asparagine	9.21	2.44	2.27
Glutamic Acid	19.73	5.23	4.87
Glutamine	12.87	3.41	3.18
Proline	12.45	3.3	3.07
Arginine	19.16	5.08	4.72
Glycine	9.06	2.4	2.24
Histidine	7.96	2.11	1.96
Serine	16.41	4.35	4.05
Other (g/kg)			
Casein	-	-	-
Soybean oil	88.5	88	88
Corn starch	240	420	420
Sucrose	100	100	100
Maltodextrin	177.56	184.6	183.06
Cellulose	90	90	90
Mineral & trace elements	35	35	35
Na hydrogen carbonate	3	3	3
Choline Cl	2	2	2
BHT	0.1	0.1	0.1