

Lowered Concentrations of Branched-Chain Amino Acids Result in Impaired Growth and Neurological Problems: Insights from a Branched-Chain α -Keto Acid Dehydrogenase Complex Kinase-Deficient Mouse Model

Malcolm Watford, DPhil

Excess circulating levels of branched-chain amino acids (BCAA), as seen in maple syrup urine disease, result in severe neuropathology. A new mouse model, deficient in the kinase that controls BCAA catabolism, shows that very low circulating levels of BCAA are also associated with neuropathology, including the development of epileptic seizures. These mice clearly demonstrate the need to control essential amino acid levels within both upper and lower limits.

Key words: alpha-keto acid dehydrogenase, branched-chain amino acids, branched-chain keto acids, maple syrup urine disease

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METABOLISM AND FUNCTION OF BRANCHED-CHAIN AMINO ACIDS

The branched-chain amino acids (BCAA), leucine, isoleucine, and valine, comprise about 40% of the indispensable (essential) amino acid intake, representing some 20% to 25% of total dietary amino acids. The average intake of BCAA is estimated to be between 14 and 35 g/d for adults, which is well above the requirement of about 7 g/d for an average adult.^{1–3} Thus, a protein-sufficient diet would almost certainly not be deficient in BCAA; however, in addition to the require-

ment for BCAA in protein synthesis, there is good evidence that BCAA have regulatory properties. Leucine, in particular, has been found to stimulate protein synthesis, to decrease protein degradation, and to increase insulin secretion, and may play a role in the regulation of food intake and the prevention of central fatigue.^{3–8} These effects have resulted in considerable interest in the use of BCAA as dietary supplements, both in clinical settings and by the lay public, particularly athletes. Such supplementation (up to 60 g/d)^{3,9} does not appear to be problematic unless there is a marked imbalance in the ratio of the three amino acids, because the body seems to have extensive capacity to deal with excess and maintain circulating concentrations within a safe range.

Excess levels of BCAA and many other amino acids are toxic, as is demonstrated in cases of maple syrup urine disease (MSUD), a genetic disorder resulting from a failure to catabolize BCAA and their respective branched-chain keto acids (BCKA).^{10–12} Untreated MSUD is associated with failure to thrive and severe neuropathology, but can be controlled by limiting BCAA intake and maintaining circulating leucine concentrations below 400 μ M.¹¹ Interestingly, a new knockout mouse model^{13,14} shows that in addition to causing, somewhat predictably, lower rates of growth, low levels of BCAA also result in neurological problems including seizures.

Apart from protein synthesis, BCAA have few known metabolic functions. They are utilized for the production of branched-chain fatty acids found predominantly in hair, their nitrogen is an important precursor of glutamate in the brain and of alanine and glutamine in skeletal muscle, and leucine carbon may be used for cholesterol synthesis in some tissues.^{2,15–17} Otherwise, and certainly from a quantitative viewpoint, BCAA metabolism is catabolic in nature, leading to energy production and nitrogenous waste products. Most indispensable amino acids are primarily catabolized in the liver, but the

Dr. Watford is with the Department of Nutritional Sciences, School of Environmental and Biological Sciences, Rutgers, The State University, New Brunswick, New Jersey.

Please address all correspondence to: Dr. Malcolm Watford, Department of Nutritional Sciences, Thompson Hall, School of Environmental and Biological Sciences, Rutgers, The State University, New Brunswick, NJ 08901; Phone: 732-932-7418; Fax: 732-932-6837; E-mail: watford@aesop.rutgers.edu.

initial reaction of BCAA catabolism is absent from the liver and is instead found in peripheral tissues, predominantly skeletal muscle.^{2,16-19} A limited amount of dietary BCAA undergo metabolism in the gastrointestinal tract, and some are taken up by the liver for protein synthesis. During the absorptive phase, however, the bulk of BCAA escape metabolism in the liver and comprise about 50% of the amino acids released by the liver into the peripheral circulation. The accompanying rise in circulating BCAA concentration is proposed to function as a signal to extra-hepatic tissues that amino acids are available, thus stimulating protein synthesis.

Branched-Chain Amino Transferase

The first two reactions of BCAA metabolism are common to all three amino acids. Catabolism begins with transamination with α -ketoglutarate to form the corresponding BCKA and glutamate, followed by decarboxylation of the BCKA to yield branched-chain acyl CoA derivatives. From this point, the pathways diverge, with leucine ultimately being metabolized to acetoacetate and acetyl CoA, isoleucine to acetyl CoA and propionyl CoA, and valine to propionyl CoA.^{2,16-19} These end products have little bearing on the study considered in this review and will not be considered in any further detail. Branched-chain aminotransferase is expressed at very low levels in the liver (about 0.5% of total body activity in the rat, 7.7% in humans), but is found in many other tissues, with skeletal muscle possessing the greatest capacity for transamination in the rat.^{16,18,19} Interestingly, the transaminase is mitochondrial in most tissues, but the brain also expresses a cytosolic form that ac-

counts for 60% to 70% of total brain activity, the significance of which is not completely clear.¹⁶

In keeping with the reversible nature of transamination reactions, and the ready availability of the cosubstrates glutamate and α -ketoglutarate, branched-chain aminotransferase is believed to operate close to equilibrium in many tissues. This means that BCAA and BCKA are in constant exchange, with net flux determined by changes in amounts of substrates and products. This also means that, strictly speaking, BCAA are not essential because they could be replaced by BCKA, which would be transaminated to BCAA. This is the basis for the therapeutic use of dietary BCKA in conditions that require low nitrogen intake, such as end-stage renal disease and inborn errors of the urea cycle.^{20,21} Normal foods, however, contain very low levels of BCKA, so this has little bearing on everyday nutrition.

Branched-Chain α -Ketoacid Dehydrogenase Complex

The second reaction in BCAA/BCKA metabolism is catalyzed by the branched-chain α -ketoacid dehydrogenase complex (BCKADHC) that is similar in structure to the pyruvate and α -ketoglutarate dehydrogenase complexes.^{16-19,22} The complex is comprised of over 150 subunits possessing at least five distinct enzymatic activities, and is located within the mitochondrial matrix. The substrate, BCKA, is decarboxylated by an irreversible thiamine pyrophosphate-dependent enzyme E1 (Figure 1). The branched-chain acyl derivative is then passed on to the lipoate group on a transferase enzyme E2 that releases branched-chain acyl CoA as an end product. The

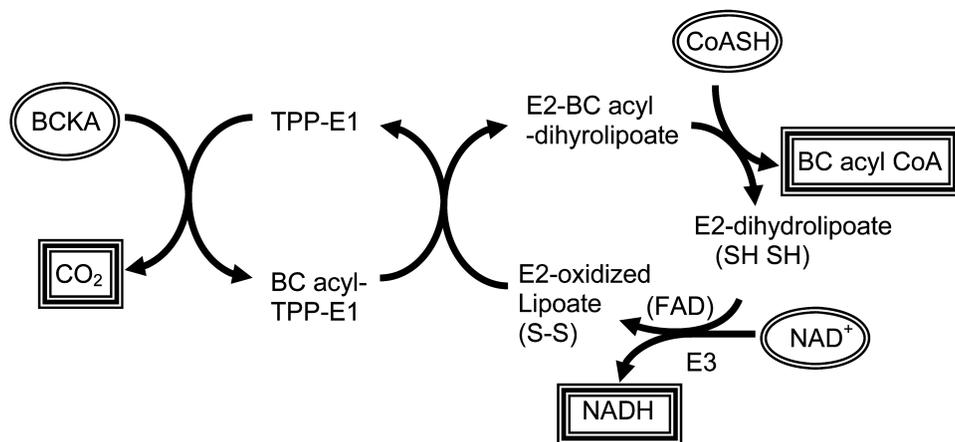


Figure 1. The branched-chain keto acid dehydrogenase complex. E1, E2, and E3 are described in the text. The equation shows the overall reaction. Substrates are shown in ovals and products in rectangles. BCKA, Branched-chain keto acids.

reduced lipoate is then oxidized through the action of a NADH-linked dehydrogenase E3, thus completing the reaction and regenerating oxidized lipoate E2 ready to pick up another branched-chain acyl intermediate. Both E2 and E3 are freely reversible, and therefore flux will be influenced by acylCoA:CoASH and NADH:NAD ratios.

In addition to such feedback inhibition, the complex is also subject to regulation by phosphorylation/dephosphorylation of E1 (Figure 2) through the action of a specific kinase (BDK) and probably a specific phosphatase, although the latter has not yet been definitively identified.¹⁸ In the phosphorylated state, E1 is inactive and this stops flux through the complex. The kinase is subject to inhibition by BCKA, and therefore a buildup of BCKA, as would occur (through the equilibrium nature of branched-chain aminotransferase) in response to increased amounts of BCAA, will decrease the amount of the complex in the phosphorylated form and thereby increase complex activity.

It is possible to assess both the total BCKADHC activity in a tissue and the proportion that is active (dephosphorylated) at the time of sampling. In the rat,^{18,19} most of the total body BCKADHC activity is present in the liver (60%) and skeletal muscle (29%), with the remainder in tissues such as kidney and brain. Moreover, in skeletal muscle the complex is predominantly (>90%) in the phosphorylated (inactive) form, while in the liver it is usually 90% to 100% in the dephosphorylated (active) form. The only time the liver enzyme is found to be significantly phosphorylated is after feeding low-protein diets, a condition where there is a need to conserve essential amino acids as much as possible. The situation in humans¹⁹ appears to be a little different in that skeletal muscle expresses more BCDHC activity (66% of total body activity), with about 50% being in the dephosphorylated (active) state. Moreover, the liver capacity is relatively low (9% of total body

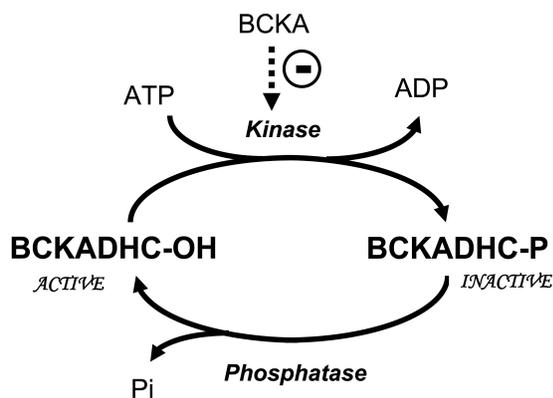


Figure 2. Regulation of branched-chain α -ketoacid dehydrogenase complex (BCKADHC) by phosphorylation/dephosphorylation. The kinase (BDK) is inhibited (-) by branched-chain keto acids (BCKA).

activity) and also predominantly (70%) in the phosphorylated (inactive) state. Thus, human BCAA metabolism shows considerable tissue-specific differences from rodents. This is reflected in studies^{23,24} of inter-organ flux in vivo, where BCAA are transaminated predominantly in skeletal muscle in both rats and humans, but where there is considerable release of BCKA into the circulation in rats but not in humans. Thus, presumably, much of the BCKA undergo further catabolism within muscle tissue in humans.

Within the body, BCAA arise from two sources, exogenously from the diet and endogenously from the breakdown of body proteins. Clearly, the degradation of BCAA must be regulated both to prevent the toxicity associated with excess and to conserve essential amino acids at times of dietary protein insufficiency. Long-term changes in the amount of BCKDHC do occur, but given the time required to synthesize all of the necessary subunits, this is not likely to play a major role in the day-to-day regulation; rather, it sets limits on the capacity for BCAA degradation. While feedback inhibition and phosphorylation/dephosphorylation both have the potential to exert control on the rate of BCAA degradation, it is difficult to determine their relative importance. In an attempt to address this question, Joshi et al.¹³ developed a BDK-knockout mouse. The resultant mice have proven very interesting and have provided much more information than simply telling us that phosphorylation is very important in the regulation of BCAA homeostasis.

Branched-Chain α -Keto Acid Dehydrogenase Kinase Knockout (BDK^{-/-}) Mice

The BDK^{-/-} mice were viable, but showed reduced fertility, as only 8 pairs out of 14 homozygotes had litters within 8 weeks of being paired for mating. Litter size was not reported, but the birth weight of the pups was similar to that of wild-type mice. Until 2 weeks of age, both BDK^{-/-} and wild-type pups had similar body weights. After that time, the growth rate of both groups slowed for about a week before rising to a peak at 4 weeks in wild-type and at 5 weeks in the BDK^{-/-} pups. Most notably, in BDK^{-/-} pups, the decrease in growth began earlier (by week 2) and was much more pronounced: they ceased to grow for about a week at 2 to 3 weeks of age. Although subsequently they showed similar rates of growth to the wild-type pups, the poor growth at 2 to 3 weeks of age resulted in a markedly lower body weight that persisted into adulthood. In addition, some of the BDK^{-/-} mice died at weaning, although survival was improved if weaning was delayed. Adult BDK^{-/-} mice were also about 15% shorter than wild-type mice, and while they had less adipose tissue

and muscle mass and smaller brains, they did have larger kidneys and liver (when expressed relative to body weight). Furthermore, the fur of $BDK^{-/-}$ mice lacked luster, which was probably related to a lack of BCAA for 18-methyleicosanoic acid synthesis in hair cuticle cells.¹⁵

The differences in body weight and growth were presumably related to low BCAA availability for protein synthesis. Joshi et al.¹³ therefore provided some $BDK^{-/-}$ mice (mothers and pups throughout the breeding and neonatal period) with a high (50%) protein diet to see if this could overcome the problems. High protein feeding normalized body weights—in fact, the $BDK^{-/-}$ mice showed higher rates of growth and slightly larger body weight prior to weaning. But even on the high-protein diet, $BDK^{-/-}$ mice ceased to grow around week 3 (presumably, weaning occurred at this time), although they subsequently caught up and there were no differences in either growth rate or body weight by week 6. It would be very interesting to know if these mice were able to grow so well prior to weaning due to increased levels of BCAA in the milk. The question of provision of milk BCAA by the dams on both normal and high-protein diets is clearly an important topic for future study.

As expected, BDK mRNA and protein were not detectable in the tissues of the $BDK^{-/-}$ mice, but, interestingly, there was an increase in the amount of the $E1\alpha$ component of the dehydrogenase complex in a number of tissues such as heart, brain, kidney, and skeletal muscle. In wild-type mice, confirming earlier work in the rat,^{18,19} BCKADHC total activity was relatively high in liver, where it was 100% in the dephosphorylated (active) form. Similarly high total activity was present in heart (61% active) and kidney (25% active), but lower in brain (65% active) and almost non-existent in skeletal muscle (and highly phosphorylated, <5% active). $BDK^{-/-}$ mice showed higher total activity in all tissues except liver. In addition, the complex was in the 100% dephosphorylated state in all tissues, confirming that only one kinase exists that is able to phosphorylate BCKADHC. Thus, the total body capacity for BCKA catabolism had increased, and all of the activity was permanently activated. This was confirmed at the tissue level by the demonstration that the rate of [^{14}C] valine oxidation was higher in isolated diaphragms from $BDK^{-/-}$ mice. There was concern that constitutively high BCKADHC activity could also decrease circulating concentrations of methionine and threonine, since both of these amino acids can produce ketoacids that are substrates for BCKADHC. However, the levels of these amino acids were not changed, confirming that, at least in the mouse, transamination is not a major pathway of methionine catabolism. It also showed that threonine catabolism is restricted to the liver and was thus not

affected, since there was no change in liver BCKADHC activity.

The permanent activation of BCKADHC would be expected to result in lower levels of BCKA and therefore lower BCAA levels. In chow-fed $BDK^{-/-}$ mice, plasma concentrations of each of the BCAA were lower by approximately 50% to 60% (Figure 3) than in wild-type animals. This was accompanied by similar lower concentrations of the BCAA in brain, kidney, heart, and muscle, but not liver (Figure 4). It would have been interesting to measure the BCAA concentrations in animals receiving the high-protein diet, because maintenance of sufficiently high concentrations of BCAA is presumably the mechanism underlying their increased growth rates.

Neurological Deficits in $BDK^{-/-}$ Mice

Perhaps the most interesting finding with the $BDK^{-/-}$ mice is that they exhibit severe neurological problems. From 3 weeks of age they exhibited a general tendency to splay their legs and to clinch their hindlimbs when held by the tail. Again, some of these symptoms could be partially prevented by the high-protein diet. But at 6 to 7 months of age, $BDK^{-/-}$ mice showed a strong predisposition to develop seizures when handled. Nine of 11 female $BDK^{-/-}$ mice developed seizures at least 2 times, some as often as 7 times, when tested 8 times over a 2-week period. Seizures were of varying duration up to 2 minutes, but some animals experienced such severe seizures that they died. Although subsequent histology did not detect any structural abnormalities, there was a general reduction in size of all parts of the brains of $BDK^{-/-}$ mice.

The mechanism responsible for the neurological problems is obviously of great interest. However, while comparisons to the neuropathology that accompanies MSUD^{3,10-12} are tempting, an importance difference must be remembered: MSUD is the result of excessive

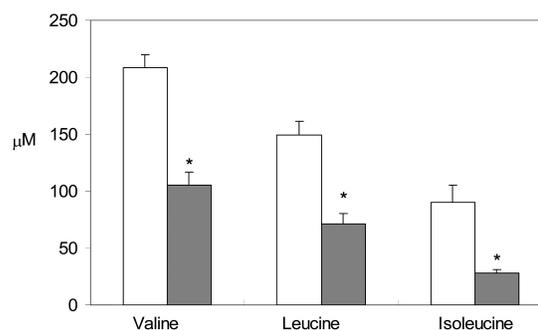


Figure 3. Plasma branched-chain amino acid concentrations are lower in $BDK^{-/-}$ mice. The results are means \pm SEM, where open bars represent wild-type mice and filled bars $BDK^{-/-}$ mice.

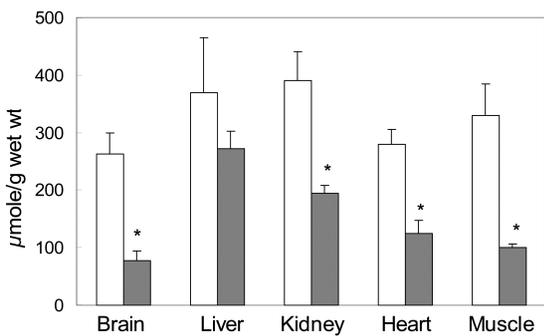


Figure 4. Tissue branched-chain amino acid concentrations are lower in BDK^{-/-} mice. Results are for the sum of all three amino acids, and are means \pm SEM, where open bars represent wild-type mice and filled bars BDK^{-/-} mice.

levels of BCAA and BCKA, whereas the BDK^{-/-} mice present with very low levels. The mechanisms underlying neurological problems in MSUD are not known, but possibilities include brain swelling and a number of disturbances linked to impaired glutamate and neurotransmitter metabolism.^{3,10-12} It been estimated that leucine provides 30% to 50% of glutamate nitrogen in the central nervous system. The problems in MSUD seem to be related to excess amounts of α -keto-isocaproic acid (the keto acid of leucine), and these would be expected to disturb glutamate levels since they are linked via transamination. Such disturbances would then affect not only the levels of glutamate, an excitatory transmitter, but also the levels of γ -amino butyric acid (GABA), an inhibitory transmitter derived from glutamate, and such mechanisms would also be expected to be perturbed in the BDK^{-/-} mice. In addition, changes in the circulating concentrations of BCAA may change the concentrations of other amino acids in the brain, since BCAA are transported on the large neutral amino acid transporter that is also responsible for the transport of tryptophan, tyrosine, and phenylalanine. Thus, a decrease in BCAA concentrations would lessen the competition for this transporter and increase uptake of other amino acids into the brain, with resultant changes in the synthesis of serotonin and catecholamines.³

An additional possible mechanism is related to the facts that hyperphosphorylation of eIF2 (eukaryotic initiation factor) in the brain has been linked to epilepsy, and low leucine abundance is known to increase phosphorylation of the α -subunit of eIF2.¹³ Phosphorylation of eIF2 α was over 2-fold higher in the brains of BDK^{-/-} than in wild-type mice, although, as Joshi et al.¹³ discuss, it is not clear if such a finding is indicative of a causal mechanism in epilepsy or if it represents a cytoprotective response. Thus, a number of putative mechanisms have been proposed that could explain the neurological problems of MSUD and/or BDK deficiency. Why such problems are delayed until adulthood in the BDK^{-/-} mice is

not clear, but these mice do provide a powerful new model for the investigation of such phenomena and epilepsy in general.

AVENUES OF FUTURE RESEARCH

Perhaps one of the biggest unanswered questions arising from these mice is, where are the BCAA being metabolized? In wild-type mice, given the low transaminase activity of the liver, dietary BCAA must undergo transamination to BCKA in peripheral tissues. Wild-type mice also appear to be similar to the rat in that skeletal muscle BCKADHC is usually very low, and therefore BCKA would be exported, probably to be oxidized in the liver. In BDK^{-/-} mice, however, peripheral tissue BCKADHC is not only increased, all of it is constitutively activated (dephosphorylated). Thus, BCKA produced within a tissue such as muscle may be oxidized within that tissue. This raises interesting questions about the fate of not only the carbon and nitrogen from BCAA catabolism, but also about tissue energy homeostasis in general. If in the BDK^{-/-} mice a tissue that previously exported BCKA now oxidizes it, this must be replacing the oxidation of some other fuel (unless there is an increase in oxygen consumption and energy production, but this is unlikely). Future work should determine indicators of related metabolism such as glucose and fatty acid use by muscle and the production of glutamine and alanine, the usual nitrogenous end products of muscle BCAA amino acid catabolism. Similarly, the mechanisms underlying the decreased growth require further investigation. Although it would be tempting to suggest that hyperphosphorylation of eIF α would result in a global decrease in protein synthesis, the investigators¹³ mention in their discussion (no results are presented) that such hyperphosphorylation was only detectable in brain, not in other tissues.

The application of transgenic and knockout technologies to the study of amino acid metabolism, compared with those developed to study carbohydrate and lipid metabolism, is still relatively uncommon. But, as with many genetically modified animals, and as pointed out by Hutson,¹⁴ these BDK^{-/-} mice raise many more questions than can be answered in their initial characterization. The results show that phosphorylation is essential in BCAA homeostasis and that feedback inhibition and long-term changes in the amount of the complex were not able to prevent BCAA (and BCKA) depletion. The data also suggest that there are considerable problems around weaning, a time of rapid growth when the body capacity for BCAA catabolism increases and control by phosphorylation becomes very important.²⁵ Interestingly, in BDK^{-/-} mice, the circulating and tissue concentrations of BCAA were only decreased by approximately

50% to 60% of those of wild-type animals. This could indicate that catabolism was now limited by transport, transamination, or some additional step in the pathway. Additionally, given the proposed role of leucine in the regulation of food intake, perhaps such mice eat continuously and thereby maintain a minimum level of BCAA. The answers to these and other questions will hopefully be forthcoming over the next few years as this very interesting mouse model is more completely characterized.

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