Impaired branched chain amino acid metabolism alters feeding behavior and increases orexigenic neuropeptide expression in the hypothalamus

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Abstract

Elevation of dietary or brain leucine appears to suppress food intake via a mechanism involving mechanistic target of rapamycin, AMPK, and/or branched chain amino acid (BCAA) metabolism. Mice bearing a deletion of mitochondrial branched chain aminotransferase (BCATm), which is expressed in peripheral tissues (muscle) and brain glia, exhibit marked increases in circulating BCAAs. Here, we test whether this increase alters feeding behavior and brain neuropeptide expression. Circulating and brain levels of BCAAs were increased two- to four-fold in BCATm-deficient mice (KO). KO mice weighed less than controls (25·9 vs 20·4 g, $P < 0.01$), but absolute food intake was relatively unchanged. In contrast to wild-type mice, KO mice preferred a low-BCAA diet to a control diet ($P < 0.05$) but exhibited no change in preference for low- vs high-protein (HP) diets. KO mice also exhibited low leptin levels and increased hypothalamic *Npy* and *Agrp* mRNA. Normalization of circulating leptin levels had no effect on either food preference or the increased *Npy* and *Agrp* mRNA expression. If BCAAs act as signals of protein status, one would expect reduced food intake, avoidance of dietary protein, and reduction in neuropeptide expression in BCATm-KO mice. Instead, these mice exhibit an increased expression of orexigenic neuropeptides and an avoidance of BCAAs but not HP. These data thus suggest that either BCAAs do not act as physiological signals of protein status or the loss of BCAA metabolism within brain glia impairs the detection of protein balance.

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Introduction

An adequate supply of protein is necessary for life, and a number of observations support the existence of regulatory systems that assess endogenous protein/amino acid demand and alter food intake to meet this demand (Peters & Harper 1984, Hannah *et al.* 1990, White *et al.* 1994, Du *et al.* 2000, Jean *et al.* 2001, Westerterp-Plantenga 2003, Anderson & Moore 2004, Lacroix *et al.* 2004, Tome 2004). This regulatory drive to consume adequate protein can be extremely powerful, resulting in protein intake being regulated as a higher priority than energy intake (Sorensen *et al.* 2008, Brooks *et al.* 2010). Yet, to date, very little is known about the mechanisms underlying 'protein balance' and its relation with energy balance.

To achieve this regulation, it seems likely that the brain responds to variations in circulating signals that reflect protein balance. To date, some evidence supports alterations in circulating hormones (Batterham *et al.* 2006), while other works suggest that individual amino acids may act locally within the brain (Panksepp & Booth 1971, Sandoval *et al.* 2008). For instance, the branched chain amino acid (BCAA) leucine suppresses food intake when locally administered into the brain (Cota *et al.* 2006, Morrison *et al.* 2007, Ropelle *et al.* 2008, Blouet *et al.* 2009), and supplementing the diet with excess leucine or BCAAs also suppresses food intake (Ropelle *et al.* 2008, Newgard *et al.* 2009). Together, these data suggest that BCAA, and particularly leucine, may be uniquely suited to serve as signals of protein balance.

To date, the majority of experiments have focused on mechanistic target of rapamycin (MTOR; also known as mammalian target of rapamycin, mTOR) as a probable mediator of brain leucine signaling (Cota *et al.* 2006, Morrison *et al.* 2007, Ropelle *et al.* 2008). However, Blouet *et al.* (2009) demonstrated that local injection of downstream products of BCAA metabolism (α-ketoisocaproic acid or α-ketoisovaleric acid), or pharmacologic manipulation of BCAA metabolism, also altered food intake. As such, it is possible that BCAA metabolism may also produce signals relevant to food intake or protein detection.

To test the effect of altered BCAA metabolism on feeding behavior and hypothalamic function, we focused on mice bearing a whole-body deletion of the mitochondrial form of branched chain aminotransferase (BCATm; She *et al.* 2007). Because these mice exhibit marked increases in circulating BCAAs, they provide a novel model system in which to test whether increased circulating BCAAs represent a signal of protein excess. We hypothesized that BCATm-KO mice would exhibit a phenotype consistent with excess protein, including reduced food intake, reduced orexigenic neuropeptide gene expression, and avoidance of high-protein (HP) diets.

Materials and Methods

Animals

BCATm-deficient mice (KO mice) were developed as described previously by She *et al.* (2007), who also described the general phenotype of increased circulating BCAAs, leanness, and increased oxygen consumption. All studies used male KO mice or wild-type (WT) littermates generated at Virginia Tech (Experiment 1) or from breeders subsequently sent to Pennington Biomedical Research Center (Experiments 2 and 3). Mice were group housed (two to four per cage) in shoebox cages under a 12 h light:12 h darkness cycle and were fed standard chow made available *ad libitum* unless otherwise noted. All experiments were approved by the Institutional Animal Care and Use Committee of Pennington Biomedical Research Center.

Experiment 1: brain amino acids and orexigenic neuropeptides in KO mice

To identify baseline differences in plasma and brain amino acids and hypothalamic neuropeptide expression, 20-week-old male homozygous BCATm-deficient mice (KO; $n=5$) or WT littermates ($n=10$) were rapidly killed, trunk blood was collected, and brains were isolated. Samples of mediobasal hypothalamus containing principally arcuate nucleus were isolated for RNA extraction, while forebrains were collected to assess brain amino acid concentrations via HPLC (see below).

Experiment 2: effects of leptin replacement on feeding behavior, body adiposity, and hypothalamic neuropeptides in KO mice

To determine whether normalization of circulating leptin levels would normalize hypothalamic neuropeptides and food intake, 8-week-old male BCATm-KO mice and WT littermates were adapted to a two-choice diet paradigm in which mice were allowed to self-select between a control diet and a diet containing BCAAs at only 2·5% of the control (BCAA-low). Mice were group housed and acclimated to the two-choice paradigm for 2 weeks and were then single-housed prior to implantation with s.c. osmotic minipumps (Alzet Minipumps, Durect, Cupertino, CA, USA) delivering

either PBS, 3 μg/day leptin, or 10 μg/day leptin ($n=9$–10 per group) (White *et al.* 2009). Leptin was procured from the National Hormone Pituitary Program (Dr A F Parlow). Food intake and body weight were measured daily, and after 6 days of infusion, mice were rapidly killed, retroperitoneal and epididymal fat pads were collected and weighed, and hypothalamus was isolated for RNA extraction.

To test whether KO mice also exhibited an altered preference for low-protein (LP)- vs HP diet, WT ($n=9$) and KO ($n=13$) mice were offered a choice between isocaloric diets providing casein protein at 10% of energy (LP) or 35% of energy (HP; Table 1). Food intake was measured daily for 10 days, but mice were otherwise not manipulated.

Experiment 3: effects of acute, high-dose leptin treatment on hypothalamic neuropeptides in control and BCATm-KO mice

To determine whether KO mice were unresponsive to leptin-dependent regulation of mRNA expression, BCATm-KO mice and WT littermates received i.p. injections of leptin in the fed or fasted state ($n=8$ per group). Mice received an i.p. injection of either leptin (5 μg/g body weight) or saline, at the beginning of the fast (Time 0) and then after 12 and 24 h. Four hours after the final injection, mice were killed and hypothalami were isolated for RNA extraction.

Amino acid analysis

Brain and plasma amino acids were measured using fluorometric HPLC via methods described previously (Wu & Knabe 1994), except that methanol extraction was used for brain tissue rather than acid precipitation. The *o*-phthaldialdehyde amino acid derivatives were separated by gradient elution using a Supelcosil LC-18 column (15 cm×4·6 mm, 3 μm, Sigma).

Hormone analysis

Trunk blood was collected and allowed to clot at 4 °C overnight, centrifuged at 3000 *g* for 30 min, and serum collected and stored at −80 °C. Serum levels of leptin were measured using RIA kit (Millipore, St Charles, MO, USA), according to the manufacturer's instructions.

RNA extraction and real-time PCR

RNA extraction and real-time PCR were conducted as described previously (Morrison *et al.* 2007, White *et al.* 2010). Total RNA was extracted from mediobasal hypothalamus using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA), according to the manufacturer's instructions. Confirmation of intact *18S* and *28S* RNA bands was achieved by ethidium bromide staining after electrophoresis of samples in 1% agarose gels. Samples were quantified by spectrophotometry, then mRNA was reverse transcribed

Table 1 Experimental diets

Ingredient (g)	Control	Low BCAA	Low protein	High protein
Casein	0	0	100	350
Corn starch	300	300	440·3	291·7
Maltodextrin	125	125	150	75
Sucrose	250	250	107·077	107·077
Cellulose	50	50	50	50
Soybean oil	50	50	25	25
Lard	0	0	75	75
Mineral Mix S10001	35	35	0	0
Mineral Mix S10022C	0	0	3·5	3·5
Sodium bicarbonate	7·5	7·5	0	0
Vitamin mix	10	10	10	10
Choline bitartrate	2	2	2·5	0
Diammonium citrate	0	24	0	0
Calcium carbonate	0	0	10	12·38
Potassium citrate	0	0	2·48	6·58
Potassium phosphate	0	0	6·86	1·6
Sodium chloride	0	0	2·59	2·59
L-arginine	8·27	8·27		
L-histidine–HCl–H_2O	6	6		
L-isoleucine	8	0·205		
L-leucine	12	0·278		
L-lysine–HCl	14	14		
DL-methionine	6	6		
L-phenylalanine	8	8		
L-threonine	8	8		
L-tryptophan	2	2		
L-valine	8	0·205		
L-alanine	10	10		
L-asparagine–H_2O	10	10		
L-cystine	4	4	1·5	5·25
L-glutamic acid	10	10		
L-glutamine	10	10		
Glycine	10	10		
L-proline	10	10		
L-serine	10	10		
L-tyrosine	4	4		
Total	987·8	984·5	990	1018
kcal/g	3·87	3·77	4·13	4·02

into cDNA, and mRNA expression determined using the SYBR green methodology in optical 384-well plates in an ABI PRISM 7900 sequence detector (Applied Biosystems, Branchburg, NJ, USA). All expression data were normalized to cyclophilin mRNA levels.

Statistical analysis

Data were analyzed using the SAS software package (SAS V9, SAS Institute, Cary, NC, USA) using a two-tailed *t*-test or ANOVA using the general linear model procedure. When experiment wide tests were significant, *post hoc* comparisons were made using the LSMEANS statement with the PDIFF option and thus represent least significant differences tests for pre-planned comparisons. For real-time PCR, expression levels were normalized to cyclophilin prior to analysis. All data are expressed as mean±S.E.M., with a probability value of 0·05 considered statistically significant.

Results

Experiment 1: impact of BCATm deletion on brain amino acids and orexigenic neuropeptides

Similar to previous experiments (She *et al.* 2007), BCATm-KO mice exhibited a marked increase in circulating BCAA levels ($P < 0·01$; Fig. 1A), with plasma leucine, isoleucine, and valine concentrations increased by four-, eight-, and seven-fold respectively. Similarly, brain BCAA levels were increased by two-, five-, and three-fold respectively ($P < 0·01$; Fig. 1B). This ratio of blood to brain levels of BCAAs is consistent with the kinetic properties of the system L amino acid transporters (LAT1) expressed in tissues forming the blood–brain barrier (Kanai *et al.* 1998). The increased BCAA concentrations had a variable effect on brain levels of non-BCAAs (Table 2). For example, Glu and Gln concentrations were statistically higher in the BCATm–KOs compared with WT controls and GABA
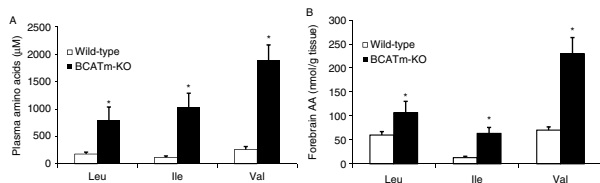
Figure 1 Plasma and brain BCAAs in KO and wild-type (WT) mice. Plasma (A) and brain (B) BCAAs were measured in BCATm-KO mice (n=5) and WT littermates (n=10; 20 weeks of age) using fluorometric HPLC methods. For brain measures, forebrains were utilized, as hypothalami were used to assess neuropeptide expression. Levels of brain and plasma BCAA were increased in KO compared with control mice. *$P<0.05$ compared to WT.

was unchanged. Amino acids that are precursors of the monoamine neurotransmitters and/or substrates for LAT1 were either unchanged (Phe, Trp, and Met) or higher (Tyr). These data do not support the possibility that elevations in BCAAs interfered with brain uptake of other large, neutral amino acids (Tews *et al*. 1979).

Agrp mRNA levels were significantly increased within BCATm–deficient mice as compared with controls ($P<0.05$; Fig. 2). *Npy* mRNA levels were roughly doubled, but this difference did not reach statistical significance ($P=0.14$). *Pomc* mRNA expression was also measured, but the results were inconsistent between experiments and as such inconclusive (data not shown). Interestingly, changes in hypothalamic *Npy* and *Agrp* mRNA expression are consistent with the profile of animals on LP diets (White *et al*. 1994, Morrison *et al*. 2007).

Experiment 2: role of hypoleptinemia in the phenotype of BCATm-deficient mice

Two weeks prior to minipump implantation, mice were transferred to a 2-choice diet paradigm offering the choice between a control diet and a diet very low in BCAAs (2·5% BCAAs compared to control; Table 1). KO mice were smaller than WT mice ($P<0.001$; Fig. 3A), and this reduction in body weight was highly consistent with previous work in these mice (She *et al*. 2007). Baseline food intake (prior to minipump) was not different between KO and WT mice on an absolute basis (Fig. 3C), but when food intake was adjusted for body weight, KO mice consumed more food than controls ($P<0.001$; Fig. 3D). While differences in absolute intake were relatively subtle, KO and WT mice exhibited marked differences in their preference between the control and low-BCAA diets ($P<0.001$; Fig. 3B). While WT mice significantly preferred the control diet, KO mice preferred the diet with very low BCAAs. KO mice therefore appear to avoid dietary BCAAs, presumably in response to their excess endogenous BCAAs.

To test whether the elevation of circulating BCAAs represents a signal of excess protein, a separate group of BCATm–deficient mice and control mice were maintained

on more generic HP (35% casein protein, Table 1) and LP diets (10% casein protein). BCATm–KO mice exhibited a subtle decrease in absolute food intake compared with controls (Fig. 4C), which was accounted for by adjusting to body weight (Fig. 4D). Despite their elevation of circulating BCAAs, KO mice did not exhibit altered selection between the LP and HP diets (Fig. 4B). Thus, the specific avoidance of BCAAs in the KO mice did not translate into a more generalized avoidance of protein.

Following this 2-week dietary acclimation period, male KO mice and their WT littermates were implanted with osmotic minipumps designed to a) return plasma leptin levels to normal following administration of leptin at a low dose (3 μg/day) and b) increase leptin above normal following administration of leptin at a higher dose (10 μg/day). Circulating leptin levels were reduced in KO mice ($P<0.05$; Fig. 5), although the difference was not as large as previously reported (She *et al*. 2007). Infusion of 3 μg/day leptin normalized circulating leptin to physiological levels in KO mice and produced a small but significant increase in the WT mice. In contrast, the higher 10 μg/day dose produced a marked increase in both KO and WT mice. Leptin treatment had little effect on body weight gain over the 6 days of infusion, as only KO mice receiving the highest dose exhibited a subtle decrease in weight ($P=0.054$; data not shown). Leptin significantly suppressed total daily food intake in both genotypes ($P<0.05$; Fig. 6A), such that there was a significant main effect of leptin treatment ($P<0.001$), but no significant treatment by genotype interaction ($P=0.53$). Although it suppressed total intake, leptin treatment had no effect on the preference ratio between the control and low-BCAA–diets in either genotype (Fig. 6B) such that KO mice

Table 2 Forebrain amino acids (μM) in BCATm-KO mice compared to wild-type littermates

| Amino acid	Wild-type	BCATm-KO	*P*
TYR	37±9	127±12	**0·001***
ORN	314±29	460±1	**0·001***
GLN	4116±497	6388±595	**0·002***
ASN	144±8	187±14	**0·01***
THR	452±54	601±73	**0·02***
ALA	823±193	724±31	**0·02***
LYS	81±5	99±12	**0·02***
GLU	9509±603	10 532±563	**0·03***
SER	840±50	916±61	**0·03***
ARG	287±29	365±32	0·07
ASP	1993±214	2264±199	0·13
MET	75±6	71±18	0·21
TRP	22±4·8	14±6·6	0·22
CIT	16±1·1	22±1·5	0·24
GLY	983±95	1178±83	0·5
TAU	9357±225	9125±125	0·67
PHE	51±3·1	50±11	0·93
GABA	2859±280	3020±126	0·97

*$P<0.05$
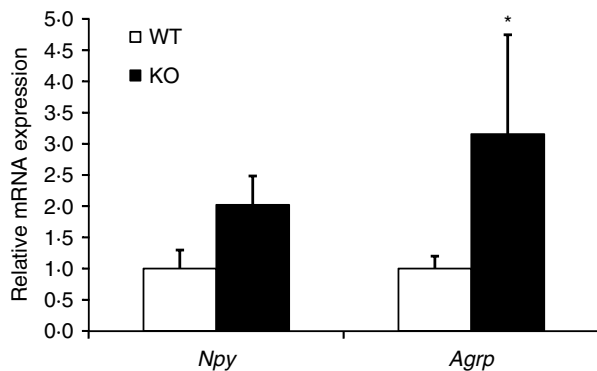
Figure 2 Hypothalamic *Npy* and *Agrp* mRNA expression in KO and wild-type (WT) mice. Total RNA was extracted from hypothalamus from 20-week-old BCATm-KO mice ($n=5$) and WT littermates ($n=10$), and expression of *Npy* and *Agrp* mRNA was measured via real-time PCR. *Npy* mRNA levels were numerically but not statistically increased, while *Agrp* mRNA expression was significantly increased in KO mice. *$P<0.05$ compared to WT.

continued to exhibit a marked shift in preference toward the low-BCAA diet despite normalization of circulating leptin.

KO mice were lean compared with WT controls, exhibiting significantly lower retroperitoneal and epididymal fat pad weights ($P<0.01$; Fig. 7). While leptin had little effect on adiposity in control mice, the KO mice exhibited significant decreases in body fat, particularly at the high dose ($P<0.05$; Fig. 7).

The primary goal of Experiment 2 was to determine whether normalization of circulating leptin levels would reduce hypothalamic *Npy* and *Agrp* mRNA expression in BCATm-KO mice. Similar to the results from Experiment 1, a significant increase in *Npy* and *Agrp* levels was detected within KO mice ($P<0.01$; Fig. 8A). However, increasing circulating leptin had no effect on either *Npy* (Fig. 8B) or *Agrp* (Fig. 8C) mRNA expression within KO mice. These data suggest that the increase in *Npy* and *Agrp* mRNA levels in BCATm-KO mice cannot be explained by reduced circulating leptin.

Experiment 3: effects of acute, high-dose leptin treatment on hypothalamic neuropeptides in control and BCATm-KO mice

It is possible that hypothalamic NPY/AgRP neurons are rendered resistant to the effects of leptin in BCATm-KO mice and that this loss of sensitivity explains the lack of effect of leptin on *Npy* and *Agrp* mRNA levels in these mice. To assess this possibility, a separate group of BCATm-KO mice was treated with acute doses of leptin every 12 h during a 28 h fast, which is a paradigm more typically used to assess the effects of leptin on hypothalamic gene expression (Baskin *et al.* 1999, Mizuno & Mobbs 1999, Korner *et al.* 2001, Morrison *et al.* 2005). While leptin had no effect on either *Npy* or *Agrp* in the fed state, fasting increased both *Npy* and *Agrp* mRNA expression ($P<0.05$; Fig. 9), and injection with leptin blocked this fasting induced increase.

Discussion

Nutrients such as glucose, fatty acids, and amino acids can act locally within the brain to regulate neuronal function, food intake, and body weight (Sandoval *et al.* 2008, Blouet & Schwartz 2010, Moran 2010). Dietary protein content or physiological protein demand influences food intake and/or food selection (White *et al.* 1994, Westerterp-Plantenga 2003, Anderson & Moore 2004, Tome 2004), as well as energy expenditure (Zhang *et al.* 2007, Smeets *et al.* 2008, Westerterp-Plantenga *et al.* 2009). Collectively, these obser-vations suggest that regulatory systems detect variations in protein balance and regulate food intake, food selection, and metabolism accordingly (Sorensen *et al.* 2008, Brooks *et al.* 2010). Significant emphasis has recently been placed on the BCAA leucine, based on its ability to suppress food intake when administered directly into the brain or following supplementation in the diet (Cota *et al.* 2006, Morrison *et al.* 2007, Ropelle *et al.* 2008, Blouet *et al.* 2009, Newgard *et al.* 2009). Yet, some uncertainty exists as to the physiological relevance and specific signaling mechanism(s) underlying brain leucine signaling. Several lines of evidence indicate that MTOR signaling is an important mediator, as central leucine activates MTOR, and rapamycin pretreatment blocks the effects of leucine (Cota *et al.* 2006, Ropelle *et al.* 2008, Blouet *et al.* 2009). However, administration of a downstream
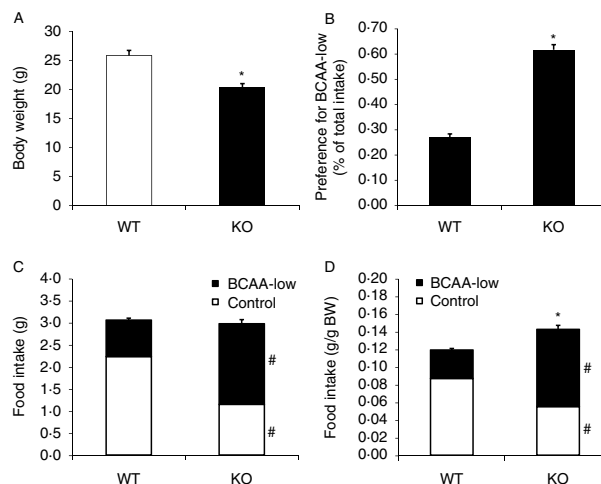

Figure 3 Body weight, food intake, and food preference in BCATm-KO mice. Eight-week-old BCATm-KO mice ($n=22$) and wild-type (WT) controls ($n=30$) were group housed (two to three per cage) and offered the choice between a control diet and a diet containing only 2·5% of control levels of BCAAs (BCAA-low). Body weights (A) and per cage food intake ($n=9-10$ per group) were measured, with food intake being expressed as both total intake (C) and intake adjusted for body weight (D). Food intake is also presented as a preference ratio for the low-BCAA diet (BCAA-low divided by total intake, B). KO mice were lighter than WT mice, and food intake relative to body weight was significantly increased. Most strikingly, KO mice exhibit a marked shift in preference toward the low-BCAA diet *$P<0.05$ compared to WT. #$P<0.05$ for individual diet choices compared with WT.
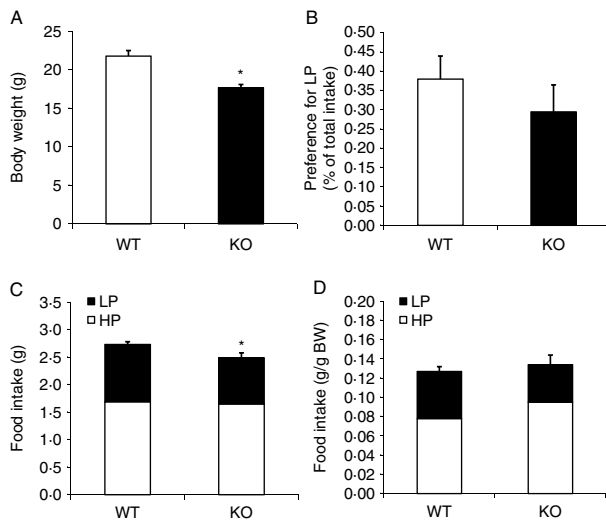
Figure 4 Food intake and selection between low-protein (LP) and high-protein (HP) diets in BCATm-KO mice. Eight-week-old BCATm-KO (n=9) and wild-type (WT) littermates (n=13) were single-housed and offered a choice between HP (35% protein energy) and LP (10% protein energy) diets. Body weight (A), preference ratio for LP diet (B), total food intake (C), and body weight adjusted food intake (D) were measured for 10 days. KO mice weighed less and consumed less total food than WT mice, but there was no difference between the genotypes regarding preference for LP vs HP diet. *P<0·05 compared to WT.

product of BCAA metabolism or pharmacological activation of BCAA oxidation was also shown to reduce food intake (Blouet *et al*. 2009), suggesting that the metabolism of BCAAs might generate regulatory signals.

Considering this evidence, we utilized BCATm–deficient mice as a unique model in which circulating BCAAs are markedly increased. BCATm serves as the initial step in the metabolism of BCAAs. Two individual isozymes of BCAT exist: BCATm, which is expressed in most peripheral tissues and brain glia, and BCATc, which is expressed almost exclusively within neurons (Hutson *et al*. 1992, Sweatt *et al*. 2004*a*,*b*). Because BCATm–deficient mice are unable to metabolize BCAAs within peripheral tissues, particularly muscle, they exhibit a marked increase in circulating BCAAs (She *et al*. 2007). Based on the previous data (Cota *et al*. 2006, Morrison *et al*. 2007, Ropelle *et al*. 2008, Blouet *et al*. 2009), one would anticipate that this elevation of circulating BCAAs would signal protein excess, resulting in a decrease in food intake and avoidance of dietary protein. In addition, because our previous work indicates that animals on a LP diet exhibit hyperphagia and increased *Npy* and *Agrp* mRNA expression (White *et al*. 1994, Morrison *et al*. 2007), our prediction was that the increased levels of BCAAs within BCATm–KO mice would act to suppress food intake and *Npy* and *Agrp* mRNA expression. Yet, the results are inconsistent with this hypothesis, as BCATm mice do not show a clear suppression of food intake, and *Npy* and *Agrp* mRNA expression was actually increased in

these mice. It should be noted that while *Agrp* mRNA expression was increased in both experiments, *Npy* was only significantly increased in Experiment 2. This lack of statistical significance in Experiment 1 is likely due to the low animal numbers and reduced power in this initial experiment because the pattern of expression was similar. Finally, we also measured *Pomc* expression in these samples, but the results were highly inconsistent across experiments. As such, the role of *Pomc* in contributing to the phenotype in these mice remains unclear.

At least two possible explanations exist for observed changes in hypothalamic neuropeptide expression: the first possibility is that BCAAs have no effect on hypothalamic neuropeptides *in vivo* and that the increases in *Npy* and *Agrp* are driven by some secondary effect (i.e. reduced circulating leptin, see below). The second possibility is that, while neuronal BCAA metabolism is intact, the loss of glia BCAA metabolism in some fashion impairs brain amino acid sensing, resulting in the increase in *Npy* and *Agrp* despite the increase in BCAAs. Considering that the observed increases in *Npy* and *Agrp* are fully consistent with the changes that we observe in animals on a LP diet, and previous *in vivo* and *in vitro* experimentation suggesting that amino acids may regulate these neuropeptides, we propose that altered glial BCAA metabolism contributes at least partly to the altered neuropeptide expression in these mice. However, early work in BCATm–KO indicated very low levels of circulating leptin (She *et al*. 2007), which could also contribute to the observed increases in *Npy* and *Agrp*. We therefore wanted to rule out the possibility that low leptin levels contribute to the phenotype of these mice. To test this question, we chronically infused BCATm–KO mice and control littermates with two
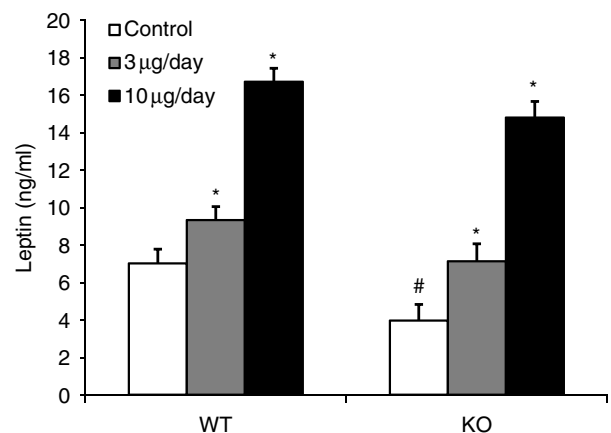

Figure 5 Leptin replacement in BCATm-KO mice. Ten-week-old BCATm-KO or wild-type mice were implanted with s.c. osmotic minipumps delivering saline, 3 μg/day leptin, or 10 μg/day leptin (nine to ten mice per group). Infusion lasted for 6 days, and on day 6 trunk blood leptin levels were measured via RIA. Infusion of serum leptin increased leptin at both doses in both genotypes. BCATm-KO mice receiving saline exhibited reduced circulating leptin levels, and infusion of the low dose served to fully normalize leptin in these KO animals. *P<0·05 compared to respective saline-infused group. #P<0·05 for WT–control vs KO–control.
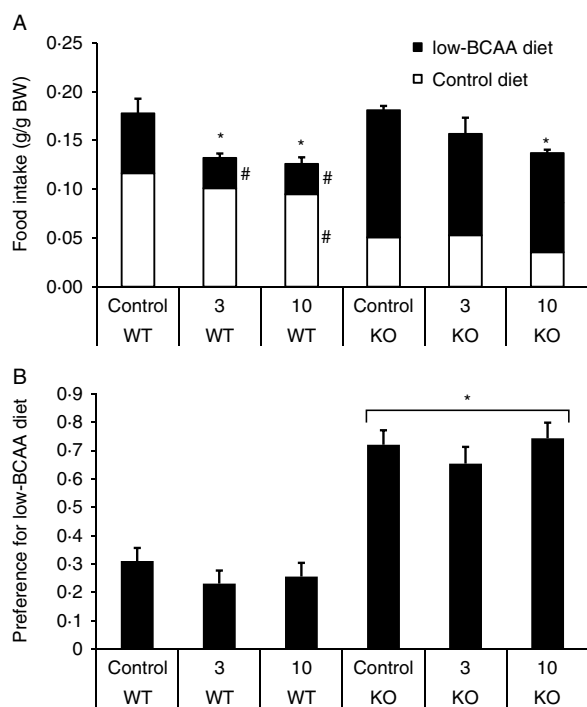
Figure 6 Food intake and food preference in BCATm-KO mice infused with leptin. BCATm-KO or wild-type mice were implanted with s.c. osmotic minipumps delivering saline, 3 μg/day leptin, or 10 μg/day leptin. Mice were offered the choice between a control diet and a diet containing only 2·5% of control levels of BCAAs (BCAA-low). Food intake normalized to body weight and preference ratio for the low-BCAA diet were measured over 6 days of leptin infusion. (A) Leptin infusion reduced food intake in both genotypes such that there was an overall treatment effect, but no genotype effect or treatment by genotype interaction. *$P<0·05$ compared to respective saline-infused group. #$P<0·05$ for individual diet choices compared with saline control. (B) KO mice exhibited a marked shift in preference toward the low-BCAA diet, and this shift was unaffected by leptin treatment. *$P<0·05$ compared to WT–control.

doses of leptin, resulting in a physiological normalization of circulating leptin levels in the low dose, and a physiological increase with the high dose.

Normalization of circulating leptin had several affects in the KO mice, including reduced food intake and body adiposity. Interestingly, the KO mice exhibited a much larger reduction in body adiposity in response to leptin compared with WT mice. Since the KO mice are lean with reduced circulating leptin, it would be logical to speculate that they are more sensitive to exogenous leptin. However, leptin produced a relatively similar decrease in food intake in both genotypes. As such, it is unclear why KO mice responded to leptin with a larger reduction in adiposity, and this difference could be due to direct effects of increased BCAAs on the adipocyte or to the metabolic consequences of increased BCAAs or deletion of BCATm. Regardless, these data clearly indicate that the doses of leptin infused into the KO mice were physiologically relevant.

While the KO mice clearly responded to leptin, neither dose of leptin had any effect to reduce *Npy* or *Agrp* expression in these mice. To confirm that leptin is capable of reducing *Npy* and *Agrp* in these mice, we injected a very high–dose leptin into fed and fasted BCATm-KO mice. At these pharmacological doses, leptin blocked the fasting–induced increase in *Agrp* and *Npy*. Thus, we conclude that BCATm-KO mice are capable of responding to leptin, but that low leptin levels are not responsible for the observed increase in *Npy* and *Agrp* expression. Instead, these data are more consistent with innate alterations in brain BCAA sensing or metabolism as contributing to the alterations in hypothalamic neuropeptide gene expression.

While BCATm-KO mice exhibit leptin–independent increases in *Npy* and *Agrp*, absolute food intake in these mice was only modestly altered. Previous work in these mice demonstrated that KO mice were smaller and leaner than controls, but that they consumed more food when that intake was normalized to body weight (She *et al.* 2007). In our hands, BCATm-KO mice are consistently smaller and leaner than controls, and initial work in Experiment 2 also indicated that they consumed similar amounts of food on an absolute basis and thus more food on a per gram body weight basis. However, subsequent experiments did not detect this difference, with KO mice showing a slight decrease in absolute food intake, but no change when intake was normalized to their reduced body weight. Taken together, we conclude that absolute food intake is relatively unperturbed in these mice, despite the markedly increased BCAAs. Thus, these data suggest that either increased BCAAs have no effect on food intake or some other defect (loss of glial BCAA metabolism) attenuates the response to increased BCAAs.

In contrast to their relatively normal absolute food intake, BCATm-KO mice exhibit a specific change in their preference for BCAAs. When given the choice between a control diet and a diet that was practically devoid of BCAAs, the low–BCAA diet constituted only 27% of the WT intake, but 62% the KO intake. Thus, the KO selected significantly more of the low-BCAA diet, suggesting that the
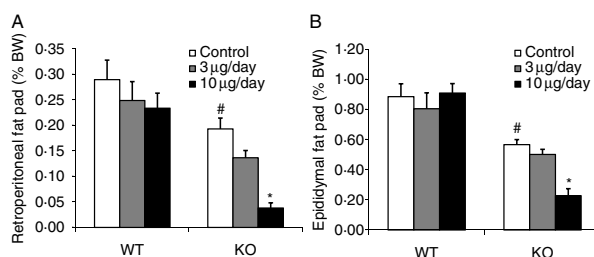

Figure 7 Fat pad weights in leptin-treated, wild-type (WT) and BCATm-KO mice. Retroperitoneal (A) and epididymal (B) fat pads were collected and weighed after 6 days of leptin infusion. Fat pads in BCATm-KO mice were reduced compared with WT, while leptin infusion further reduced fat pad weight in KO mice but not in WT mice. *$P<0·05$ compared to respective saline-infused group; #$P<0·05$ for WT–control vs KO–control.
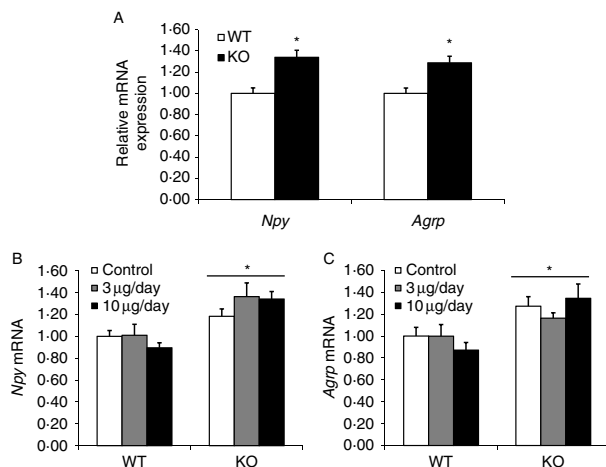
Figure 8 Hypothalamic *Npy* and *Agrp* gene expression in BCATm-KO mice infused with leptin. Following 6 days of s.c. leptin infusion, hypothalamus was removed, total RNA was extracted, and *Npy* and *Agrp* expression was measured via real-time PCR. (A) Main effect of genotype on mRNA expression, demonstrating that KO mice have increased *Npy* and *Agrp* (*$P<0.05$). (B) *Npy* expression following leptin treatment. Leptin replacement has no effect on *Npy* expression. *$P<0.05$ compared to WT–control. (C) *Agrp* expression following leptin treatment. Leptin replacement has no effect on *Agrp* expression. *$P<0.05$ compared to WT–control.

BCATm-KO mice detect their abnormally high BCAAs and avoid consuming these amino acids. Surprisingly, this altered preference did not translate to protein in general. Thus, despite the evidence that leucine or BCAAs may signal excess protein, the KO mice chose normal amounts of protein when given the choice between low or high in protein. Because the low-BCAA and control diets were isocaloric and contained normal amounts of all amino acids besides BCAAs, BCATm mice had the luxury of avoiding BCAAs without negatively impacting energy or protein intake. However, when maintained on the standard LP and HP diets, avoiding BCAAs would result in a marked reduction in overall protein intake. Taken together, these data suggest that an elevation of BCAAs does not represent an overall signal of excess protein (Anderson *et al.* 1990) and instead suggests that the altered preference was limited to the three BCAAs. Although further work is required to define why KO mice avoid BCAAs, these data are consistent with previous work demonstrating that neuronal systems detect imbalances in dietary and/or circulating amino acids and alter food intake and selection to counteract these imbalances (Fromentin & Nicolaidis 1996, Gietzen & Rogers 2006, Rudell *et al.* 2011). In addition, this shift in preference was insensitive to leptin treatment, suggesting that the regulation of protein intake and selection is regulated separately from energy intake.

Finally, these data also highlight the possibility that glia may contribute to amino acid detection within the brain. Astrocytes are classically associated with neuronal support, functioning to maintain the extracellular environment by both regulating nutrient and metabolite balance and

contributing to the uptake and break down of secreted neuropeptides (Volterra & Meldolesi 2005). Yet, recent evidence indicates that astrocytes may also act in a regulatory role by detecting and secreting various signaling molecules, influencing the activity of nearby neurons, and having direct effects on synaptic plasticity (Araque *et al.* 1999, Newman 2003, Haydon & Carmignoto 2006, Hermann *et al.* 2009). In particular, studies on brain glucose sensing have implicated astrocytes as a functional mediator of glucose detection (Young *et al.* 2000, Guillod–Maximin *et al.* 2004, Marty *et al.* 2005), while BCAA metabolism in astrocytes contributes to the glutamate–glutamine cycle and neuronal glutamate turnover (Yudkoff 1997, Lieth *et al.* 2001). Because BCATm–KO mice exhibit elevated circulating BCAAs but intact neuronal BCAA metabolism, we anticipated a phenotype more consistent with excess protein availability (i.e. reduced food intake and orexigenic neuropeptide expression). Instead, BCATm–KO mice exhibit a phenotype that is more representative of animals on LP diets. Therefore, while the current data do not provide unequivocal proof for the importance of glial BCAA metabolism in amino acid detection, these data together with previous work are consistent with the possibility that impaired glial BCAA metabolism may alter neuronal function and hypothalamic neuropeptide expression, and future experiments utilizing glial and neuronal specific knockouts will be required to directly test this hypothesis.

In summary, we have demonstrated that loss of BCAA transamination results in specific changes in diet selection and hypothalamic neuropeptide gene expression. These changes in neuropeptide expression and protein selection are independent of changes in circulating leptin, indicating not only that mechanisms other than leptin drive the increased *Npy* and *Agrp* but also that protein intake and selection may be unrelated to the regulation of energy intake. Finally, considering that hypothalamic *Npy* and *Agrp* expression is increased despite elevations in circulating amino acids, these data raise the possibility that impaired glial BCAA metabolism results in a loss of amino acid sensing within the brain.
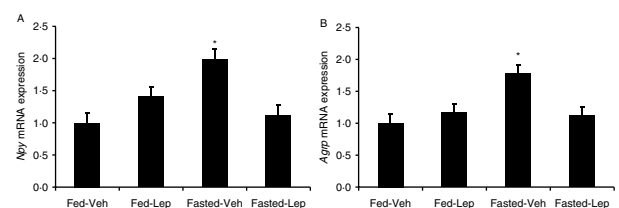

Figure 9 Effect of acute, high-dose leptin on *Npy* and *Agrp* mRNA. BCATm-KO mice were fed or fasted for 28 h and received i.p. injections of either leptin (5 μg/g body weight) or saline at the beginning of the fast (time 0), and then after 12 and 24 h (eight mice per group). Four hours after the final injection, hypothalamus was isolated to assess *Npy* and *Agrp* expression via real-time PCR. As expected, fasting significantly increased both *Npy* (A) and *Agrp* (B). In both cases, leptin treatment blocked the fasting-induced increase in neuropeptide expression. *$P<0.05$ compared fed–vehicle.

Declaration of interest

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

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