

Published in final edited form as:

J Endocrinol. 2012 January ; 212(1): 85–94. doi:10.1530/JOE-11-0270.

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 mTOR, AMPK and/or branched chain amino acid (BCAA) metabolism. Mice bearing a deletion of mitochondrial branched chain amino transferase (BCATm), which is expressed in peripheral tissues (muscle) and brain glia, exhibit marked increases in circulating BCAAs. Here we test whether this increase in circulating BCAAs alters feeding behavior and brain neuropeptide expression. Circulating and brain levels of BCAAs were increased 2–4 fold in BCATm-deficient mice (KO). KO mice weighed less than controls (25.9 vs. 20.4g, $P < 0.01$), but absolute food intake was relatively unchanged. In contrast to wildtype 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 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, an avoidance of dietary protein, and a reduction in neuropeptide expression in BCATm-KO mice. Instead, these mice exhibit increased expression of orexigenic neuropeptides and an avoidance of BCAAs but not high protein. These data thus suggest either that BCAAs do not act as physiological signals of protein status, or that the loss of BCAA metabolism within brain glia impairs the detection of protein balance.

Keywords

protein intake; amino acid metabolism; hypothalamus; leucine

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Declarations of Interest

The authors have nothing to disclose.

Introduction

An adequate supply of protein is necessary for life, and a number of observations support the existence of regulatory systems which assess endogenous protein/amino acid demand and alter food intake to meet this demand (Anderson and Moore 2004; Du, et al. 2000; Hannah, et al. 1990; Jean, et al. 2001; Lacroix, et al. 2004; Peters and Harper 1984; Tome 2004; Westerterp-Plantenga 2003; White, et al. 1994). This regulatory drive to consume adequate protein can be extremely powerful, resulting in protein intake being regulated at a higher priority than energy intake (Brooks, et al. 2010; Sorensen, et al. 2008). Yet to date very little is known about the mechanisms underlying “protein balance” and its relationship to 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 work suggests that individual amino acids may act locally within the brain (Panksepp and Booth 1971; Sandoval, et al. 2008). For instance, the branched-chain amino acid (BCAA) leucine suppresses food intake when locally administered into the brain (Blouet, et al. 2009; Cota, et al. 2006; Morrison, et al. 2007; Ropelle, et al. 2008), and supplementing the diet with excess leucine or BCAAs also suppresses food intake (Newgard, et al. 2009; Ropelle et al. 2008). 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 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 and colleagues (Blouet et al. 2009) demonstrated that local injection of downstream products of branched-chain amino acid metabolism (alpha-ketoisocaproic acid or alpha-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 amino-transferase (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 elevated circulating BCAAs represent a signal of protein excess. We hypothesized that BCATm KO would exhibit a phenotype consistent with excess protein, including reduced food intake, reduced orexigenic neuropeptides gene expression and an avoidance of high protein diets.

Materials and Methods

Animals

BCATm-deficient mice (KO mice) were developed as previously described by She and colleagues (She et al. 2007), who also described the general phenotype of elevated circulating BCAAs, leanness and increased oxygen consumption. All studies used male KO mice or wildtype 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 (2-4/cage) in shoebox cages under 12:12 light/dark cycle and were fed standard chow 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 BCAT^m-deficient mice (KO; n = 5) or wildtype littermates (WT; n=10) were rapidly sacrificed, trunk blood collected and brains 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 BCAT^m KO mice and wildtype 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 2-choice paradigm for 2 weeks, and were then single housed prior to implantation with subcutaneous osmotic minipumps (Alzet) delivering either PBS, 3 ug/day leptin, or 10 ug/day leptin (n=9-10/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 sacrificed, retroperitoneal and epididymal fat pads collected and weighed, and hypothalami isolated for RNA extraction.

To test whether KO mice also exhibited an altered preference for low vs. high protein diet, wildtype (n=9) and KO mice (n=13) were offered a choice between isocaloric diets providing casein protein at 10% of energy (low protein) or 35% of energy (high protein; 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 BCAT^m KO mice

To determine if KO mice were unresponsive to leptin-dependent regulation of mRNA expression, BCAT^m-KO mice and wildtype littermates received IP injections of leptin in the fed or fasted state (n=8 per group). Mice received an IP injection of either leptin (5ug/g body weight) or saline, at the start of the fast (Time 0), and then after 12 and 24hrs. 4 hours after the final injection, mice were sacrificed and hypothalami isolated for RNA extraction.

Amino Acid Analysis

Brain and plasma amino acids were measured using fluorometric HPLC via methods described previously (Wu and Knabe 1994), except that a methanol extraction was used for brain tissue rather than acid precipitation. Separation of the o-phthalaldehyde amino acid derivatives was made by gradient elution from a Supelcosil LC-18 column (15cm × 4.6 mm, 3μm, Sigma).

Hormone Analysis

Trunk blood was collected at sacrifice and allowed to clot at 4°C overnight, centrifuged at 3000xg for 30 minutes, and serum collected and stored at -80°C. Serum levels of leptin were measured via radioimmunoassay kit (Millipore, St. Charles, MO), according to the manufacturer's instructions.

RNA extraction and real-time PCR

RNA extraction and real-time PCR was 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), 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 reversed transcribed 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). 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) using a two-tailed t-test or ANOVA using the general linear model (GLM) procedure. When experiment wide tests were significant, post-hoc comparisons were made using the LSMEANS statement with the PDIF 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 \pm SEM, with a probability value of 0.05 considered statistically significant.

Results

Experiment 1

Impact of BCATm deletion on brain amino acids and orexigenic neuropeptides. Consistent with previous experiments (She et al. 2007), BCATm KO mice exhibited a marked increase in circulating BCAA levels ($P < 0.01$; Figure 1A), with plasma leucine, isoleucine, and valine concentrations increased 4, 8 and 7 fold, respectively. Similarly, brain BCAA levels were increased by 2, 5 and 3 fold, respectively ($P < 0.01$; Figure 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 elevated 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 to wildtype controls and GABA was unchanged. Amino acids that are precursors of the monoamine neurotransmitters and/or substrates for LAT1 were either unchanged (Phe, Trp, 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 to controls ($P < 0.05$; Figure 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, the changes in hypothalamic NPY and AgRP mRNA expression are consistent with the profile of animals on low protein diets (Morrison et al. 2007; White et al. 1994).

Experiment 2: Role of hypoleptinemia in the phenotype of BCATm deficient mice

Two weeks prior to minipump implantation, mice were placed on a choice diet paradigm, which offered the choice between a control diet and a diet very low in branched chain amino acids (2.5% BCAAs relative to control; Table 1). KO mice were smaller than wildtype mice ($P < 0.001$; Figure 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 wildtype mice on an absolute basis (Figure 3C), but when food intake was adjusted for body weight, KO mice consumed more food than controls ($P < 0.001$; Figure 3D). While differences in absolute intake were relatively subtle, KO and wildtype mice exhibited marked differences in their preference between the control and low BCAA diet (Figure 3B; $P < 0.001$). While wildtype 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 branched chain amino acids.

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

Following this two week dietary acclimation period, male KO mice and their wildtype littermates were implanted with osmotic minipumps designed to a) return plasma leptin levels to normal following administration of leptin at a low dose (3ug/day), and b) increase leptin above normal following administration of leptin at a higher dose (10ug/day). Circulating leptin levels were reduced in KO mice ($P < 0.05$; Figure 5), although the difference was not as large as previously reported (She et al. 2007). Infusion of 3ug/day of leptin normalized circulating leptin to physiological levels in KO mice, and produced a small but significant increase in the wildtype mice. Contrastingly, the higher 10ug/day dose produced a marked increase in both KO and wildtype 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 ($P < 0.05$; Figure 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 BCAA-low diet in either genotype (Figure 6B), such that KO mice continued to exhibit a marked shift in preference toward the low BCAA diet despite normalization of circulating leptin.

KO mice were lean relative to wildtype controls, exhibiting a significant lower retroperitoneal and epididymal fat pad weights ($P < 0.01$; Figure 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 ($P < 0.05$; Figure 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 ($P < 0.01$; Figure 8A). However, increasing circulating leptin had no effect on either NPY (Figure 8B) or AgRP (Figure 8C) mRNA expression within KO mice. These data suggest 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 BCAT^m KO mice were treated with acute doses of leptin every 12 hours during a 28-hour fast, which is a paradigm more typically used to assess the effects of leptin on hypothalamic gene expression (Baskin, et al. 1999; Korner, et al. 2001; Mizuno and Mobbs 1999; 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 ($P < 0.05$; Figure 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 (Blouet and Schwartz 2010; Moran 2010; Sandoval et al. 2008). Dietary protein content or physiological protein demand influences food intake and/or food selection (Anderson and Moore 2004; Tome 2004; Westerterp-Plantenga 2003; White et al. 1994), as well as energy expenditure (Smeets, et al. 2008; Westerterp-Plantenga, et al. 2009; Zhang, et al. 2007). Collectively, these observations suggest that regulatory systems detect variations in protein balance and regulate food intake, food selection and metabolism accordingly (Brooks et al. 2010; Sorensen et al. 2008). Significant emphasis has recently been placed on the branched chain amino acid (BCAA) leucine, based on its ability to suppress food intake when administered directly into the brain or following supplementation in the diet (Blouet et al. 2009; Cota et al. 2006; Morrison et al. 2007; Newgard et al. 2009; Ropelle et al. 2008). 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 (Blouet et al. 2009; Cota et al. 2006; Ropelle et al. 2008). However, administration of a downstream 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 BCAT^m-deficient mice as a unique model in which circulating BCAAs are markedly elevated. Branched-chain aminotransferase serves as the initial step in the metabolism of BCAAs. Two individual isozymes of BCAT exist: BCAT^m, which is expressed in most peripheral tissues and brain glia, and BCAT^c, which is expressed almost exclusively within neurons (Hutson, et al. 1992; Sweatt, et al. 2004a; Sweatt, et al. 2004b). Because BCAT^m-deficient mice are unable to metabolize branched-chain amino acids within peripheral tissues, particularly muscle, they exhibit a marked increase in circulating BCAAs (She et al. 2007). Based on previous data (Blouet et al. 2009; Cota et al. 2006; Morrison et al. 2007; Ropelle et al. 2008), 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 low protein diet exhibit hyperphagia and increased NPY and AgRP mRNA expression (Morrison et al. 2007; White et al. 1994), our predication was that the elevated levels of BCAAs within BCAT^m KO mice would act to suppress food intake and NPY and AgRP mRNA expression. Yet the results are inconsistent with this hypothesis, as BCAT^m 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. Lastly, 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 glial 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 low protein 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 contributes to the phenotype of these mice. To test this question, we chronically infused BCATm KO mice and control littermates with two 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 effects 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 as compared to wildtype 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 elevated 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 marked elevated BCAAs. Thus these data suggest either that increased BCAAs have no effect on food intake, or that some other defect (loss of glial BCAA metabolism) attenuates the response to elevated 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 BCAA-low diet constituted only 27% of the wildtype intake, but 62% the KO intake. Thus the KO selected significantly more of the BCAA-low diet, suggesting that the BCATm KO mice detect their abnormally high BCAAs and avoid consuming these amino acids. Surprisingly, this altered preference did not translate 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 BCAA-low 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 placed 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 suggest 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 and Nicolaidis 1996; Gietzen and 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.

Lastly, 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 and 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; Haydon and Carmignoto 2006; Hermann, et al. 2009; Newman 2003). In particular, studies of brain glucose sensing have implicated astrocytes as a functional mediator of glucose detection (Guillod-Maximin, et al. 2004; Marty, et al. 2005; Young, et al. 2000), while BCAA metabolism in astrocytes contributes to the glutamate-glutamine cycle and neuronal glutamate turnover (Lieth, et al. 2001; Yudkoff 1997). 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 low protein 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. Lastly, 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.

Acknowledgments

Funding

This work was supported by NIH Grants R01-DK081563 and P20-RR021945 to CDM, as well as core facilities supported in part by COBRE (NIH P20-RR021945) and NORC (NIH 1P30-DK072476) center grants from the National Institutes of Health.

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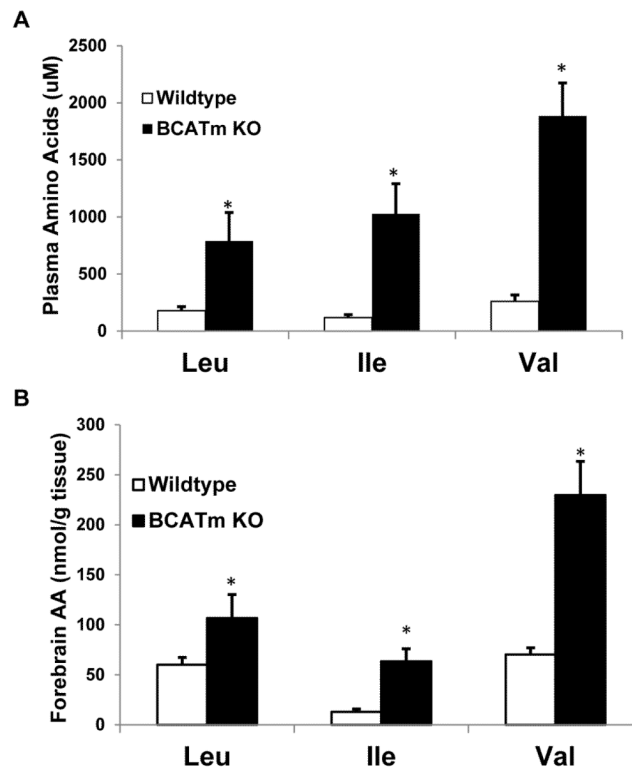


Figure 1. Plasma and brain BCAAs in KO and wildtype mice

Plasma (A) and brain (B) BCAAs were measured in BCATm KO mice (n=5) and wildtype littermates (n=10; 20 wks 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 elevated in KO relative to control mice. * $P < 0.05$ compared to wildtype.

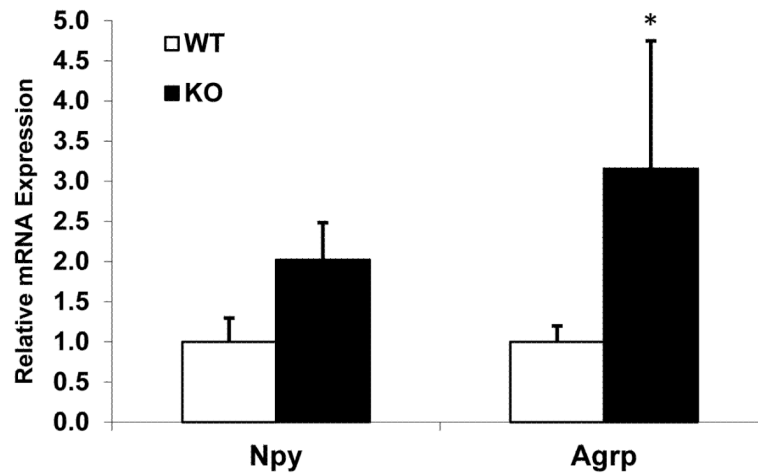


Figure 2. Hypothalamic NPY and AGRP mRNA expression in KO and wildtype mice
Total RNA was extracted from hypothalami from 20 week old BCAT^m KO mice (n=5) and wildtype littermate (n=10), and expression of NPY and AGRP mRNA was measured via real-time PCR. NPY mRNA levels were numerically but not statistically increased, while AGRP mRNA expression was significantly increased in KO mice. *P<0.05 compared to wildtype.

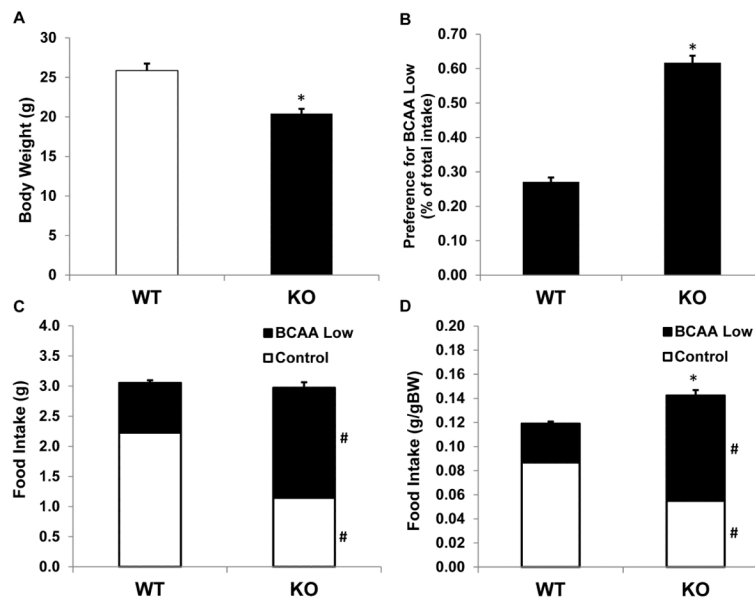


Figure 3. Body weight, food intake and food preference in BCATm KO mice

8 week old BCATm KO mice (n=22) and wildtype controls (n=30) were group housed (2-3 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 BCAA low diet (BCAA-Low divided by Total intake, B). KO mice were lighter than wildtype mice, and food intake relative to body weight was significantly increased. Most strikingly, KO mice exhibit a marked shift in preference toward the BCAA low diet *P<0.05 compared to wildtype. #P<0.05 for individual diet choices compared to wildtype.

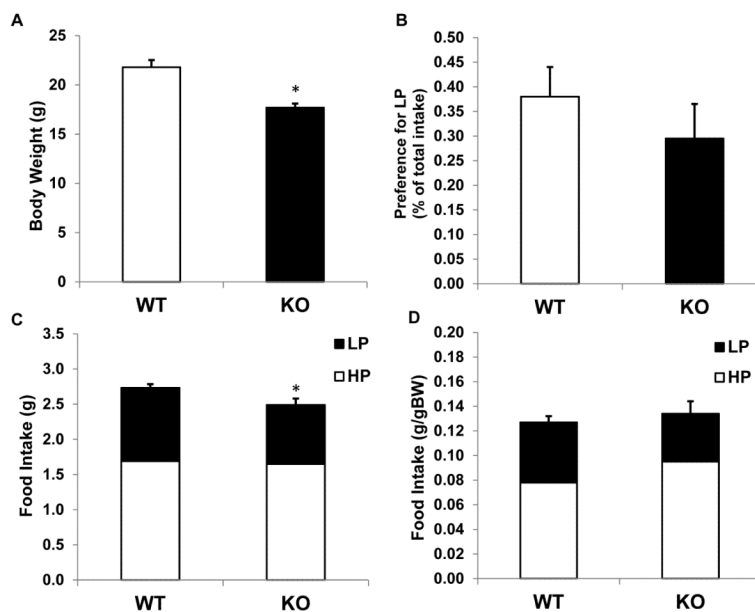


Figure 4. Food intake and selection between low and high protein diets in BCATm KO mice 8-week old BCATm KO (n=9) and wildtype littermates (n=13) were single housed and offered a choice between high protein (35% protein energy) and low protein (10% protein energy) diets. Body weight (A), preference ratio for LP diet (B), total food intake (C), body weight adjusted food intake (D) were measured over 10 days. KO mice weighed less and consumed less total food than wildtype mice, but there was no difference between the genotypes regarding preference for low vs high protein diet. *P<0.05 compared to wildtype.

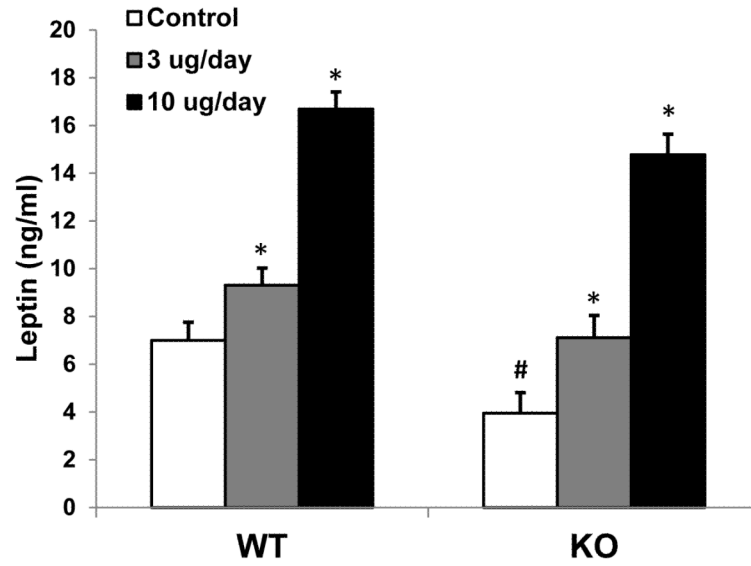


Figure 5. Leptin replacement in BCATm KO mice

10 week old BCATm KO or wildtype mice were implanted with subcutaneous osmotic minipumps delivering saline, 3ug/day leptin, or 10ug/day leptin (9-10 mice per group). Infusion lasted for 6 days, and at sacrifice 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.

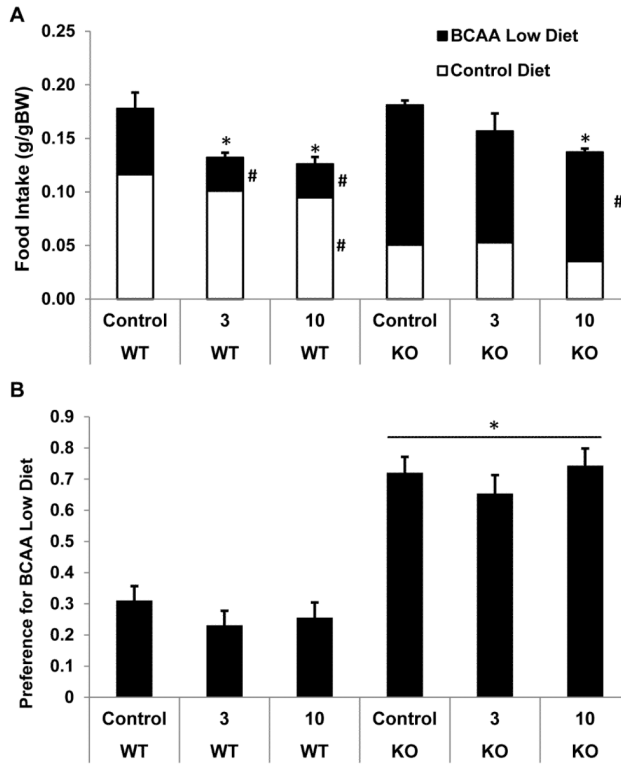


Figure 6. Food intake and food preference in BCATm KO mice infused with leptin
 BCATm KO or wildtype mice were implanted with subcutaneous osmotic minipumps delivering saline, 3ug/d leptin, or 10ug/d leptin. Mice were offered the choice between a control diet and a diet containing only 2.5% of control levels of BCAAs (BCAA-Low). Food intake normalized to body weight and preference ratio for the BCAA low 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*genotype interaction. *P < 0.05 compared to respective saline infused group. #P < 0.05 for individual diet choices compared to saline control. **B)** KO mice exhibited a marked shift in preference toward the BCAA-low diet, and this shift was unaffected by leptin treatment. *P < 0.05 compared to WT-Control.

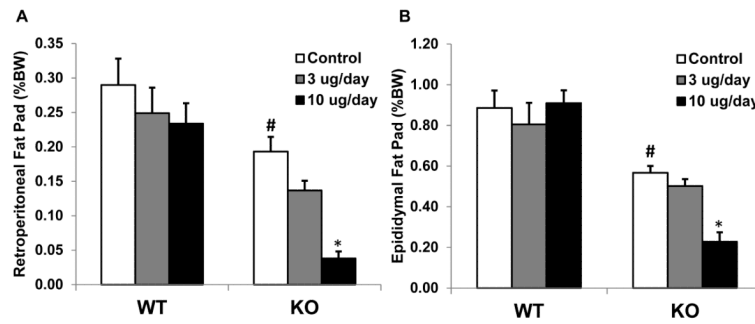


Figure 7. Fat pad weights in leptin-treated wildtype and BCATm KO mice
 Retroperitoneal (A) and Epididymal (B) fat pads were collected and weighed at sacrifice. Fat pads in BCATm KO mice were reduced compared to wildtype, while leptin infusion further reduced fat pad weight in KO mice but not wildtype mice. * $P < 0.05$ compared to respective saline infused group; # $P < 0.05$ for WT-Control vs. KO-Control.

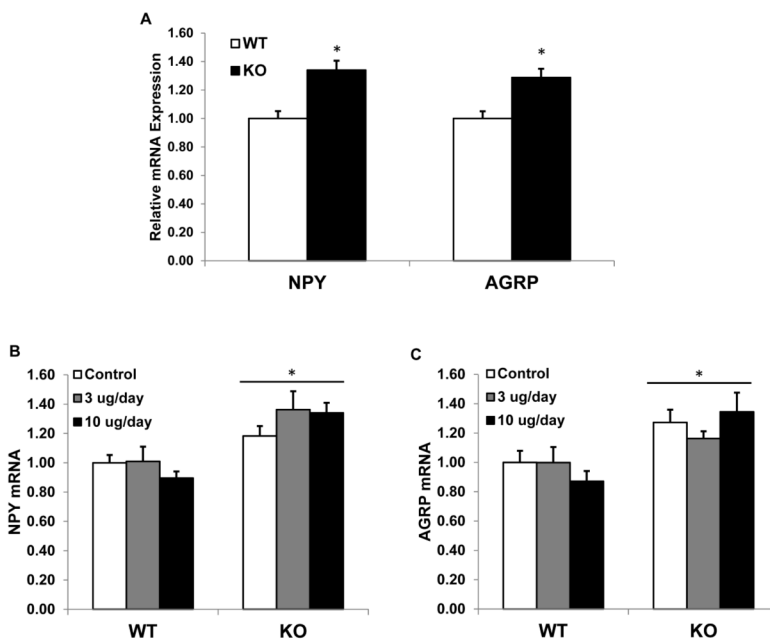


Figure 8. Hypothalamic NPY and AGRP gene expression in BCATm KO mice infused with leptin

Following 6 days of subcutaneous leptin infusion, hypothalami were removed, total RNA extracted, and NPY and AgRP expression 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.

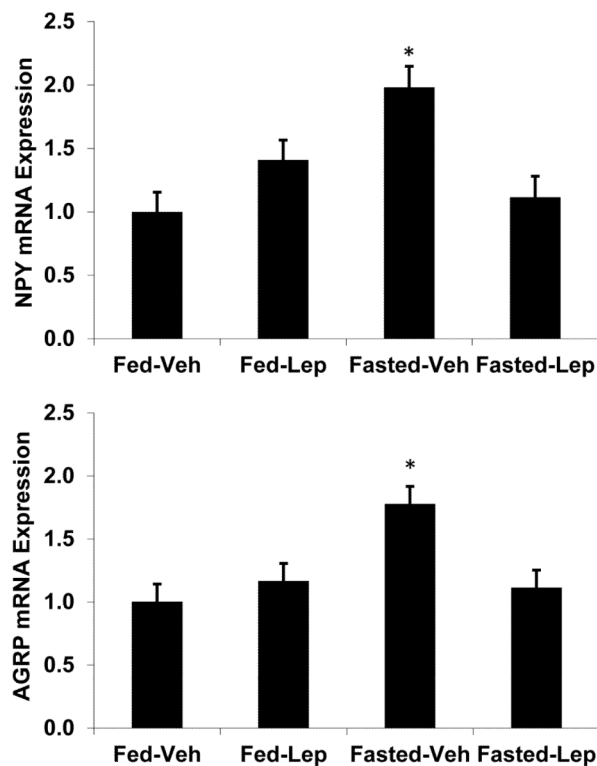


Figure 9. Effect of acute, high dose leptin on NPY and AGRP mRNA

BCAT^m KO mice were fed or fasted for 28 hours, and received IP injections of either leptin (5ug/g body weight) or saline at the start of the fast (Time 0), and then after 12 and 24hrs (8 mice per group). 4 hours after the final injection, mice were sacrificed and hypothalami 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

Table 1
Experimental Diets

Ingredient (gram)	Control	BCAA Low	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 Bitrartrate	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-H2O	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-H2O	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/gm	3.87	3.77	4.13	4.02

Table 2
Forebrain Amino Acids (uM) in BCATm KO mice compared to wildtype littermates

Amino Acid	WildType	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	10532 ± 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