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Adipose transplant for inborn errors of branched chain amino acid metabolism in mice

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Abstract

Liver transplantation appears to be quite beneficial for treatment of Maple Syrup Urine Disease (MSUD, an inherited disorder of branched chain amino acid metabolism); however, there is a limited availability of donor livers worldwide and the first year costs of liver transplants are quite high. Recent studies have suggested that intact adipose tissue, already widely used in reconstructive surgery, may have an underappreciated high capacity for branched chain amino acid (BCAA) metabolism. Here we examined the potential for adipose tissue transplant to lower circulating BCAAs in two models of defective BCAA metabolism, BCATm and PP2Cm [branched chain keto acid dehydrogenase complex (BCKDC) phosphatase] knockout (KO) mice. After 1–2 g fat transplant, BCATm and PP2Cm KO mice gained or maintained body weight 3 weeks after surgery and consumed similar or more food/BCAAs the week before phlebotomy. Transplant of fat into the abdominal cavity led to a sterile inflammatory response and nonviable transplanted tissue. However when 1–2 g of fat was transplanted subcutaneously into the back, either as small (0.1–0.3g) or finely minced pieces introduced with a 18 ga. needle, plasma BCAAs decreased compared to Sham operated mice. In two studies on BCATm KO mice and one study on PP2Cm KO mice, fat transplant led to 52–81% reductions in plasma BCAAs compared to baseline plasma BCAA concentrations of untreated WT type siblings. In PP2Cm KO mice, individual BCAAs in plasma were also significantly reduced by fat transplant, as were the alloisoleucine/Phe ratios. Therefore, subcutaneous fat transplantation may have merit as an adjunct to dietary treatment of MSUD. Additional studies are needed to further refine this approach.

Keywords

Alloisoleucine; Adipose tissue; Branched chain amino acids; Maple Syrup Urine Disease; Transplantation; Mice

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1. Introduction

MSUD is an autosomal recessive inborn error of branched chain amino acid (BCAA) metabolism named for the burnt sugar smell of the urine due to accumulation of incompletely metabolized metabolites in the urine [1]. During their metabolism, BCAAs are transaminated to α-keto acids in a rapid and reversible reaction catalyzed by isoforms of branched chain amino acid transaminase (BCATc or BCATm), principally BCATm in most peripheral tissues. Subsequently, the α-keto acids are oxidized by the branched-chain alpha-keto acid dehydrogenase complex (BCKD). This step is irreversible and rate-controlled by phosphorylation. BCKD kinase inactivates the complex after phosphorylation of the BCKD E1-α subunit at Ser283 and this phosphorylation is reversed by the recently discovered BCKD phosphatase [PP2cm, PPM1K gene, see 2, 3–12]. In humans, several forms of MSUD result from mutations in genes encoding the E1, E2 or E3 subunits of BCKD [see reviews: 13, 14–18]. Dysfunction of this enzyme complex results in whole body accumulation of BCAAs and branched chain keto acids (BCKAs) along with potential depletion of essential amino acids in the brain through amino acid transport competition [19]. In a subset of patients, MSUD may also be caused by a mutation of BCKD phosphatase leading to inactivation and phosphorylation of the E1-α subunit of BCKD [6, 20].

Loss of the ability to metabolize BCAAs can cause neurological symptoms and seizures through multiple mechanisms as first described by Menkes *et al* [21]. At the molecular level these include competition for transport of other critical amino acids into the brain, increased reactive oxygen species generation, activation of several stress kinases, mitochondrial transition pore opening leading to Krebs cycle disruption, apoptosis and cell death in neurons and other cell types [4, 6, 19, 22–25]. Intracellular accumulation of the keto acid of leucine, α-ketoisocaproate, has been linked to some of these toxicities, while the ketoacid of valine, α-ketoisovalerate, may cause seizures. On the other hand, BCAAs have been linked to neuronal degeneration or growth restriction [for reviews see, 19, 22]. However, further studies are needed to definitively determine the relative contributions of BCAAs versus their rapidly formed metabolites, the α-ketoacids, to these toxicities.

If untreated, MSUD can precipitate seizures, coma, and early death usually before 3 months of age [14, 19, 25]. Standard treatment for MSUD patients involves nutritional interventions designed at monitoring plasma BCAAs and adjusting dietary intake. Under normal conditions, plasma BCAAs concentrations are derived both from the diet and from whole body protein turnover, and their levels are relatively steady in plasma. Unfortunately, rates of protein catabolism can increase significantly in stress situations such as infection, psychosocial stress, trauma, food deprivation, etc.). Thus, even with excellent dietary control, stress can precipitate episodic rises in BCAAs leading to acute “metabolic crises” in MSUD patients. Reoccurrences of these episodes and/or poor nutritional control over time can accelerate the rate of progressive neurological damage [for reviews see 15, 26, 27–29]. Thus while dietary intervention is lifesaving, its efficacy can be diminished during stress or infection through the normal activation of protein catabolism and/or diminished rates of protein synthesis.

A new experimental treatment for MSUD involves liver transplantation [14, 17, 30–36]. The liver is an important site for BCAA metabolism that expresses higher concentrations of BCKD and lower concentrations of the inhibitory BCKD kinase compared to other tissues like muscle [37]. Thus, the total activity and percent of the enzyme that is active is higher in liver than muscle. Experimental treatment with liver transplant has been described in case reports and a recent clinical trial [17, 30, 31, 33, 34, 36]. The results of these transplants have been quite encouraging with experiences ranging from greatly decreased incidences of

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metabolic crises to subjects being able to resume normal diets. Despite this success, there may be two obstacles for the broad adoption of this procedure. One is that demand for donor livers exceeds availability in the U.S. and worldwide -- roughly 17,000 people were waiting for liver transplants in the U.S alone last year. Fortunately, livers from MSUD patients may sometimes be donated to patients with liver disease in a so-called domino transplant. However, another potential barrier may be the cost of a liver transplant. Some estimates of U.S. billed charges including admission, surgery and one-year post-transplant treatment range from around four to six hundred thousand dollars per transplant [38, 39].

Another tissue that expresses enzymes for BCAA metabolism is fat. During white and brown adipocyte differentiation BCKD is induced [40, 41] and recent studies have suggested that human and rodent adipose tissues may possess an under-appreciated capacity for catalyzing the first steps in BCAA metabolism [42, 43]. Thus while adipose tissue lysates have very low BCKD activity *in vitro*, Western blots suggest that BCATm and BCKD are expressed at comparable concentrations as found in other tissues. Consistent with Western blot findings, adipose tissue explants possess higher activity than muscle [43]. Several aspects of adipose tissue biology facilitate its use in reconstructive surgery and thereby make it amenable for use in transplantation [e.g., 44, 45–50]. First, adipocytes exhibit a high capacity for neovascularization and low oxygen requirements. Second, approaches for repair of adipose precursors *in vitro*, coupled with *in vitro* or *in vivo* production of adipose tissue from those cells are available using current technologies. This could open the door for autogenic transplant after repair of the affected BCKD complex gene or genes. Third, recent studies are focused on approaches to convert white fat into so-called beige fat that has higher numbers of mitochondria, the organelle where BCAA oxidation occurs [51–60]. Approaches to stably initiate such conversion before transplant might be available in the future. Finally, if used for MSUD, finding adipose tissue donors could be easier than finding liver donors and the first year costs of adipose tissue transplantation might also be significantly lower.

Consequently, we explored the use of adipose transplant in mitigating elevated BCAAs in two mouse models. Mouse models of MSUD present additional challenges compared to human MSUD. For example, a formula feeding intervention with a purified amino acid formula, as might be used in patients with classic MSUD, would be difficult to achieve with newborn mice. Furthermore, blood volume is too limited in neonatal mice to allow frequent monitoring of blood metabolites and formula adjustment. For transplant studies a mouse model needs to be sufficiently robust not only survive to adulthood without formula intervention, but as adults must be sufficiently healthy to survive a surgical intervention.

While there are no recorded human cases of MSUD resulting from BCAT2 mutation, BCAT2 deletion has nevertheless been proposed as an MSUD model [16, 61]. BCATm catalyzes the transamination of BCAAs in most peripheral tissues and glia; the KO of this enzyme in mice leads to elevated circulating BCAAs but normal BCKAs. These mice survive to weaning at which time a dietary intervention can be used to lower BCAAs [62].

PP2Cm KO mice are another recently described MSUD model. These mice display greatly increased phosphorylation on the BCKD E1-α subunit (pSer293) leading to inactivation of BCKD, along with elevated plasma concentrations of BCAAs and BCKAs [2–6]. PP2Cm KOs and patients with homozygous for PPm1K mutation display similar toxicities at the cellular level and have elevations of the pathognomonic plasma biomarker of MSUD, alloisoleucine [2–6, 25].

Three studies were conducted. In the first experiment, fat was transplanted into a subcutaneous pocket on the back and into the peritoneum of BCATm KO mice. As peritoneal fat did not become vascularized in the first study, the second experiment entailed

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finely minced subcutaneous adipose tissue that was deposited with a wide gauge needle only along the back. In the final experiment, this second approach was repeated in PP2Cm KO mice. In all cases plasma BCAA concentrations were significantly reduced in mice with a fat transplant compared to those who received sham surgery treatment.

2. Materials and Methods

2.1. Mice

All procedures were conduced after review and approval by the Penn State Hershey Institutional Animal Care and Use Committee (IACUC). The Animal Resource Program, operated by the Department of Comparative Medicine is accredited by AAALAC, International. All animal living conditions are consistent with standards laid forth in the *Guide for the Care and Use of Laboratory Animals*, 8th edition, published by the National Research Council.

BCATm KO and PP2Cm KOs lines were maintained by heterozygote breeding strategies, backcrossed to C57BL/6J more than 10 times and maintained by backcrossing to new mice from Jackson Laboratories approximately every 3–4 generations after that. The creation and genotyping strategy for BCATm (aka BCAT2 gene) KOs from the floxed allele (MGI: 3772355, $BCAT2^{tm1.1Clyn}$) has been previously described [62]. The metabolic phenotype and genotyping strategy of the second mouse strain used, PP2Cm KO (a generous gift from Yibin Wang, UCLA), has also been previously described [2]. This strain arises from KO of the Ppm1K allele (MGI: 3850971, $Ppm1k^{tm1Yiwa}$) the gene that codes for PP2Cm, the intramitochondrial BCKD phosphatase.

Animals for these studies were housed in open-top, solid-bottom polycarbonate cages (Max75, Alternative Design, Siloam Springs, AR) with wire bar lids and corncob bedding (Teklad 7097 Corn Cob Bedding, Harlan, Frederick, MD). Lighting was controlled with a 12 h: 12 h light: dark cycle with lights on at 0700 h and off at 1900 h. Water and food were available *ad libitum* unless otherwise noted for study purposes. Prior to surgery and afterwards to facilitate regular perioperative and postoperative monitoring, they were moved to a satellite animal facility next to the main lab. Food intake was determined at 24 h intervals and Leu intake was calculated from information provided by the diet manufacturer on the Leu content of the diets. Elevated BCAA concentrations were verified in the animals used for these studies by measuring BCAAs before the surgery intervention. Only BCATm KO or PP2Cm KO mice with total BCAA concentrations greater than 1900 μM or 1000 μM, respectively were used.

In BCATm KO model (Experiments B1 and B2, see Fig 1 and Table 1), we used our experience on how various diets alter circulating BCAAs to design the experiments. To maintain plasma BCAAs at lower concentrations in the BCATm KO mice and their WT siblings and heterozygous mice are maintained on one of two choice diets. When BCATm KO mice are provided a choice between two food pellets, one containing and one missing BCAAs, they tend to consume most of their diet as BCAA-free food leading to lower plasma BCAAs in contradistinction to WT mice which eat mostly BCAA containing pellets when provided this choice. We have developed two versions of the choice diet for BCATm KO mice that lead to different plasma BCAAs. In Choice Diet 1 the mice choose between an apparently more palatable 18% Protein rodent chow (Teklad 2018 Global 18% Protein Rodent Diet, Harlan, Frederick, MD) and a less palatable BCAA-free purified diet (product #510081, Dyets Inc., Bethlehem, PA). Choice diet 2 is a choice between two purified amino acid diets. One pellet is BCAA-containing (product #510090, Dyets Inc.) and the other BCAA-free (product #510081, Dyets Inc). The BCATm mice eat ~70% (Choice diet 1) or 80% (Choice diet 2) of their food as BCAA-free pellets. Consequently the plasma BCAAs

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observed with the two diets differ in BCATm KO mice (e.g., for one cohort of BCATm KOs on Choice diet 1: plasma Leu concentrations were 1920 ± 400 μM compared to 115 ± 9 μM for WT. For Choice diet 2: plasma Leu concentrations were 420 ± 200 μM in BCATm KO mice compared to 116 ± 13 μM for WT mice). If these mice are switched to 100% normal 18% protein rodent chow, the BCATm KO mice develop exceedingly high plasma BCAA. For example, after three days, plasma Leu concentrations were approximately 5760 ± 430 μM compared to ~176 ± 11 μM for WT in one cohort. While the BCAA concentrations rise quickly when the animals are provided chow diet, they tend to level off due to greatly diminishing food intake associated with decreasing body weight. Therefore, to help reduce the circulating BCAAs before and after surgery in the BCATm KO mice, both the KO mice and reference WT mice were maintained on Choice Diet 2 at first. To provide a BCAA challenge, two weeks after surgery, all of the mice in experiments B1 and B2, including WT siblings that did not receive surgery were switched to Choice Diet 1 as shown in Fig 1. In this case, mice were provided access to two pellets containing different purified amino acid diets, one BCAA- containing (product #510090, Dyets Inc.) and the other BCAA-free (product #510081, Dyets Inc., i.e. same as in Choice Diet 1). The rationale for the two diets is that Choice Diet 1 is less expensive for maintenance of the colony compared to Choice Diet 2. Palatability or other differences in the choices in these two diets appear to alter the relative selection of the BCAA free diet [62]. That is important because whereas WT mice eat mostly BCAA containing food, BCATm KO mice eat ~70–80% of their food as the BCAA free diet [62]. The two purified amino acid diets used in Choice Diet 2 have better nutritional and palatability equivalency. BCATm parents along with all offspring were maintained on Choice Diet 1 during pregnancy and until two weeks after surgery of the KO mice and then both KO and WT were switched to Choice Diet 2 during the third week of the studies.

PP2Cm mice (Experiment P1, see Fig 1 and Table 1) were maintained on Teklad 2018 Global 18% Protein Rodent Diet. Five weeks before surgery, they were switched to a Harlan Teklad Research 50% protein diet (TD.94266, Harlan Teklad Research 50% protein diet, Harlan) to increase their BCAAs as previously described [2]. The 50% protein diet continued following surgery. Reference WT mice were matched for diet.

2.2. Adipose tissue transplantation

Two variations of the adipose tissue transplants were conducted in three experiments (B1, B2 and P1, outlined in Table 1). In each of these experiments two grams of fat was transplanted. As summarized in Table 1, the variables included the mouse strain, the site of the transplant (visceral and subcutaneous or only subcutaneous), along with whether the ~2 g of fat was transferred to recipients as 0.1–0.3 g pieces or injected through a wide gauge (18-ga.) needle after being finely minced into pieces that could be accommodated by the needle. Because previous studies have found that retention of transplanted fat is facilitated by removal of fat from the recipient [63], we removed the gonadal fat from transplant recipient animals, albeit the amount of fat in BCATm mice is quite small [62]. In each of the studies, a group of sibling WT mice were maintained on identical diets but received no surgical interventions in order to obtain reference “normal” plasma BCAA values.

All surgical procedures were performed in a dedicated surgical space under aseptic conditions. Animals were placed on warmed circulating heating pads while under anesthesia and during recovery. Donor WT animals were deeply anesthetized with isoflurane gas anesthesia before being euthanized via cervical dislocation. Following aseptic preparation of the abdomen, peri-gonadal white adipose tissue was removed and placed within a sterile Petri dish containing approximately 2.0 mL of sterile saline. That adipose tissue was then either cut into pieces 0.1–0.3 g in size (Exp. B1) or finely minced to accommodate passage through a 18-ga. needle (Exp.s B2 and P1)

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Recipient (KO) animals were anesthetized using isoflurane gas anesthesia and the abdomen from xyphoid to pubis and a 2–3 cm section of the dorsum was prepared in an aseptic fashion.

In experiment B1, the BCATm KO mice were placed in dorsal recumbency and native perigonadal adipose tissue was removed before 1.0 g of donor (WT) fat (0.1–0.3 g pieces) was placed in the abdomen. Prior to closure, a few drops (less than 0.05 ml) of a 50:50 lidocaine: bupivacaine mix was instilled into the incision. The body wall and skin were closed in separate layers, using an absorbable 4-0 polydioxanone suture (PDS II, Ethicon) in an interrupted pattern. Following abdominal closure the animal was moved to sternal recumbency and a subcutaneous pocket created. Approximately 1.0 g of donor (WT) fat in 0.1–0.3 g pieces was placed within the subcutaneous pocket followed by skin closure, again using 4-0 PDS in an interrupted pattern. Sham controls were also KO mice. The Sham Surgery (Sham Sx) group had adipose tissue removed and BCATm KO fat was transplanted instead of the WT fat (Table 1).

In Exp.s B2 and P1, transplant group animals did not receive intra-abdominal fat transplants; all of the tissue was placed in the subcutaneous dorsal space after blunt dissection to open the space. In those two experiments, the donor fat was finely minced and injected into the pocket created by blunt dissection via a 18-ga. needle. However, since KO fat was in limited supply, in these experiments Sham Sx controls did not receive transplanted tissue or experience tissue removal, but did receive anesthesia and blunt dissection of the dorsal subcutaneous space (Table 1). In order to compare these mice to Exp B1, they also received a laparotomy with manipulation of the tissues.

In each experiment, peri-operative care of mice included 0.1 mg/kg buprenorphine for pain control given subcutaneously during surgical preparation, and 1 mL of warmed saline was given subcutaneously prior to discontinuing anesthesia to alleviate consequences of any blood loss or dehydration. Animals were subsequently checked on postoperative days 1–4 for signs of pain or infection and on subsequent days for pain or illness (tenderness, swelling or weight loss etc.) when other measures such as food intake or body weight were taken.

2.3 Plasma BCAA assays

Phlebotomy was performed via the facial vein using 5mm Goldenrod animal lancets (Medipoint, Inc., Mineola, NY) and was preceded by 2h of food but not water deprivation. Approximately 100–200 μl of blood was collected in a lithium-heparinized capillary collection tube (CB 300 LH, Microvette®, SARSTEDT INC., Newton, NC). After phlebotomy, the animals were given 0.5 ml of warmed normal saline subcutaneously before being returned to their home cage. Animals were closely observed for at least 15 minutes following blood collection to assess any adverse effects such as bleeding. Plasma was obtained by centrifugation and stored at −75–84°C. Plasma was thawed on ice for determination of total BCAAs concentration using a previously described enzymatic assay [2, 62].

Analysis of individual plasma BCAAs (including alloisoleucine) and phenylalanine (as the ratio denominator) was performed on a Waters Synapt HDMS hybrid QTOF with Ion Mobility, housed in the Penn State College of Medicine Macromolecular Core Facility. Amino acids were extracted from plasma samples as follows: to 10 μl of plasma sample, 1 μl of internal standard mixture (L-alanine (2,3,3,3-D4), L-phenylalanine (Ring 13C6), L-leucine (5,5,5,-D3), L-valine (2,3,4,4,4,5,5,5-D8)) was added, followed by 20 μl of methanol. The samples were vortexed, incubated on ice for 10 minutes, centrifuged at 10,000xg for 10 min, and the supernatants were dried in a vacuum centrifuge, and then re-suspended in 5 mM heptafluorobutyric acid (HFBA), 1% formic acid (FA). Five μl of each

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sample was injected onto a Waters UPLC T3 column (2.1×100 mm) with the column temperature kept at 35°C. Solvent A was 1 mM HFBA and B was 100% methanol with a 0.4 mL/min flow rate. The following gradient program was used: 8% B for 3 min; to 50% B for 1 min; to 95% B for 1 min; then initial conditions for 1 min. The standard curve encompassed a range of 1 to 1,000 μM for all amino acids. The mass spectrometer was operated in positive product ion mode, with a source temperature of 150°C, desolvation temperature of 450°C, and cone gas flow of 30.0 L/hr.

At each time point blood was also collected from a small number of otherwise untreated WT siblings of the mice on the same diet regimen as the transplant and Sham Sx group.

2.4. Surgical site examination

At the conclusion of the experiments, animals were euthanized by CO_2 asphyxiation followed by cervical dislocation and the surgical sites examined grossly for signs of integration of the transplant (i.e., vascularization, visceral attachment). Animals in experiment B1 were subjected to whole body perfusion of saline followed by toluidine blue to examine vascular patterns of the transplantation.

2.5. Statistical analysis

Normality tests and Student t-tests were performed using GraphPad Prism statistical software. Significance was set at $p < 0.05$. Potential differences between transplant and Sham Sx operated groups by sex were analyzed by ANOVA, using a Bonferonni post-test. All statistical tests were two tailed. We did not incorporate WT mice into statistical analyses for three reasons. First, increasing to a three-arm study would have decreased our sensitivity to see differences. Since a number of WT and KO mice were used as donors, the number of mice available to increase the n to accommodate a 3-arm study was not feasible. Second, the best practice for experimental treatment of the WT mice was not clear-cut. Comparisons of human MSUD liver transplant efficacy has been studied by comparing BCAAs longitudinally before and after surgery in transplant subjects. These values are either compared to established normal value or to plasma donated by non-phenotypic family or community members without a dietary intervention [Therefore in our experiments the sibling WT mice did not receive any surgery However, we did examine the effect of fat transplant on BCAAs of a different cohort of WT mice using the experiment B2 protocol. Two hr fasted UPLC-determined BCAAs were not significantly different before and three weeks after surgery (Pre-surgery: Leu 138 ± 13 vs Post-surgery: 158 ± 9; Ile: 71 ± 7 vs 79 ± 6; Val: 205 ± 17 vs 222 ± 11, N=8, N.S., p=2.4–6.6)]. Finally, while WT mice were provided access to the same choice diets it is difficult to control for their BCAA intake. They eat approximately 80–90% of the BCAA-containing food in contrast to the KO mice that eat a far smaller proportion of this diet in favor of the BCAA-free diet [62]. The WT mice would develop a deficiency if pair fed to the low amount of BCAAs that BCATm KO consume on the choice diet. That in turn would trigger processes, such as GCN-2 activation or amino acid imbalance toxicity, that occur when some essential amino acids are insufficient in the diet [64]. Our goal was to compare the Transplant and Sham Sx group; however, we recognized the importance of providing “normal reference values”. Therefore given the small number of available mice, the differences in their diet, BCAA metabolic capacity and the fact that the WT sibling mice did not receive any sham anesthesia or surgical treatments, plasma BCAA values for the WT mice are provided for reference only.

3. Results

Three experiments were performed as outlined in Fig 1 and Table 1. Two of these used the BCATm KO mouse model (B1, B2) and one involved the PP2Cm KO mouse model (P1).

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3.1. Exp B1 – Visceral and Subcutaneous transplant of adipose tissue in BCATm KO mice

We first examined the effect of transplanting 2.0 g of WT fat divided as 1.0 g into the peritoneum and 1.0 g into the subcutaneous dorsal space of male (n=5) and female (n=5) BCATm KO mice (n= 10 total). These mice were compared to a surgical control (Sham Sx, n=8, 4 per sex) in which BCATm KO adipose tissue was transplanted. Notably, this design decreased the number of available WT mice and KO mice. More KO than WT mice were needed as donors to obtain 2.0 g of KO fat because BCATm KO mice have smaller amounts of adipose tissue [62]. Mice were maintained on before and for two weeks after surgery on Choice Diet 2. Plasma obtained at wk 2 had 45% lower BCAAs in the Transplant group (data not shown). The mice were then switched to Choice Diet 1 for the next week at which time BCATm KOs transplanted with WT fat had a 71% reduction in their BCAAs compared to those receiving KO fat (Fig 2A). Notably, the goal of the transplant is not to decrease the plasma BCAA concentrations to zero, but to align them with normal concentrations. Simultaneous reference values for WT mice plasma BCAA concentrations using siblings on the same choice diet protocol but not subjected to other interventions were 710 μM. If this is taken as the target baseline for normalization then the reduction of the BCAAs toward normal values is 81% (The BCATm enzyme is needed to form alloisoleucine, a marker for MSUD, so it was not assayed in studies involving BCATm KO mice.)

Although body weight did slightly increase in the Transplant group, these changes were not significant and were not affected by treatment; as expected, female mice weighed less (Fig 2B). Between weeks 2 and 3, the Sham Sx and Transplant groups ate similar amounts of food and their intake of BCAA determined from consumption of the 18% protein pellets was not different (Fig 2C–D).

Subsequently, the mice were euthanized for examination of the surgical sites (data not shown). The appearance of the transplanted abdominal fat was firm, encapsulated and non-vascular with a distinct whitish color. It was often found freely floating in the abdomen surrounded by a mature fibrotic capsule of varying thickness. The transplanted subcutaneous fat differed in appearance. Vascular growth on the surface of the subcutaneous transplanted fat was noted in the majority of cases; however, some centers appeared necrotic, which is consistent with previous studies in rabbits [48]. There was a tendency for the transplanted subcutaneous fat to accumulate together in one or two locations and it was unclear therefore which fat was from the recipient and which was from the donor animals.

3.2. Exp B2 – Subcutaneous transplant of finely-minced adipose tissue via needle in BCATm KOs

The results of Exp B1 supported use of the subcutaneous part of the dorsum for adipose tissue transplant. In Exp B2, we examined the effects of introducing all of the fat into the dorsal subcutaneous spaces of male BCATm KO mice (Transplant group, n=5). To facilitate this treatment, the fat was finely minced and introduced with a 18-ga. needle after blunt dissection of the space. The Sham Sx group (n=5) received anesthesia and blunt dissection of the back and laparotomy with tissue manipulation to mimic the fat removal in the Transplant group. The post surgery diet regimen was the same as in Exp B1. At two (data not shown) and three weeks (Fig 3A) after surgery the Transplant group had a ~50% decrease in their BCAAs. The mean reference BCAA value for WT mice on the same diet protocol but not subjected to other interventions was 620 μM. The wk 3 data demonstrated that BCAAs were 6-fold higher in the Sham Sx group compared to the reference value of the WT mice group, but the Transplant group mice were only 3-fold higher than the WT reference value.

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Body weight did not change significantly in these mice, however their overall average body weight was larger at the start of the experiment compared to Exp B1 (Fig 3B). There was no significant difference in the food intake or BCAA intake the week preceding the 3-week phlebotomy (Fig 3C–D). Gross examination of the fat was similar to experiment B1 (data not shown).

3.3. Exp P1 – Subcutaneous transplant of finely-minced adipose tissue via needle in PP2Cm KO mice

We also explored PP2Cm mice as an MSUD model for the transplant studies (Fig 4). On the 18% protein diet, PP2Cm mice had statistically higher plasma BCAAs compared to WT siblings, however the differences were only 56% (data not shown). Therefore, as in previous studies, they were maintained on a high protein diet [2]. PP2Cm mice used in this study all had detectable plasma alloisoleucine concentrations above the 2 μM cut off used for newborn screening [65], in contrast to WT mice on the same diet (data not shown); however these were variable, ranging from ~3–13 μM. Surgery was performed 5 weeks after switching to the high protein diet. Mice were randomized to Transplant or Sham SX groups. These did not differ significantly in their plasma BCAA concentrations prior to surgery (2254 + 74 vs 2200 + 191 μM respectively, compared to a diet and time controlled reference value of 964 μM for WT mice. This reference value is somewhat elevated compared to Exp B1 and B2 due to the high protein diet all the animals received).

In experiment P1, male and female KO mice were subjected to subcutaneous WT adipose tissue transplant as in Exp B2. Sham Sx mice in this study were PP2Cm mice that received the same sham treatments as in Exp B2 mice (Table 1). At week 4, plasma BCAAs in the Sham Sx mice were 182% higher than the WT average reference value compared to only 85% higher in the Transplant group (Fig 4A). The average reference value for WT mice for the same time on the diet was 871 μM. Alloisoleucine/Phe ratios were reduced 32% and undetectable in the WT mice. The Transplant group had a ~65% reduction in plasma Leu, Ile and Val concentrations compared to WT average reference amino acids values (Table 2).

Immediately after surgery, food intake decreased as expected and normalized several days afterward (Fig 4B). Thereafter it remained stable and not significantly different between the groups, including between males and females, which was unexpected (Fig 4B). Overall, there were no significant differences in the body weights at any time between the groups (Fig 4C), however mice with fat transplant tended to have higher weights after surgery.

Transplanted mice were euthanized for gross examination of the transplanted fat (data not shown). The transplanted subcutaneous fat was similar in appearance to the subcutaneous fat in Exp B1. Some tissue was discretely organized attached to the skin or back whereas other tissue coalesced into one or two groupings with a lipomatous appearance with some areas of discrete fibrosis and other areas with surface vascularization evident. The reorganization of this tissue into discrete masses is consistent with our previous studies showing that adipocytes and clusters of fat can organize into tissue *in vitro* [66, 67]. Other smaller groupings of fat were also evident. The color of the tissue ranged from the normal color of mouse adipose tissue to a more yellow appearance seen in human fat. It was not possible to discriminate between native and transplanted fat owing to the reorganization.

4. Discussion

In this paper, we show that subcutaneous transplant of visceral adipose tissue into either BCATm or PP2Cm KO mice leads to significant reductions in circulating BCAAs. BCAA lowering occurred either when the fat was transplanted as small pieces introduced by blunt

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dissection or as finely minced pieces introduced via a cannula. The procedure and BCAA lowering seemed to work equally well in male or female animals.

We were disappointed that simple transfer of fat into the peritoneum led to a sterile inflammatory response and encapsulation of the transplanted tissue. In contrast, several recent studies have employed fat transplant into the peritoneum to explore the role of white and brown fat in metabolic syndrome or obesity. For example, Tran et al [68], successfully transplanted fat into the visceral compartment by introducing the transplanted fat within existing adipose tissue. We did not use that approach because we removed visceral adipose tissue to prevent a previously reported reduction in all adipose tissue when removal is not performed during transplant [63]. Konrad et al. [69] used another approach that involved stitching a whole fat pad to the wall of the visceral cavity. In the future these approaches should be explored.

Several observations may point to the importance of vascularization in the efficacy of this procedure, since simply increasing the amount of fat transplanted in the mouse's back from 1 g to 2 g did not lower plasma BCAAs any further. It may be valuable in the future therefore, to introduce smaller amounts of fat at multiple locations subcutaneously and not to introduce additional fat until the existing transplanted fat has had a chance to vascularize.

While the BCATm KO mouse has been proposed as a MSUD model, it is not ideal. Patients with classic or intermediate MSUD have elevated BCKAs and alloisoleucine arising from the blocked at the next step in metabolism, normally catalyzed by the BCKD complex. Plasma BCAAs in BCATm KO mice are higher than would be expected from a block at BCKD because BCATm normally acts to balance BCAAs and BCKAs through reversible transamination. In addition because BCATm is missing in these mice, circulating BCKAs are lower not higher as in MSUD [62]. That is important because BCKAs are thought to contribute to neurotoxicity and may also be toxic in other tissues [70]. Thus their negative influence would not be captured in a BCATm KO model, which also has a metabolic phenotype [62]. Nevertheless, from our previous experience, when BCATm are placed on the 18% protein chow diet and cannot lower their BCAAs through a choice diet, they lose appetite and body weight and can develop a moribund appearance. Over time, BCAAs in BCAT KO mice can accumulate to concentrations that may approach BCAA solubility associated with formation of leucine crystals in the urine sediment and pupillary opacities (unpublished data). In addition they have exercise intolerance [71].

Compared to the BCATm KO model of MSUD, the PP2Cm KO mouse is a better model by having both elevated BCAAs and BCKAs [2]. PP2Cm mutation leads to MSUD in humans, and so is a valid model, but because this enzyme is only recently discovered, it is unclear what frequency it contributes to human MSUD relative to other types at this time [6, 20]. While PP2Cm KO mice meet and exceed the >2 μM alloisoleucine cutoff value for newborn diagnosis of MSUD, their plasma BCAAs are not as high on an 18% protein diet as one might expect from KO of the only BCKD phosphatase. KO of PP2Cm may therefore cause some adaptation that affects the expression or stability of the BCKD kinase and/or BCKD expression leading to lower BCKD kinase concentrations and residual BCKD activity [2]. On the other hand they have improved survivability in our hands and appear healthier in terms of body weight than two other models of MSUD that involve disruption of the E2 subunit of BCKD from the Homanics group [for review see 16].

In conclusion, subcutaneous adipose tissue transplant may have some value in mitigating elevations in BCAAs in response to stress-induced elevations in those who are receiving standard treatment for MSUD. Additionally, adipose transplants may provide a less expensive alternative to liver transplants, although further refinements are needed to

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improve efficacy relative to the impressive results of liver transplantation. A number of refinements have been suggested from studies to improve “take” of fat in reconstructive surgery that we could explore as part of such efficacy improving efforts [e.g., 44, 45–50].

Acknowledgments

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Abbreviations

BCAA	branched-chain amino acids
BCATc or BCATm	the cytosolic or mitochondrial isoform of branched-chain amino acid transaminase respectively (gene names: BCAT1 for BCATc, BCAT2 for BCATm)
BCKA	branched chain keto acids
BCKD	branched-chain keto acid dehydrogenase complex
Exp	experiment
KO	knock out mouse
MSUD	Maple Syrup Urine Disease
PP2Cm	branched-chain keto acid dehydrogenase phosphatase (gene name: PPM1K)
WT	wildtype sibling mice homozygous for wild type allele

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Highlights

- White fat transplantation was tested as a potential MSUD intervention.
- BCATm (*BCAT2*) and PP2cM (*PPm1K*) knock out (KO) mice were used as MSUD models.
- Different fat transplant sites and methods of introduction were examined.
- Transplant into subcutaneous space led to 52–81% plasma BCAA reductions
- Transplant lowered BCAAs and alloisoleucine, the pathognomonic MSUD biomarker

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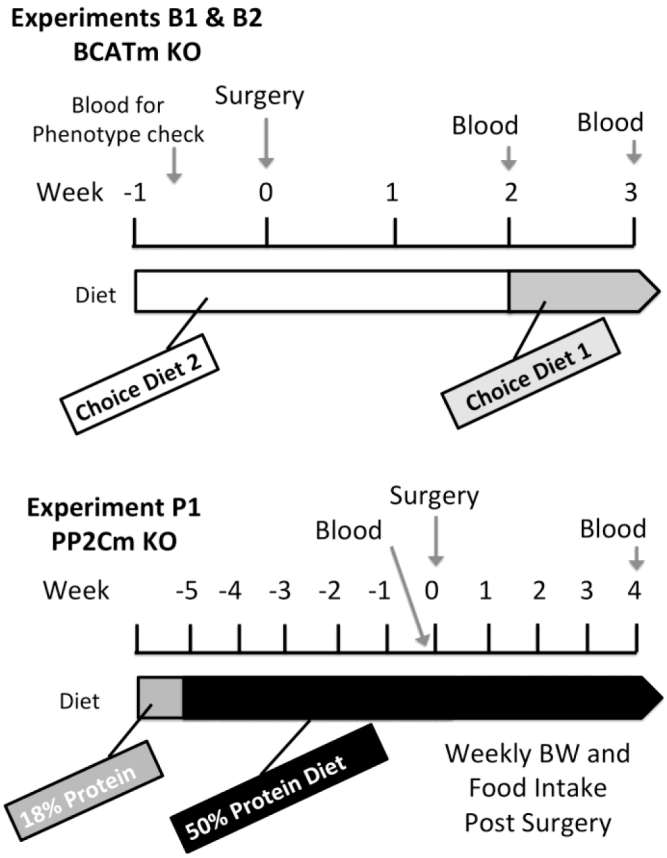

Figure 1.
Schematic representation of interventions and diets used in experiments B1, B2 and P1. Table 1 shows the types of surgery and surgical controls used.

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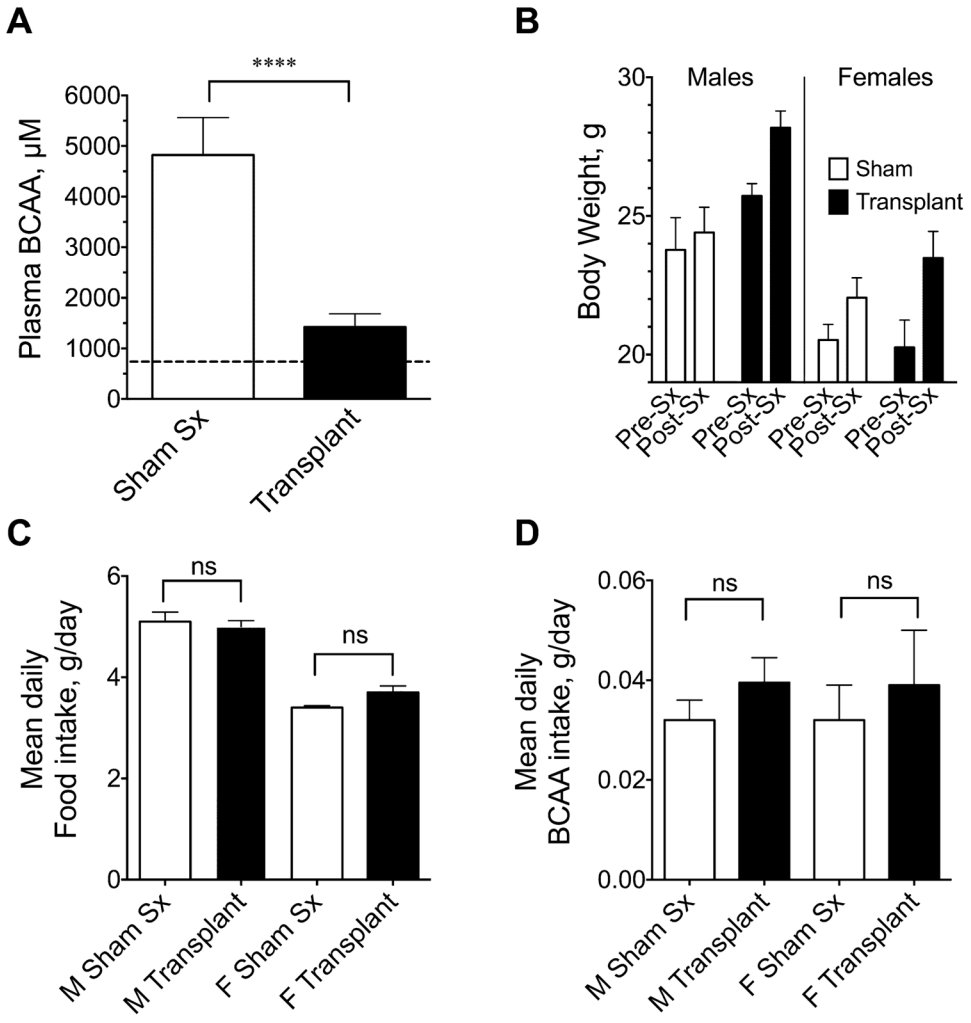

Figure 2.

Exp B1, Transplant of WT fat into the subcutaneous and peritoneal spaces of BCATm KO mice. A. 2 h food-deprived plasma BCAA concentrations 21 d after Transplant or Sham Sx in mice. An asterisk (*) indicates $p<0.05$ when Sham Sx and Transplant groups are compared. A dotted line indicates the reference BCAA concentration of sibling WT mice, 710 μM. B. Body weights of mice before and after surgery. C. Average daily food intake of mice by treatment and sex, days 14–21. D. Average daily BCAA intake based on BCAA content of food days 14–21.

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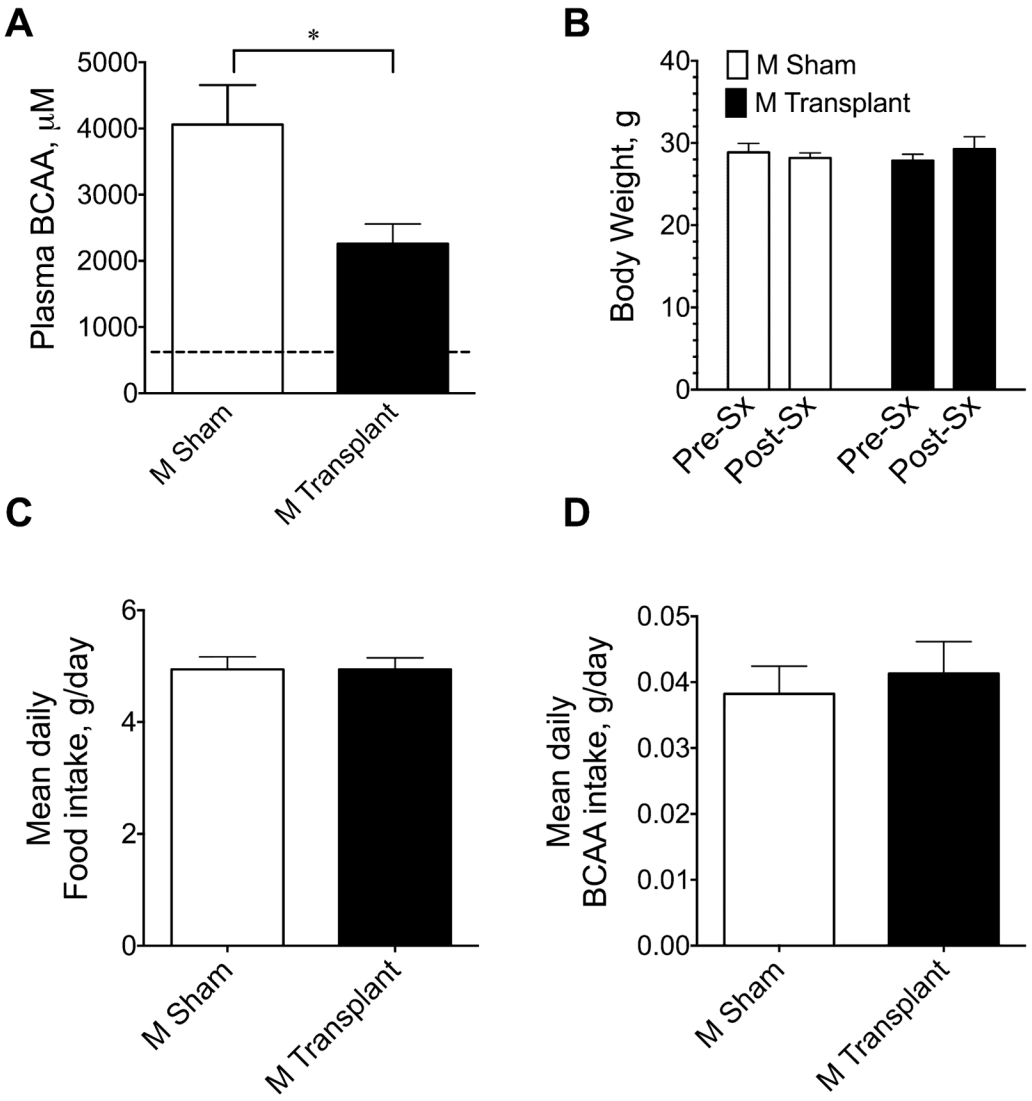

Figure 3.

Exp B2, Transplant of finely minced adipose tissue in the subcutaneous space of BCATm KO mice via a 12 g needle. A. 2 h food-deprived plasma BCAA concentrations determined 21 days after surgery in mice. An asterisk (*) indicates significant difference between the Sham Sx and Transplant groups ($p<0.05$). A dotted line indicates the reference BCAA concentration of sibling WT mice, 620 μM. B. Body weights before and after surgery. C. Average daily food intake, days 14–21. D. Average daily BCAA intake from BCAA containing diet, days 14–21.

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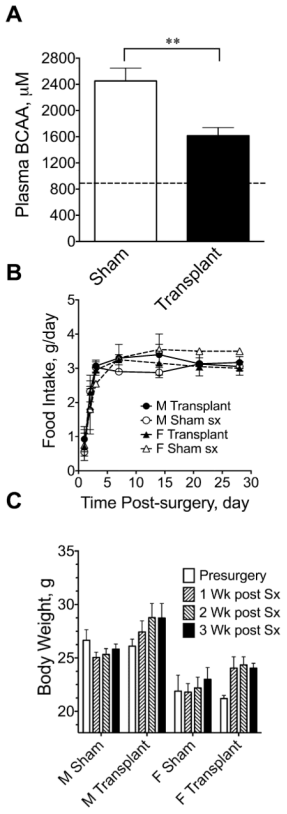

Figure 4.

Exp P1, Transplant of finely minced adipose tissue in the subcutaneous space of PP2Cm KO mice via a 12 g needle. A. 2 h food-deprived plasma BCAA concentrations 4 wk after Transplant or Sham Sx (** indicates $p<0.01$ when Sham Sx and Transplant groups are compared). A dotted line indicates the reference BCAA concentration of sibling WT mice, 1075 μM. B. Food intake of mice. C. Body weights of mice before and after surgery.

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Table 1

Sex, strain and conditions of transplant experiments*

Experimental group						
Experiment designation	**KO Strain**	**Sex**	**Fat transplanted as 0.1–0.3g pieces**	**Minced fat transplanted via 12-ga. needle**	**Transplant site: 1g Dorsal subcutaneous space and 1g Peritoneum**	**Transplant site: Dorsal subcutaneous space only**
B1	BCATm	M & F	X		1g	1g
B2	BCATm	M		X		2 g
P1	PP2Cm	M & F		X		2 g

Sham group treatments							
Experiment designation	**KO Strain**	**Sex**	**Anesthesia**	**Laparotomy**	**BCATm KO fat transplanted**	**Manipulation of visceral tissues only**	**Subcutaneous blunt dissection**
B1	BCATm	M & F	X	X	X		X
B2	BCATm	M	X	X		X	X
P1	PP2Cm	M & F	X	X		X	X

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Table 2

Plasma amino acid values 28 d after surgery in PP2Cm mice*

	Leu (μM)	Ile (μM)	Val (μM)	Alloisoleucine/Phe ratio
PP2Cm KO, Sham Surgery	735 ± 42	411 ± 17	1287 ± 72	0.035 ± 0.003
PP2Cm KO, Transplant	383 ± 33	220 ± 14	712 ± 71	0.024 ± 0.001
P value	0.0003	< 0.0001	0.0008	0.02

* Reference plasma values for wildtype siblings (n=7) on same diet but without surgical intervention were as follows: Leu, 216 ± 49μM; Ile, 110 ± 23μM; Val, 382 ± 84μM; Alloisoleucine, Not Detected.