THE CONSEQUENCES OF EXCESS BCAA CATABOLISM DUE TO BCKDK DEFICIENCY

Laura Ohl

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2023

Supervisor of Dissertation

Rebecca Ahrens-Nicklas MD, PhD.

Assistant Professor of Pediatrics

Graduate Group Chairperson

Bruce D. Freedman, VMD, PhD.

Associate Professor of Veterinary Medicine

Dissertation Committee

Zoltan Arany MD, Ph.D., Professor of Medicine

Elizabeth Bhoj MD, Ph.D., Assistant Professor of Human Genetics

Eric Marsh MD, Ph.D., Professor of Neurology

Yuanquan Song, Ph.D., Assistant Professor of Pathology and Laboratory Medicine

This work is dedicated to my Mom, this is for us. This work is additionally dedication to my Dad, who inspired me to do this and would have loved to be here today to see it come to fruition. This work is also dedicated to Dr. Santiago-Blay, whose constant curiosity and passion for his research ignited and fueled my own. Thank you for always being there for me, for teaching me all of life's many lessons, and for constantly reminding me to, "do what's in your heart."

ACKNOWLEDGMENT

I would like to thank Dr. Rebecca Ahrens-Nicklas for allowing me to be the natural leader I am, for always being a cohesive front in all our endeavors, and for enabling me to achieve this goal towards my dreams.

Thank you to the BCKDK KO team. You were the most intelligent, thoughtful, and collaborative colleagues I've ever had. Your dedication to supporting each other was novel and revolutionary to this body of work. Each of you are so different from each other, yet so delightfully wonderful in your own ways. A special thank you to Amanda. Your dedication and drive motivated me in hard times, you tolerated and fulfilled my many requests, and you always became my lab hands when I needed to write. I don't think I could have made this ambitious timeline without your superhuman assistance.

Thank you to the members of my thesis committee. You have helped me develop into a stronger scientist with your humor, feedback, guidance, and unwavering support. I appreciate all you have done for me.

Thank you to the MSUD Foundation Support Group for their funding that supported this research on the mechanism of BCKDK disease pathophysiology. Our findings from this work have helped us further understand the pathway impacted in MSUD and our understanding of potential therapeutic targets.

Thank you to the CAMB coordinators, particularly Anna Kline. Your kindness, friendliness, and helpfulness made every step of this journey more achievable and less daunting. Every question has been answered, sometimes repeatedly, with the utmost respect and altruism. We all admire your compassion.

Importantly, thank you to my friends and family who kept me on track. A special thank you to my parents who encouraged my hard work and education, to my siblings who always reminded me of the end-goal, and to my partner for keeping me grounded, focused, and always moving forward, quite literally. To my closest friends who have been there for every high and low of this journey, it is not lost on me how precious you all are. You mean the most to me.

ABSTRACT

THE CONSEQUECES OF EXCESS BCAA CATABOLISM DUE TO BCKDK DEFICIENCY

Laura Ohl

Rebecca Ahrens-Nicklas

BCAAs are essential proteogenic amino acids required for protein synthesis and can be utilized for energy production when catabolized. BCAA catabolic rate is mediated through inhibitory phosphorylation of the rate limiting enzyme branched-chain alpha-ketoacid dehydrogenase complex by branched-chain alpha-ketoacid dehydrogenase complex kinase (BCKDK). Pathogenic variants throughout the BCKDK protein structure have been associated with intellectual disability, global developmental delay (motor milestone delay, EEG abnormalities, language impairment), atypically autism, and progressive microcephaly. Patients with loss of function variants had severe depletion of BCAAs, presumably due to increased BCAA catabolism. To test if a previously published *Bckdk*$^{-/-}$ mouse model had clinically relevant signs of neurodevelopmental delay and postnatal microcephaly with developmental assessments and MicroCT analyses. Our results of these studies implicating increased catabolism of BCAAs, neurodevelopmental delay in *Bckdk*$^{-/-}$ mice, and postnatal microcephaly. Next, to determine if increased BCAA catabolism is the molecular mechanism of BCKDK deficiency, we measured key BCAA catabolite intermediates of the BCAA pathway and downstream TCA cycle intermediates with LC-MS. Our results implying increased catabolism of BCAAs. After understanding the disease mechanism, two interventions were tested to determine if they could correct disease pathophysiology and biochemical changes in the *Bckdk*$^{-/-}$ mouse model. These interventions included BCAA repletion and reduced BCAA catabolism through reducing levels of one of the BCKDH subunits, *Dbt*. BCAA repletion has minimal impact on developmental delay or postnatal microcephaly, although brain size was partially restored. Genetic modulation trended towards correction of these phenotypic changes. Intriguingly genetic modulation of BCAA catabolic rate corrected biochemistry and partially improved neurodevelopmental delay to a larger extent than BCAA repletion, further supporting that the BCAA catabolic rate is the driving cause of metabolic imbalances and the mechanism of action. These findings reveal a novel target to restore biochemical and neurological phenotypes in BCKDK deficiency that

should be further explored in controlled, randomized trials. Furthermore, these studies reveal that BCAA catabolism needs to be tightly regulated to maintain normal progression of developmental milestones and homeostasis of brain biochemistry, further solidifying the importance of BCAA metabolism in brain function and neurodevelopment.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 1: THE EMERGING ROLE OF BRANCHED CHAIN KETOACID DEHYDROGENASE KINASE IN NEURODEVELOPMENT

This chapter was written by Laura Ohl, with input from Rebecca Ahrens-Nicklas.

1.1 **Abstract**

Branched chain ketoacid dehydrogenase kinase (BCKDK) deficiency is an autosomal recessive inherited neurometabolic disorder of excess branched chain amino acid (BCAA) catabolism. The rate of BCAA catabolism is regulated by BCKDK through inhibition of BCKDH through phosphorylation. It has been hypothesized that a severe reduction in BCAAs underlies the disease pathophysiology, and that BCAA supplementation may ameliorate disease phenotypes. However, robust efficacy studies in preclinical models and human studies are lacking. Collectively, this review discusses the current literature revealing the function of BCKDK in the brain, the possible mechanisms for BCKDK deficiency, and the emerging role of BCKDK as a master regulator of macronutrient metabolism. Currently there is no established therapy for BCKDK deficiency. However, patients may benefit from therapies that modulate BCAA metabolism and could be identified through newborn screening once an approved therapy is established.

1.2 **Introduction**

1.2.1 **BCAAs role in cellular and molecular biology**

BCAA levels modulate essential metabolic, molecular, and cellular pathways in the cell. First, they act as substrates for both BCAA oxidation and protein synthesis and play an important role in nutrient sensing and response to cellular stress. These essential processes are regulated by a number of signaling cascades including the 5' AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways and the integrated stress response (ISR)[1]. Recent data also suggest that BCAAs play an additional role in regulating mitochondrial biogenesis[1]. Given that BCAAs regulate a large

array of fundamental cellular reactions, pathologic changes in BCAA levels, can lead to severe cellular dysfunction.

BCAAs are not able to be stored for later use. Instead, they must be immediately utilized as building blocks for *de novo* synthesized proteins or broken down for energy production. In fact, BCAAs are so essential that under BCAA depleted conditions, the body will catabolize endogenous proteins to prolong normal homeostasis. This is most noticeable in large biomasses where amino acids are stored as proteins, such as in the skeletal muscle. Alternatively, under conditions where BCAAs are in excess they are catabolized.

1.2.2 **BCKDK regulates the rate of BCAA catabolism**

The biochemistry of branched-chain amino acid (BCAA) catabolism has been well studied in several organisms and cell types. Under hypoglycemic conditions, BCAAs are first broken down into alpha-keto acids by branched-chain aminotransferase and then further broken down by the branched-chain ketoacid dehydrogenase complex (BCKDH). This complex consists of four different subunits, BCKDHA, BCKDHB, DBT, and DLD[2]. BCKDH breaks down the alpha-keto acids into intermediates, including acylcarnitine intermediates that eventually break down into acetyl-CoA. Acetyl-CoA can enter the TCA cycle which is coupled with the electron transport chain for energy production in the mitochondria. Within the mitochondria, the rate of BCAA catabolism is regulated by branched-chain keto-acid dehydrogenase kinase (BCKDK). BCKDK achieves this regulation by phosphorylation of the BCKDH-E1a subunit of the BCKDH complex to inhibit its activity and prevent excessive catabolism of BCAAs. Therefore, BCKDK acts as a modulator of the BCAA catabolic rate.

Pathogenic variants within essential enzymes in the BCAA catabolism pathway have previously been associated with pediatric neurocognitive and neurodegenerative disorders including BCAT2 deficiency and Maple Syrup Urine Disease (MSUD)[3,4]. BCAT2 deficiency is caused by pathogenic variants in the upstream reversible enzymatic reaction that converts branch chain amino acids to alpha-keto acids, while MSUD is caused by mutations in pathogenic variants in any of the four subunits of the BCKDH complex, although some pathogenic variants are more prevalent than others (BCKDHA, BCKHDB)[3,5]. Both

disorders lead to the accumulation of BCAAs, and thus diet intervention to prevent excess BCAAs and BCKAs has been the standard of care[4]. Inversely, loss of function of BCKDK has been associated with BCAA depletion from plasma amino acid profiles[4,5]. In the last two decades, these pathogenic variants have been associated with neurodevelopmental delay and intellectual disability[6–9]. From these disorders it is evident that finely tuned regulation of the BCAA pathway is essential for the proper development of the central nervous system.

1.3 **BCKDK pathogenic variants and implications for its role in brain dysfunction**

1.3.1 **Emerging role for BCKDK in neurodevelopmental delay**

Biallelic loss of function pathogenic variants in BCKDK lead to intellectual disability, autism, and epilepsy in human patients[6]. Additionally, pathogenic variants in BCKDK are associated with low plasma levels of BCAAs, which has subsequently been confirmed as a metabolic marker of loss of BCKDK function[6,7,9].

In the first BCKDK study to assess neurodevelopmental assessments were performed on two patients throughout the first 5 years of their life. Patients with *BCKDK* c.C520G (p.R174G*) and c.T1166C (p. L389P) pathogenic variants had impaired motor function, speed, vision and hearing, social, behavioral, and severe developmental delay with greater than 1 year in developmental delay according to the Brunet-Lezine test[7]. Since this study, a total of 32 patients with BCKDK pathogenic variants have been reported.

In a recent large study of 21 cases, 100% of patients with pathogenic variants in BCKDK were found to have motor milestone delay. Additional findings included: global developmental delay, intellectual disability language impairment, progressive microcephaly, EEG abnormalities, and autism. Additionally, most patients had behavioral abnormalities, while approximately half of patients presented with epilepsy. Analysis of available clinical data from all 32 reported cases shows a high burden of neurological symptoms (Figure 1A). Patients display a spectrum of disease severity and phenotypes.

Additionally, high protein diet with BCCAA supplementation has had variability in the impacts on these neurological phenotypes.

These clinical manifestations clearly illustrate that BCKDK plays an important role in brain function. Patients with epilepsy are 2-3 times more likely to prematurely die, posing a major risk to these infants[5]. Newborn screening using low branched chain amino acid levels as a marker could detect patients with this potentially treatable disorder. Currently, some patients are being treated with a high protein diet combined with BCAA supplementation, with the goal of repleting BCAA levels[9]. If a successful therapy is established, newborn screening could optimize outcomes and reduce or prevent irreversible neurological damage, as has been seen in MSUD since it was added to the US Recommended Uniform Screening Panel[3].

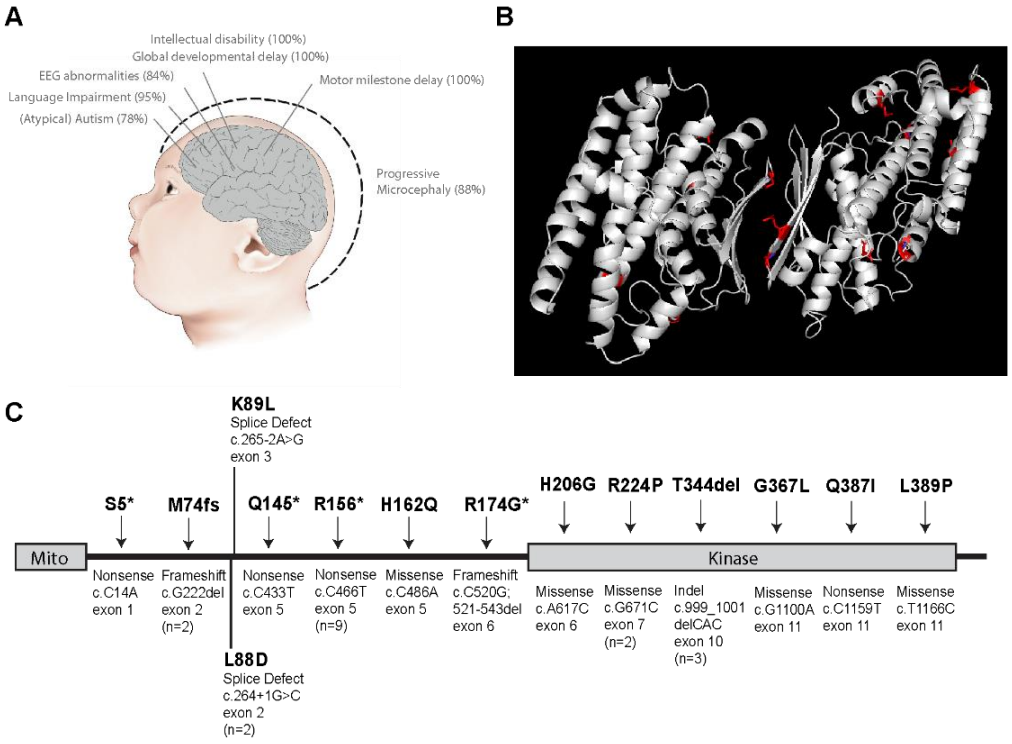

Figure 1 Clinical spectrum of BCKDK deficiency and pathogenic variants

(A) Most prevalent clinical symptoms in patients with BCKDK deficiency. Clinical symptomology with highest documented incidence. (B) Location of BCKDK pathogenic variants throughout the 3D dimerization structure**.** Emphasis on their localization to binding sites, dimerization interface, and important protein domains that may affect BCKDK function. (C) Location of BCKDK pathogenic variants throughout the protein structure**.** Nonsense, frameshift, missense, splice, and indel pathogenic variants have been discovered throughout BCKDK structure, including within the Kinase domain.

1.3.2 **Expansion of the clinical spectrum of BCKDK disorders**

Pathogenic variants within *BCKDK* are continuously emerging due to better diagnostic methods such as clinical exome sequencing. To date there have been 14 pathogenic variants found within *BCKDK*. The first pathogenic variants in *BCKDK* (c.22del (p.M74fs), c.C466T (p.R156*), c.G671C (p.R224P)) were discovered in 2012[6]. Although the nonsense pathogenic variant, specifically *BCKDK* c.C466T (p.R156*), was further investigated in this work, the mechanism of the missense pathogenic variant *BCKDK* c.G671C (p.R224P) remains to be elucidated. Since the discovery of these initial pathogenic variants, García-Cazorla et al. also discovered a frameshift *BCKDK* c.C520G (p.R174G*) and a second missense pathogenic variant *BCKDK* c.T1166C (p.L389P)[7]. The novel missense pathogenic variant was the first pathogenic variant discovered within the kinase domain of BCKDK. An additional pathogenic variant *BCKDK* c.999_1001delCAC (p.T344del) was later found within the kinase domain, supporting that the kinase domain of BCKDK is essential for normal protein function.

Thus far, pathogenic variants within the BCKDK protein are located throughout the BCKDK protein structure (Figure 1C). These pathogenic variants span across the regulatory domain and the kinase domain. Inversely to what has previously been found, a gain of function heterozygous pathogenic variant (c.C486A (p.H162Q)) in BCKDK with clinical presentation of MSUD was recently discovered in 2022[10]. This variant is predicted to impact the *BCKDK* inhibitor binding site resulting in continuous inactivation of BCKDC and accumulation of BCAAs. This expansion of pathogenic variants in *BCKDK* has broadened the clinical spectrum of BCKDK disorders and challenged our previous assumptions that BCKDK was solely a loss of function disorder.

Since the initial discovery, the largest cohort to date of patients with BCKDK deficiency was published, adding 7 novel pathogenic variants to the genetic spectrum (*BCKDK* c.C14A (p.S5*), c.264+1G>C (p.L88D), c.265-2A>G (p.K89L), c.C433T (p.Q145*), c.A617C (p.H206G), c.G1100A (p.G367L), c.C1159T (p.Q387I))[9]. This work drastically broadened the clinical spectrum of BCKDK disorders and further strengthened the association between BCKDK deficiency and neurodevelopmental delay.

How missense and indel variants lead to loss of BCKDK function remains to be elucidated. Genotype-phenotype correlation of the recent 21 patient cohort of BCKDK patients revealed that missense or in frame mutations had later onset as compared to nonsense or splice mutations that had earlier onset of neurological symptoms[9]. It is possible that these pathogenic variants alter active binding sites, enzymatic activity, or the dimerization of BCKDK, all of which are essential for the enzyme's normal homeostatic function[11]. To better understand if pathogenic variants mapped to similar regions, the kinase domains, or the dimer interface, 3D mapping of the pathogenic variants onto the protein structure was performed. From this map, it is evident that pathogenic variants are distributed throughout the entire protein structure and are not localized to one region of the protein (Figure 1B). A majority of pathogenic variants are located within the regulatory domain, while a minority that have been discovered are located within the kinase domain. Upon closer look at the 3D protein structure 3 of the 14 published pathogenic variants are located at the dimer interface. Furthermore, previous studies have more extensively shown predictive 3D protein modeling to illustrate structural changes of particular pathogenic variants impact on allosteric binding sites, substrate binding sites, and co-factor binding sties[9]. This 3D rendering illustrates that pathogenic variants are throughout the protein structure and reiterate the importance of both regulatory and enzymatic regions in BCKDKs function.

1.3.3. **The role of BCKDK in brain function**

BCAA metabolism has been well studied in the peripheral tissues of a variety of organisms[12]. Abnormal BCAA metabolism in peripheral organs has been associated with diabetes and cardiac disease[13]. However, how BCAAs are utilized and perturbed in disease states in the brain is not nearly as well studied.

Clinical findings in disorders of BCAA metabolism reveal that correction of peripheral BCAA metabolism but does not fully correct cognitive or neuropsychiatric deficits [14,15]. Therefore, whether CNS BCAA levels are restored after peripheral correction, should be evaluated in future studies. Research into this area is of great interest to these patients since neuropsychiatric impairment lasts a lifetime. Addressing this unmet clinical care need would greatly improve the quality of life for these detrimental

pediatric onset disorders that cause long-term intellectual disability. Basic research into BCKDK loss of function mouse models have helped us better understand that BCKDK plays a role in brain dysfunction and that the current standards of care are limited by the neuroprotective qualities of the blood brain barrier.

Bckdk$^{-/-}$ mouse models have been developed and studied with a focus on reproducing clinical phenotypes primarily motor deficits and seizures. The original paper revealed that in the absence of BCKDK, BCKDH activity was higher in brain with higher overall expression of BCKDH-E1a. Due to this increased expression and activity of the BCKDH complex, BCAA levels were significantly lower with a decrease equal to or greater than 68% for each individual branch chain amino acid. Due to this decrease the authors attempted to replete overall amino acid levels with a high protein diet and were able to restore body weight at the endpoint of the study as compared to normal chow diets. However, a high protein diet did not rescue their growth curves either revealing that developmental delay is not restored, and that additional metabolic pathways may be impacted by BCKDK that are essential to normal weight gain with growth, such as an fatty acid synthesis. More specifically, the high protein diet increased brain volume sizes significantly in *Bckdk*$^{-/-}$ mice. Prior to treatment, these mice had hindlimb splaying, hindlimb clasping, and epileptic seizures. The rescue of these neurological phenotypes was not assessed in these mice in this initial study.

A subsequent study performed a more targeted approach by supplementing BCAAs through the chow. This work was completed in a second *Bckdk*$^{-/-}$ mouse model in 2012[6]. This new model demonstrated hindlimb clasping and seizures, similar to the previous model. BCAA supplementation in the new model significantly reduced hindlimb clasping and seizures within 24-48h after diet intervention[6]. Limited data are available about changes in seizure stage or duration in this model, and sample size was limited with only 3-4 mice per genotype. Therefore, more data is needed to fully evaluate the therapeutic benefit of BCAA supplementation on seizure stage, duration, and overall burden. Other behavioral analyses of these *Bckdk*$^{-/-}$ mice demonstrated defects in motor coordination and autism-related behaviors[16]. However, rescue of these clinically relevant phenotypes with BCAA dietary supplementation

was not assessed. Therefore, more studies are needed to fully evaluate the therapeutic benefit of BCAAs in preclinical models of BCKDK deficiency.

1.3.4. **Contribution of altered brain intrinsic BCAA catabolism in BCKDK deficiency**

Patients and animal models of BCKDK deficiency have neurological phenotypes. It is not known if changes in peripheral metabolism are driving these symptoms, or if loss of BCKDK activity in the CNS is also contributing to disease pathogenesis. In addition, it is unclear what role specific cell types in the brain play in the disease.

To date, one study has been published evaluating a BCKDK conditional knockout (CKO) model targeting the brain. This study revealed that knockout of BCKDK specifically in cortical cells of the forebrain using the Emx1 driver was sufficient to significantly reduce BCAA levels in the forebrain without affecting peripheral serum levels. Interestingly, knockout of BCKDK in the forebrain was sufficient to increase aspartate, glutamate, lysine, serine, and threonine levels in the brain[17]. There were also slight increases in alanine, and glycine levels[17]. These findings implicate that loss of BCKDK in the forebrain is likely sufficient to alter amino acid levels, which was also previously investigated in the first BCKDK full body KO mouse model[6]. These studies clearly illustrate that brain biochemistry is drastically altered due to loss of BCKDK, and unequivocally reveals the importance of BCKDK activity in neurons to maintain homeostasis in the brain.

1.3.5. **Efficacy of BCAA supplementation in BCKDK patients**

In patients, significant depletion of BCAAs in the plasma are the only metabolic signatures that have been well characterized in BCKDK deficiency patients.[6–8] BCAA levels have also been shown to be significantly reduced in the CSF of BCKDK patients with the novel p.Thr344del variant, although not all loss of function BCKDK variants have significantly reduced BCAA levels in the CSF as is shown in Tangeraas et al.[8]

To evaluate if BCAA supplementation can increase BCAA levels in BCKDK deficiency patients, researchers evaluated two different dosages of BCAAs. Lower dosages of intravenous administration

(0.5-0.64g/Kg/day) did not elevate BCAAs to within reference ranges in the plasma. However, higher dosages (0.7-1g/kg/BCAA) overshot BCAA reference ranges in plasma. Combination of high protein diet with high dose IV supplementation allowed patients to have short periods of time where BCAA plasma levels were in the normal range, approximately 2-4 hours after a meal[6]. However, in general supplementation strategies lead to drastic peaks and troughs in BCAA levels throughout the day. Low BCAAs levels are the fundamental problem of BCKDK deficiency. However, periods of excess BCAAs in the bloodstream and large swings in levels could also have unintended neurological consequences that have not been fully evaluated.

A number of observational studies of BCAA supplementation in BCKDK deficiency patients have been reported. A small cohort study of 3 patients with the same causal pathogenic variant (c.999_1001delCAC (p.Thr334del)) showed improvements in communication and social skills as measured by the Vineland Adaptive Behavioral Scale over an 18 month period. However, as the authors note the natural history of BCKCK deficiency is poorly understood so this could be the normal developmental trajectory of the disorder[8]. More clearly, a high protein diet with BCAA supplementation significantly reduced seizure frequency in these patients.

The molecular or biochemical effects of BCAA supplementation that induced these clinical changes were not assessed. This recent study also revealed our lack of understanding of the pharmacodynamics of BCAA metabolism in the blood and how this impacts the brain. This was evident when dosage escalation was necessary in these studies to achieve physiologically relevant levels of BCAAs in the serum of BCKDK deficient patients.[8] However, if this leads to elevations in the brain, obviously can't be assessed due to physical limitations of human studies.

Together, these data in mice and patients support that BCKDK plays an essential role in the brain, and that targeting the brain directly for therapeutic intervention is of great importance to improve neurological dysfunction.

1.4 **Possible molecular mechanisms of loss of BCKDK**

Although BCAA supplementation has demonstrated variable and incremental amelioration of disease phenotypes in small studies of BCKDK deficiency patients, unfortunately patients still suffer from significant neurological symptoms. A full molecular understanding of disease mechanisms is lacking and will be essential to inform the development of more effective targeted therapies. BCKDK may regulate several fundamental cellular processes as outlined below that may be promising avenues for novel therapy development.

BCAAs are essential amino acids, that must be ingested through the diet. Therefore, the supplementation of BCAAs in BCKDK patients, who have severely reduced BCAA levels, is understandable as the first step of therapeutic action. However, these approaches focused solely on repleting peripheral levels of BCAAs, despite major neurological symptoms known to be driven by the CNS. Once BCAAs are ingested, they are able to cross over from the bloodstream through the blood brain barrier (BBB)[18]. The BBB is composed of primarily endothelial cells with accessory cells to protect the brain from unnecessary insult and regulate the uptake of nutrients into the brain[19]. These key nutrients include the BCAAs, which competitively cross the BBB through the large-neutral amino acid transporter (LAT1)[20–22]. Importantly, the preferential transport of BCAA through LAT1 can inhibit aromatic amino acids from crossing into the BBB when BCAA levels are high[23,24]. Reduced transport of these aromatic compounds into the brain has functional consequences on the biochemistry and function of the brain[23]. Therefore, maintaining normal homeostasis of these transport systems is of great importance to maintain proper neurologic function. Once BCAAs cross the BBB they are also transported intracellularly by the same transporter (LAT1) to perform their cellular functions.

Intracellular BCAAs modulate a number of cellular pathways that may contribute to the pathogenesis of BCKDK deficiency (Figure 2)[25]. One key pathway regulated by the bioavailability of BCAAs is the amino acid response, which allows cells to sense when amino acids are depleted. An additional pathway that could be impacted is mTOR signaling, which is regulated by leucine levels. Metabolically, BCAAs also serve as major nitrogen donors for glutamate and glutamine neurotransmitter biosynthesis in the

brain[25]. Therefore, BCAAs play a key role in neurotransmitter biosynthesis and recycling. Additionally, BCAAs modulate a number of cell signaling pathways[25]. One key pathway regulated by the bioavailability of BCAAs is the amino acid response, which allows cells to sense when amino acids are depleted and conserve energy. Energy is also directly produced from BCAAs due to their carbon backbone skeletons being structural precursors for TCA cycle intermediates, which feed into the electron transport chain for energy production in the mitochondria[25].

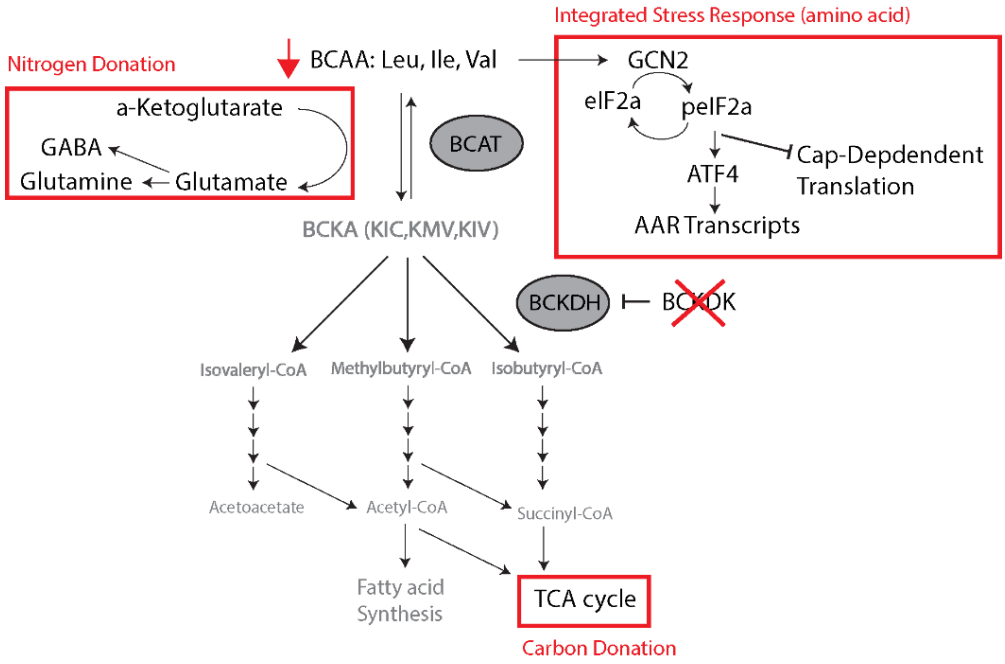

Figure 2 Potential Mechanism of loss of BCKDK and Potential Targets for Therapeutics

(A) Depletion of BCAAs due to loss of BCKDK could lead to three potential mechanisms, activation of the amino acid response, increase in nitrogen donation to neurotransmitter biosynthesis, or perturbations in carbon skeleton donation to TCA cycle intermediates essential for ATP energy production. (B) BCAA repletion has been proposed as a treatment target in the clinic, but rescue of neurological phenotypes are subdued (C) Re-regulation of the BCAA catabolic pathway by reducing enzyme levels in the pathway likely will slow down the catabolic rate and restore regulation of the BCAA catabolic rate.

Secondary changes have also been discovered in mouse models of BCKDK deficiency including changes in amino acid transporter transcript level alterations and global transcriptional changes[6]. These changes may also partially contribute to the mechanism of disease but would be challenging to correct since they are likely an indirect consequence of metabolic and molecular changes due to loss of BCKDK

function. Instead, direct correction of BCAA catabolic rate would more directly address the underlying mechanism of disease and likely correct these secondary changes.

Below we highlight details of each of the major proposed mechanisms that may contribute to BCKDK deficiency pathogenesis.

1.4.1 **Activation of the integrated stress response**

The integrated stress response is a common adaptive pathway that is triggered by multiple diverse types of stress stimulus[26]. In the case of loss of BCKDK deficiency, amino acid deprivation likely initiates the cascade. Upon amino acid deprivation, GCN2 phosphorylates eIF2a, which in turn reduces cap-dependent translation and initiates ATF4 dependent transcripts[27]. ATF4 acts as a transcription factor that binds to specific C/EBP-ATF response elements (CARE) within the genome to regulate transcription of multiple CAP independent transcripts that are less energy intensive during cellular stress, for example SNAT2, 4E-BP1, and CHOP[28,29]. These target genes that are activated during the integrated stress response allow cells to adapt to low amino acid levels mediated by transcriptional changes to reduce transport, non-essential metabolism, and energy consumption.

Although the integrated stress response is well understood at the molecular level, how the integrated stress response plays a role in brain function is not fully understood. The integrated stress response has been shown to be a causal mechanism for multiple diverse brain disorders and neurodegenerative diseases that contain symptomology of cognitive dysfunction[30]. Interestingly, preventing the cascade of the integrated stress response can slow down the aging process and increase survival[27]. These correlations and reversal of aging with its modulation suggest that preventing the initiation of the ISR is likely essential to maintain normal cognitive function and importantly increase survival. Furthermore, previous data showing an increase in peIF2a in a *Bckdk*$^{-/-}$ mouse model, suggests that triggering the ISR is a possible mechanism for brain dysfunction in BCKDK deficiency[31]. Although, more work is needed to evaluate if this pathway is activated in BCKDK deficiency, and how this may alter metabolism and energy

production. Furthermore, other nutrient sensing cellular signaling pathways may also be impacted in BCKDK deficiency.

1.4.2 Dysregulated mTOR1 signaling

The mTOR signaling pathway plays its essential role in cellular growth. *Bckdk*$^{-/-}$ mice have delayed growth rates in early postnatal neurodevelopment[31]. Growth is regulated by the major nutrient-sensitive regulator mTOR, which is activated by the availability of amino acids. Amino acids aid in many cellular processes including lipid synthesis, nucleotide synthesis, ribosome biogenesis, lysosome biogenesis, and mRNA translation[32]. These cellular processes are essential to cell growth and proliferation of cells, which is an essential part of neurodevelopment.

Branched chain amino acids can activate mTOR signaling. Specifically, sestrin2 serves as a leucine sensor [33]. In *Bckdk*$^{-/-}$ mouse models and patients, leucine levels are significantly depleted[6,7,9,31]. This chronic low leucine level likely alters mTOR regulation and activation. In addition to leucine, several other amino acids and nutrients are abnormal in BCKDK deficiency which may have additional effects on mTOR signaling.

1.4.3. Nitrogen donation to glutamate/glutamine neurotransmitter cycling

BCAAs play a major role in the synthesis of glutamate and glutamine mean. Approximately 30% of the nitrogen groups required for the synthesis of these key neurotransmitters come from BCAAs [18,34,35]. BCAAs donate nitrogen groups to alpha-ketoglutarate (aKG) to form glutamate in neurons, which can be further cycled into glutamine and GABA in astrocytes[25,36]. Glutamate is the major excitatory neurotransmitter in the brain, while GABA is the major inhibitory neurotransmitter in the brain[37]. The balance of excitatory and inhibitory neurotransmitters in the brain is essential to maintain neurologic function. Previous literature suggests that an imbalance in this axis has been associated with human epilepsy[38]. If glutamate levels and neurotransmission abnormalities occur early in development, this can induce circuit-level dysfunction in brain that can result in a variety of neurologic phenotypes including intellectual disability and epilepsy [39–41].

Neurons and astrocytes function together to regulate neurotransmitter levels through the glutamate-glutamine cycle [34,42,43]. The exact roles each cell type plays in the pathophysiology of BCAA disorders, such as BCKDK deficiency, is poorly understood[42]. Interestingly perturbations in glutamate-glutamine homeostasis in iPSC models has been shown between these coupled cells in other neuronal diseases including frontotemporal dementia[44]. Therefore, understanding which cell type or if the coupling of these two cell types is essential for proper BCAA catabolism and glutamate-glutamine cycling is warranted and will inform therapeutic development efforts[45].

Theoretically, in BCKDK deficiency increased BCAA catabolism may increase nitrogen donation and subsequent glutamate production in the brain. However, in previous literature, there were no changes in glutamate levels in a *Bckdk*$^{-/-}$ mouse model and in BCKDK deficient patients[6,9]. However the initial BCAA transamination step is reversible; therefore, elevated glutamate levels could be partially compensated for by reversing this reaction to replete both aKG and BCAA levels (Figure 3). It is important to note that aKG is a key metabolite, in multiple pathways specifically as a TCA cycle intermediate and several amino acid catabolic pathways. Therefore, alterations in aKG could likely alter many cellular metabolites.

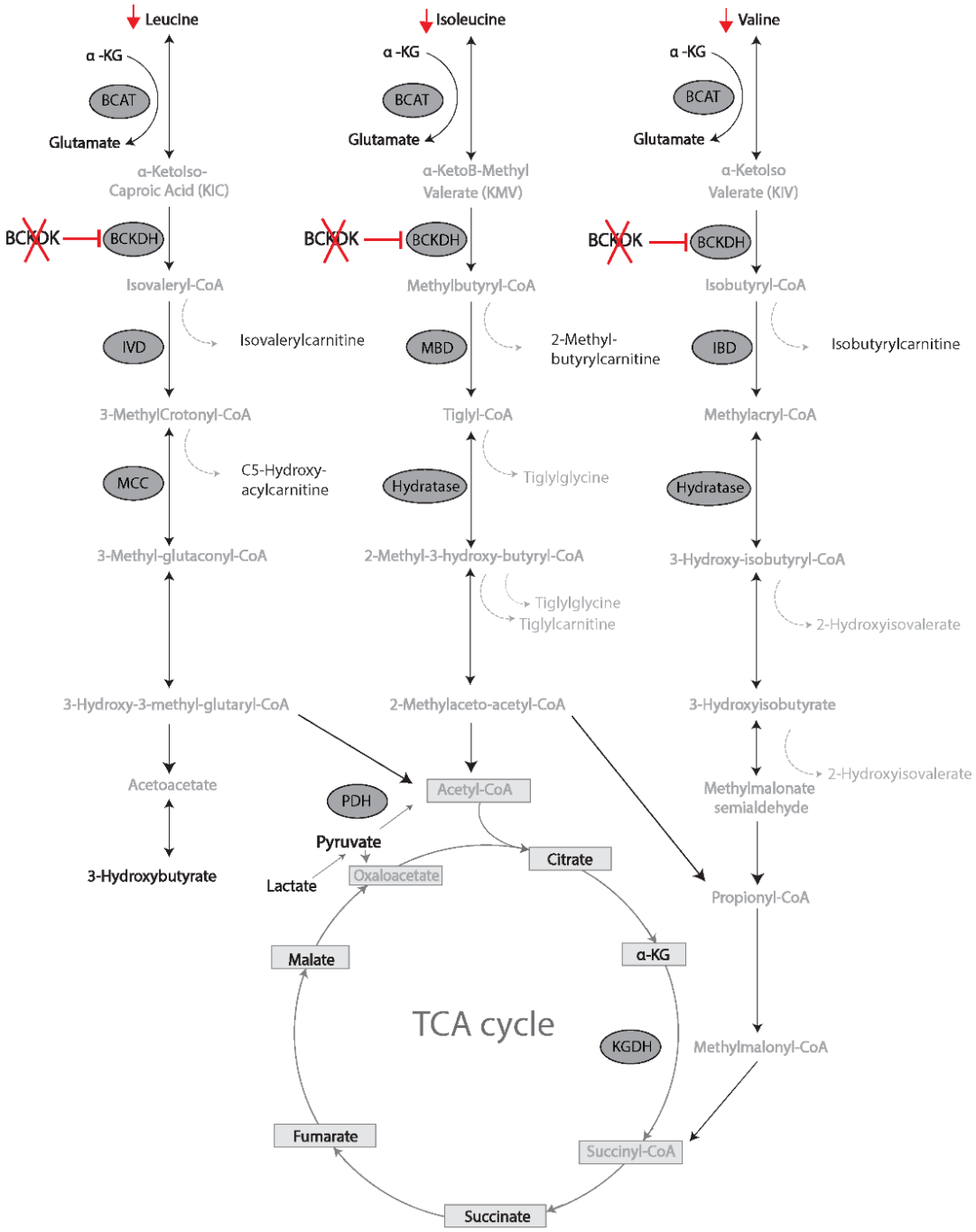

Figure 3 Integrated Pathway of Downstream Metabolites and Enzymes in the BCAA catabolic pathway and TCA Intermediates

BCAA catabolites break down into intermediates that eventually feed into the TCA cycle via acetyl-CoA and succinyl-CoA. These TCA cycle intermediates can further be broken down through coupling of the TCA cycle to the electron transport chain for ATP energy production.

1.4.4. Carbon donation to TCA Cycle intermediates for energy production

Products of BCAA catabolism also aid in energy production in the mitochdonria[46] (Figure 3). Byproducts created from the BCAA pathway, including acetyl-CoA and succinyl-CoA feed into TCA cycle, which is coupled to the electron transport chain for ATP energy production in the mitochondria[46].

Although many stable isotope flux experiments have been performed mapping the contribution of BCAAs to the TCA cycle in peripheral organs[12,13], very few tracing experiments have been done in CNS-derived cells, particularly in human tissue.

It has long been debated in the field if BCAAs could be fully catabolised throughout the human brain, as it was thought that BCAT was not present in human astrocytes[47,48]. Salcedo et al. demonstrated that BCAA catabolism rates are similar in mouse and human neurons and astrocytes, revealing that astrocytes are in fact functionally coupled with neurons in the human brain. Furthermore, they found that BCAA-derived carbons enter the TCA cycle and stimulate ATP synthesis in human neurons[25]. This discovery further supports the importance of BCAAs utilization as a precusor for energy production in brain.

1.4.5. Secondary changes: amino acid transporter upregulation and transcriptional changes

In addition to these mechanisms, reduction of BCAA levels in BCKDK deficiency likely allows for increased uptake of other amino acids that use the same amino acid transporters across the BBB and intracellularly. Intriguingly, upregulation of amino acid transporters are also seen in a *Bckdk*$^{-/-}$ mouse model, likely due to depletion of BCAAs[6]. This upreglution of amino acid transporters following BCAA depletion may be a compensatory mechanism to try to increase their uptake. However, this may further worsen imbalances in amino acid levels since there is less competition for these transporters when BCAAs are depleted.

In the original *Bckdk*$^{-/-}$ mouse model there were significant increases in multiple different amino acids including y-transported amino acids (serine, glycine, ornithine, and arginine) and L-system transported amino acids (threonine, phenylalnine, tyrosine, histidine, and methionine)[6]. BCAAs can be

transported through diffusion, facilitated diffusion, or active transport due to their small size and charge [49]. While the Y system transporters allow for the transport of cationic amino acids, the L-system allows for large neutral amino acids[50]. Both transporter systems can transport branch chains. Therefore, when branch chains are reduced in BCKDK deficiency there is a lack of competative uptake of other amino acids that allows for preferrential increases in other amino acid levels in the brain.

Alterations in a several amino acid levels in brain are associated with human disorders. For example, BCAAs are elevated in MSUD [51], while elevations in glycine and lysine are seen in a number of different inherited metabolic epilepsy syndromes[52] [53,54]. More work is needed to elucidate how additional amino acid imbalances, beyond BCAA abnormalities, are contributing to BCKDK deficiency.

In BCKDK deficiency there are alterations in transport and uptake of several amino acids, especially throug the SLC7A5 transporter. Interestingly, loss of the SLC7A5 transporter at the blood brain barrier leads to global changes in amino acid levels in the brain of the Slc7a5 conditional knockout mouse model[16]. Interestingly these SLC7A5 CKO mice also had finemotor coordination and locomotion issues, similarly to *Bckdk*$^{-/-}$ mouse models[16].

Interestingly, amino acid changes in previously published *Bckdk*$^{-/-}$ models are inconsistent with patient-derived fibroblast data, the latter of which showed only trend increases in glutamine and methonine but not outisde of standard ranges[6,31,55]. Cell culture conditions likely heavily influence these levels, as most medias contain excess amino acids. Interestingly, metabolic changes in humans are also dissimilar to mouse models with only depletion of BCAAs as a consistent amino acid change. Although, this is likely due to tissue specific metabolic differences between serum and CSF collected from human patients compared to brain tissue collected from mice.

In addition to amino acid levels changes in *Bckdk*$^{-/-}$ mice, transcriptional profiles were drastically altered compared to WT mice cortices[6]. Gene ontology analysis identified differences in a number of gene networks including those regulating cell junction, synaptic transmission, synapse part, aminoacyl-tRNA synthetase, neuron projection development, and amino acid transmembrane transporter activity.

Together, overall dysregulation of amino acid levels likely contribute to the pathophysiology of BCKDK deficiency, but our understanding of this mechnaism is limited. Future research should focus on which amino acid changes contribute most to disease pathogenesis and if amino acid transporter modulation could improve phenotypes in animal models and human patients.

1.4.6. Therapeutic targets of BCKDK deficiency

BCKDK Deficiency has been assumed to be a BCAA depletion, due to hypercatabolism of upstream catabolites. However, therapeutic interventions have solely focused on BCAA replenishment, without addressing the underlying cause of disease pathology, which is a lack of regulation of the BCAA catabolic pathway (Figure 4A). Re-regulating the BCKDK catabolic pathway could be done through enzymatic inhibition or transcript reduction. The first target of interest would be BCKDH, since BCKDK directly regulates this step (Figure 4B). Additionally, BCKDH has been shown to interact in a metabolon with BCAT, suggesting that this could also be another target worth investigating as a therapeutic target for BCKDK deficiency (Figure 4C)[56]. Additionally, combinatorial approaches of genetic modulation of the BCAA pathway and dietary repletion of BCAAs could be beneficial, particularly during metabolic decompensation when metabolism is dysregulated. Alternative to replenishment and modulation therapies, gene replacement of BCKDK would likely achieve the maximum therapeutic benefit, since BCKDK has additional metabolic targets besides BCKDH (Figure 4D)[57].

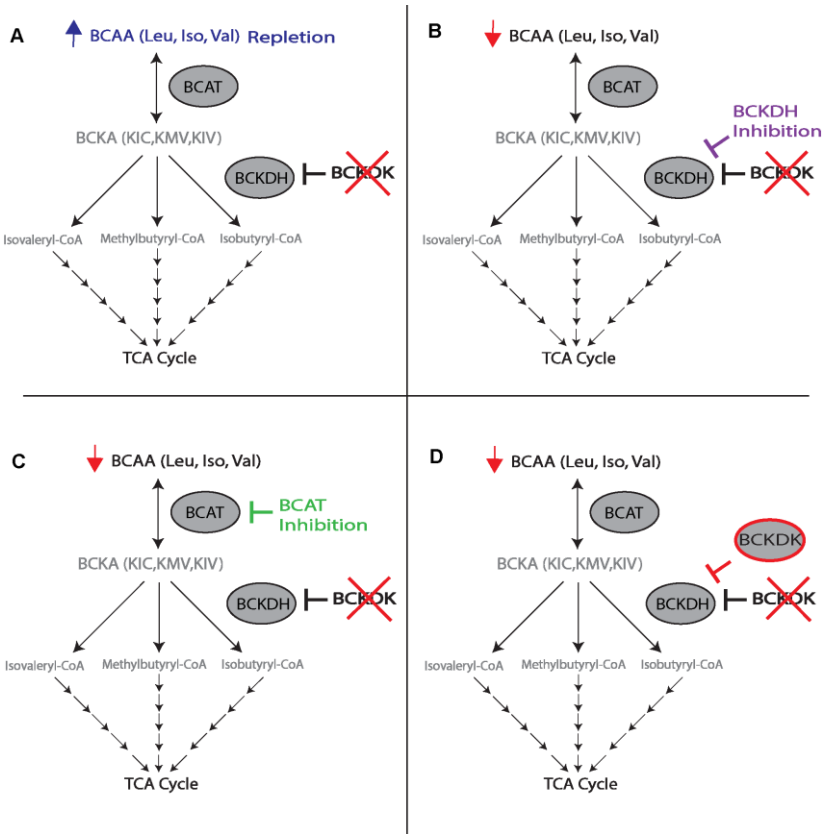

Figure 4 Novel Therapeutic Targets of BCKDK Deficiency

(A) BCAA depletion is the most consistent metabolic marker of BCKDK deficiency, therefore therapeutic repletion has been the adopted therapeutic intervention (B) BCKDH reduction or inhibition is a novel direct target for BCKDK deficiency to re-regulate the rate of BCAA catabolism. (C) BCAT reduction or inhibition is also a novel upstream target for BCKDK deficiency which would also likely re-regulate the rate of BCAA catabolism (D) Ideally, gene replacement of BCKDK would be the ideal therapeutic replacement (immune response may be elicited).

1.5 **BCKDK as a master regulator of macronutrient metabolism**

BCAAs are proteinogenic amino acids indicating their importance for protein synthesis in an anabolic state. This cellular state of anabolism is achieved when the availability of essential amino acids (EAAs) are sufficient to synthesize new proteins. Simutaneously, catabolism can also be occurring, but to a lesser extent than the rate of anabolism. The metabolic transition from a predominantly anabolic state to a primarily catabolic state transitions after the absorption of EAAs from the bloodstream into the skeletal

muscle, the first site of protein anabolism after ingestion. After this first pass in the muscle, EAAs become available for other tissues for anabolism, including the brain[1,58].

Interestingly, the current standard of care for BCKDK deficiency relies on this concept that keeping the body in a predominantly anabolic state with a high protein diet and BCAA supplementation, will prevent the excess catabolism of BCAAs. However, since these two states occur simutaneously and at different rates depending on nutrient availability, it's important to challenge this assumption since there is likely an imbalance between these two states leading to excess BCAA catabolism due to BCKDK deficiency.

In addition to feeding state, cellular and molecular feedback mechanisms regulate these states. For cells to know when BCAA retention or catabolism needs to occur, nutrient sensors within the cell assess the microenvironment for key nutrients. These nutrients include pathways previously presented as potential mechanisms for BCKDK deficiency including the amino acid response and the mTOR pathway, which sense AA levels and activates under cellular stress conditions, respectively. Metabolic substrates also play a role in feedback mechanisms to regulate the switch between anabolism and catabolism. One of these substrates is KIC, which negatively regulates BCKDK. From these examples, it is clear that our assumptions regarding metabolic state should not be oversimplified or assumed in BCKDK deficiency, but instead assessed before and after treatment. Furthermore, our understanding of the regulation of BCKDK and feedback mechanisms involved in this pathway, should also be closely considered to better understand their potential contribution to BCKDK deficiency and uncover potential therapeutic targets.

1.5.1 Regulation of BCKDK

BCKDK is regulated at multiple steps on the way to becoming a functional kinase. The tight regulation of BCKDK levels helps maintain BCAA catabolic rates and cellular energy homeostasis. First, BCKDK is transcriptionally regulated by the Carbohydrate Responsive-Element Binding Protein (ChREBP-β) transcription factor. Increased expression of the ChREBP-β transcription factor correlates with increased expression of the BCKDK transcript levels[57]. Simultaneously, increased ChREBP-β

expression repressed PPM1K expression, the key dephosphorylase of BCKDH. Interestingly, both BCKDH post-translational modifiers can also utilize ATP-citrate lyase (ACL) as an alternate substrate[57]. Specifically, BCKDK phosphorylation activates *de novo* lipogenesis, while PPM1K dephosphorylation of ACL deactivates lipogenesis. Together, these findings reveal that ChREBP-β integrates BCAA and lipid metabolism through transcriptional regulation. In addition to transcriptional regulation of BCKDK, an *in vitro* model of colorectal cancer metastasis has been shown to be driven by the posttranslational modification of Src at the 246th tyrosine of BCKDK[59]. This specific modification was shown to enhance BCKDK activity and stabilize its structure. However, if this modification is relevant to normal physiological function of the brain, remains unknown.

BCKDK is also regulated by the bioavailability of key metabolic cofactors and substrates. BCKDK contains structural domains including its regulatory domain (1-184 AA) and a histidine kinase domain (185-C terminus). Importantly, BCKDK also contains a mitochondrial localization domain (N-terminus) to localize the protein to the mitochondrial matrix, where it performs its catabolic function[6]. Cofactors that bind to BCKDK and aid in its enzymatic function consist of ATP, ADP, Mg^{2+}, and K^{+}. Substrates also enhance BCKDK enzymatic activity, for example KIC. Most importantly, dimerization of BCKDK is essential to its function. Thus, it is evident that BCKDK is highly regulated by cofactors, substrates, and feedback mechanisms. Interestingly, BCKDK is structurally similar to other metabolic enzymes. In fact, BCKDK is a non-canonical functional paralog of pyruvate dehydrogenase kinase (PDK) and shares structural homology with alpha-ketoglutarate dehydrogenase complex (KGDHC)[60,61]. PDH is the key enzyme to convert pyruvate into acetyl-CoA prior to entrance into the TCA cycle. KGDHC aids the conversion of alpha-ketoglutarate into succinyl-CoA in the TCA cycle. Each of these enzymes are essential to macronutrient metabolism of pyruvate oxidation, BCAA catabolism, and the TCA cycle. Once BCKDK performs its regulatory function of phosphorylating its respective targets, it is ubiquitinated by UBE3 for degradation by the proteasome[62].

1.5.2. Inhibition of BCKDK

Our understanding of how loss of BCKDK variants are pathogenic comes from a body of basic research into the structural biology of BCKDK[11,63]. These studies have used crystal structures to discover the location of specific substrate binding sites, cofactor binding sites, and inhibitor binding sites that impact BCKDKs function. A majority of this work has focused on developing inhibitors of BCKDK as a therapeutic target primarily for cancer, but also metabolic disease.

BCKDK is inhibited by the BCKDH substrate alpha-ketoisocaproate (KIC). The negative regulation by KIC ensures that there isn't aberrant overoxidation of BCAAs and alpha ketoacids. In addition to this feedback loop, other inhibitors of BDK have been discovered in parallel through structural studies. KIC is a weak inhibitor of BDK similarly to the pharmacological inhibitor PB. Upon investigation for additional inhibitors of BDK, (S)-CPP was identified from precursor compounds including PB and the enantiomers (S)-CIC, (R)-CIC. The most potent inhibitors being (R)-CIC and (S)-CPP. Their inhibition is further enhanced by the co-binding of adenine nucleotides such as ATP, ATPgammaS, and ADP. KICs binding to the allosteric site in the N-terminal domain of each BDK dimer subunit has been well characterized. Therefore, pathogenic variants within this region would be predicted to lead to a permanent activation of BCKDK likely leading to a toxic gain of function mechanism, similar to the novel BCKDK pathogenic variant recently discovered in 2022[10,11].

Additionally, site-directed mutagenesis studies have been used to understand the structural importance of particular binding sites and interactions between bonds formed in the BDK dimer. The results from Tso et al. revealed that conformational changes such as hydrogen bonds between the Tyr99 sidechains and the carboxylate-oxygen atom are essential for allosteric inhibitors to bind to BCKDK. Additionally, they show that the salt bridges formed between the side chains Arg167 and Arg171 are essential for this binding. More importantly, they reveal that due to confirmational changes phenylalanine side chains can block the N-terminal binding sites that leads to steric hinderance preventing allosteric inhibitor binding. Therefore, inhibition of BCKDK may prove to be more challenging than previously

thought due to the oligomeric structure, confirmational changes, and requirement for adenosine nucleotides to enhance BDK inhibition[11].

During a follow up high throughput screen, benzothiphene carboxylate derivatives, including BT1 and BT2, were discovered as novel BDK inhibitors [63]. BT2 is the most commonly used and was the most metabolically stable allosteric inhibitor of BCKDK in the field before 2021. It is slightly more potent than the previously developed (S)-CPP with a half-life of approximately 240 minutes. One of the limitations of this BT2 inhibitor is that is it charged due an exposed carboxylic acid. Therefore, the prodrug BT3 was developed to increase its half-life and increase any residual BCKDK activity. Although this prodrug has been tested in multiple model systems, to our knowledge it has not been tested in the clinic[64]. In summary, structural studies into the inhibition of BCKDK has led us to better understand its metabolic regulation and helped us better understand its emerging role in regulating multiple metabolic pathways.

1.5.3. Novel pathways regulated by BCKDK

BCKDK also has allosteric inhibitors that inhibit its activity and allow for increased BCAA catabolism[64]. Inhibition of BCKDK has been used to discover novel targets of BCKDK, suggesting that it may play a larger role in integrating multiple metabolic pathways. Of these metabolic pathways, BCKDK has been well studied in the context of BCAA catabolism. However, its role in other metabolic pathways has not been as extensively investigated.

BCKDK has been shown to regulate the TCA cycle through phosphorylation of PDC in the absence of PDK in the early stages of embryonic development[52]. This work revealed the importance of limiting input into the TCA cycle, particularly since loss of the regulators *Bckdk* and *Pdk* lead to embryonic lethality in mouse models[60]. Interestingly, this also reveals that *Bckdk* is expressed during embryonic development and likely regulates the BCAA catabolic pathway during gestation. While pathogenic variants in BCKDK lead to autism, intellectual disability, and seizures, pathogenic variants in PDC lead to chronic neurological dysfunction and seizures[65]. Therefore, excess input of catabolites into the TCA cycle

coorelates with impaired neurological function, but if this is due to increased entry of catabolites into the TCA cycle needs to be further investigated in BCKDK deficiency.

Phosphoproteomics in Zucker fatty rats treated with the BT2, an allosteric inhibitor of BCKDK, revealed additional phosphorylation targets of BCKDK. These targets are involved in multiple different metabolic pathways including BCAA catabolism (BCKDHA), de novo fatty acid biosynthesis (ACC1), de novo cholesterol biosynthesis (ACC1, ACL), oxidation-reduction, reactive oxygen species homeostasis, amino acid degradation, L-phenylalanine degradation, and tyrosine aminotransferase activity (Figure 5). Although not many of these targets have been validated. BCKDK also has non-canonical roles such as regulating crosstalk between other metabolic pathways, such as pyruvate oxidation since BCKDK is a functional homolog of PDK when it is dysfunctional (Figure 5)[60]. Importantly, since a lot of these additional phosphorylation targets of BCKDK have yet to be validated, this leaves ambiguity as to if these changes are of biological importance.

In summary, BCKDKs regulation of BCKDHA through inhibitory phosphorylation is the most well established and studied role of BCKDK, acting as the brakes of the BCAA catabolism pathway (Figure 5). This is essential in the brain, since BCAAs act as nitrogen donors for glutamate cycling. In addition, it acts as a carbon skeleton donor to BCAA-derived catabolites and downstream TCA cycle intermediates that aid in energy production. The relevance of these novel phosphorylation targets of BCKDK performed in the liver after BT2 inhibition of BCKDK, are yet to be investigated in the context of the brain. However, the validation of at least one of these targets in addition to other recent publications reveals that BCKDK may play a larger role in macronutrient metabolism than previously thought. Therefore, replacing BCKDK when it is lost due to pathogenic variants, may be the best possible treatment to restore brain function in BCKDK deficiency.

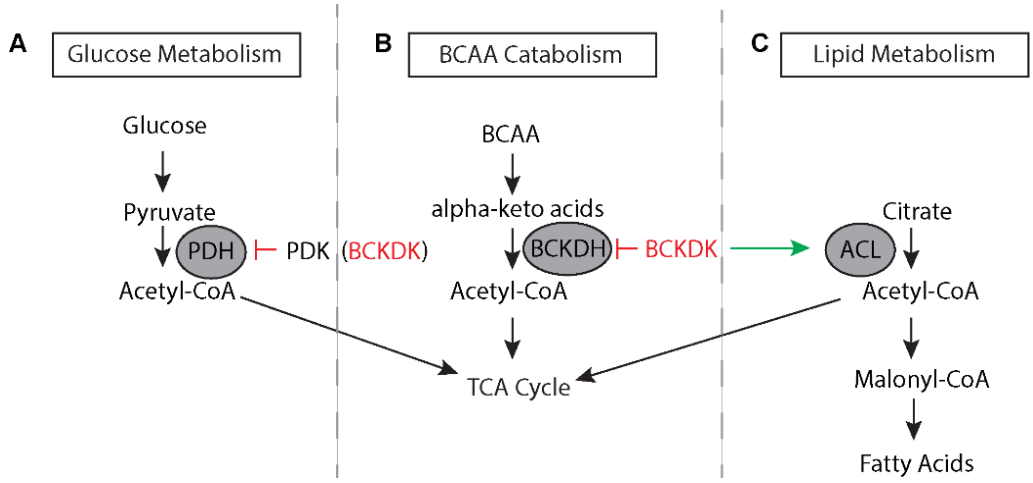

Figure 5 Integration of novel pathways regulated by BCKDK

(A) In the recent literature, BCKDK has been shown to be a functional homolog of PDK, a key kinase involved in glucose metabolism. (B) BCKDK has its established role in regulating BCAA catabolic rate and (C) BCKDK has also been shown to be instrumental as a key regulator of lipid metabolism through activating ACL for fatty acid synthesis.

1.6. Conclusion

Determining the molecular mechanism of disease in BCKDK deficiency is warranted. Further investigation is needed to understand if excess BCAA catabolism is driving disease pathology. A shift in focus from temporarily repleting BCAAs, to directly addressing the causes of neurocognitive dysfunction and seizures will impact the quality of life of patients. This switch in focus is necessary, since BCAA repletion has been proven to be incremental in improving cognitive function and has not been shown to stably restore molecularly changes in human biochemistry. Therefore, studying and understanding the importance of BCAA catabolic rate in neurological function will be instrumental to developing more beneficial therapeutic targets for BCKDK patients. Furthermore, better understanding the role of BCKDK in BCAA catabolism will facilitate advances in therapeutic development for BCAA accumulation disorders that may have a similar mechanism of action, particularly for rare but higher prevalence disorders such as MSUD.

Additional studies will be necessary to assess to what extent plasma amino acids in the periphery contribute to CNS levels and further clarify therapeutically beneficial doses. Thus far, individualized BCAA

dosing strategies have been employed in BCKDK patients. However, even with these tailored dosages clinical data of BCAA supplementation in loss of BCKDK patients reveals that peripheral administration is not sufficient, even at early ages, to correct cognitive dysfunction due to dysregulated BCAA catabolism. Part of this is that in the field we have not simutaneously addressed the over catabalization of BCAA due to loss of BCKDK. Potential approaches could include titration of inhibitors in the pathway to BCAT or BCKDH. Additionally, the field should be cautious in administering excess BCAAs to patients during critical period of brain development, as has been seen in MSUD patients who develop cognitive defects due to an accumulation of BCAAs[14]. All this clinical data supports that BCAA catabolism needs to remain in fine equilibrium and that the rate limiting step of BCKDH and its regulation by BCKDK are imperative to neurodevelopment and cognitive function. Investigating the molecular mechanism of loss of BCKDK will be essential to inform targeted therapeutic approaches.

CHAPTER 2: PARTIAL SUPRESSION OF BCAA CATABOLISM AS A POTENTIAL THERAPY FOR BCKDK DEFICIENCY

Laura Ohl[1,2], Amanda Kuhs[1], Ryan Pluck[1], Emily Durham[1], Michael Noji[2,3], Nathan D Philip[1], Zoltan Arany[3], Rebecca C Ahrens-Nicklas[1,4*]

This chapter is in review for publication.

2.1. **Abstract**

Branched chain ketoacid dehydrogenase kinase (BCKDK) deficiency is a recently described inherited neurometabolic disorder of branched chain amino acid (BCAA) metabolism implying increased BCAA catabolism. It has been hypothesized that a severe reduction in systemic BCAA levels underlies the disease pathophysiology, and that BCAA supplementation may ameliorate disease phenotypes. To test this hypothesis, we characterized a recent mouse model of BCKDK deficiency and evaluated the efficacy of enteral BCAA supplementation in this model. Surprisingly, BCAA supplementation exacerbated neurodevelopmental deficits and did not correct biochemical abnormalities despite increasing systemic BCAA levels. These data suggest that aberrant flux through the BCAA catabolic pathway, not just BCAA insufficiency, may contribute to disease pathology. In support of this conclusion, genetic re-regulation of BCAA catabolism, through *Dbt* haploinsufficiency, partially rescued biochemical and behavioral phenotypes in BCKDK deficient mice. Collectively, these data raise into question assumptions widely made about the pathophysiology of BCKDK insufficiency and suggest a novel approach to develop potential therapies for this disease.

2.2. **Introduction**

Branched-chain amino acids (BCAA), leucine, isoleucine, and valine, are essential amino acids, required for cellular function and growth. Abnormalities of BCAA metabolism have been implicated in a variety of human diseases including inborn errors of metabolism, diabetes, cancer, heart failure, and neurodegenerative diseases[12,51]. BCAA levels are controlled by a highly regulated catabolic pathway. In

the first step of the pathway, BCAAs are converted into alpha-keto acids by a branched-chain aminotransferase. These alpha-keto acids are then further broken down by the rate limiting enzyme branched-chain ketoacid dehydrogenase complex (BCKDH). The BCKDH complex and subsequent downstream enzymes convert the alpha-keto acids into intermediates, including acetyl-CoA and succinyl-CoA. These CoA species can feed into the tricyclic acid (TCA) cycle, which is coupled to the electron transport chain in the mitochondria. Within the mitochondria, branched-chain keto-acid dehydrogenase kinase (BCKDK) phosphorylates BCKDH and inhibits its activity to prevent excessive catabolism of BCAAs and the downstream branch chain keto acids(BCKAs).[51]

BCAAs cross the blood brain barrier and regulate several key processes in the central nervous system. BCAAs provide approximately 30% of the nitrogen groups required for the synthesis of glutamate, the major excitatory neurotransmitter of the brain[25]. As such, BCAAs provide essential precursors for neurotransmitter biosynthesis and recycling. Additionally, BCAAs serve as key energy sources, as they donate their carbon skeleton to TCA cycle intermediates when catabolized[25]. Although the biochemistry of BCAA catabolism is well studied, how perturbation of this pathway leads to brain dysfunction is not well understood.

Pathogenic variants in BCKDK lead to a syndrome characterized by intellectual disability, postnatal microcephaly, autism, and seizures in human patients[66]. To date, there are 14 reported unique pathogenic variants located throughout the protein structure[7,8,66]. BCKDK deficiency patients all have reduced BCAA levels in the blood, which has resulted in BCAA supplementation being proposed as a potential therapy[66]. However, recent studies of high protein diets with BCAA supplementation and its impact on brain function reveal nominal improvement of neurodevelopmental skills and neurodevelopment[8,9].

Furthermore, how BCKDK pathogenic variants induce neurological symptoms remains understudied. Previous studies of BCKDK deficiency have focused on behavioral characterization of mouse models, amino acid level changes in mice and humans, and mitochondrial defects observed in patient-derived fibroblasts[31,66,67]. However, an explanation as to how these changes alter human brain function remains to be elucidated.

Here, we investigate the consequences of loss of BCKDK function in the brain by utilizing a recently published *Bckdk*$^{-/-}$ mouse model[13]. In addition, we evaluate if implementation of BCAA repletion therapy in the postnatal period can prevent early neurodevelopmental deficits and postnatal microcephaly. Finally, we compare BCAA supplementation to an alternative therapeutic approach, re-regulating BCAA catabolism through genetic inhibition.

2.3. **Results**

2.3.1 ***Bckdk*$^{-/-}$ mice have reduced survival, developmental delay, and postnatal microcephaly.**

To determine if a previously published *Bckdk*$^{-/-}$ model had clinically relevant neurological phenotypes of BCKDK deficiency, a panel of neurodevelopmental assessments and micro computed tomography (μCT) analysis for microcephaly was performed[13]. We validated molecular loss of BCKDK by PCR and western blot in addition to the phosphorylation site of BCKDH-E1a subunit indicating functional loss of BCKDK. A subset (38%) of *Bckdk*$^{-/-}$ mice died prior to weaning at postnatal day 21 (p21) (Figure 1A). Prior to p21, neurodevelopmental assessments revealed that ectodermal-derived tissues have delayed development including delayed fur development (Figure 1B-C) and eye opening (Figure 1D). Motor development was also reduced with delayed age to reach the maximum screen hold times in both a vertical and horizontal assay (Figure 1E-G). The initial weight of *Bckdk*$^{-/-}$ mice was not significantly different from WT controls (Figure 1H). *Bckdk*$^{-/-}$ mice gained weight during the first two weeks of life, but then started losing weight in the third week, indicative of developmental regression. Final weight at p21 was significantly reduced in *Bckdk*$^{-/-}$mice (Figure 1I). Additional metrics assessed were not significantly altered including mouse length, grasping ability, pinnae development, incisor protrusion, maximum time for T-bar suspension, surface righting, and edge avoidance. Microcephaly was assessed by μCT at p0, and there was an initiation of a subtle reduction in skull height to skull length at this early age (Figure J-M). μCT derived endocasts of skulls at p0 revealed no differences in endocranial volume, normalized to skull length, at this early age. However, by p21 *Bckdk*$^{-/-}$ mice had significantly reduced skull height to skull length, revealing a flattened skull characteristic of postnatal microcephaly and suggesting a delay in cortical development (Figure 1N-Q)[68]. Similarly, *Bckdk*$^{-/-}$ mice had noticeably smaller brains seen by 3D

endocasts and significantly reduced endocranial volumes at p21, revealing that microcephaly arises postnatally and that brain size is affected. Quantification of physical brain size revealed reduced brain weight recapitulating µCT results at p21. Therefore, this *Bckdk*$^{-/-}$ mouse effectively models clinically relevant neurological phenotypes of human disease including neurodevelopmental delay and postnatal microcephaly.

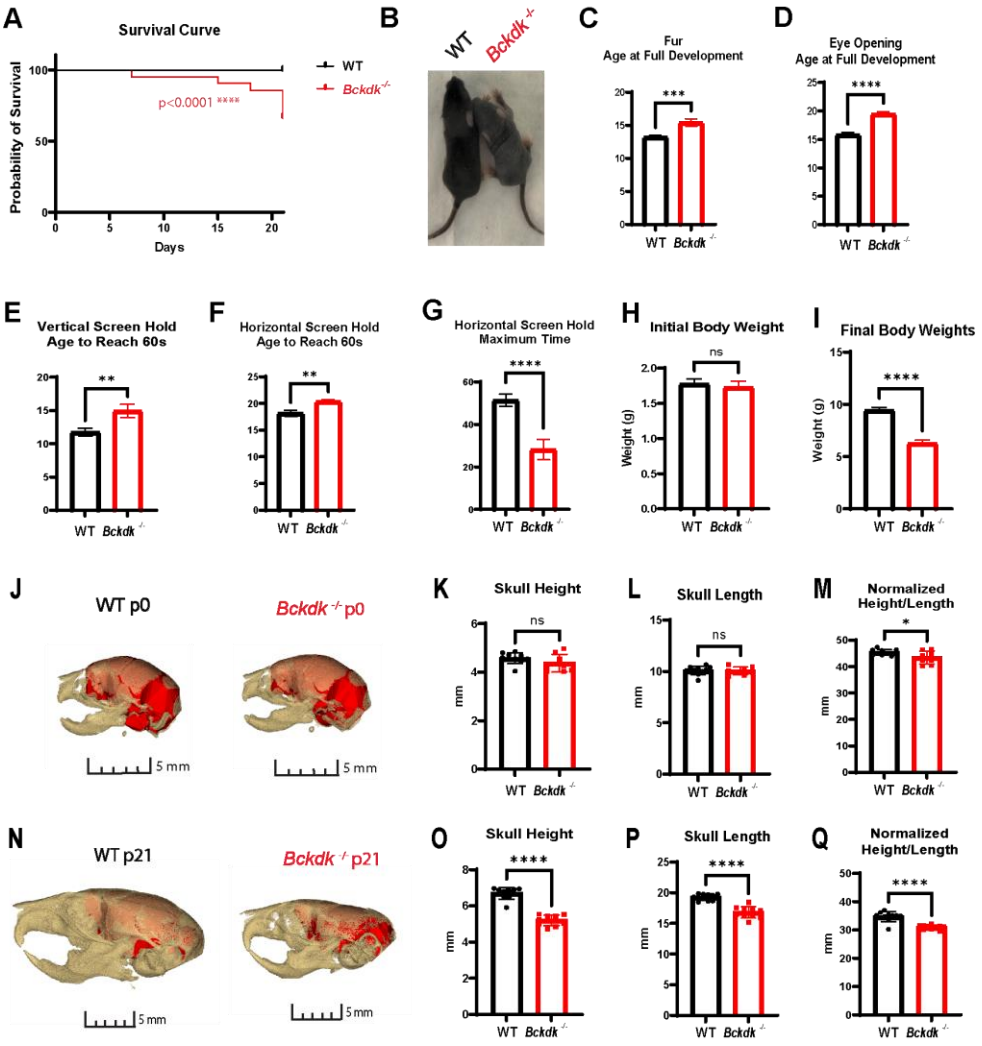

Figure 1 Reduced survival, neurodevelopmental delay, weight loss, and postnatal microcephaly in *Bckdk*$^{-/-}$ mice.

A sex balanced cohort of WT (n=27) and *Bckdk*$^{-/-}$ (n-21) mice were compared for neurodevelopmental assessment. An unpaired t-test was used for statistical comparison. In all panels, data are represented as mean with s.e.m. (* $p < 0.05$, ** $p<0.005$, *** $p < 0.001$, **** $p<0.0001$, ns=non-significant). A proportion (8/21) *Bckdk*$^{-/-}$ mice died before p21. (A) Fur development is delayed in *Bckdk*$^{-/-}$ mice as seen by a representative image at p9. (A) A proportion (8/21) *Bckdk*$^{-/-}$ mice died before p21. (B) Fur development is delayed in *Bckdk*$^{-/-}$ mice as seen by a representative image at

p9. (C) Full fur coat development is significantly delayed in *Bckdk*$^{-/-}$ mice. (D) Age at full eye development was significantly delayed *in Bckdk*$^{-/-}$ mice. (E) Delayed age to reach maximum time in *Bckdk*$^{-/-}$ mice on vertical screen hold. (F) The age to reach the maximum time for horizontal screen hold is significantly delayed in *Bckdk*$^{-/-}$ mice. (G) The maximum hold time is significantly reduced in *Bckdk*$^{-/-}$ mice on the horizontal screen hold (max 60 seconds). (H) Similar initial body weights for *Bckdk*$^{-/-}$ and control mice at p1. (I) Decrease in final body weight in *Bckdk*$^{-/-}$ mice compared to WT mice at p21. (J) No changes in brain size at p0 between WT (n=10) and *Bckdk*$^{-/-}$ (n=10), as seen by 3D reconstructed overlay of skull and brain from CT endocasts. (K) Minimal changes in skull height in *Bckdk*$^{-/-}$ mice compared to WT mice at p0. (L) No changes in skull length in *Bckdk*$^{-/-}$ mice at p0. (M) Slight decrease in a normalized metric of microcephaly (height/length*100) in *Bckdk*$^{-/-}$ mice at p0. (N) Drastically smaller skull sizes in *Bckdk*$^{-/-}$ (n=10) mice compared to WT (n=10) mice at p21 as seen by 3D reconstructed overlay of skull and endocast from µCT. (O) Reduced skull height in *Bckdk*$^{-/-}$ mice relative to WT mice at p21. (P) Decreased skull length in *Bckdk*$^{-/-}$ mice at p21 compared to WT mice. (Q) Significantly reduced skull size in *Bckdk*$^{-/-}$ mice at p21 evidence of the normalized metric of microcephaly comparing skull measurements (height/length*100).

2.3.2 **Acylcarnitine and TCA cycle intermediate pooling in *Bckdk*$^{-/-}$ brains**

Previous literature revealed depletion of BCAAs in human patients and a decrease in downstream adenosine triphosphate (ATP) levels in BCKDK deficient patient-derived cells.[6,7,9,13,31,55,67] Given the unique role of BCAAs in the brain, we sought to elucidate how BCKDK deficiency impacts BCAA catabolism and downstream TCA cycle intermediate levels in the brain. We hypothesized that there would be increased levels of intermediates derived from BCAA nitrogen donation (glutamate, glutamine) and carbon donation (acylcarnitine species, TCA cycle intermediates) due to loss of BCKDK. Brain BCAA levels were significantly reduced at p21, as has previously been shown at a similar timepoint in another *Bckdk*$^{-/-}$ mouse model (Fig2A-C)[6]. Glutamate and glutamine levels were unchanged from WT levels at p21, implying that either insignificant changes in nitrogen donation or compensation after increased nitrogen donation to regulate levels in the brain (Figure 2D-E). Either possibility suggests it is an unlikely mechanism of biochemical dysregulation. Acylcarnitine conjugates of downstream intermediates below the BCKDH step accumulated, supporting that there is increased carbon donation to direct downstream intermediates (Figure 2F-H). Interestingly, the leucine-derived intermediate C5-OH carnitine is depleted, two steps below the BCKDH step, suggesting a blockade in catabolism and pooling of upstream intermediates (Figure 2I)[69].

To bridge the gap between pooling BCAA acylcarnitine intermediates and their downstream impact on downstream metabolism in the brain, we investigated if organic acids, particularly TCA-cycle

intermediates, were altered. 3-HBA organic acid levels were elevated in *Bckdk*$^{-/-}$ mice. Pyruvate levels, derived from both glucose and BCAA breakdown, were elevated while lactate levels were similar between groups, suggesting that cytosolic NAD/NADH ratios may be increased (Figure 2J). All TCA cycle intermediate levels increase, except succinate, implying that there is increased anaplerosis in *Bckdk*$^{-/-}$ mice (Figure J-O). These results are consistent with higher flux through the anaplerotic BCAA catabolic pathways.

Interestingly, we also found a blockade in other amino acid degradation pathways that have been previously implicated in neurologic disorders, specifically lysine and glycine degradation. Lysine and tryptophan levels accumulated in *Bckdk*$^{-/-}$ mice. Accumulation of lysine degradation intermediates have previously been associated with pyridoxine-dependent epilepsy[70–72]. Therefore, we looked at downstream C5-DC levels, which are also significantly depleted implying that reduced lysine degradation. In addition, glycine levels were significantly elevated at p21. Glycine has been associated with glycine encephalopathy, a severe seizure disorder[52]. Clinical biomarkers of BCKDK deficiency, tyrosine, phenylalanine, and alanine, were significantly and trend increased in *Bckdk*$^{-/-}$ mice. Subtle abnormalities in additional amino acid levels were also observed.

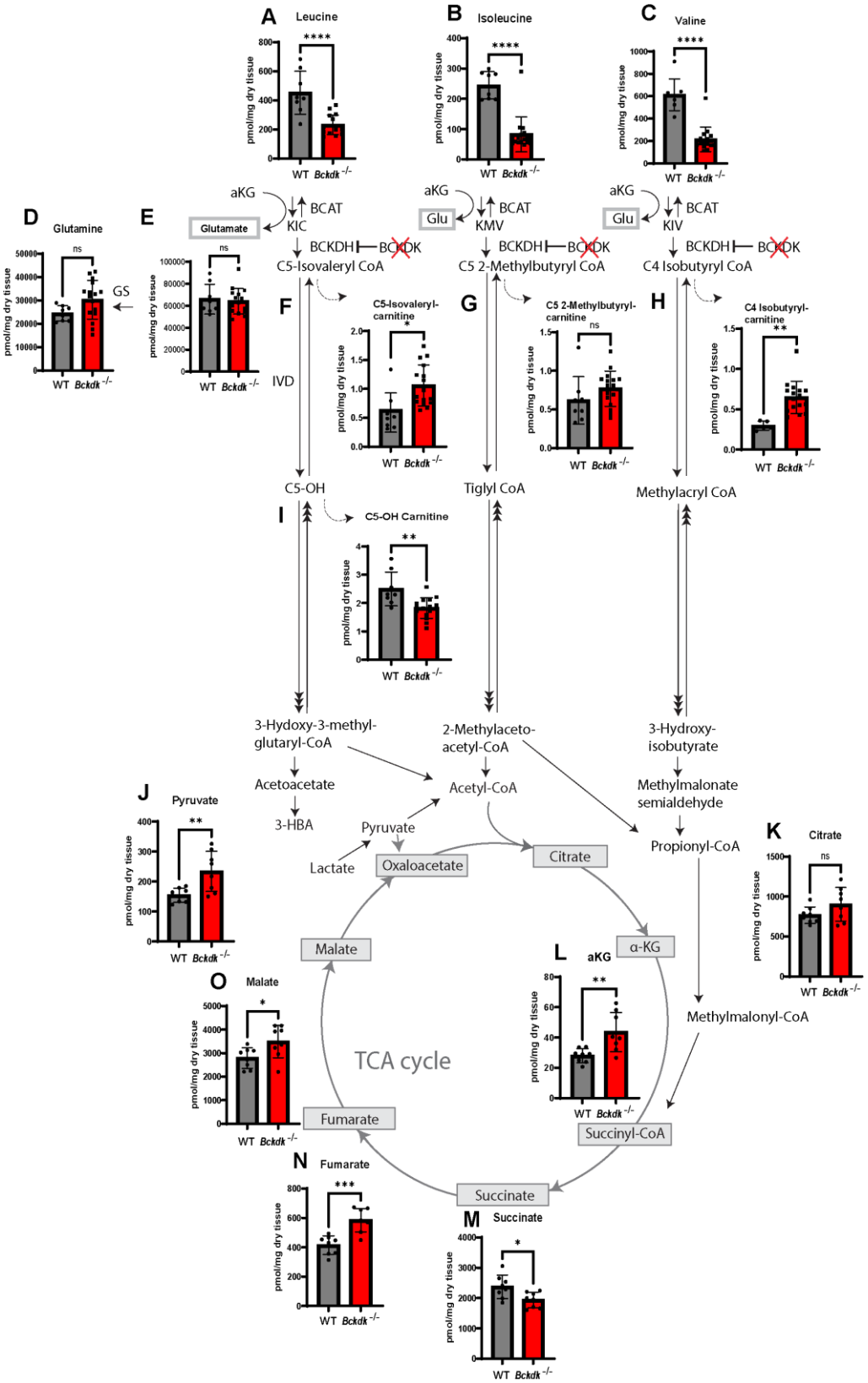
A
Leucine
B
Isoleucine
C
Valine
pmol/mg dry tissue
WT Bckdk -/-
aKG
BCAT
KIC
KMV
KIV
Glu
Glutamate
BCKDH
BCKDK
C5-Isovaleryl CoA
C5 2-Methylbutyryl CoA
C4 Isobutyryl CoA
D
Glutamine
GS
E
F
C5-Isovaleryl-carnitine
IVD
G
C5 2-Methylbutyryl-carnitine
H
C4 Isobutyryl-carnitine
C5-OH
Tiglyl CoA
Methylacryl CoA
I
C5-OH Carnitine
3-Hydoxy-3-methyl-glutaryl-CoA
2-Methylaceto-acetyl-CoA
3-Hydroxy-isobutyrate
Acetoacetate
3-HBA
Acetyl-CoA
Methylmalonate semialdehyde
J
Pyruvate
Lactate
Oxaloacetate
Citrate
Propionyl-CoA
K
Citrate
Malate
α-KG
L
aKG
Methylmalonyl-CoA
O
Malate
TCA cycle
Fumarate
Succinyl-CoA
N
Fumarate
Succinate
M
Succinate

Figure 2 Acylcarnitine's accumulate downstream of BCKDH in the brain of *Bckdk*$^{-/-}$ mice at p21.

A sex balanced cohort of WT (n=8) and *Bckdk*$^{-/-}$ (n=15) mice were sent for amino acid and acylcarnitine analysis, and a subset of *Bckdk*$^{-/-}$ mice (n=7) for organic acids measured by mass spectrometry. Unpaired t-tests were performed to compare the two genotypes. In all panels, data are represented as mean with s.e.m. (* $p < 0.05$, ** $p<0.005$, *** $p < 0.001$, **** $p<0.0001$, ns=non-significant). (A-C) Significant reduction of BCAA levels in *Bckdk*$^{-/-}$ mice. (D-E) No changes in glutamate and glutamine levels in *Bckdk*$^{-/-}$ mice. (F-H) Increased pooling of acylcarnitine intermediates one step below BCKDH in *Bckdk*$^{-/-}$ mice. (I) Reduced levels of an acylcarnitine two steps below BCKDH (C5-OH) in *Bckdk*$^{-/-}$ mice, specifically in the leucine degradation pathway. (J) Elevated pyruvate levels in *Bckdk*$^{-/-}$ mice relative to WT levels. (K) No significant changes in citrate levels in *Bckdk*$^{-/-}$ mice. (L) Increased aKG levels in *Bckdk*$^{-/-}$ mice. (M) Decreased succinate levels in *Bckdk*$^{-/-}$ mice. (N) Significant increase in fumarate levels in *Bckdk*$^{-/-}$ mice. (O) Increased malate levels in *Bckdk*$^{-/-}$ mice.

2.3.3 **BCAA repletion does not rescue survival, developmental delay, or microcephaly.**

To determine if BCAA repletion could impact clinically relevant neurological phenotypes in our *Bckdk*$^{-/-}$ model, we performed the same panel of neurodevelopmental assessments and µCT evaluation for microcephaly in BCAA treated mice and compared them to our untreated mice[13]. BCAAs were diluted in reverse osmosed water to a supersaturated 2% solution, as previously described[73]. BCAAs were administered through lactation to treat pups continuously throughout the day to maximize BCAA repletion from p0-p21 (Figure 3A). BCAAs did not significantly impact overall water intake and daily dosage was adjusted for litter size by maternal intake. Through this administration route, BCAAs were significantly increased in the liver of WT pups (Figure 3B). BCAA treated *Bckdk*$^{-/-}$ mice still had 15% reduced survival, revealing peripherally administered BCAAs did not fully rescue survival deficits (Figure 3C). Developmental delay of ectodermal-derived tissues persisted in BCAA treated *Bckdk*$^{-/-}$ pups including delayed fur development (Figure 3D-E), and eye opening (Figure 3F). BCAA treated *Bckdk*$^{-/-}$ mice initially gained weight similarly to control littermates, but also developmentally regressed around week 2 of life. Final body weight was not significantly altered by BCAA administration as compared to untreated *Bckdk*$^{-/-}$ mice (Figure 3G). Motor development was also delayed despite BCAA treatment, with reduced age to reach the maximum time in the horizontal screen hold and reduced screen hold times in both the vertical and horizontal assay (Figure 3H-K). BCAA administration worsened maximum time on the vertical screen hold, pinnae development, and edge avoidance in BCAA treated versus untreated *Bckdk*$^{-/-}$ mice (Figure 3K-M). Furthermore, differences in edge avoidance, grasping, and maximum T-bar suspension time were new defects elicited by BCAA administration to *Bckdk*$^{-/-}$ mice (Figure 3M). Similar to our initial

characterization, body length, incisor protrusion, and surface righting did not differ between groups. Microcephaly was not drastically improved by BCAA treatment, as skull height and lengths were still reduced (Figure 3N-Q)[68]. BCAA treatment did increase brain weight, which recapitulates what was seen in the first *Bckdk*$^{-/-}$ mouse model supplemented with a high protein diet[31]. However, BCAA treatment was not sufficient to normalize endocranial volumes. Together, these data indicate that postnatal peripheral BCAA repletion is not sufficient alone to rescue neurodevelopmental delay in *Bckdk*$^{-/-}$ mice and can even worsen some neurological phenotypes.

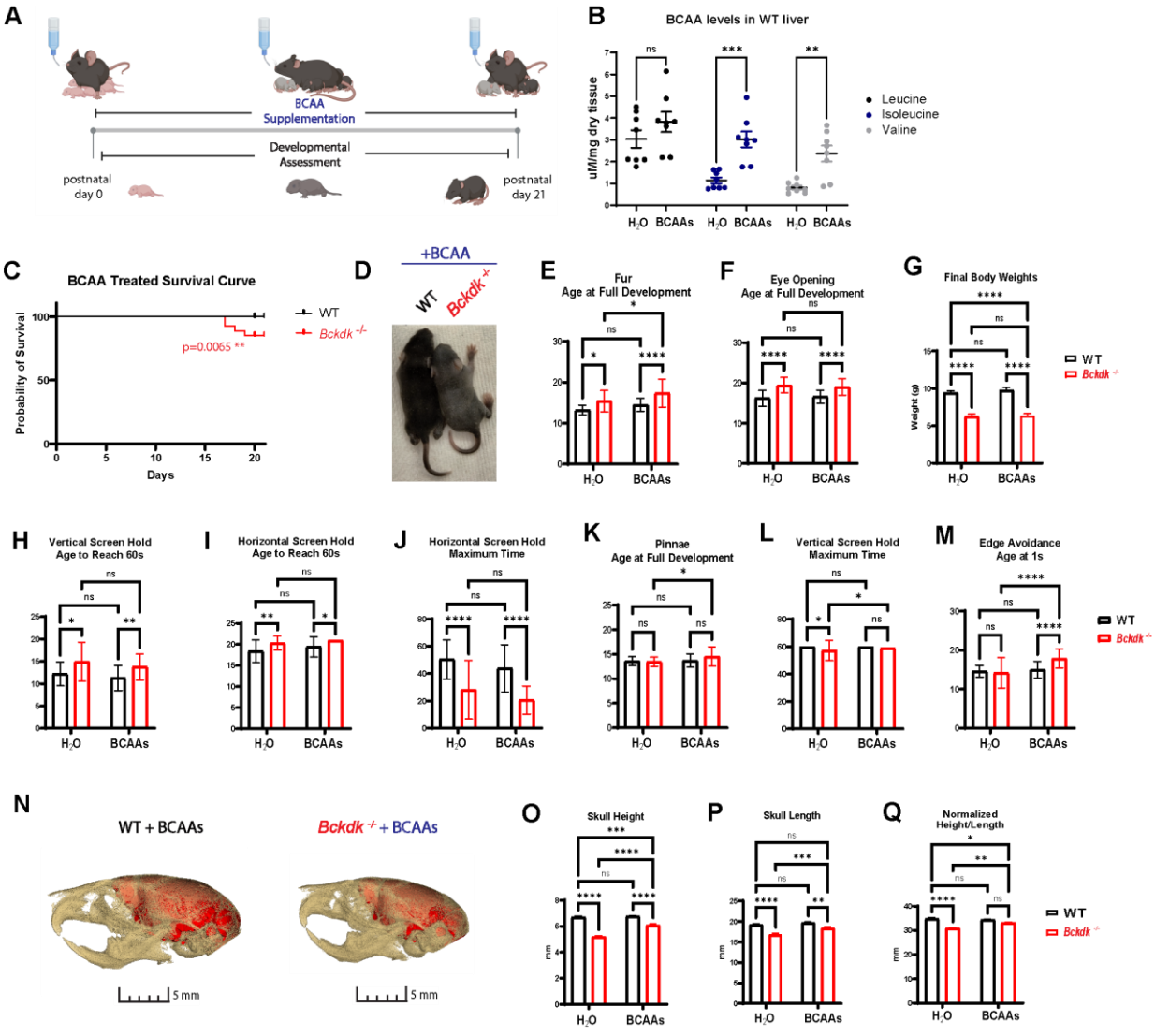

Figure 3 BCAA supplementation did not significantly impact survival, neurodevelopmental delay, weight loss, or microcephaly in *Bckdk*$^{-/-}$ mice.

A sex balanced cohort of WT (n=39) and *Bckdk*$^{-/-}$ (n=28) mice were compared for all neurodevelopmental assessments. A 2-way ANOVA with multiple comparisons was used for all statistical analyses. In all panels, data are represented as mean with s.e.m. (* $p < 0.05$, ** $p<0.005$, *** $p < 0.001$, **** $p<0.0001$, ns=non-significant). Experimental design of BCAA administration through dams through lactation, with simultaneous neurodevelopmental assessment of treated pups. (A) Significant elevation of BCAAs in the livers of BCAA treated WT mice (n=8). (B) Reduced survival (4/28; 14%) of BCAA treated *Bckdk*$^{-/-}$ mice prior to p21. (C) Delayed fur development of BCAA treated *Bckdk*$^{-/-}$ mice as seen by representative images at p9. (D) Delayed age of full coat development of BCAA treated *Bckdk*$^{-/-}$ mice. (E) Delayed age of full eye opening in BCAA treated *Bckdk*$^{-/-}$ mice. (F) Significantly reduced final body weight in BCAA treated *Bckdk*$^{-/-}$ mice at p21. Final body weight was taken from mice collected for experiments (WT(n=8), BCKDK$^{-/-}$(n=16), BCAA treated WT (n=9), BCAA treated *Bckdk*$^{-/-}$ (n=8)). (G) Delayed age to reach the maximum hold time for vertical screen in BCAA treated *Bckdk*$^{-/-}$ mice. (H) Delayed age to reach the maximum hold time for horizontal screen in BCAA treated *Bckdk*$^{-/-}$ mice. (I) Decreased maximum hold time on horizontal screen hold in BCAA treated *Bckdk*$^{-/-}$ mice. (J) Further delayed age to reach full pinnae development in BCAA treated *Bckdk*$^{-/-}$ mice compared to untreated *Bckdk*$^{-/-}$ mice. (K) Further increased maximum time on vertical screen hold in BCAA treated *Bckdk*$^{-/-}$ mice compared to untreated *Bckdk*$^{-/-}$ mice. (L) Further worsening of edge avoidance in BCAA treated *Bckdk*$^{-/-}$ mice compared to untreated *Bckdk*$^{-/-}$ mice. (M) Smaller skull sizes in BCAA treated *Bckdk*$^{-/-}$ (n=10) mice compared to BCAA treated WT (n=10) mice at p21 as seen by 3D reconstructed overlay of skull and endocast from µCT. (N) Reduced skull height in BCAA treated *Bckdk*$^{-/-}$ mice (n=10 per group). (O) Decreased skull length in BCAA treated *Bckdk*$^{-/-}$ mice (n=10 per group). (P) Reduced skull size in BCAA treated *Bckdk*$^{-/-}$ mice at p21 evidence of the normalized metric of microcephaly comparing skull measurements.

2.3.4 **BCAA repletion reduced catabolic pathway pooling but not downstream TCA cycle changes in the brain of *Bckdk*$^{-/-}$ mice.**

Although BCAA repletion did not significantly rescue neurodevelopmental delay in mice, it remained unclear if BCAA repletion would be sufficient to prevent pooling of pathway intermediates and normalize downstream TCA cycle intermediate levels. Peripheral BCAA supplementation through lactation did not drastically elevate BCAA levels in the brain despite elevated levels in the liver (Figure 4A-C), revealing a limitation of peripheral BCAA intervention also seen in a lack of restoration of BCAA levels in the central nervous system in human patients[9]. Again, glutamate and glutamine levels were not altered by this intervention (Figure 4D-E). BCAA supplementation did reduce the amount of pooling of intermediates directly downstream of the BCKDH step, as measured through acylcarnitine analysis (Figure 4F-H). The intermediate two steps down from BCKDH, C5-OH carnitine, was further depleted in *Bckdk*$^{-/-}$ mice with BCAA supplementation (Figure 4I).

Since BCAA supplementation corrected levels of upstream intermediates, we investigated if BCAA supplementation also normalized organic acid levels, particularly downstream intermediates of the TCA

cycle. 3-HBA levels were elevated upon BCAA supplementation in *Bckdk*$^{-/-}$ mice. Pyruvate levels were further elevated by BCAA supplementation (Figure 4J), while lactate levels remained unchanged. Citrate levels were increased compared to WT untreated controls (Figure 4K). Interestingly, aKG levels were further increased on BCAA supplementation in *Bckdk*$^{-/-}$ mice (Figure 3L). Inversely, succinate levels were still depleted in *Bckdk*$^{-/-}$ mice even in the presence of BCAAs (Figure 4M). Fumarate and malate levels were elevated with similar levels compared to untreated *Bckdk*$^{-/-}$ mice (Figure 4N-O). The pooling of citrate and aKG is consistent with increased catabolism upon BCAA supplementation in *Bckdk*$^{-/-}$ mice, although this is not reflected in the fumarate-malate span of the TCA cycle. Together, these data show that BCAA supplementation has identifiable effects on levels of various brain metabolic intermediates, but it alone does not normalize carbon donation to downstream intermediates nor restore TCA cycle changes due to loss of BCKDK.

Lysine accumulation in BCAA treated *Bckdk*$^{-/-}$ mice remained increased while lysine degradation remained restricted as evidence by reduced C5-DC carnitine. Similarly, glycine levels remained elevated in *Bckdk*$^{-/-}$ mice despite BCAA treatment. Additional amino acid markers of BCKDK deficiency remained elevated in BCAA treated *Bckdk*$^{-/-}$ mice compared to BCAA treated WT mice. Multiple amino acids were not impacted by BCAA supplementation including arginine, histidine, and aspartate. Specific amino acids, proline and asparagine, were similarly elevated in *Bckdk*$^{-/-}$ mice with or without BCAA supplementation. BCAA supplementation further exacerbated differences in amino acid levels particularly for serine between *Bckdk*$^{-/-}$ and WT treated mice. Together this data suggests that peripheral repletion of BCAAs does not restore all metabolic changes in the brain nor neurodevelopmental delay phenotypes associated with BCKDK deficiency, implying that an alternative mechanism of disease pathology besides BCAA depletion alone.

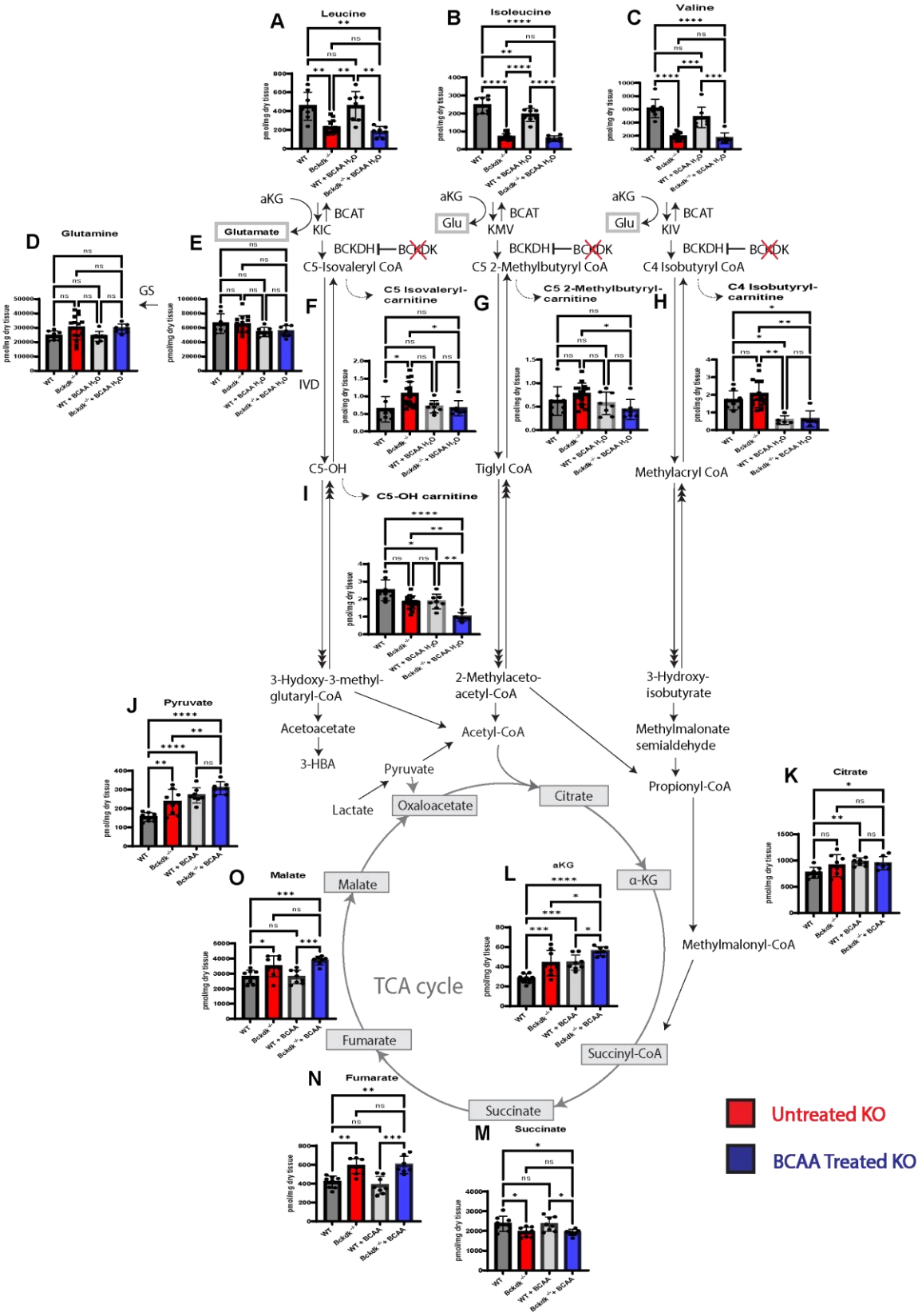
A
Leucine
B
Isoleucine
C
Valine
aKG
BCAT
KIC
Glu
KMV
KIV
BCKDH
BCKDK
C5-Isovaleryl CoA
C5 2-Methylbutyryl CoA
C4 Isobutyryl CoA
D
Glutamine
E
Glutamate
GS
IVD
F
C5 Isovaleryl-carnitine
G
C5 2-Methylbutyryl-carnitine
H
C4 Isobutyryl-carnitine
C5-OH
Tiglyl CoA
Methylacryl CoA
I
C5-OH carnitine
3-Hydoxy-3-methyl-glutaryl-CoA
2-Methylaceto-acetyl-CoA
3-Hydroxy-isobutyrate
Acetoacetate
3-HBA
Acetyl-CoA
Methylmalonate semialdehyde
Propionyl-CoA
J
Pyruvate
Lactate
Oxaloacetate
Citrate
K
Citrate
Malate
O
Malate
L
aKG
α-KG
TCA cycle
Methylmalonyl-CoA
Fumarate
Succinyl-CoA
N
Fumarate
Succinate
M
Succinate
Untreated KO
BCAA Treated KO

Figure 4 Amino Acids and Acylcarnitine levels in the brain of WT and *Bckdk*$^{-/-}$ mice at p21.

A sex balanced cohort of BCAA treated WT (n=8) and *Bckdk*$^{-/-}$ (n=7) mice were compared to untreated WT (n=8) and *Bckdk*$^{-/-}$ (n=15) data from figure 2. Brain amino acid, acylcarnitine, and organic acid levels were measured by mass spectrometry. 2-way ANOVAs were performed to compare all four groups for genotype and BCAA intervention differences. In all panels, data are represented as mean with s.e.m. (* $p < 0.05$, ** $p<0.005$, *** $p < 0.001$, **** $p<0.0001$, ns=non-significant). (A-C) BCAA levels are significantly reduced in BCAA treated *Bckdk*$^{-/-}$ mice at p21. (D-E) No differences in glutamate and glutamine in BCAA treated *Bckdk*$^{-/-}$ mice. (F-H) Reduced pooling of acylcarnitine intermediates one step below BCKDH in BCAA treated *Bckdk*$^{-/-}$ mice compared to untreated *Bckdk*$^{-/-}$ mice. (I) Further reduced levels of acylcarnitine species two steps below BCKDH (C5-OH) in BCAA treated *Bckdk*$^{-/-}$ mice relative to untreated *Bckdk*$^{-/-}$ mice. (J) Further elevated pyruvate levels in BCAA treated *Bckdk*$^{-/-}$ mice compared to untreated *Bckdk*$^{-/-}$ mice and untreated WT mice. (K) Increase in citrate levels in BCAA treated *Bckdk*$^{-/-}$ mice relative to untreated WT mice. (L) Further increased aKG levels in BCAA treated *Bckdk*$^{-/-}$ mice compared to WT levels and untreated *Bckdk*$^{-/-}$ levels. (M) Similarly decreased succinate levels in BCAA treated *Bckdk*$^{-/-}$ mice compared to untreated *Bckdk*$^{-/-}$ mice. (N) Similar increase in fumarate levels in BCAA treated *Bckdk*$^{-/-}$ mice relative to *Bckdk*$^{-/-}$ mice. (O) Further increase in malate levels in BCAA treated *Bckdk*$^{-/-}$ mice compared to untreated *Bckdk*$^{-/-}$ mice, both relative to WT levels.

2.3.5 **DBT haploinsufficiency rescues neurodevelopmental delay in *Bckdk*$^{-/-}$ mice.**

An alternative hypothesis to low levels of BCAAs being the cause of neurological defects in *Bckdk*$^{-/-}$ mice is the hypothesis that high flux of BCAA catabolism is in fact causal. To test this hypothesis, we reduced BCAA catabolism by haploinsufficiency of *Dbt*, encoding the critical E2 subunit of the BCKDH complex. We crossed *Bckdk*$^{+/-}$ mice with a novel *Dbt*$^{+/-}$ mouse model to obtain *Bckdk* $^{+/-}$ *Dbt* $^{+/-}$ mice for breeding. These mice were further crossed to obtain "genetic rescue" mice (*Bckdk*$^{-/-}$*Dbt*$^{+/-}$*)* and littermate controls for neurodevelopmental assessment and metabolic analysis (Figure 5A). *Dbt* haploinsufficiency was detected by PCR, and DBT mRNA levels in genetic rescue mice were decreased 32% relative to normalized WT levels in the brain (Figure 5B-C). DBT haploinsufficiency slightly increased *Bckdk*$^{-/-}$ mice survival with 12% death prior to our endpoint at 21 (Figure 5D). Prior to this endpoint, neurodevelopmental assessment revealed a trend rescue of fur development (Figure 5E-F), but no change in the age at full eye opening (Figure 5G). Motor development was partially rescued with slightly earlier ages to reach the maximum time of vertical screen hold (Figure 5H). Although the age to be able to reach the maximum time on the horizontal screen was delayed compared to WT mice, their average time on the horizontal screen was more similar to WT mice than *Bckdk*$^{-/-}$ mice indicative of motor improvement (Figure 5I-J). Genetic rescue mice tracked with WT mice in their weight gain throughout the 3-week assessment. Additionally, final body weights at p21 were partially rescued (Figure 5K). Genetic rescue mice had similar lengths, grasping, pinnae development, incisor protrusion, max vertical screen

hold time, maximum suspension time on T-bar, surface righting, and edge avoidance compared to both WT and *Bckdk*$^{-/-}$ mice. Microcephaly analyses revealed highly significant partial rescue of skull length (Figure 5L-O)[68]. Estimation of brain size through µCT analysis indicated higher endocranial volume relative to *Bckdk*$^{-/-}$ mice. Partial rescue of brain weight recapitulated CT studies with a trend towards increased brain weight relative to *Bckdk*$^{-/-}$ mice. These data thus support the use of *Dbt* inhibition as a novel therapeutic target, which partially rescues neurodevelopmental delay and microcephaly in *Bckdk*$^{-/-}$ mice.

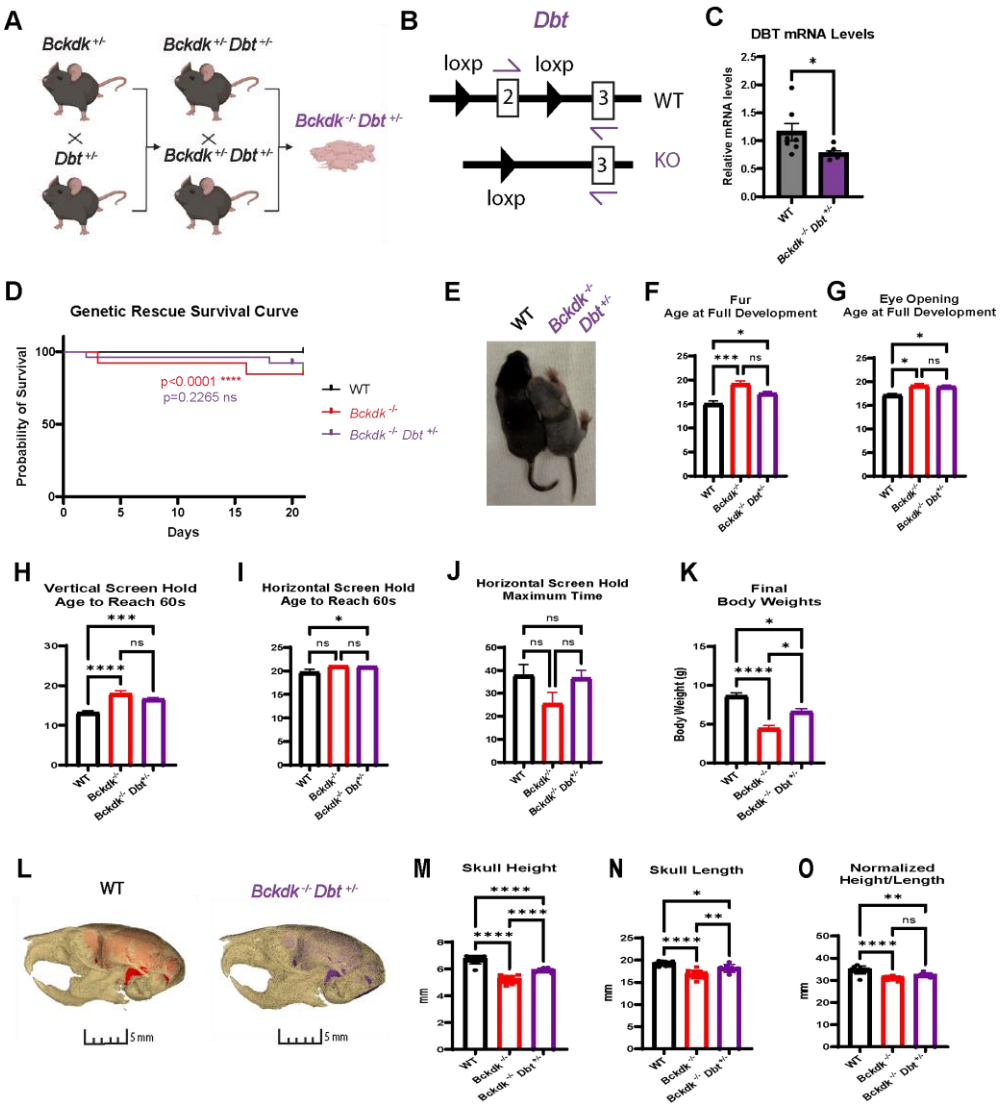

Figure 5 Higher survival, less severe neurodevelopmental delay, increased weight gain, and partial restoration of microcephaly in *Bckdk$^{-/-}$ Dbt$^{+/-}$* mice.

A sex balanced cohort of WT (n=12), *Bckdk$^{-/-}$* (n=13), and *Bckdk$^{-/-}$Dbt$^{+/-}$* (n=26) mice were compared for all neurodevelopmental assessments. A one-way ANOVAs with multiple comparisons was used for comparisons between the three groups. In all panels, data are represented as mean with s.e.m. (* $p < 0.05$, ** $p<0.005$, *** $p < 0.001$, **** $p<0.0001$, ns=non-significant). (A) Breeding scheme to obtain *Dbt* haploinsufficient *Bckdk$^{-/-}$* mice. *Bckdk$^{+/-}$* and *Dbt$^{+/-}$* mice were crossed to obtain *Bckdk$^{+/-}$Dbt$^{+/-}$*. These mice were further crossed to obtain *Bckdk$^{-/-}$Dbt$^{+/-}$* mice. (B) Design of qRT-PCR for DBT KO and WT allele to measure mRNA transcript levels. (C) Decreased relative DBT transcript levels in *Bckdk$^{-/-}$Dbt$^{+/-}$* (n=7) mice compared to WT (n=7) mice measured by qRT-PCR and normalized to Actin levels. (D) Higher survival rates in *Bckdk$^{-/-}$Dbt$^{+/-}$* (3/25;12%) mice compared to littermate matched *Bckdk$^{-/-}$* mice (3/13;23%). (E) Delayed fur development in *Bckdk$^{-/-}$Dbt$^{+/-}$* as seen by representative images at p10. (F) Partial rescue of delayed age of full coat development of *Bckdk$^{-/-}$Dbt$^{+/-}$* mice relative to *Bckdk$^{-/-}$* mice, both compared to WT mice. (G) Similarly delayed age of full eye opening in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice compared to *Bckdk$^{-/-}$* mice. (H) Similarly delayed age to reach the maximum hold time for vertical screen in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice relative to *Bckdk$^{-/-}$* mice. (I) Later age to reach the maximum hold time for horizontal screen in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice compared to WT mice. (J) Similar maximum time on horizontal screen hold in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice relative to WT mice. (K) Final body weights are partially rescued in *Bckdk$^{-/-}$Dbt$^{+/-}$* compared to WT and *Bckdk$^{-/-}$* matched controls. (L) Partial rescue of skull size in *Bckdk$^{-/-}$Dbt$^{+/-}$* (n=10) mice compared to WT (n=10) mice at p21 as seen by 3D reconstructed overlay of skull and endocasts from µCT. (M) Partially restored increase in skull height in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice (n=10 per group) compared to *Bckdk$^{-/-}$* mice. (N) Partial rescue of skull length in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice (n=10 per group) compared to WT mice. (O) Partial restoration of skull size in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice at p21 compared to WT mice evidence of the normalized metric of microcephaly comparing skull measurements.

2.3.6 **DBT haploinsufficiency reduces BCAA catabolite pooling and partially rescues TCA cycle changes in the brain of *Bckdk$^{-/-}$* mice.**

Next, we evaluated if *Dbt* haploinsufficiency could improve metabolic markers of BCKDK deficiency in the brain. DBT haploinsufficiency in genetic rescue mice did not significantly increase BCAA levels in the brain (Figure 6A-C), further supporting that BCAA depletion alone is not the sole cause of disease pathology. However, DBT haploinsufficiency was sufficient to reduce pooling of intermediates one step below BCKDH, partially normalizing the BCAA catabolic pathway (Figure 6F-I). Glutamate and glutamine levels were not significantly changed similar to control groups (Figure 6D-E), again supporting that excess glutamate is an unlikely cause of disease pathology in BCKDK deficiency.

Lastly, we investigated if DBT haploinsufficiency in BCKDK deficiency restored organic acid including TCA cycle intermediate changes. Pyruvate levels were restored back to WT levels (Figure 6J). Citrate levels remained unchanged (Figure 6K). aKG levels resolved back to WT levels in genetic rescue mice (Figure 6L), suggesting there is partial correction of the TCA cycle. However, downstream changes in succinate, fumarate, and malate were unchanged in genetic rescues compared to *Bckdk$^{-/-}$* mice (Figure

6M-O). Together, partial rescue of BCAA catabolic intermediates and resolution of initial intermediates of the TCA cycle are therefore achievable with *Dbt* haploinsufficiency in BCKDK deficiency.

Dbt haploinsufficiency also corrected elevations of key amino acids in the CNS, including lysine and glycine. Several additional amino acid markers of disease were similar in genetic rescue mice as compared to WT mice. Together these data support that there is partial restoration of amino acid levels, acylcarnitine levels, and organic acid levels through DBT haploinsufficiency in *Bckdk*$^{-/-}$ mice.

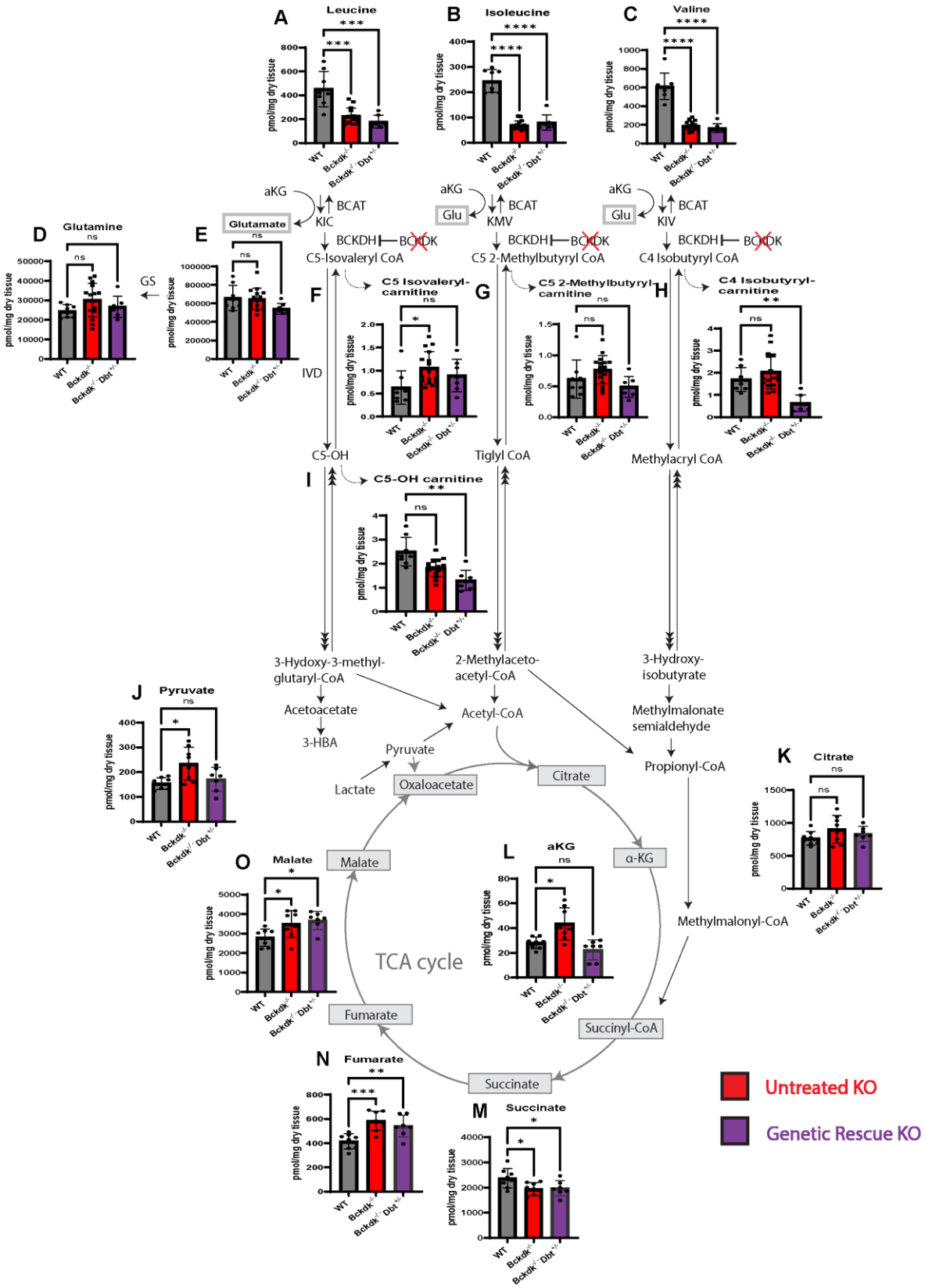
A
Leucine
B
Isoleucine
C
Valine
pmol/mg dry tissue
WT
Bckdk-/-
Bckdk-/- Dbt+/-
aKG
BCAT
KIC
Glu
KMV
KIV
BCKDH
BCKDK
C5-Isovaleryl CoA
C5 2-Methylbutyryl CoA
C4 Isobutyryl CoA
D
Glutamine
E
Glutamate
GS
F
C5 Isovaleryl-carnitine
G
C5 2-Methylbutyryl-carnitine
H
C4 Isobutyryl-carnitine
IVD
C5-OH
Tiglyl CoA
Methylacryl CoA
I
C5-OH carnitine
3-Hydoxy-3-methyl-glutaryl-CoA
2-Methylaceto-acetyl-CoA
3-Hydroxy-isobutyrate
J
Pyruvate
Acetoacetate
3-HBA
Acetyl-CoA
Methylmalonate semialdehyde
Lactate
Oxaloacetate
Citrate
Propionyl-CoA
K
Citrate
Malate
α-KG
L
aKG
O
Malate
TCA cycle
Methylmalonyl-CoA
Fumarate
Succinyl-CoA
N
Fumarate
Succinate
M
Succinate
Untreated KO
Genetic Rescue KO

Figure 6 Amino Acids and Acylcarnitine levels in the brain of WT and Bckdk$^{-/-}$Dbt$^{+/-}$ mice at p21.

A sex balanced cohort of *Bckdk$^{-/-}$Dbt$^{+/-}$* (n=7) mice were compared to untreated WT (n=8) and *Bckdk$^{-/-}$* (n=15) metabolic data from the original characterization shown in Figure 2. Amino acid, acylcarnitine, and organic acids levels were measured by mass spectrometry. One-way ANOVA with multiple comparisons was used to compare genotypes. In all panels, data are represented as mean with s.e.m. (* $p < 0.05$, ** $p<0.005$, *** $p < 0.001$, **** $p<0.0001$, ns=non-significant). (A-C) Significant reduction of BCAA levels in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice at p21. (D-E) No changes in glutamate and glutamine in all groups at p21. (F-H) Reduced pooling of acylcarnitine species one step below BCKDH are relatively similar in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice compared to WT mice, and C4-Isobutyryl carnitine was even further reduced. (I) Further reduced levels of acylcarnitine species two steps below BCKDH (C5-OH) in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice relative to WT mice. (J) Normalization of pyruvate levels back to WT levels in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice. (K) Citrate levels were similar in all groups. (L) aKG levels resolved in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice back to WT levels. (M) Succinate levels were similarly decreased in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice compared to WT mice. (N) Fumarate levels remained elevated in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice relative to WT, although to a lesser extent than *Bckdk$^{-/-}$* levels. (O) Malate levels were also elevated in *Bckdk$^{-/-}$Dbt$^{+/-}$* mice compared to WT mice, having similar levels to *Bckdk$^{-/-}$* mice.

2.4. **Discussion**

Pathogenic variants in BCKDK lead to a syndrome characterized by intellectual disability, postnatal microcephaly, autism, and seizures in human patients[66]. Previous models of *Bckdk* deficiency focused solely on the presence of seizures and hindlimb clasping as a phenotypic readout of *Bckdk* deficiency[6,31]. Therefore, in our study we characterized additional early onset, clinically-relevant neurological phenotypes and found significant neurodevelopmental delay and postnatal microcephaly in a recently published *Bckdk$^{-/-}$* mouse model[13]. From these assessments we can conclude that this mouse is a useful tool to investigate disease pathophysiology and assess potential therapies.

Since the discovery of BCKDK pathogenic variants, the mechanism of disease pathology was assumed to be hypercatabolism of BCAAs leading to their severe depletion. Our study revealed that there are multiple imbalances in BCAA catabolism and the TCA cycle due to BCKDK deficiency. First, we showed that there is accumulation of acylcarnitine derivatives downstream of the BCKDH step and decreased C5-OH levels further downstream suggesting a partial blockade in the BCAA catabolic pathway. Additionally, we found loss of BCKDK leads to imbalances in precursors to and intermediates of the TCA cycle with elevated pyruvate, aKG, fumarate, and malate levels but decreased succinate levels. Together, these data suggest that abnormal upstream BCAA metabolites induce imbalances in TCA cycle intermediates. These findings led us to question whether BCAA supplementation could both correct BCAA levels and normalize downstream TCA cycle changes.

Clinical studies of BCAA supplementation in patients with pathogenic variants of BCKDK are limited, and no randomized-control trials have been conducted. Available data suggest that in small cohorts early BCAA supplementation can partially improve disease phenotypes[7,9].

Therefore, we systematically tested the efficacy of enteral BCAA supplementation in our mouse model of BCKDK deficiency. Our approach to replete BCAA levels was two-fold. The first was to test if peripheral administration would be sufficient to replete BCAA levels in the brain, since this had not yet been systematically proven[7,9]. Secondly, we aimed to increase the frequency of BCAA dosage to maximize repletion of BCAAs. Clinical studies revealed that multiple injections with BCAA supplementation (up to 6x) a day would be required, and even then, levels were insufficiently maintained within physiological ranges for most of the day[9]. Therefore, BCAA supplementation through dams via lactation was the best option to maximize frequent dosing of pups during the developmental timeframe without negatively impacting simultaneous neurodevelopmental assessments. In order to supplement BCAAs without them precipitating out of solution or reducing pup intake, we used a published protocol utilizing a 2% supersaturated solution of a ratio of BCAAs (2 Leu:1 Iso:1 Val) to maximize supplementation to pups[73]. BCAA supplementation did increase brain weight and size in *Bckdk*$^{-/-}$ mice, likely through BCAAs known role to increase protein synthesis required for tissue growth[31]. However, BCAA supplementation did not rescue neurodevelopmental deficits, prevent postnatal microcephaly, or drastically correct biochemical abnormalities despite increasing systemic BCAA levels. The only correction BCAAs made to the BCAA catabolic pathway in *Bckdk*$^{-/-}$ mice was to reduce pooling of the acylcarnitine derivatives one step below BCKDH.

These results reveal limitations in the ability of BCAA supplementation to rescue neurodevelopmental and biochemical changes in BCKDK deficiency. First, enteral administration of BCAAs does not significantly increase BCAA levels in the cerebral spinal fluid of patients[9] nor in the brain of *Bckdk*$^{-/-}$ mice. Therefore, restoration of BCAAs in the brain is not likely achievable through a peripheral administration route. Second, enteral supplementation may be insufficient to sustainably increase BCAAs in the periphery, as

seen in patients where levels dropped to below the reference range 3-5 hours after dosing and in mice where leucine levels trended toward an increase but demonstrated great variability (Figure 3B)[9].

As BCAA supplementation was insufficient to normalize disease biomarkers and neurodevelopment, we explored alternative therapeutic approaches. Under normal physiological conditions BCKDK serves to decrease activity of the BCKDH complex[31]. Therefore, we sought to mimic its inhibitory function by reducing expression of DBT, a key component of the BCKDH complex. Specifically, we tested if DBT haploinsufficiency, could normalize BCAA pathway and TCA cycle intermediates in the absence of BCKDK. Interestingly, early genetic re-regulation of BCAA catabolism, through *Dbt* haploinsufficiency, partially rescued microcephaly, behavioral phenotypes and restored most biochemical changes in *Bckdk*$^{-/-}$ mice. Although intervention timing was not directly investigated in this work, our genetic rescue study supports that earlier intervention can affect outcomes such as survival and developmental phenotypes, both of which were improved with *Dbt* haploinsufficiency in *Bckdk*$^{-/-}$ mice. In genetic rescue mice, *Dbt* mRNA transcript levels were only reduced by 32%, suggesting that future directions of this work could aim to further reduce these levels to potentially maximize phenotypic outcomes.

Still, caution should be taken to not fully deplete DBT levels in BCKDK deficiency, as the loss of DBT function would lead to a Maple-Syrup Urine Disease (MSUD), which induces severe neurological dysfunction[51]. *Dbt* haploinsufficiency may modulate this pathway by additional mechanisms by reducing BCAT activity, as they have been shown to interact in a metabalon[56]. This further illustrates that BCAA catabolism is fine-tuned to maintain metabolic homeostasis and neurodevelopment. Interestingly, not all changes in the downstream TCA cycle were changed by *Dbt* haploinsufficiency in *Bckdk*$^{-/-}$ mice, suggesting the changes downstream of succinyl-CoA within the TCA cycle are inherent to absence of *Bckdk*. Previous studies have shown that pharmacologic inhibition of BCKDK with BT2 affected additional protein targets beyond BCKDH[57]. Therefore, a key future direction of this research is to investigate the impact of BCKDK in regulating the TCA cycle and overall macronutrient metabolism beyond BCAA catabolism.

In summary, we found that regulation of the rate of BCAA catabolism is essential to maintain metabolic homeostasis and neurodevelopment. As universal newborn screening has been transformative in other BCAA disorders, as is the case for MSUD[3], the possibility of screening for BCKDK deficiency has been raised[9]. For screening to be effective, we must have therapeutics that improve clinical phenotypes and treat the underlying disease pathophysiology. Early studies of BCAA supplementation in patients have yielded some signs of benefit, yet outcomes vary and neurologic deficits remain in most if not all patients[9]. Here we also found that early BCAA supplementation does not fully correct and even worsens some disease phenotypes in a *Bckdk*$^{-/-}$ mouse model. In addition, our data suggest that decreasing flux through the BCAA pathway may provide an alternative therapeutic approach. Future, additional, well-controlled studies of BCAA supplementation and additional potential therapies are needed to improve outcomes in BCKDK deficiency.

2.5. **Methods**

2.5.1 **Animals**

All experiments with animals were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and guidelines set by the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals. *Bckdk*$^{-/-}$ Mice were generated, published, and gifted from the Arany lab.[13] Mice were collected after cryoanesthetization (p0) followed by decapitation or euthanasia with isoflurane (p21) and cervical dislocation followed by decapitation. Heads were collected for µCT analysis for microcephaly at p0 and p21. Brains and livers were collected, and flash frozen for metabolic analysis at p21. Mice were perfused with PBS followed by 4% PFA for representative images of brains.

Dbt $^{-/+\ \text{mice}}$ were generated from a novel floxed *DBT* allele mouse on the C57BL/6J background. *Dbt* $^{Flox/Flox}$ mice with loxP sites flanking exon 2 of *Dbt* were generated by the University of Pennsylvania CRISPR/Cas9 Mouse Targeting Core using the Alt-R™ CRISPR-Cas 9 genome editing system (Integrated DNA Technologies, Coralville, IA). The target sequences were as follows: 5′ - GTGAAGGTTATTGACAGAGTGGG; 3′ - CATTGTGGGAAACTATGGAGCGG. CRISPR guides were

designed, and the crispr RNA (crRNA) sequences were as follows: 5′ - GTGAAGGTTATTGACAGAGT; 3′ - CATTGTGGGAAACTATGGAG. The trans-activating crRNA (tracrRNA, Integrated DNA Technologies, #1072533) then hybridizes to crRNA to activate the Cas9 enzyme. After oocyte injection, founders were confirmed via Sanger sequencing of genomic DNA. Founders were backcrossed to C57BL/6J mice, which were then bred to homozygosity (*Dbt* $^{Flox/Flox}$). *Dbt* $^{Flox/Flox}$ mice were then crossed with *Nestin* $^{Cre+}$ mice (The Jackson Laboratory, Bar Harbor, ME; B6.Cg-Tg(Nes-cre)1Kln/J, Stock No: 003771), which express Cre in both the nervous system and oocytes, resulting in the frequent germline recombination of Cre in pups[31]. Offspring that demonstrated germline recombination of the floxed allele were then bred to *Dbt*$^{+/+}$ mice to produce *Dbt* $^{+/-}$ (*Nestin* $^{Cre-}$) mice, which were used for breeding.

2.5.2 **Genotyping**

Mouse tail snips were obtained for genotyping. DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany, #69506).

For *Bckdk* genotyping, a combination of primers that flank the *Bckdk* Flox and KO sites were used to discern the *Bckdk* Flox, WT, and KO alleles. The sequence of the flox primers were 5'-CTGCTTAAGCCCTTCCCTCT -3' and 5'-AAGAGCACTTGCCCTTCCTT -3'. The sequence of the BCKDK KO primers were 5'- TACTGCCAGCTGGTGAGACA -3' and 5'-GCAACACTTCCACCCAACTT -3'. The relevant product sizes were 442 bp for the WT allele, and 253 bp for the KO allele. The flox allele size is 1301 bp. The PCR was performed with the KAPA2G Fast HotStart ReadyMix (Roche, Switzerland, #KK 5609).

For *Dbt* genotyping, we selected primers which flanked the 5’ and 3’ LoxP’s to distinguish Flox, WT, and KO allele. The sequences for the forward and reverse primer are 5’-ACCGGAGCATCAGCCTAAA, and 3’- TGTGCACAAGGACATACAGG. The product sizes are 621 bp for the wild-type allele, 311 bp for the knockout allele, and 689 bp for the flox allele.

2.5.3 **Developmental Assessment**

A panel of developmental assessments was performed on *Bckdk*$^{-/-}$mice and age matched littermate controls from postnatal day 1-21, unless otherwise indicated. After assessment at p1, pups were tattooed for identification throughout the study. Developmental milestones were assessed on scales or time-based assessments with a maximum cut-off time of 60 seconds. Body weight and body length from snout to tail base were measured for developmental progression. Eye opening was assessed on a scale of 0-3; 0 indicates that both eyes are closed with no slits present, 1 indicates the presence of slits but the eyes are still fully closed or one eye is partially open, 2 indicates that both eyes are partially open or one eye is fully open, and 3 indicates that both eyes are fully open. Incisor eruption was assessed as the age of full protrusion of the lower incisors through the gums. Grasping reflexes were scored on a scale of 0-2; 0 indicates no movement from either paw (no grasp), 1 indicates partial grasp or full grasp from only one paw, and 2 indicates a full grasp from both paws. Surface righting time was assessed from p4-p14 by placing mice in a supine position on a flat surface and recording the time it takes for mice to fully roll over with all four paws flat on the surface. Edge avoidance was assessed from p4-p14 by placing a mouse on a raised platform in a prone position with both the forepaws and head placed over the edge and recording the time for the mouse to retreat onto the platform with no part of the body remaining over the edge. Mice that fell from the platform could be retested a maximum of 3 times. Vertical hold time was assessed from p7-p21 by placing each mouse on top of a screen rotated 90^{o} until vertical relative to the table. The time at which the mouse fell off the apparatus was recorded. Horizontal hold time was assessed from p10-p21 by placing each mouse on top of a horizontal screen rotated 180^{o} relative to the table, so that the mouse was hanging below the screen. The time to fall off the apparatus was recorded. T-bar hold time was assessed from p10-p21 by placing the forepaws of the mouse on a thin horizontal bar. Proper grip was ensured, and the time suspended on the bar was recorded. The age at maximum score or time, on scale-based or time-based assays, respectively, was also represented to illustrate developmental delay of the indicated developmental milestone. Pups that did not survive to p14 were excluded from the study.

2.5.4 **μCT Analysis for Microcephaly**

Bckdk$^{-/-}$ mice and age matched wildtype littermate controls were collected at p21 after euthanasia with isoflurane followed by cervical dislocation and decapitation. Heads were stored in paraformaldehyde for 48 hours, then transitioned to 70% ethanol at 4°C until μCT images were obtained. Postnatal day 21 mouse skulls were scanned with a Scanco μCT35 (SCANCO Medical AG, Switzerland, Penn Center for Musculoskeletal Disorders MicroCT Imaging Core, Perelman School of Medicine, University of Pennsylvania) at a resolution of 15μm. Skulls were reconstructed with Scanco software and analysis of images was performed with 3DSlicer (V5.2.2, Slicer.org).[74] Threshold settings were optimized to visualize only bone volume. Virtual endocasts of the skull were created to compare skull volumes by using 3DSlicer Software and the Wrap Solidify extension. Cephalometric quantifications from these images were measured from the 3D skull models including length, width, height, and endocranial volume. These measurements were inferred using previously published landmarks[75,76]. Genotype specific measurements were compared for normality and homogeneity of variance. Unpaired t-test was used for significance ($p \leq 0.05$) in the original characterization (Figure 1), 2-way ANOVA was used for BCAA treatment studies (Figure 3), and 1-way ANOVA was used for genetic rescue studies (Figure 5). Non-parametric assessment was used as needed for analyses (SPSS 26.0, IBM, Armonk, NY, USA).

2.5.5 **Metabolite levels analysis**

Flash frozen mouse brains were homogenized in 50% acidified acetonitrile for targeted LC/MS metabolomics (acylcarnitines, amino acids, organic acids) according to validated, optimized protocols in our previously published studies[77,78]. These protocols use cold conditions and solvents to arrest cellular metabolism and maximize the stability and extraction recovery of metabolites. Each class of metabolites was separated with a unique HPLC method to optimize their chromatographic resolution and sensitivity. Quantitation of metabolites in each assay module was achieved using multiple reaction monitoring of calibration solutions and study samples on an Agilent 1290 Infinity UHPLC/6495 triple quadrupole mass spectrometer[77,78]. Raw data was processed using Mass Hunter

quantitative analysis software (Agilent). Calibration curves (R2 = 0.99 or greater) were either fitted with a linear or a quadratic curve with a 1/X or 1/X2 weighting.

Liver tissue from BCAA supplemented animals were snap frozen in liquid nitrogen. Frozen tissue was homogenized, and 30 mg was weighed out. Tissue was mixed with 1 mL 40:40:20 methanol:acetonitrile:water (extraction solvent) and homogenized with a benchtop lyser (SciLogex OS20-S) at 1600 mhZ for approximately 15 sec. U-13C-propionate was spiked in to the extraction solvent as an internal standard. Samples were then immediately centrifuged at 13.3g for 20 mins at 4C. Supernatant was saved and dried down in a speedvac (ThermoFisher Savant SpeedVac SPD130DLX) overnight at room temp. Samples were resuspended in 50 uL 60:40 methanol:water and spun down again at 13.3g for 20 mins at 4C. Supernatant was collected for LC-MS analysis.

A quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, San Jose, CA) operating in negative ion mode was coupled to hydrophilic interaction chromatography on a Vanquish UHPLC System (Thermo Fisher Scientific) via electrospray ionization and used to scan from m/z 65-425 at 1 Hz and 140,000 resolution. LC separation was on a ACQUITY Premier BEH Amide VanGuard FIT Column, 1.7 µm, 2.1 mm X 100 mm (Water, Milfod, MA) using a gradient of solvent A (20 mM ammonium acetate, 20 mM Ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45) and solvent B (acetonitrile). Flow rate was 300 µL/min. The LC gradient was: 0 min, 95% B; 9 min, 40%; 11 min, 40%; 11.1 min, 95%; 20 min, 95%. Autosampler was set at 4°C and injection volume was 3 µL. LC-MS was used to measure BCAAs and BCKAs. A standard curve for BCAAs/BCKAs was run in parallel to allow for quantitation. Data analysis was done using El-MAVEN software[79].

2.5.6 **BCAA Supplementation**

BCAA powder was ordered at a premixed 2:1:1 ratio of Leu:Iso:Val (BCAA 5000 Natural Powder, Nutrabio, Inc., Middlesex, NJ). A previous publication verified this biochemical ratio with assistance from an independent 3rd party vendor (ALS Global, Salt Lake City, UT).[73] A 2% percent BCAA solution (2g/100mL) was introduced via drinking water to dams with a new litter at age p0/p1 and maintained until p21. Bottles were weighed alternate days to monitor intake and the solution was refreshed weekly. IACUC approval was received for BCAA supplementation.

2.5.7 **Realtime qPCR**

RNA extraction, cDNA synthesis, and real-time qPCR were performed as described[31]. Briefly, RNA was extracted and purified using a combination of TRIzol reagent and QIAGEN RNeasy columns (QIAGEN, #74004); reversed transcribed into cDNA using the qScript cDNA SuperMix (Quantabio, Beverly, MA, #95047). The cDNA was then diluted and used as the template for real-time PCR analysis using the Power up SYBR Green PCR Master Mix (ThermoFisher Scientific, # A25779) on the QuantStudio3 Real-Time PCR System (ThermoFisher Scientific). The list of primers and sequences used is found in Supplementary Table 1.

2.5.8 **Western Blot**

Whole brains were homogenized in 1X RIPA buffer followed by BCA assay (ThermoFisher Scientific, #23227). Equivalent protein quantities for all genotypes were loaded onto a Nupage 4-12% Bis-Tris Protein Gels (ThermoFisher Scientific) for 1 hour. Subsequently, resolved proteins were transferred to a nitrocellulose membrane for 1.5 hours (ThermoFisher Scientific). Membranes were blocked with 5% bovine serum albumin in tris-buffered saline with 0.1% tween (TBS-T) for 1 hour, followed by incubation of primary antibody dilutions in blocking solution overnight at 4°C. The following primary antibodies were used for Western blot including BCKDK, pBCKDH-E1a, BCKDH-E1a, and GAPDH. Additional antibody information can be found in Supplementary Table 2. Membranes were washed with TBS-T, incubated with HRP-conjugated secondary antibodies (1:10,000) diluted in blocking solution for 1 hour, and washed with TBS-T prior to imaging. SuperSignal™ West Pico PLUS reagents were used (ThermoFisher Scientific) to image relative protein amounts using a ChemiDoc Gel Imaging System (BioRad, Hercules, CA). After acquisition, protein levels were quantified using Image J software's band analysis feature (NIH).

2.5.9 **Statistical analysis**

Data are presented as mean ± SEM. Statistical analysis was performed using Prism8 software (GraphPad, San Diego, CA). The respective statistical test for each experiment is indicated in the figure legends. Regardless of statistical test performed, statistical significance was annotated with

asterisks within the figures as follows for all tests and results, unless otherwise stated (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$).

CHAPTER 3: DISCUSSION

3.1. **BCKDK role in BCAA metabolism and the clinical spectrum of BCKDK deficiency**

BCAA catabolism has been well studied under its normal physiological conditions. Although, its regulation is far less understood. Previous studies suggest that BCKDK may play a key role in regulating macronutrient metabolism, specifically influencing transitions between glucose utilization, amino acid catabolism, and lipid anabolism. This recently discovered role in lipid anabolism may reveal why *Bckdk*$^{-/-}$ mice do not gain weight like littermate controls throughout development. In addition to these validated phosphorylation targets of BCKDK, other phosphorylation targets of BCKDK have been identified through phosphoproteomic screenings. These findings suggest that there is still more is to be understood regarding BCKDKs regulation of other proteins in the cell. Importantly, from these studies we found that loss of BCKDK impacts key TCA cycle steps following succinate that are likely regulated by BCKDK, since they are not corrected by *Dbt* haploinsufficiency. This reveals that BCKDK also likely regulate the TCA cycle, although further investigation is necessary to determine how it is regulating these particular steps.

Previous research into BCKDK deficiency focused on recapitulating patient-associated phenotypes, for example seizures, rather than characterizing the full spectrum of neurodevelopmental delay associated with BCKDK deficiency. In fact, our comprehensive look at the clinical spectrum of BCKDK deficiency reveals that the documented 32 patients to date all have intellectual disability, global developmental delay, and motor milestone delay. A majority also have language impairment, EEG abnormalities, progressive microcephaly, and atypical autism. Whereas only approximately 50% of patients with BCKDK deficiency experience overt clinical seizures. Therefore, our research focused on the most predominant features of BCKDK deficiency and recapitulated neurodevelopmental delay in a *Bckdk*$^{-/-}$ mouse model. Our findings revealed profound neurodevelopmental delay in this model, specifically impacting the nervous system. Therefore, this model can be used to test novel therapeutic targets for correction of these more predominant neurological phenotypes. Future research is needed to

elucidate the cause of seizures in BCKDK deficiency, to guide therapeutic development efforts. Our research suggests that elevated glycine levels may contribute to seizure generation, such as seen for glycine encephalopathy. Therefore, repurposing of therapeutics for this disorder could be investigated in BCKDK deficiency. Further research to determine if these therapeutics can reduce seizure burden in BCKDK deficiency in preclinical research studies, is warranted and a promising avenue to improve quality of life.

3.2. **Restoration of the regulation of BCAA catabolism is likely more essential than BCAA repletion in BCKDK deficiency**

It has long been understood that BCKDK regulates the catabolic rate of BCAA catabolism. However, the current utilization of BCAA repletion as a therapy does not directly address this function. BCAA disorders have often been treated with diet manipulation, without a full understanding of the metabolic and molecular mechanisms behind these diseases. Importantly, BCAA repletion without correction of BCAA catabolic rate in BCKDK deficiency is risky, as adding additional precursor will overload the downstream metabolic pathway potentially further impacting metabolic dynamics and causing further brain dysfunction. Additionally, extremely high levels of BCAAs have also been shown to be detrimental to the brain in disorders such as MSUD. This is important to note, since BCAA accumulation also leads to cognitive dysfunction. It is also important to note that these disorders have the inverse issue of BCKDK, in that there is assumed to be decreased flux through the BCAA pathway due to pathogenic variants in the key rate limiting enzyme BKCDH. Therefore, elevated levels of BCAAs are neurotoxic in the brain, especially without correction of the accelerated rate of BCAA catabolism due to loss of BCKDK.

Instead, re-regulation of the pathway is warranted, prior to proportionate supplementation with BCAAs through the diet. BCKDK directly acts upon BCKDH, therefore reducing BCKDH subunit levels or activity would directly re-regulate the rate limiting metabolic step of BCAA catabolism. While there are not

currently approved BCKDH inhibitors, our work provides rationale for their development for BCKDK deficiency.

Alternatively, studies have also shown that BCKDH and the upstream enzyme BCAT interact in a metabolon. Therefore, disrupting this interaction could also prove beneficial in BCKDK deficiency. Finally, directly reducing BCAT levels would likely also be beneficial. BCAT2 inhibitors are being developed for clinical applications of cancer immunotherapies. Intriguingly, BCAT2 is expressed both in the periphery and in the CNS allowing for systemic targeting of all phenotypes associated with BCKDK deficiency. Further research should be done to determine if BCAT can be a therapeutic target for BCKDK deficiency.

Our research did not investigate further modulation of BKCDH, through reduction of *Dbt* levels since we approached modulation of the pathway with genetic haploinsufficiency. This approach was used to avoid any potential off target effects of pharmacological intervention in our proof-of-concept study. Additionally, our study did not investigate if only postnatal modulation of the BCAA catabolic pathway would be able to rescue neurodevelopmental deficits. Therefore, future work should focus on and answer these questions to better understand the therapeutic landscape for BCKDK deficiency.

3.3. **Major findings**

From this work there are a few key findings that should be reiterated. First and foremost, this is the first time it has been shown that loss of BCKDK leads to the accumulation of downstream metabolites supporting the hypothesis that loss of BCKDK leads to increased carbon donation and flux into the downstream BCAA catabolic and TCA cycle pathways. This finding reveals that modulation of the BCAA catabolic rate is warranted to correct loss of BCKDK changes. Our study also fully characterized the spectrum of clinically relevant phenotypic changes in a *Bckdk*$^{-/-}$ mouse model. Our study revealed that ectodermal derived tissues and organs are most impacted by BCKDK deficiency, including fur development, eye development, and brain development. These findings reiterate that BCKDK plays a key functional role in early germ layer development, as was also shown in the PDH study. Our research

furthers this work by directly showing that early developmental changes due to BCKDK deficiency, negatively impacts and slows down the development of these ectodermal derived tissues (skin, eye, brain). Also, our study is the first to show postnatal microcephaly in a *Bckdk*$^{-/-}$ mouse model, a key clinical phenotype that can be assessed in preclinical and clinical trials in mouse models and in human patients, respectively. Therefore, our study has aided in the understanding of the human symptomology of disease by looking at the full spectrum of patients and utilizing a mouse model to narrow down what phenotypes and tissues are of highest prevalence and most impacted by BCKDK deficiency through cross-comparison. Additionally, this study has uncovered the mechanism of disease pathophysiology, which could accelerate preclinical work into therapeutic targets for BCKDK deficiency.

Once we recreated a clinically relevant model of disease, we investigated potential therapeutic targets for BCKDK to aid in our further understanding of the disease mechanism and further advance effective therapeutic options for BCKDK deficiency. This work revealed that BCAA repletion was not sufficient to correct neurodevelopmental changes in our *Bckdk-/-* model, nor postnatal microcephaly. Instead, BCAA supplementation assisted with overall brain size, reiterating the known role of BCAAs in protein synthesis and tissue growth. In addition to this finding, we showed that BCAA repletion did not correct any of the biochemical changes seen due to BCKDK deficiency. Instead, BCAA supplementation simply reduced pooling of the downstream acylcarnitine intermediates directly downstream of the BCKDH step, suggesting further increased flux through the pathway likely through excess BCAA utilization. Therefore, overloading the metabolic pathway with more precursors for metabolic flux is likely not helpful to neurodevelopment and does not assist with the key underlying metabolic mechanism of disease pathology. It is important to note that BCAA supplementation may be beneficial to some milestones of development, such as weight gain and tissue development. However, these gains may come at a detriment to metabolic dysregulation that likely leads to brain dysfunction. Therefore, the risk benefit ratio should be weighed when considering BCAA supplementation in BCKDK deficiency. Additionally, BCAA supplementation in BCKDK deficiency may not be necessary if re-regulation of the pathway can be achieved through genetic modulation of the BCAA pathway or gene replacement therapy of BCKDK.

Finally, we tested the hypothesis that slowing down the rate of BCAA catabolism, through *Dbt* haploinsufficiency, would improve neurodevelopmental phenotypes and biochemical changes. First, haploinsufficiency was expected to reduce DBT mRNA levels in half based on the assumptions of gene dosage. However, they were only reduced approximately to 1/3 of the levels. Therefore, reduction of DBT levels may be transcriptionally compensated for in the brain in BCKDK deficiency. Interestingly, this result suggests that although further modulation of this pathway is likely possible by reducing DBT levels further it may be better to perform a combinatorial intervention to further modulate the rate of BCAA catabolism through upstream enzymatic modulation or reduction of BCKDH's other subunits. Since DBT forms a complex with other components of BCKDH (BCKDHA and BKCDHB) to form a functional unit, any imbalance in the ratio of these subunits impacts overall BCKDH complex formation and function. Additionally, caution should be taken to not fully deplete DBT or BCKDH levels, as this would cause an MSUD phenotype. Full depletion of *Dbt* levels in BCKDK deficiency would likely be pathogenic if not lethal, since *Bckdk* $^{-/-}$*Dbt* $^{-/-}$ double knockout pups from our *Bckdk*$^{+/-}$ *Dbt*$^{+/-}$ crosses did not survive. Therefore, further investigation into the ideal modulation of BCKDH levels and its impact on BCKDH activity in BCKDK deficiency is warranted and optimization is clearly necessary to maximize therapeutic benefit.

Interestingly, even with partial reduction of *Dbt* levels, there was partial rescue of neurodevelopmental changes and postnatal microcephaly in these genetic rescue mice. Although it is not understood what percentage of the BCKDH complex is active throughout neurodevelopment, even partial depletion of *Dbt* levels may re-regulate the catabolic rate in BCKDK deficiency and induce positive neurodevelopmental changes. In addition to partially reversing phenotypic changes, *Dbt* haploinsufficiency restored key metabolites in the BCAA pathway and the initiation of the TCA cycle, supporting that reduction of BCKDH can reregulate the pathway when BCKDK is lost. Incidentally, we also found TCA cycle intermediates that were unaltered by this intervention, implying that BCKDK somehow regulates these key steps of the TCA cycle. Although this role is less clear, the recent literature revealed that BCKDK has non-canonical functions such as a regulator of pyruvate oxidation through being a functional paralog to PDH [60]. Intriguingly, BCKDK also shares structural homology with a key

enzyme in the TCA cycle, alpha-ketoglutarate dehydrogenase complex (KGDHC), which is upstream of these metabolic changes in succinate, fumarate, and malate. Therefore, it would be interesting to understand if BCKDK can also act as a functional paralog to KGDHC, particularly when the input to the TCA cycle is overloaded.

3.4. **Future directions**

The research described in this dissertation revealed key insights into the metabolic mechanism underlying cellular and molecular changes in BCKDK deficiency and the discovery of a novel therapeutic target for intervention. This work thoroughly characterized a *Bckdk*$^{-/-}$ mouse model as a clinically relevant preclinical model for BCKDK deficiency to test therapeutic targets. Importantly, this work uncovered multiple potential therapeutic targets for intervention that should be further investigated. Further transcriptional decrease of DBT would likely be beneficial in BCKDK deficiency, based on the partial biochemical correction seen even with a one-third reduction of DBT mRNA levels. Additionally, reduction of upstream enzymes of BCKDK, such as BCAT2, would likely also be a beneficial therapeutic target based on the recently published interactions shown between the two enzymes in a metabolon[56]. Furthermore, reduction of enzymatic activity with inhibitors would likely prove to be phenotypically and biochemically beneficial in BCKDK deficiency. In conclusion, our study has shown that slowing down the rate of BCAA catabolism through *Dbt* haploinsufficiency is sufficient to restore biochemical changes, although phenotypic changes could be further improved through additional genetic modulation. Although there are not currently commercially available inhibitors, small molecule inhibitors could rapidly be developed and tested in BCKDK deficiency to improve clinical outcomes.

In addition to this future direction of re-regulating the BCAA catabolic pathway, further research is warranted into the non-canonical roles of BCKDK that could impact clinical phenotypes in BCKDK deficiency. BCKDK clearly has an emerging role in regulation of macronutrient metabolism. In addition to BCKDKs clear role in BCAA catabolism, BCKDK also inhibits and activates key enzymes within pyruvate oxidation (PDH) and lipid metabolism (ACL). However, BCKDKs role as a functional paralog of PDH in

pyruvate oxidation never would have been discovered if not for a double knockout model of PDH and BCKDK. Therefore, a further investigation into BCKDK potential role as a paralog to other enzymes with structural homology is warranted.

3.5. **Conclusion**

Mechanisms of BCKDK deficiency have been poorly understood. However, this work reframes and enlightens the field to take a closer look at the basic biology of the function of BCKDK and its importance in regulating the BCAA catabolic pathway for input into the TCA cycle. Additionally, this work brings together key findings in the recent literature that BCKDK is more than just a kinase for the BCAA catabolic pathway. Our study highlights these findings since modulation of the BCAA catabolic pathway did not correct all neurodevelopmental phenotypes. In fact, these trends towards correction of neurodevelopmental changes further supports that BCKDK has non-canonical roles in the cell that are essential, particularly under stressful times of metabolic decompensation. Furthermore, future research into BCKDK potential role as a functional paralog of KGDHC is warranted since our study illustrated that loss of BCKDK regardless of genetic modulation, could not correct TCA cycle intermediate levels downstream of this key enzyme in the pathway. Finally, this work advanced both our understanding of this neurogenetic disorder due to advancements in diagnosis of this disorder and advanced our biological understanding of BCKDK. Specifically, our work reframes BCKDK deficiency as a BCAA hypercatabolic disorder and recontextualizes the recently discovered phosphorylation targets of BCKDK that assist with regulation of macronutrient metabolism. In conclusion, our research clearly revealed the mechanism of BCKDK deficiency in brain, and tested novel therapeutic targets that are likely to improve neurodevelopmental delay and biochemical changes in BCKDK deficiency.

BIBLIOGRAPHY

1. Bifari, F., and Nisoli, E. (2017). Branched-chain amino acids differently modulate catabolic and anabolic states in mammals: a pharmacological point of view. Br J Pharmacol *174*, 1366–1377. 10.1111/bph.13624.

2. Maple Syrup Urine Disease (Branched-Chain Ketoaciduria) McGraw Hill Medical. https://ommbid.mhmedical.com/content.aspx?bookId=2709&sectionId=225084607.

3. Naylor, E.W., and Guthrie, R. (1978). Newborn screening for maple syrup urine disease (branched-chain ketoaciduria). Pediatrics *61*, 262–266.

4. Knerr, I., Colombo, R., Urquhart, J., Morais, A., Merinero, B., Oyarzabal, A., Pérez, B., Jones, S.A., Perveen, R., Preece, M.A., et al. (2019). Expanding the genetic and phenotypic spectrum of branched-chain amino acid transferase 2 deficiency. J Inherit Metab Dis *42*, 809–817. 10.1002/jimd.12135.

5. Strauss, K.A., Carson, V.J., Soltys, K., Young, M.E., Bowser, L.E., Puffenberger, E.G., Brigatti, K.W., Williams, K.B., Robinson, D.L., Hendrickson, C., et al. (2020). Branched-chain α-ketoacid dehydrogenase deficiency (maple syrup urine disease): Treatment, biomarkers, and outcomes. Molecular Genetics and Metabolism *129*, 193–206. 10.1016/j.ymgme.2020.01.006.

6. Novarino, G., El-Fishawy, P., Kayserili, H., Meguid, N.A., Scott, E.M., Schroth, J., Silhavy, J.L., Kara, M., Khalil, R.O., Ben-Omran, T., et al. (2012). Mutations in BCKD-kinase lead to a potentially treatable form of autism with epilepsy. Science *338*, 394–397. 10.1126/science.1224631.

7. García-Cazorla, A., Oyarzabal, A., Fort, J., Robles, C., Castejón, E., Ruiz-Sala, P., Bodoy, S., Merinero, B., Lopez-Sala, A., Dopazo, J., et al. (2014). Two novel mutations in the BCKDK (branched-chain keto-acid dehydrogenase kinase) gene are responsible for a neurobehavioral deficit in two pediatric unrelated patients. Hum Mutat *35*, 470–477. 10.1002/humu.22513.

8. Boemer, F., Josse, C., Luis, G., Di Valentin, E., Thiry, J., Cello, C., Caberg, J.-H., Dadoumont, C., Harvengt, J., Lumaka, A., et al. (2022). Novel Loss of Function Variant in BCKDK Causes a Treatable Developmental and Epileptic Encephalopathy. Int J Mol Sci *23*, 2253. 10.3390/ijms23042253.

9. Tangeraas, T., Constante, J.R., Backe, P.H., Oyarzábal, A., Neugebauer, J., Weinhold, N., Boemer, F., Debray, F.G., Ozturk-Hism, B., Evren, G., et al. (2023). BCKDK deficiency: a treatable neurodevelopmental disease amenable to newborn screening. Brain, awad010. 10.1093/brain/awad010.

10. Maguolo, A., Rodella, G., Giorgetti, A., Nicolodi, M., Ribeiro, R., Dianin, A., Cantalupo, G., Monge, I., Carcereri, S., De Bernardi, M.L., et al. (2022). A Gain-of-Function Mutation on BCKDK Gene and Its Possible Pathogenic Role in Branched-Chain Amino Acid Metabolism. Genes (Basel) *13*, 233. 10.3390/genes13020233.

11. Tso, S.C., Qi, X., Gui, W.J., Chuang, J.L., Morlock, L.K., Wallace, A.L., Ahmed, K., Laxman, S., Campeau, P.M., Lee, B.H., et al. (2013). Structure-based design and mechanisms of allosteric inhibitors for

mitochondrial branched-chain α-ketoacid dehydrogenase kinase. Proceedings of the National Academy of Sciences of the United States of America *110*, 9728–9733. 10.1073/pnas.1303220110.

12. Neinast, M., Murashige, D., and Arany, Z. (2019). Branched Chain Amino Acids. Annu Rev Physiol *81*, 139–164. 10.1146/annurev-physiol-020518-114455.

13. Murashige, D., Jung, J.W., Neinast, M.D., Levin, M.G., Chu, Q., Lambert, J.P., Garbincius, J.F., Kim, B., Hoshino, A., Marti-Pamies, I., et al. (2022). Extra-cardiac BCAA catabolism lowers blood pressure and protects from heart failure. Cell Metabolism *34*, 1749-1764.e7. 10.1016/j.cmet.2022.09.008.

14. Shellmer, D.A., Dabbs, A.D., Dew, M.A., Noll, R.B., Feldman, H., Strauss, K., Morton, D.H., Vockley, G., and Mazariegos, G.V. (2011). Cognitive and Adaptive Functioning after Liver Transplantation for Maple Syrup Urine Disease: A Case Series. Pediatr Transplant *15*, 58–64. 10.1111/j.1399-3046.2010.01411.x.

15. Mazariegos, G.V., Morton, D.H., Sindhi, R., Soltys, K., Nayyar, N., Bond, G., Shellmer, D., Shneider, B., Vockley, J., and Strauss, K.A. (2012). Liver Transplantation for Classical Maple Syrup Urine Disease: Long-Term Follow-Up in 37 Patients and Comparative United Network for Organ Sharing Experience. J Pediatr *160*, 116-21.e1. 10.1016/j.jpeds.2011.06.033.

16. Tărlungeanu, D.C., Deliu, E., Dotter, C.P., Kara, M., Janiesch, P.C., Scalise, M., Galluccio, M., Tesulov, M., Morelli, E., Sonmez, F.M., et al. (2016). Impaired Amino Acid Transport at the Blood Brain Barrier Is a Cause of Autism Spectrum Disorder. Cell *167*, 1481-1494.e18. 10.1016/j.cell.2016.11.013.

17. Mizusawa, A., Watanabe, A., Yamada, M., Kamei, R., Shimomura, Y., and Kitaura, Y. (2020). BDK Deficiency in Cerebral Cortex Neurons Causes Neurological Abnormalities and Affects Endurance Capacity. Nutrients *12*, 2267. 10.3390/nu12082267.

18. Yudkoff, M. (1997). Brain metabolism of branched-chain amino acids. Glia *21*, 92–98. 10.1002/(SICI)1098-1136(199709)21:1<92::AID-GLIA10>3.0.CO;2-W.

19. Dotiwala, A.K., McCausland, C., and Samra, N.S. (2023). Anatomy, Head and Neck: Blood Brain Barrier. In StatPearls (StatPearls Publishing).

20. Christensen, H.N. (1990). Role of amino acid transport and countertransport in nutrition and metabolism. Physiological Reviews *70*, 43–77. 10.1152/physrev.1990.70.1.43.

21. Yanagida, O., Kanai, Y., Chairoungdua, A., Kim, D.K., Segawa, H., Nii, T., Cha, S.H., Matsuo, H., Fukushima, J., Fukasawa, Y., et al. (2001). Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines. Biochimica et Biophysica Acta (BBA) - Biomembranes *1514*, 291–302. 10.1016/S0005-2736(01)00384-4.

22. Verrey, F., Closs, E.I., Wagner, C.A., Palacin, M., Endou, H., and Kanai, Y. (2004). CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch - Eur J Physiol *447*, 532–542. 10.1007/s00424-003-1086-z.

23. Fernstrom, J.D. (2005). Branched-chain amino acids and brain function. J Nutr *135*, 1539S-46S. 10.1093/jn/135.6.1539S.

24. Smith, Q.R., Momma, S., Aoyagi, M., and Rapoport, S.I. (1987). Kinetics of Neutral Amino Acid Transport Across the Blood-Brain Barrier. Journal of Neurochemistry *49*, 1651–1658. 10.1111/j.1471-4159.1987.tb01039.x.

25. Salcedo, C., Andersen, J.V., Vinten, K.T., Pinborg, L.H., Waagepetersen, H.S., Freude, K.K., and Aldana, B.I. (2021). Functional Metabolic Mapping Reveals Highly Active Branched-Chain Amino Acid Metabolism in Human Astrocytes, Which Is Impaired in iPSC-Derived Astrocytes in Alzheimer's Disease. Front Aging Neurosci *13*, 736580. 10.3389/fnagi.2021.736580.

26. Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., and Gorman, A.M. (2016). The integrated stress response. EMBO Rep *17*, 1374–1395. 10.15252/embr.201642195.

27. Derisbourg, M.J., Hartman, M.D., and Denzel, M.S. (2021). Perspective: Modulating the integrated stress response to slow aging and ameliorate age-related pathology. Nat Aging *1*, 760–768. 10.1038/s43587-021-00112-9.

28. Palam, L.R., Baird, T.D., and Wek, R.C. (2011). Phosphorylation of eIF2 Facilitates Ribosomal Bypass of an Inhibitory Upstream ORF to Enhance CHOP Translation♦. J Biol Chem *286*, 10939–10949. 10.1074/jbc.M110.216093.

29. Kilberg, M.S., Shan, J., and Su, N. (2009). ATF4-dependent transcription mediates signaling of amino acid limitation. Trends Endocrinol Metab *20*, 436–443. 10.1016/j.tem.2009.05.008.

30. Costa-Mattioli, M., and Walter, P. (2020). The integrated stress response: From mechanism to disease. Science *368*, eaat5314. 10.1126/science.aat5314.

31. Joshi, M.A., Jeoung, N.H., Obayashi, M., Hattab, E.M., Brocken, E.G., Liechty, E.A., Kubek, M.J., Vattem, K.M., Wek, R.C., and Harris, R.A. (2006). Impaired growth and neurological abnormalities in branched-chain alpha-keto acid dehydrogenase kinase-deficient mice. Biochem J *400*, 153–162. 10.1042/BJ20060869.

32. Sabatini, D.M. (2017). Twenty-five years of mTOR: Uncovering the link from nutrients to growth. Proceedings of the National Academy of Sciences *114*, 11818–11825. 10.1073/pnas.1716173114.

33. Wolfson, R.L., Chantranupong, L., Saxton, R.A., Shen, K., Scaria, S.M., Cantor, J.R., and Sabatini, D.M. (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. Science *351*, 43–48. 10.1126/science.aab2674.

34. Yudkoff, M. (2017). INTERACTIONS IN THE METABOLISM OF GLUTAMATE AND THE BRANCHED-CHAIN AMINO ACIDS AND KETOACIDS IN THE CNS. Neurochem Res *42*, 10–18. 10.1007/s11064-016-2057-z.

35. Sperringer, J.E., Addington, A., and Hutson, S.M. (2017). Branched-Chain Amino Acids and Brain Metabolism. Neurochem Res *42*, 1697–1709. 10.1007/s11064-017-2261-5.

36. Rowley, N.M., Madsen, K.K., Schousboe, A., and Steve White, H. (2012). Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochemistry International *61*, 546–558. 10.1016/j.neuint.2012.02.013.

37. Zhou, Y., and Danbolt, N.C. (2014). Glutamate as a neurotransmitter in the healthy brain. J Neural Transm *121*, 799–817. 10.1007/s00702-014-1180-8.

38. Sarlo, G.L., and Holton, K.F. (2021). Brain concentrations of glutamate and GABA in human epilepsy: A review. Seizure *91*, 213–227. 10.1016/j.seizure.2021.06.028.

39. Montanari, M., Martella, G., Bonsi, P., and Meringolo, M. (2022). Autism Spectrum Disorder: Focus on Glutamatergic Neurotransmission. Int J Mol Sci *23*, 3861. 10.3390/ijms23073861.

40. Moretto, E., Murru, L., Martano, G., Sassone, J., and Passafaro, M. (2018). Glutamatergic synapses in neurodevelopmental disorders. Progress in Neuro-Psychopharmacology and Biological Psychiatry *84*, 328–342. 10.1016/j.pnpbp.2017.09.014.

41. Nisar, S., Bhat, A.A., Masoodi, T., Hashem, S., Akhtar, S., Ali, T.A., Amjad, S., Chawla, S., Bagga, P., Frenneaux, M.P., et al. (2022). Genetics of glutamate and its receptors in autism spectrum disorder. Mol Psychiatry *27*, 2380–2392. 10.1038/s41380-022-01506-w.

42. Conway, M.E., and Hutson, S.M. (2016). BCAA Metabolism and NH3 Homeostasis. In The Glutamate/GABA-Glutamine Cycle: Amino Acid Neurotransmitter Homeostasis Advances in Neurobiology., A. Schousboe and U. Sonnewald, eds. (Springer International Publishing), pp. 99–132. 10.1007/978-3-319-45096-4_5.

43. Schousboe, A., Scafidi, S., Bak, L.K., Waagepetersen, H.S., and McKenna, M.C. (2014). Glutamate Metabolism in the Brain Focusing on Astrocytes. Adv Neurobiol *11*, 13–30. 10.1007/978-3-319-08894-5_2.

44. Aldana, B.I., Zhang, Y., Jensen, P., Chandrasekaran, A., Christensen, S.K., Nielsen, T.T., Nielsen, J.E., Hyttel, P., Larsen, M.R., Waagepetersen, H.S., et al. (2020). Glutamate-glutamine homeostasis is perturbed in neurons and astrocytes derived from patient iPSC models of frontotemporal dementia. Molecular Brain *13*, 125. 10.1186/s13041-020-00658-6.

45. Chen, Y.H., Claflin, K., Geoghegan, J.C., and Davidson, B.L. (2012). Sialic acid deposition impairs the utility of AAV9, but not peptide-modified AAVs for brain gene therapy in a mouse model of lysosomal storage disease. Mol Ther *20*, 1393–1399. 10.1038/mt.2012.100.

46. Martínez-Reyes, I., and Chandel, N.S. (2020). Mitochondrial TCA cycle metabolites control physiology and disease. Nat Commun *11*, 102. 10.1038/s41467-019-13668-3.

47. Xing, G., Ren, M., and Verma, A. (2018). Divergent Induction of Branched-Chain Aminotransferases and Phosphorylation of Branched Chain Keto-Acid Dehydrogenase Is a Potential Mechanism Coupling Branched-Chain Keto-Acid–Mediated-Astrocyte Activation to Branched-Chain Amino Acid Depletion-Mediated Cognitive Deficit after Traumatic Brain Injury. Journal of Neurotrauma *35*, 2482–2494. 10.1089/neu.2017.5496.

48. Bixel, M.G., Shimomura, Y., Hutson, S.M., and Hamprecht, B. (2001). Distribution of Key Enzymes of Branched-chain Amino Acid Metabolism in Glial and Neuronal Cells in Culture. J Histochem Cytochem. *49*, 407–418. 10.1177/002215540104900314.

49. Stevens, B.R., Kaunitz, J.D., and Wright, E.M. (1984). Intestinal Transport of Amino Acids and Sugars: Advances Using Membrane Vesicles. Annual Review of Physiology *46*, 417–433. 10.1146/annurev.ph.46.030184.002221.

50. Zaragozá, R. (2020). Transport of Amino Acids Across the Blood-Brain Barrier. Frontiers in Physiology *11*.

51. Xu, J., Jakher, Y., and Ahrens-Nicklas, R.C. (2020). Brain Branched-Chain Amino Acids in Maple Syrup Urine Disease: Implications for Neurological Disorders. Int J Mol Sci *21*, 7490. 10.3390/ijms21207490.

52. Hamosh, A., McDonald, J.W., Valle, D., Francomano, C.A., Niedermeyer, E., and Johnston, M.V. (1992). Dextromethorphan and high-dose benzoate therapy for nonketotic hyperglycinemia in an infant. The Journal of Pediatrics *121*, 131–135. 10.1016/S0022-3476(05)82559-4.

53. Sacksteder, K.A., Biery, B.J., Morrell, J.C., Goodman, B.K., Geisbrecht, B.V., Cox, R.P., Gould, S.J., and Geraghty, M.T. (2000). Identification of the alpha-aminoadipic semialdehyde synthase gene, which is defective in familial hyperlysinemia. Am J Hum Genet *66*, 1736–1743.

54. Crowther, L.M., Mathis, D., Poms, M., and Plecko, B. (2019). New insights into human lysine degradation pathways with relevance to pyridoxine-dependent epilepsy due to antiquitin deficiency. Journal of Inherited Metabolic Disease *42*, 620–628. 10.1002/jimd.12076.

55. Oyarzabal, A., Bravo-Alonso, I., Sánchez-Aragó, M., Rejas, M.T., Merinero, B., García-Cazorla, A., Artuch, R., Ugarte, M., and Rodríguez-Pombo, P. (2016). Mitochondrial response to the BCKDK-deficiency: Some clues to understand the positive dietary response in this form of autism. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease *1862*, 592–600. 10.1016/j.bbadis.2016.01.016.

56. Patrick, M., Gu, Z., Zhang, G., Wynn, R.M., Kaphle, P., Cao, H., Vu, H., Cai, F., Gao, X., Zhang, Y., et al. (2022). Metabolon formation regulates branched-chain amino acid oxidation and homeostasis. Nat Metab *4*, 1775–1791. 10.1038/s42255-022-00689-4.

57. White, P.J., McGarrah, R.W., Grimsrud, P.A., Tso, S.-C., Yang, W.-H., Haldeman, J.M., Grenier-Larouche, T., An, J., Lapworth, A.L., Astapova, I., et al. (2018). The BCKDH Kinase and Phosphatase Integrate BCAA and Lipid Metabolism via Regulation of ATP-Citrate Lyase. Cell Metab *27*, 1281-1293.e7. 10.1016/j.cmet.2018.04.015.

58. Wolfe, R.R. (2017). Branched-chain amino acids and muscle protein synthesis in humans: myth or reality? J Int Soc Sports Nutr *14*, 30. 10.1186/s12970-017-0184-9.

59. Tian, Q., Yuan, P., Quan, C., Li, M., Xiao, J., Zhang, L., Lu, H., Ma, T., Zou, L., Wang, F., et al. (2020). Phosphorylation of BCKDK of BCAA catabolism at Y246 by Src promotes metastasis of colorectal cancer. Oncogene *39*, 3980–3996. 10.1038/s41388-020-1262-z.

60. Heinemann-Yerushalmi, L., Bentovim, L., Felsenthal, N., Vinestock, R.C., Michaeli, N., Krief, S., Silberman, A., Cohen, M., Ben-Dor, S., Brenner, O., et al. (2021). BCKDK regulates the TCA cycle through PDC in the absence of PDK family during embryonic development. Developmental Cell *56*, 1182-1194.e6. 10.1016/j.devcel.2021.03.007.

61. Chang, C.-F., Chou, H.-T., Chuang, J.L., Chuang, D.T., and Huang, T.-H. (2002). Solution structure and dynamics of the lipoic acid-bearing domain of human mitochondrial branched-chain alpha-keto acid dehydrogenase complex. J Biol Chem *277*, 15865–15873. 10.1074/jbc.M110952200.

62. Cheon, S., Kaur, K., Nijem, N., Tuncay, I.O., Kumar, P., Dean, M., Juusola, J., Guillen-Sacoto, M.J., Bedoukian, E., Ierardi-Curto, L., et al. The ubiquitin ligase UBE3B, disrupted in intellectual disability and absent speech, regulates metabolic pathways by targeting BCKDK. 6.

63. Tso, S.-C., Gui, W.-J., Wu, C.-Y., Chuang, J.L., Qi, X., Skvorak, K.J., Dorko, K., Wallace, A.L., Morlock, L.K., Lee, B.H., et al. (2014). Benzothiophene Carboxylate Derivatives as Novel Allosteric Inhibitors of Branched-chain α-Ketoacid Dehydrogenase Kinase. J Biol Chem *289*, 20583–20593. 10.1074/jbc.M114.569251.

64. East, M.P., Laitinen, T., and Asquith, C.R.M. (2021). BCKDK: an emerging kinase target for metabolic diseases and cancer. Nature Reviews Drug Discovery *20*, 498–498. 10.1038/d41573-021-00107-6.

65. Patel, K.P., O'Brien, T.W., Subramony, S.H., Shuster, J., and Stacpoole, P.W. (2012). The spectrum of pyruvate dehydrogenase complex deficiency: Clinical, biochemical and genetic features in 371 patients. Mol Genet Metab *106*, 385–394.

66. Novarino, G., Fenstermaker, A.G., Zaki, M.S., Hofree, M., Silhavy, J.L., Heiberg, A.D., Abdellateef, M., Rosti, B., Scott, E., Mansour, L., et al. (2014). Exome sequencing links corticospinal motor neuron disease to common neurodegenerative disorders. Science *343*, 506–511. 10.1126/science.1247363.

67. Zigler, J.S., Hodgkinson, C.A., Wright, M., Klise, A., Sundin, O., Broman, K.W., Hejtmancik, F., Huang, H., Patek, B., Sergeev, Y., et al. (2016). A Spontaneous Missense Mutation in Branched Chain Keto Acid Dehydrogenase Kinase in the Rat Affects Both the Central and Peripheral Nervous Systems. PLoS One *11*, e0160447. 10.1371/journal.pone.0160447.

68. Garcez, P.P., Stolp, H.B., Sravanam, S., Christoff, R.R., Ferreira, J.C.C.G., Dias, A.A., Pezzuto, P., Higa, L.M., Barbeito-Andrés, J., Ferreira, R.O., et al. (2018). Zika virus impairs the development of blood vessels in a mouse model of congenital infection. Sci Rep *8*, 12774. 10.1038/s41598-018-31149-3.

69. Vockley, J., Parimoo, B., and Tanaka, K. (1991). Molecular characterization of four different classes of mutations in the isovaleryl-CoA dehydrogenase gene responsible for isovaleric acidemia. Am J Hum Genet *49*, 147–157.

70. Al-Shekaili, H.H., Petkau, T.L., Pena, I., Lengyell, T.C., Verhoeven-Duif, N.M., Ciapaite, J., Bosma, M., van Faassen, M., Kema, I.P., Horvath, G., et al. (2020). A novel mouse model for pyridoxine-dependent epilepsy due to antiquitin deficiency. Hum Mol Genet *29*, 3266–3284. 10.1093/hmg/ddaa202.

71. van Karnebeek, C.D.M., Stockler-Ipsiroglu, S., Jaggumantri, S., Assmann, B., Baxter, P., Buhas, D., Bok, L.A., Cheng, B., Coughlin, C.R., Das, A.M., et al. (2014). Lysine-Restricted Diet as Adjunct Therapy for Pyridoxine-Dependent Epilepsy: The PDE Consortium Consensus Recommendations. JIMD Rep *15*, 47–57. 10.1007/8904_2014_296.

72. Kava, M.P., Bryant, L., Rowe, P., Lewis, B., Greed, L., and Balasubramaniam, S. (2020). Beneficial outcome of early dietary lysine restriction as an adjunct to pyridoxine therapy in a child with pyridoxine dependant epilepsy due to Antiquitin deficiency. JIMD Rep *54*, 9–15. 10.1002/jmd2.12121.

73. Platt, K.M., Charnigo, R.J., Shertzer, H.G., and Pearson, K.J. (2016). Branched-Chain Amino Acid Supplementation in Combination with Voluntary Running Improves Body Composition in Female C57BL/6 Mice. J Diet Suppl *13*, 473–486. 10.3109/19390211.2015.1112866.

74. Fedorov, A., Beichel, R., Kalpathy-Cramer, J., Finet, J., Fillion-Robin, J.-C., Pujol, S., Bauer, C., Jennings, D., Fennessy, F., Sonka, M., et al. (2012). 3D Slicer as an image computing platform for the Quantitative Imaging Network. Magn Reson Imaging *30*, 1323–1341. 10.1016/j.mri.2012.05.001.

75. Cray, J., Kneib, J., Vecchione, L., Byron, C., Cooper, G.M., Losee, J.E., Siegel, M.I., Hamrick, M.W., Sciote, J.J., and Mooney, M.P. (2011). Masticatory hypermuscularity is not related to reduced cranial volume in myostatin-knockout mice. Anat Rec (Hoboken) *294*, 1170–1177. 10.1002/ar.21412.

76. Durham, E., Howie, R.N., Warren, G., LaRue, A., and Cray, J. (2019). Direct Effects of Nicotine Exposure on Murine Calvaria and Calvarial Cells. Sci Rep *9*, 3805. 10.1038/s41598-019-40796-z.

77. Lanfear, D.E., Gibbs, J.J., Li, J., She, R., Petucci, C., Culver, J.A., Tang, W.H.W., Pinto, Y.M., Williams, L.K., Sabbah, H.N., et al. (2017). Targeted Metabolomic Profiling of Plasma and Survival in Heart Failure Patients. JACC Heart Fail *5*, 823–832. 10.1016/j.jchf.2017.07.009.

78. Hahn, V.S., Petucci, C., Kim, M.-S., Bedi, K.C., Wang, H., Mishra, S., Koleini, N., Yoo, E.J., Margulies, K.B., Arany, Z., et al. (2023). Myocardial Metabolomics of Human Heart Failure With Preserved Ejection Fraction. Circulation *147*, 1147–1161. 10.1161/CIRCULATIONAHA.122.061846.

79. Agrawal, S., Kumar, S., Sehgal, R., George, S., Gupta, R., Poddar, S., Jha, A., and Pathak, S. (2019). El-MAVEN: A Fast, Robust, and User-Friendly Mass Spectrometry Data Processing Engine for Metabolomics. Methods Mol Biol *1978*, 301–321. 10.1007/978-1-4939-9236-2_19.