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Supporting Online Material for

Mutations in *BCKD-kinase* Lead to a Potentially Treatable Form of Autism with Epilepsy

Gaia Novarino,* Paul El-Fishawy, Hulya Kayserili, Nagwa A. Meguid, Eric M. Scott, Jana Schroth, Jennifer L. Silhavy, Majdi Kara, Rehab O. Khalil, Tawfeg Ben-Omran, A. Gulhan Ercan-Sencicek, Adel F. Hashish, Stephan J. Sanders, Abha R. Gupta, Hebatalla S. Hashem, Dietrich Matern, Stacey Gabriel, Larry Sweetman, Yasmeen Rahimi, Robert A. Harris, Matthew W. State, Joseph G. Gleeson*

*To whom correspondence should be addressed. E-mail: gnovarino@ucsd.edu (G.N.); jogleeson@ucsd.edu (J.G.G.)

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MATERIALS AND METHODS

Human subjects

Patients were enrolled according to standard local practice in approved human subjects protocols at the University of California and Yale University.

Linkage and homozygosity analysis

DNA was extracted from peripheral blood leukocytes using salt extraction. For family 1435 all the informative members were genotyped with the Infinium iSelect24 mapping panel (Center for Inherited Disease Research) and analyzed with easyLINKAGE-Plus (*22*), software to generate multipoint LOD scores for assembling exclusion maps. For families 558 and 18 blocks of homozygosity were determined by HomozygosityMapper (*3*).

Whole-Exome Sequencing and variant analysis

WES was performed using solution hybrid SureSelect reagents (Agilent, Mountain View, CA) and sequenced on an Illumina GAIIx or HiSeq2000 instrument (*23*).

For probands of families 558 and 1435 the sequence reads were aligned to the human genome (hg19), genetic variants were delineated using the Genome Analysis ToolKit (GATK 1.1) software and SAMTools (v1.4-r985) algorithms, for both SNPs and Indels (*24*), then sequentially filtered for variants that were: 1] in coding regions and/or splice sites, 2] non-synonymous, 3] not found out of Hardy-Weinberg equilibrium with the disease frequency in control populations, 4] homozygous, 5] and within linkage intervals or in blocks of homozygosity. The remaining variants were ranked by type of mutation (indels > nonsense >

missense), amino acid conservation across species and protein damage prediction based upon location of the mutation in a specialized protein domain.

For Family 18 the sequence reads obtained were aligned to the human genome (hg18) using BWA; duplicate reads were removed and SNVs and indels were called using SAMtools. Shared homozygous segments of the affected individuals were detected using PLINK software version 1.06. The variants were annotated for novelty and compared with dbSNP (build 132) and control samples analyzed by whole-exome sequencing experiments performed by our human genomics groups. Variants were analyzed against the RefSeq hg18 gene definitions, a list that includes 18,933 specific genes. Where multiple isoforms gave varying results the one most likely to lead to protein disruption was chosen. Filtering and ranking of variants proceeded as described above for families 558 and 1435.

Sanger sequencing

Primers were designed using the Primer3 program and tested for specificity using ENSEMBL's BLAST software. PCR products were treated using Exonuclease I (Fermentas) and Shrimp Alkaline Phosphatase (USB Corporation) and sequenced using the Big Dye terminator cycle sequencing Kit v.3.1 (Applied Biosystems) on an ABI 3100 DNA analyzer (Applied Biosystems). The sequence data were analyzed using Sequencher 4.9 (Gene Codes).

Protein modeling

The structural model of the mutant rat BCKDK was derived from the structure of the rat wildtype protein (PDB ID codes 1gjv) (*4*) using Protein Local Optimization Program (PLOP) from the Matt Jacobson Lab and modified with PYMOL

(http://pymol.sourceforge.net/).

Expression analysis

Total RNA was extracted with RNeasy Mini Kit (Qiagen), in accordance with the manufacturer's instructions. A total of 2µg RNA was transcribed to cDNA using the SuperScriptIII cDNA kit (Invitrogen) with random hexamers. For RT-PCR primers were designed to amplify the entire BCKDK transcript. For quantitative real time PCR primers were designed using Primer Express Software (Applied Biosystems) on the exon-exon junction and tested for specificity using NCBI's BLAST software. Real-time PCR was performed using the Syber Green PCR master mix (Applied Biosystems) and the ABI 7900 Sequence Detection System (Applied Biosystems). Data were normalized to TBP (TATA box-binding protein) and analyzed by the comparative CT method (*25*).

Mouse Gene Expression 12X135K Arrays (NimbleGen) were used for gene expression analysis of wildtype and *Bckdk* knockout mouse cortices.

Second strand synthesis and hybridization to the NimbleGen array were performed following the NimbleGen expression array user's guide.

Probe normalized expression values were calculated using the NimbleScan software (Nimblegen). Raw chip images were processed to derive intensity values and normalization was performed using Robust Multichip Average (RMA) analysis (Bolstad et al. 2003). Subsequent analysis was performed using the Bioconductor project (http://www.bioconductor.org). Normalized intensities were grouped in wildtype and knockout and fit to a linear model to shrink pooled variances. An empirical bayes methodology was used to calculate moderated t-

statistical and p-values. Probes were considered significant if p-values met a threshold of <=.05. Heatmaps were generated using heatmap2 command from the gplots package. QC analysis was performed on all data to check for array artifacts or a bimodal distribution of intensities. Arrays that failed these QC metrics were removed from the comparison. Gene annotation enrichment was performed using DAVID (http://david.abcc.ncifcrf.gov/).

Western Blot

Right hemispheres of mouse brains or cell pellets were lysed in ice-cold RIPA buffer supplemented with protease inhibitor cocktail (Complete, Roche). After 1 hour of incubation at 4 °C the lysate was centrifuged at 14.000 rpm for 15 minutes. The protein concentration of the supernatant was measured using the BioRad protein assay method (BioRad). For the dephosphorylation experiment protein cell lysates were incubated with calf intestine phosphatase (CIP) (New England Biolabs) according to the manufacturer's instructions prior to gel electrophoresis. For the Western blot 30 µg proteins were separated in 10% SDS-PAGE gels and transferred to a PVDF membrane using a Bio-Rad MicroCell Western blotting apparatus. The membranes were blocked with 5% milk in 1x TBS-T, and blotted with primary antibody overnight at 4 °C. Detection used a peroxidase-coupled anti-IgG antibody (Pierce) and an enhanced chemiluminescence substrate (Thermo Scientific Pierce ECL). The following primary antibodies were used: rabbit anti-BCKDK (1:500, Acris or 1:1000, Sigma), rabbit anti-BCKDH-E1α (1:100, (*10*)), rabbit anti-BCKDH-E1α-pSer293 (1:1000, (*10*)), mouse anti-tubulin (1:1000, Sigma), rabbit anti-GAPDH (1:1000,

Millipore).

Plasma amino acid analysis

Patient blood samples were collected under fasting or non-fasting conditions (as labeled) in sodium heparin or potassium EDTA treated tubes. After collection the blood was centrifuged for 10 minutes at 1,000-2,000 x g using a refrigerated centrifuge and plasma was immediately frozen and shipped on dry ice. Plasma amino acids were analyzed at the Baylor Research Institute (USA) and at Bioscientia Laboratories (Germany) by HPLC on an amino acid analyzer or at Mayo Clinic (USA) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Due to the Libyan revolution dry ice shipment was not available in Libya, for family 1435. Therefore, blood was collected on Guthrie cards and analyzed by LC-MS/MS and flow injection analysis-tandem mass spectrometry (FIA-MS/MS).

Brain amino acids analysis

The left hemisphere of mouse brain was collected, weighed and immediately homogenized in 1 ml of ice-cold RIPA buffer in the presence of protease inhibitors. The tissue was disrupted using a glass homogenizer, transferred into a collecting tube, and left on ice for 1 hour. The homogenate was centrifuged for 40 minutes at 100,000 X g at 4 °C, the supernatant was collected and deproteinized by the addition of an equal volume of a solution of 8% (wt/vol) 5-sulfosalicylic acid and stored at -70°C until assayed for amino acid concentrations. At the time of the assay, the samples were thawed, mixed, centrifuged for 30 min at 5,000 Xg at 4°C to remove the precipitated protein and assessed by High Performance

Liquid Chromatography (HPLC). Amino acid concentrations were normalized using the wet tissue weight.

Cell culture

Mutant and control fibroblasts were generated from explants of dermal biopsies following informed consent under protocols approved by the University of California San Diego.

Induced pluripotent stem cells (iPS cells) were generated as previously described (*13*). Briefly, primary skin fibroblasts from affected and unaffected passage-matched control were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Gemini) and mesenchymal stem cell growth medium (MSCGM), respectively. Three micrograms of expression plasmid mixtures (*OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, *LIN28 and MYCL1)* were electroporated into $6X10^5$ of cells.

The cells were trypsinized 7 d after transduction, and $1.5X10^5$ cells were re-plated onto 100-mm dishes with $1.5X10^5$ irradiated CF-1 mouse embryonic fibroblasts (MEF) feeder layer. The culture medium was replaced the next day with standard hESC/iPSC media, DMEM:F12 (Invitrogen) supplemented with 20% KOSR (Invitrogen) and 20 ng/ml bFGF (Invitrogen). Colonies with good appearance were selected for further cultivation and evaluation.

After 3 passages iPS cells were transferred to MEFs free plates and growth in mTeSer media (Stem Cells Technologies).

Neural progenitors cells (NPCs) were obtained as previously described (*26*). Briefly, embryoid bodies (EBs) were formed by mechanical dissociation of cell clusters and plated in hESC medium without bFGF and kept shaking at 95 rpm

for 7 days. On the 7th day, EBs were plated onto poly-ornithine/laminin (Sigma)-coated dishes in DMEM/F12 plus 1XN2 (Invitrogen). Rosettes were visible to collect after 7 to 10 days, dissociated with Accutase (Chemicon), and plated again onto laminin-coated dishes with NPC media (DMEM/F12; 0.5X N2; 0.5X B27 and FGF2). To ensure reproducible and consistent data, homogeneous populations of NPCs were achieved by FACS sorting, as previously described (*14*). Briefly, cells were dissociated with Accutase for 20 minutes and CD184^{+}, CD24^{+}, CD44^{-}, CD271^{-} NPCs were FACS-purified and plated onto poly-ornithine/laminin-coated plates and cultured with bFGF.

To obtain differentiated neurons 1X10^{6} NPCs were plated in 1X10 cm plated coated with poly-ornithine/laminin and differentiated in DMEM:F12 supplemented with 1X B27, 20ng/ml BDNF and 20 ng/ml GDNF for at least 4 weeks before experiments. Media was replaced every 2-3 days.

Immunocytochemistry and immunohistochemistry

Cells were fixed in 4% paraformaldehyde for 10 minutes and subsequently permeabilized with 0.5% Triton X-100 in PBS. Cells were then incubated for 1 hour with the blocking solution (PBS containing 0.5% Triton X-100 and 5% donkey serum) followed by incubation with primary antibody, in PBS:blocking solution (1:1), overnight at 4 °C. After three washes with PBS, cells were incubated with fluorescently labeled secondary antibodies (Jackson ImmunoResearch) for 1 hr at room temperature. Fluorescent signal was detected using an Olympus FluoView 1000 inverted confocal microscope and images were processed with Photoshop CS5 (Adobe Systems). For

immunohistochemistry mice were transcardially perfused with 4% paraformaldehyde in phosphate buffer (pH 7.4). Tissues were further fixed in the same buffer for additional 2 hours and embedded sucrose-based media.

The following primary antibodies were used: Tra1-81 (1:200, Chemicon), Sox-2 (1:1000, Millipore), Nanog (1:200, Santa Cruz), SSAE3 (1:200, Chemicon), Tra1-60 (1:200, Chemicon), Nestin (1:2000, Chemicon), VGlut1 (1:400, Synaptic System), GABA-A (1:200, Millipore), Tuj1 (1:500, Covance), Map2(1:1000, Sigma), Cux1 (1:400, SantaCruz), Ctip2 (1:400, Abcam).

Mice

Experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. The mice were fed diets containing either 2% or 7% BCAAs (W/W) consisting of a ratio of 2:1:1.2 (leucine:isoleucine:valine). Typical chow diets contain 2-3.5% BCAAs. Mice were observed for hind-limb clasping and/or seizures, twice a day. When indicated in the figures, the diet was changed to either 2% or 7% BCAA content. The mice were observed twice a day for the additional time indicated.

Human Branched Chain Amino Acid Supplementation

A powdered branched chain amino acid mixture with a ratio of leucine:isoleucine:valine 1:1:1 (Jo Mar Laboratories, Campbell, California, USA) or 2:1:1 (Ajinomoto) were used to supplement the affected individuals in Family 18 and 558, respectively.

Based on a protocol accepted by the human investigations committee, we assayed pre-supplementation amino acids in the affected individuals and began adding BCAA supplement to their beverages three (Family 558) or four times (Family 18) daily during breakfast, lunch, dinner, and a night-time snack.

For 18-IV-1, the total daily starting amount of supplement was 0.5 grams/kg/day. Approximately 4 weeks later this was increased to 0.7 grams/kg/day.

For 18-IV-2, the total daily starting amount of supplement was 0.64 grams/kg/day. Approximately 4 weeks later this was increased to 0.8 grams/kg/day.

For 558-IV-2 and 558-IV-3 the starting amount was 0.6 g/Kg/day. Approximately 2 weeks later this was increased to 1 g/Kg/day.

Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

SeattleSeq Annotation, http://gvs.gs.washington.edu/

Genome browser, http://www.genome.ucsc.edu/

UCSC gene ID for BCKDK cDNA: uc010cai.3

SUPPLEMENTARY TEXT

558-IV-2 was born at full term by normal delivery to a healthy mother (G5P2Ab3) after an uneventful pregnancy. There was no history of intellectual disability or epilepsy in the family. The mother and the healthy brother (IV-1) reported episodes of migraine headaches. At 4 months of age 558-IV-2 had bilateral cataracts that were corrected at the age of 2. She developed normally until the age of 18 months, when she had her first seizure. Parents reported that there was also a single febrile seizure before the age of 2. She walked at the age of 2 and said her first word at the age of 4. She tested negative for known metabolic disorders. No chromosomal abnormalities were identified. Cranial MRI was normal. EEG showed epileptic activity in the left hemisphere, and she was treated with oxcarbazepine. At the time of our first examination she was 15 years and 7 months old. The parents reported difficulties with communication, including the inability to speak in sentences, social disabilities, including the inability to play with children her age, repetitive behaviors, and symptoms of intellectual disability, including deficits in the activities of daily living. She met the DSM-IV-TR (*27*) criteria for autism. Physical examination was normal except for hyperactive deep tendon reflexes. Head circumference was 49 cm (-3 SD) (Table S8), indicating a mild microcephaly with bi-frontal narrowing. New EEGs were performed at the age of 20 and 21, and both reported extra temporal (bilateral frontal) and generalized epileptiform abnormalities. She is currently taking risperidone and valproate. Despite these medications, she has generalized,

tonic-clonic seizures, every 3 months, that last 3-4 minutes each.

At the age of 21, no substantial improvements were observed. For instance, she was still unable to speak in sentences, to take care of herself, to follow general safety rules at home, to cross the street alone, to say “hello” and “good-bye” at the appropriate time, to control anger, and she could not complete a simple puzzle.

Plasma amino acids levels were within the normal range except for significant reductions in BCAA levels (Table S3). Urine amino acids and plasma organic acids are reported in Table S6 and S7, respectively.

558-IV-3 was born at full term to a healthy mother (G6P3Ab3). She was generally healthy until the age of 40 days when she had hypocalcemic convulsions. At 18 months she began walking and said her first word. She was toilet trained at the age of 6. She tested negative for known metabolic disorders. No chromosomal abnormalities were identified. Cranial MRI was normal. At the time of our first examination she was 11 years and 9 months old. The parents reported symptoms of difficulties with communication, including the inability to speak in sentences, social disabilities, including the inability to play with children her age, repetitive behaviors, and symptoms of intellectual disability, including deficits in the activities of daily living. She met the DSM-IV-TR criteria for autism. Physical examination was normal except for hyperactive deep tendon reflexes. Head circumference was 51 cm (Table S8). A previous EEG reported epileptic

discharges while she was sleeping and focal spikes in the left hemisphere. She was treated with oxcarbazepine.

At the age of 16 years, no substantial improvements were observed. The parents reported that she was still unable to speak in sentences, to take care of herself, to follow general safety rules at home, to cross the street alone, to say “hello” and “good-bye” at the appropriate time, and to control her anger. She could not complete a simple puzzle.

Plasma amino acids levels were within the normal range except for significant reductions in BCAA levels (Table S3). Urine amino acids and plasma organic acids are reported in Table S6 and S7 respectively.

18-IV-1 was born at full term by normal vaginal delivery to a G1P0, healthy mother who received antibiotics during the first 2 weeks of pregnancy for an upper respiratory infection that resolved immediately. There were no other medications or exposures during the pregnancy. The pregnancy and delivery were uneventful. 18-IV-1 weighed 3.8 kg with normal APGAR scores. She had physiological jaundice that resolved after several days. She fed normally and had no difficulties with transitioning to solid foods and has eaten a normal, varied diet since. There were no growth abnormalities during infancy or toddlerhood. In terms of milestones, she did not sit until one year. She never crawled and began to walk at two years. She said her first words at two years old and never spoke in sentences.

Her parents brought her to medical attention at two years of age secondary to concerns about delays in walking, language development, intellectual ability, and social interaction. Upon interview when she was 5 years 11 months old, the parents reported significant social difficulties including that she would not play normally with same-age peers and did not interact normally with adults either. She did not engage in make-believe play. She could use only approximately 10 partial words meaningfully and could not use two-word sentences. Her parents indicated that she seemed to be able to understand more words than she could utter but many fewer than they expected for her age. She reportedly could use some gestures like waving “bye-bye,” but did not nod her head to reply “yes” or point to an object that she desired. She manifested a number of repetitive behaviors, including flapping her arms, whirling her head about, and staring for long periods at her hands as she moved them in front of her eyes. She began receiving speech and behavioral therapy twice a week at age two. At 5 years and 11 months she could feed herself, pull a chair over to reach something on a high shelf, and imitate actions. She was becoming toilet trained for urine and feces. She could not participate in schooling. Upon interview when she was 7 years and 7 months, the parents noted no significant changes in her presentation.

She had one febrile seizure at one year of age and a generalized, non-febrile seizure at age 8. There were no significant head traumas. She was treated with risperidone that was withdrawn secondary to hyperprolactemia and atomexetine that was discontinued because it had no benefit. After her non-febrile seizure she was started on carbamazepine.

In terms of family history, the parents are normal. The younger brother, 18-IV-2, has autism. A paternal uncle has schizophrenia. A second degree cousin has intellectual disability. A distant cousin reportedly has schizophrenia. Full physical and neurological examinations at age eight were normal. There were no obvious dysmorphologies. She was left-hand dominant. Stature, weight, and head circumference were normal during the course of development (Table S8). Hearing by ABR was normal bilaterally. CT brain, Fragile X, chromosomal analysis, and CNV analysis were all negative. EEG was normal at age 4 and abnormal at age 8, with “frequent focal spike wave complexes over the left temporal region consistent with left temporal epileptogenic dysfunction.” Plasma amino acids were essentially normal except for significant reductions in BCAAs (Table S4). Repeat fasting plasma amino acids using a dried blood spot card and LC-MS and FIA-MS/MS at the Mayo Clinic confirmed these initial results. (Table S10). Pre-albumin and CRP were analyzed on the same day at the same laboratory. Pre-albumin was minimally decreased at 0.19 g/L (0.2-0.4). CRP was normal. Fasting urine amino acids obtained at the same laboratory were clinically unremarkable and BCAAs in the urine were not elevated. (Table S6). Electrolytes and liver function tests were normal. Lipid profile was normal except for a mild elevation in LDL to 127 mg/dl (0-100). Nutritional analysis at age 8 was normal (Table S9).

ADIR (administered in Arabic) scores were as follows at 5 years 11 months: Qualitative abnormalities in reciprocal social interaction - 26 (autism cutoff is 10), Qualitative abnormalities in communication - 14 (autism cutoff is 7 if non verbal

and 8 if verbal), and Restricted, repetitive and stereotyped pattern of behavior - 3 (autism cutoff is 3). CARS (Administered in Arabic) score at age 5 years 11 months was 34 (autism cutoff is 30). At age 8 it was 33. ADOS at age 5 years and 11 months, reported by the original scoring algorithm was as follows: Communication - 8 (autism cutoff 4), Reciprocal Social Interaction - 9 (autism cutoff 7), Communication and Social Interaction Combined - 17 (autism cutoff 12), Play- 2, and Stereotyped Behaviors and Restricted Interests - 3. The parents report that at age 4 years old she received IQ testing and a score of 59. The type of test is unknown. At 7 years 7 months, a Vineland administered in Arabic gave a composite adaptive behavior score of 31.3 – in the low range.

Based on interviews at age 5 years and 11 months and 7 years and 7 months she met DSM–IV-TR criteria for autism and intellectual disability. The diagnosis of autism was corroborated by the structured interviews listed above conducted by the authors.

18-IV-2 was born at full term by normal vaginal delivery to a G2P1, healthy mother. The mother took no medications and had no exposures during the uneventful pregnancy. Delivery was uneventful and 18-IV-2 weighed 4 kg at birth with normal APGAR scores. There were no medical issues at birth. He breastfed normally and had no difficulties with transitioning to solid foods. He has eaten a normal, varied diet since. There were no noted growth abnormalities. In terms of milestones, he was delayed in sitting. He never crawled and began to walk at

age 26 months. He said his first words at two years old and never spoke in sentences.

His parents brought him to medical attention at two years of age secondary to concerns about delays in walking, language development, intellectual ability, and social interaction. Upon interview when he was 2 years 11 months old, the parents stated that he never brought others objects including toys to show enjoyment or to engage them in play. They reported that he never pointed to objects. His parents said that he could use only around 5 words meaningfully and could not use simple, two-word sentences. He could not understand simple commands. He reportedly could use some gestures like waving “bye-bye,” but did not nod his head to reply “yes.” He would repetitively bite himself. Upon interview when he was 4 years and 8 months of age, the parents reported no significant differences in presentation except that he had increased repetitive movements, including intermittent hand stereotypies and flapping of his arms. He received twice weekly behavioral therapy starting at age two. At age 2 years 11 months, he could imitate others, feed himself, and was starting to clothe himself. He was not toilet trained for urine or feces. At age 4 years 8 months he understood the concept of a puzzle but could not complete even a simple one. He could not unscrew a disk from a peg. He was starting to be trained to toilet and clothe himself.

In terms of medical history,18-IV-2 had 4 closely spaced febrile seizures at age 12 months. He was started on valproate and had a recurrence of febrile seizure once every 4 to 5 months subsequently. At age 2 years and 5 months he started

phenobarbital and has not had seizures since. At 2 years and 9 months of age the patient underwent a surgery to “release his tongue” because he was “tongue tied.” There were no significant head injuries.

Full physical and neurological examination at age 5 were normal and revealed no gross dysmorpholigies. He was left-hand dominant. Hearing by ABR was normal. CT brain, Fragile X, Cytogenetics, and CNV analysis were all negative. EEG was normal at age 5 but abnormal on multiple previous occasions with a report of “Center encephalic epileptogenic dysfunction.” Fasting plasma amino acids were essentially normal except for significant reductions in BCAAs (Table S4). Repeat fasting plasma amino acids using a dried blood spot card and LC-MS and FIA-MS/MS at the Mayo Clinic confirmed these initial results. (Table S10). Pre-albumin and CRP were analyzed on the same day at the same laboratory. Pre-albumin was minimally decreased at 0.16 g/L (0.2-0.4). CRP was normal. Fasting urine amino acids obtained at the same laboratory were clinically unremarkable and BCAAs in the urine were not elevated. (Table S6). Urine organic acids obtained at the same laboratory were normal. Electrolytes and liver function tests were normal. Lipid profile was normal. Nutritional analysis at age 5 was normal (Table S9).

ADIR (*28*)(administered in Arabic) scores at age 2 years 11 months were as follows: Qualitative abnormalities in reciprocal social interaction - 18 (autism cutoff is 10), Qualitative abnormalities in communication - 9 (autism cutoff is 7 if non-verbal and 8 if verbal), and Restricted, repetitive and stereotyped pattern of behavior - 4 (autism cutoff is 3). CARS (*29*)(administered in Arabic) score at age

5 years 1 month was 34.5 (autism cutoff is 30). ADOS (*30*)(administered in Arabic) scores at age 2 years 11 months and reported by the original scoring algorithm were as follows: Communication - 7 (autism cutoff 4), Reciprocal Social Interaction - 11 (autism cutoff 7), Communication and Social Interaction Combined - 18 (autism cutoff 12), Play - 3, and Stereotyped Behaviors and Restricted Interests - 2. At age 4 years and 8 months ADOS reported by the original scoring algorithm was as follows: Communication - 5 (autism cutoff 4), Reciprocal Social Interaction - 11 (autism cutoff 7), Communication and Social Interaction Combined - 16 (autism cutoff 12), Play - 3, and Stereotyped Behaviors and Restricted Interests - 1. There is no formal IQ testing. In terms of adaptive behavior, a Vineland was administered when he was 4 years and 8 months old in Arabic, and the composite adaptive behavior score was 40.5 – in the low range.

Based on the clinical presentation he met DSM-IV-TR criteria for autism and intellectual disability. The diagnosis of autism was corroborated by the structured interviews listed above conducted by the authors.

1435-IV-6 was born at full term after an uneventful pregnancy. The parents are healthy. There is no history of epilepsy or intellectual disability in the family. 1435-IV-6 has 8 healthy siblings who are doing well at school, have normal motor skills, and socialize normally. The five healthy male siblings all have below average head circumference (-1.5 SD). However, none of them has signs of intellectual disability. 1435-IV-6 was brought to medical attention when he was 6

years old secondary to parental concern about his development. In particular, the parents reported that he could not speak. At the time of our first visit, he was 9 years old. The parents reported that he had no words and that they were concerned about his intellectual ability. During our visit, he understood and was able to follow simple directions. However, he never spoke. He was unable to reproduce a circle, a square, or two intersecting rhombi. Physical examination was normal. Head circumference was 47.5 cm (-3SD) (Table S8). Cranial MRI was normal. An EEG at 11 years showed no abnormalities. ADI-R (administered in Arabic) scores were as follows at 7 years: Qualitative abnormalities in reciprocal social interaction - 27 (autism cutoff is 10), Qualitative abnormalities in communication - 18 (autism cutoff is 7 if non verbal and 8 if verbal), and Restricted, repetitive and stereotyped pattern of behavior - 7 (autism cutoff is 3). CARS (*29*)(administered in Arabic) score at age 7 years was 39.5 (autism cutoff is 30). Based on the clinical presentation he met DSM-IV-TR criteria for autism and intellectual disability. The diagnosis of autism was corroborated by the structured interviews listed above conducted by the authors.

Plasma amino acids levels were essentially normal except for a decrease in arginine and an increase in aspartate that were also present in all healthy family members and are of unknown clinical significance. While there were no internal laboratory controls available for reference, BCAAs were markedly reduced compared with normal siblings and fell at the very low end of normal or below normal compared to published reference ranges (*31*) (*32*) (Table S5).

1435-IV-8 was born at full term after an uneventful pregnancy. He was brought to medical attention, when he was 4 years old by his parents who were concerned about his development. In particular, the parents reported that 1435-IV-8 could not speak. Upon our examination at age 5 years, the parents reported that he still had no words and that they were concerned about his intellectual ability. He understood and was able to follow simple directions, however, he never spoke. He was unable to reproduce a circle, a square, or two intersecting rhombi. Physical examination was normal. Head circumference was 47 cm (-3SD) (Table S8). He had no history of epilepsy. During our visit he understood and was able to follow simple directions. MRI was normal. An EEG at 7 years was described as abnormal, with right temporal slowing and rare surface negative frontal sharp waves. ADI-R (administered in Arabic) scores were as follows at 11 years: Qualitative abnormalities in reciprocal social interaction - 28 (autism cutoff is 10), Qualitative abnormalities in communication - 21 (autism cutoff is 7 if non verbal and 8 if verbal), and Restricted, repetitive and stereotyped pattern of behavior – 10 (autism cutoff is 3). CARS (*29*)(administered in Arabic) score at age 9 years was 46 (autism cutoff is 30). Based on the clinical presentation he met DSM-IV-TR criteria for autism and intellectual disability. The diagnosis of autism was corroborated by the structured interviews listed above conducted by the authors.

Plasma amino acids levels were essentially normal except for a decrease in arginine and an increase in aspartate that were also present in all healthy family members and are of unknown clinical significance. While there were no internal

laboratory controls available for reference, BCAAs were markedly reduced compared with normal siblings and fell at the very low end of normal or below normal compared to published reference ranges (Table S5).

Supplementation results

Dietary supplementation of the affected individuals in families 558 and 18 with BCAAs was initiated as soon as low BCAAs were confirmed and human subjects approval was obtained. Our initial aim was to determine whether plasma BCAA levels could be increased with supplementation. Currently, we continue to make adjustments in supplementation amount and frequency in order to achieve stable normalization of plasma BCAAs throughout the day to the greatest extent possible. When maximal stabilization has been achieved, our ultimate goal will be to determine whether measurable phenotypic amelioration can be attained in signs and symptoms of autism, intellectual disability, and/or epilepsy.

Transient super-elevations in plasma BCAAs were observed at 1 hour after supplementation taken with a meal at the highest supplementation amount given to date for each affected individual with supplementation amounts ranging between 0.7 and 1 g/kg/day (Table S10). At this supplementation amount a trend toward normalization of plasma BCAAs was observed in the fasting state for all affected individuals. These improvements in plasma levels of BCAAs occurred without measurable alterations in the plasma levels of other amino acids. Urine organic acids in the affected individuals from family 18 showed no accumulation of BCAA-related organic acids. Results from urine organic acid tests from family 558 are pending. To date, no adverse side effects have been reported. When

BCAAs are maximally stabilized, we plan to undertake phenotypic follow-up in both families, including serial EEG's, serial neuropsychiatric assessments, including cognitive tests and structured assessments of autism, and serial physical and neurological examinations. Supplementation of the affected individuals in family 1435 was delayed as a result of the Libyan revolution and will be initiated as soon as logistically possible.

Novarino et al. Figure S1

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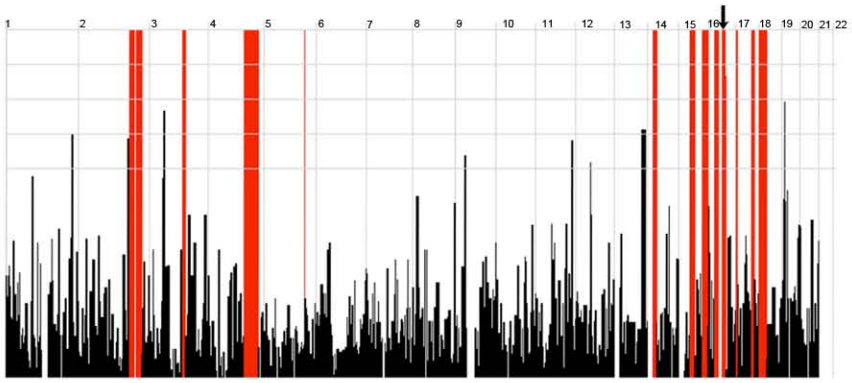

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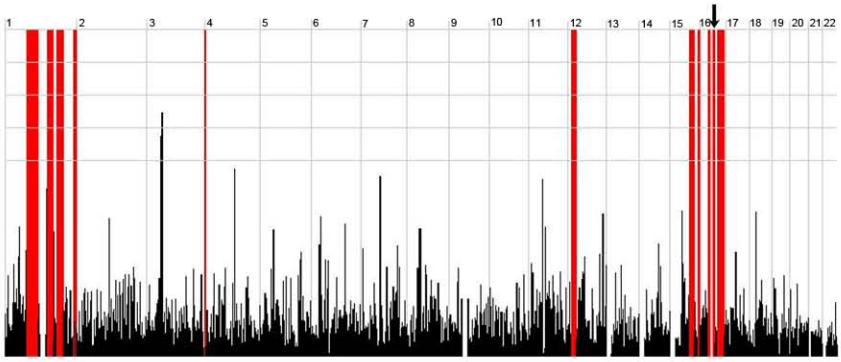
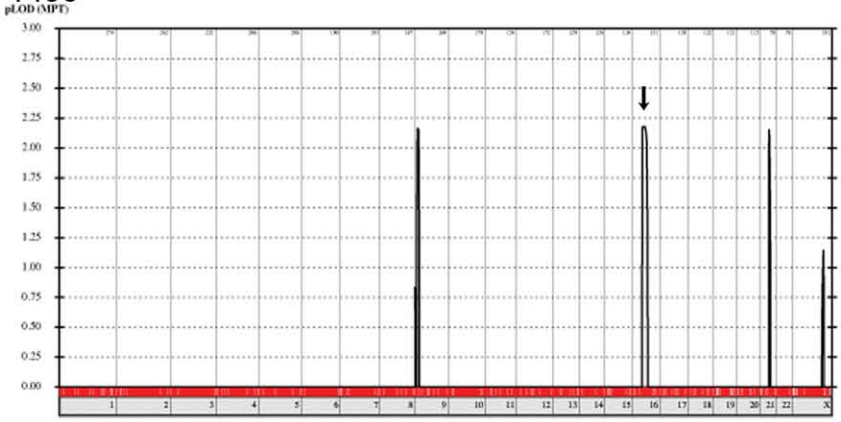


1435



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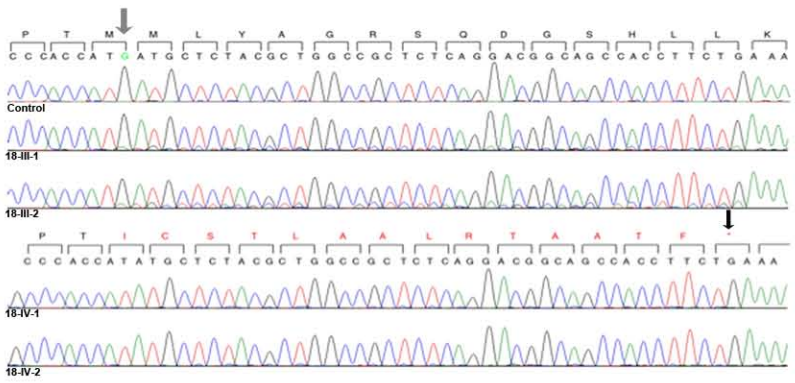
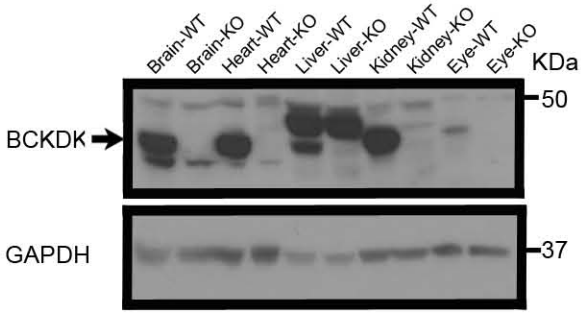
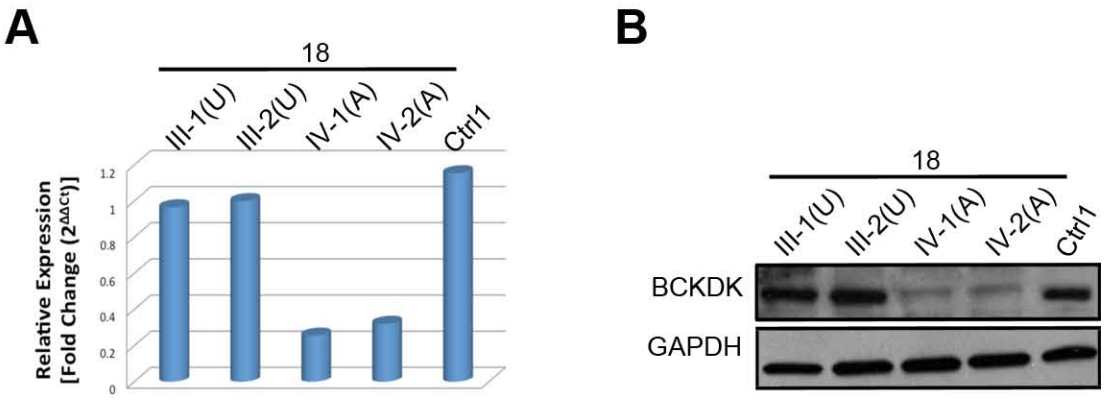


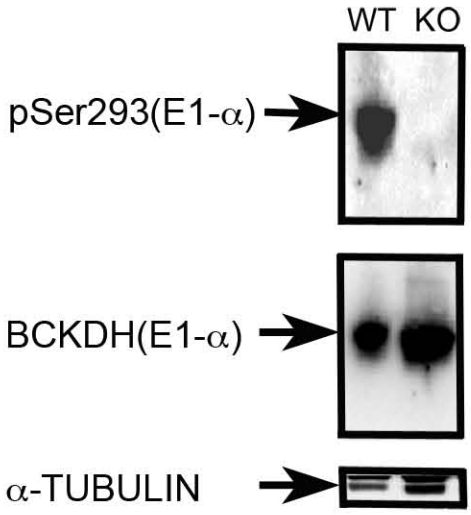
Novarino et al. Figure S2



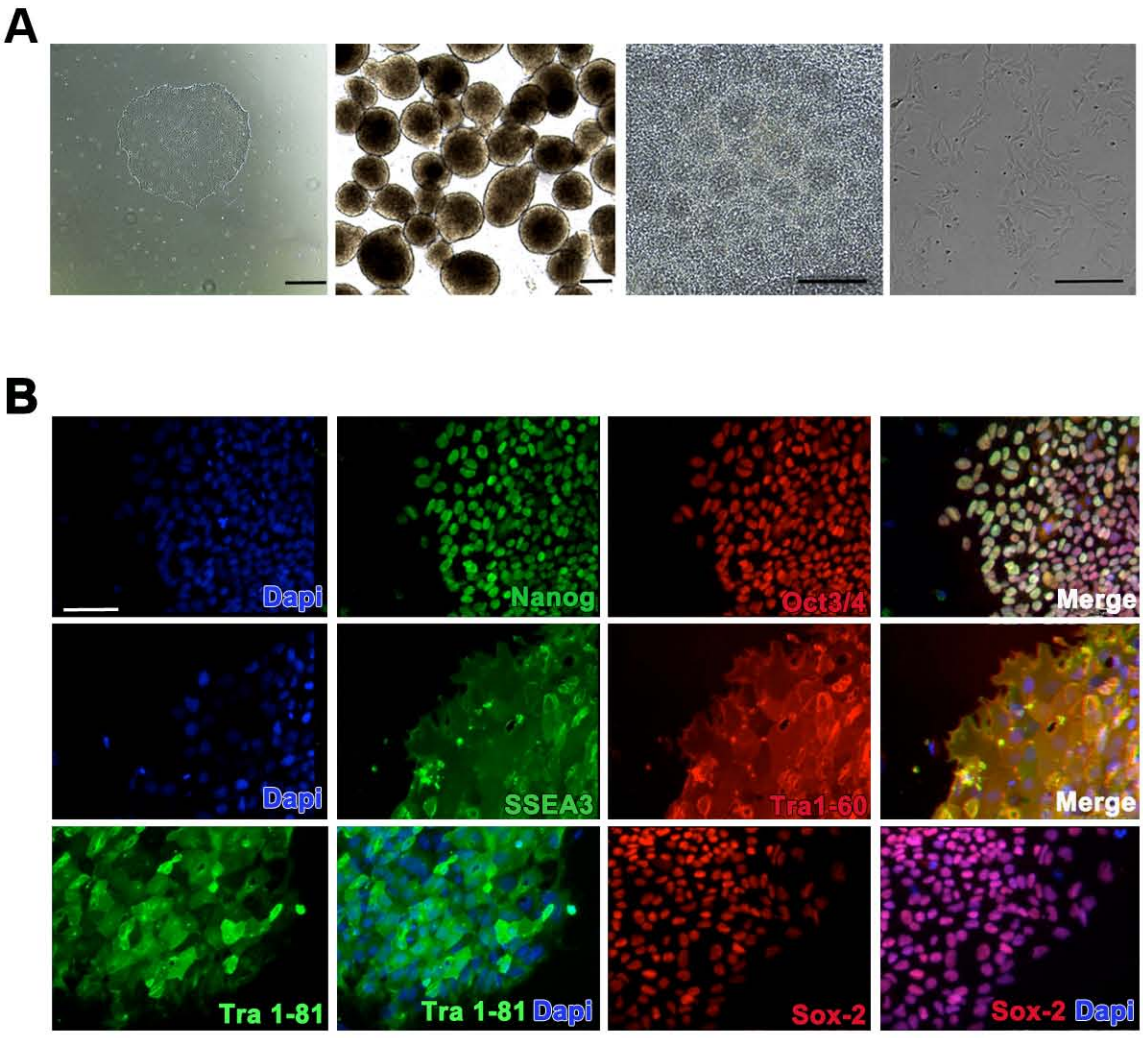
Novarino et al. Figure S3



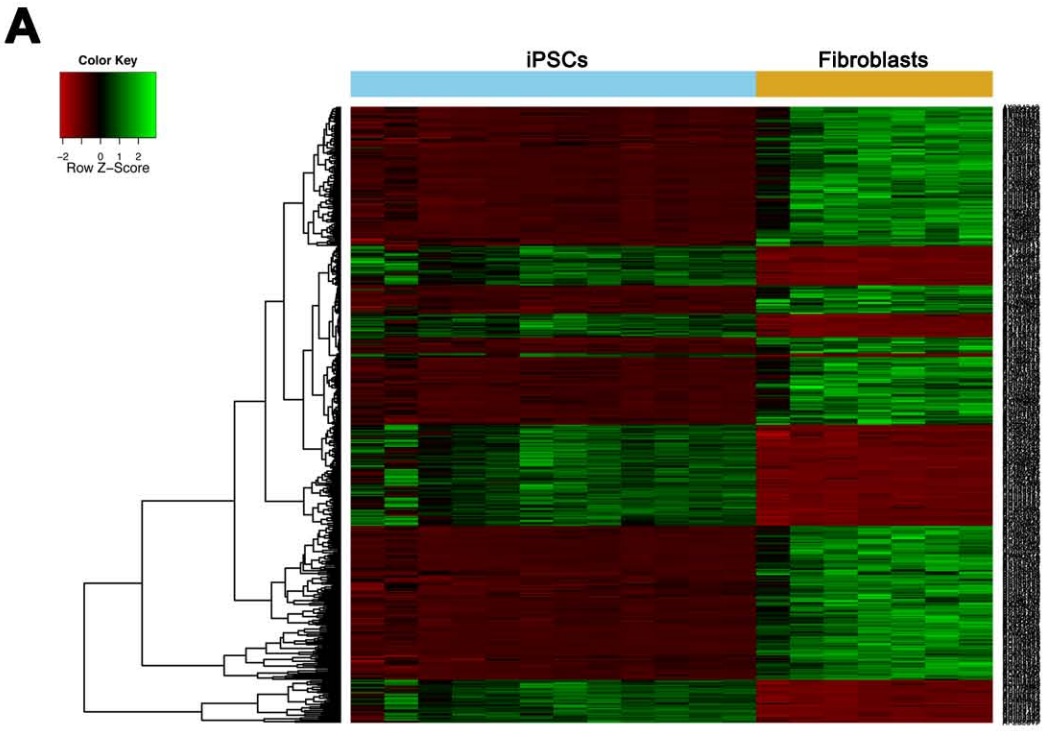
Novarino et al. Figure S4

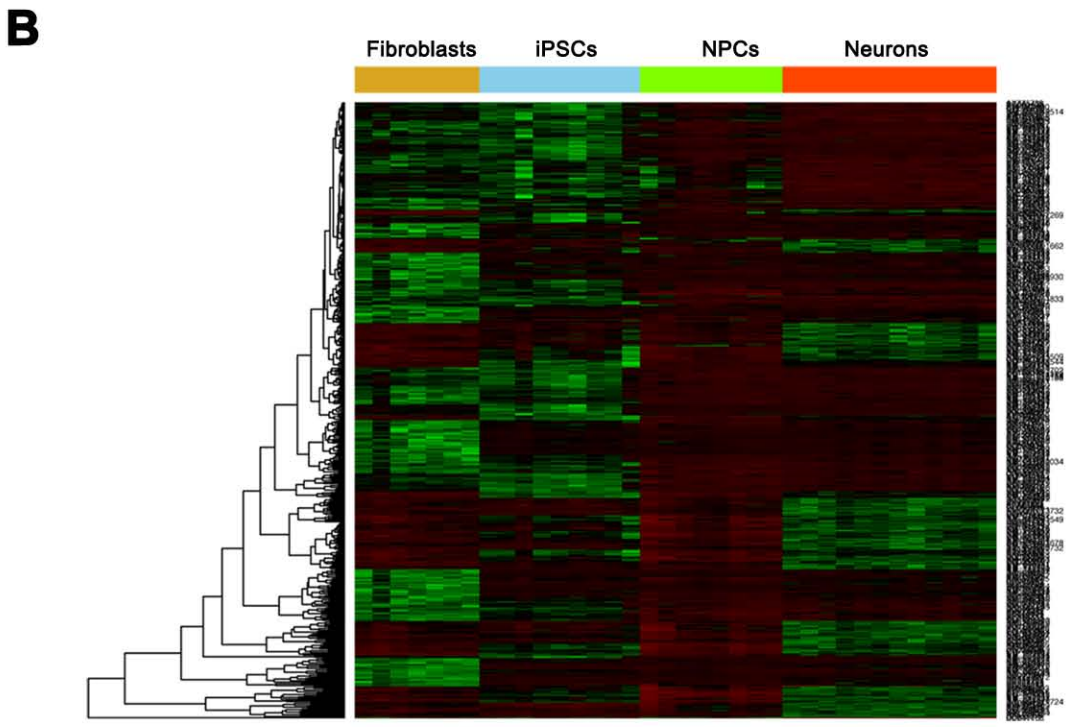


Novarino et al. Figure S5

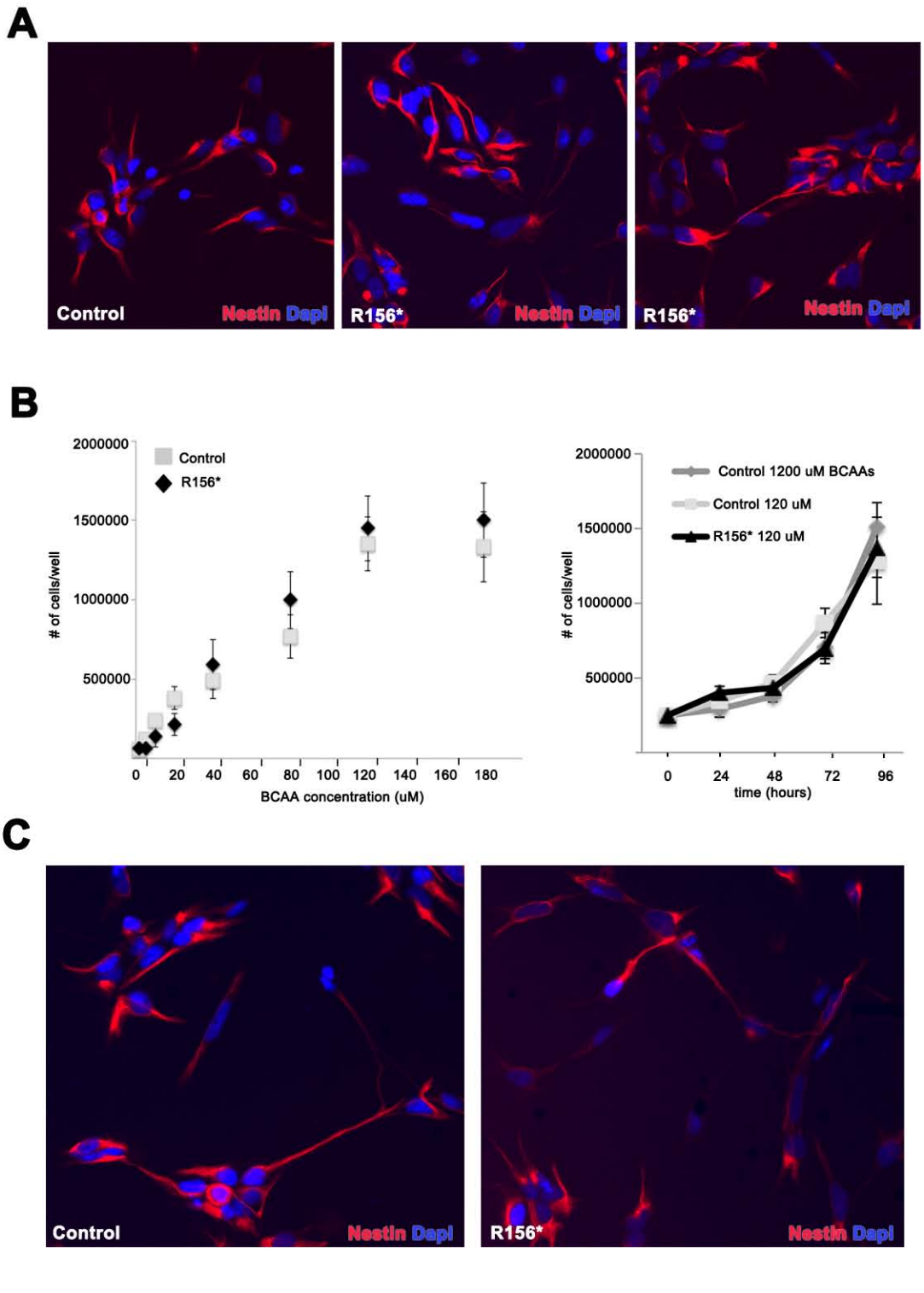


Novarino et al. Figure S6

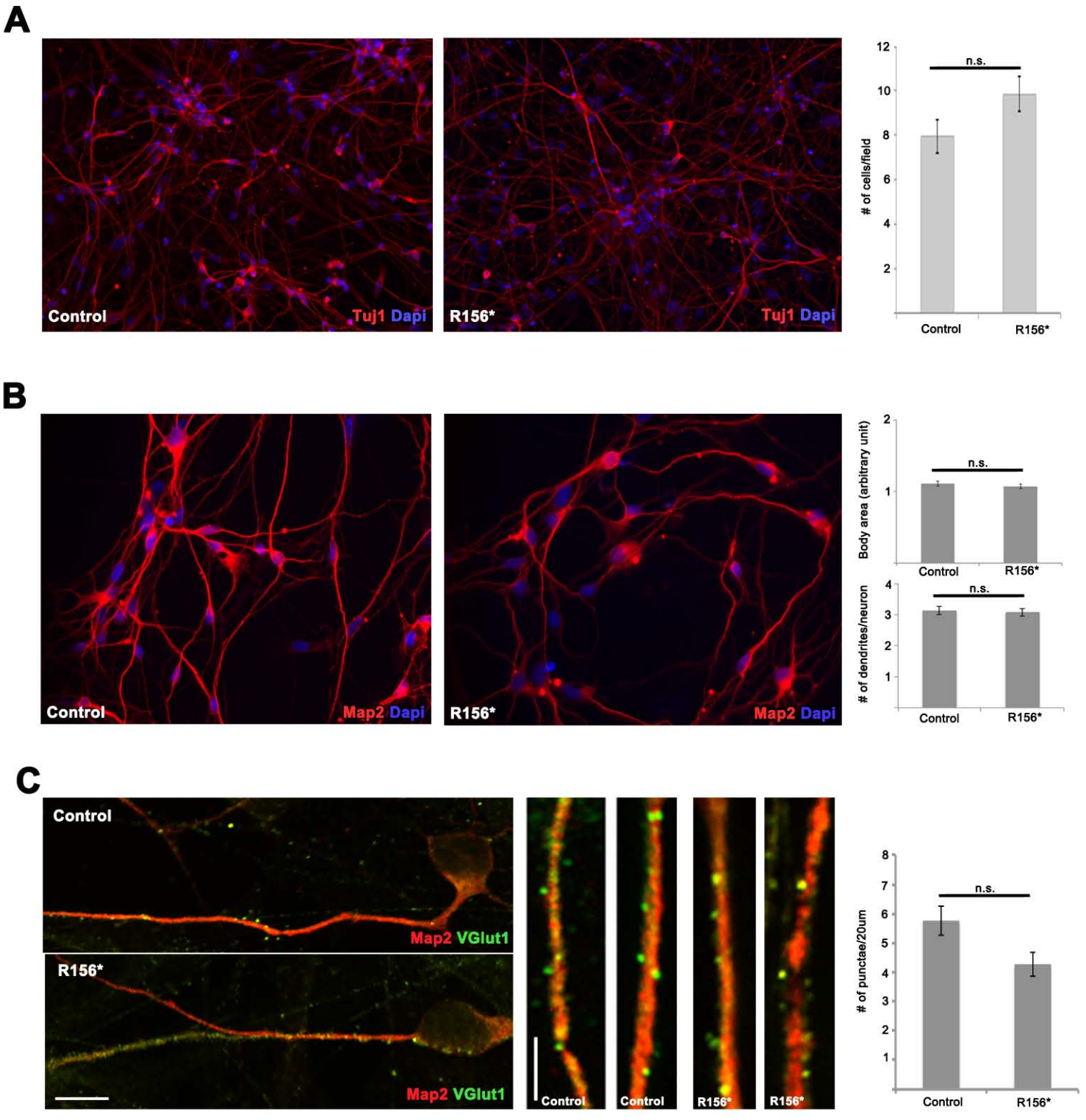




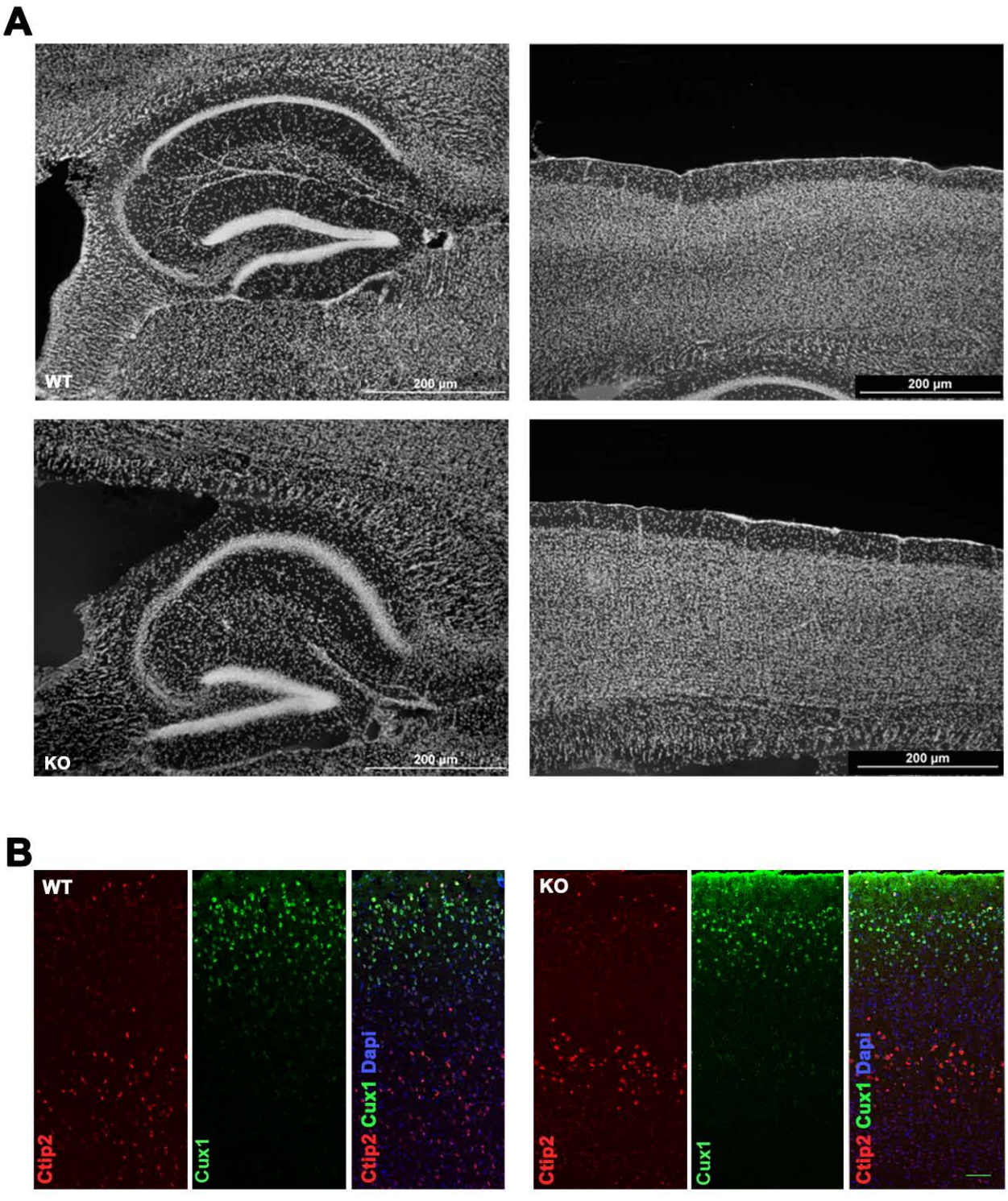
Novarino et al. Figure S7



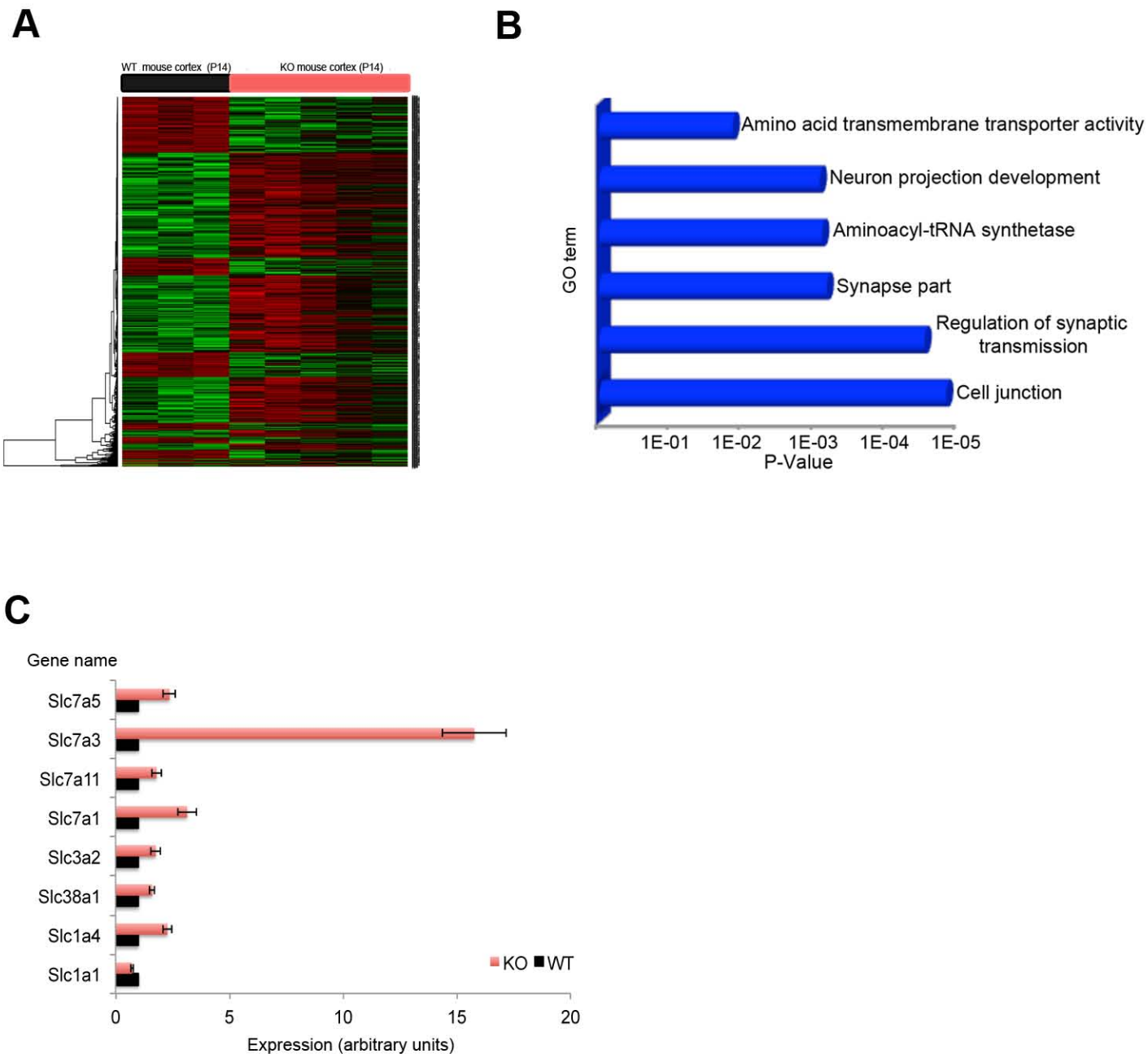
Novarino et al. Figure S8



Novarino et al. Figure S9



Novarino et al. Figure S10



Supplementary Figure Legends

Fig. S1: Genotyping information for families 558, 18, and 1435. A) Homozygosity map (558 and 18) and linkage plot (1435) for the consanguineous families with mutations in the *BCKDK* gene. Blocks of homozygosity are depicted as red peaks. Arrows indicate the interval that includes *BCKDK*. B) cDNA sequences of all the individuals of Family 18. Arrows point to the deleted base (grey) and to the premature stop codon in the two affected individuals (black).

Fig. S2: Tissue distribution of BCKDK. Western blot analysis of BCKDK expression in mouse tissues. GAPDH was used as loading control.

Fig. S3: Deleterious effect of M74fs mutation in affecteds of Family 18. A) Real-Time RT-PCR demonstrates a 3 to 4 fold decrease in BCKDK mRNA levels in the probands (IV-1 and IV-2) relative to their parents (III-1 and III-2) and an unrelated control (Ctrl1). B) BCKDK protein expression in peripheral lymphocytes determined by Western blot. The two affected (A) individuals (IV-1 and IV-2) show less expression than the parents (III-1 and III-2) and an unrelated control (Ctrl1).

Fig. S4: BCKDH-E1-α subunit hypophosphorylation in *Bckdk* KO animals. Western blot analysis shows the absent Serine-293 phospho-specific band (pSer932(E1-α) in the *Bckdk* KO brain lysate despite a normal expression level of BCKDHA-E1a.

Fig. S5: Generation of iPSCs and NPCs. A) Representative images of iPSCs clone, embryoid bodies, neural rosettes, and neural progenitors cells (left to right). B) iPSCs from affected individuals show expression of the pluripotent markers Nanog, Oct3/4, SSEA3, Tra1-60, Tra1-81 and Sox-2. Scale bars: 500 um (A) (iPSCs), 200 um (A) (EBs, rosettes and NPCs), 100um (B).

Fig. S6: Gene expression analysis of cells generated during fibroblast reprogramming. A) Heatmap depicting the expression of genes differentially regulated between fibroblasts and iPSCs. B) Heatmap of differentially regulated genes in fibroblasts, iPSCs, NPCs, and neurons. Each column represents a single cell line. Genes that are highly expressed are in red and poorly expressed genes in green.

Fig. S7: Cell proliferation and morphology of R156* NPCs. (A) Neural progenitors cells from the unaffected (left) and affecteds (center and right) were stained with the NPC marker nestin (red) and DAPI (blue) (20X objective). B) 2.5 X 10^5 cells from unaffected and affected patients were plated in media containing different concentrations of BCAAs. Media was changed every day, and cells from multiple clones and wells were counted after 96 hours (left). NPC proliferation curve in normal (1200 uM BCAA) and modified (120 uM BCAA) media (right). Error bars indicate standard deviation. (C) Representative images of control and R156* mutant NPCs in 120 uM BCAA (20X objective).

Fig. S8: R156* neurons display unremarkable morphology and presynaptic marker density. Representative images of Tuj1 (A) and Map2 (B) staining of iPSC-derived neurons of unaffected (left) and affected (right) individuals and quantification of cell density (A), neuron body size (ctrl n=153, R156* n=164) and number of dendrites per neuron (ctrl n=109, R156* n=131) (B). Error bars represent standard error. Dapi (blue) was used to stain nuclei. Magnifications: 20X (A), 40X (B). C) VGlut1 (green) stained presynaptic vesicular glutamate transporter. No major differences were observed between control and R156* neurons. Scale bars 10 and 5 um. Quantification of VGlut1 positive punctae density is shown on the right . Error bars represent standard deviation.

Fig. S9: Absence of gross alterations in brain architecture in *Bckdk*$^{-/-}$ mice. A) Nuclei staining of hippocampus (left) and cerebral cortex (right) in *Bckdk*$^{-/-}$ and their wildtype littermates showing no gross morphological abnormalities. B) Cerebral cortex of wildtype and *Bckdk* knockout animals stained with Cux1 and Ctip2 showing no major abnormalities in cell layers distribution. Scale bars 60 um.

Fig. S10: Gene expression analysis of *Bckdk*$^{-/-}$ animals brain cortex. (A) Heatmap depicting the expression of genes differentially regulated between wildtype and Bckdk$^{-/-}$ brain cortices. Each column represents a single mouse. Genes that are highly expressed in knockout cells relative to controls are shown

in red. (B) Relevant gene ontology categories enriched in differentially expressed genes. C) Normalized expression of brain amino acids transporters in *Bckdk*$^{-/-}$ relative to wildtype mice.

Novarino et al. Table S1

Table S1: Phenotypic and genetic information of the patients carrying mutations in the *BCKDK* gene.

Abbreviations: ID, Intellectual Disability; EEG, electroencephalogram; BCAAs, branched chain amino acids

Family data				Clinical data				Genetic data	
Identification	Age (y)	Sex	Origin	Diagnoses	EEG	Seizures	BCAAs	Nucleotide change	Amino acid change
558-IV-2	22	F	Turkey	autism[a], ID[a]	abnormal	no	low	C466T	R156*
558-IV-3	18	F	Turkey	autism[a], ID[a]	abnormal	yes	low	C466T	R156*
18-IV-1	8	F	Egypt	autism[a,b,c,d], ID[a]	abnormal	yes	low	G222del	M74fs
18-IV-2	5	M	Egypt	autism[a,b,c,d], ID[a]	abnormal	yes	low	G222del	M74fs
1435-IV-6	11	M	Libya	autism[a,b,c], ID[a]	normal	no	low	G671C	R224P
1435-IV-8	7	M	Libya	autism[a,b,c], ID[a]	abnormal	no	low	G671C	R224P

Method of Diagnosis
a Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) criteria (American Psychiatric Association (2000))
b Childhood Autism Rating Scale (CARS)(Schopler, Reichler et al. 1986)
c Autism Diagnostic Interview Revised (ADI-R) (Lord, Rutter et al. 1994)
d Autism Diagnostic Observation Schedule (ADOS) (Lord, Risi et al. 2000)

Novarino et al. Table S2

Table S2: Other segregating, rare variants discovered in the affected individuals using whole-exome sequencing. Despite being novel relative to dbSNP, most of the mutations were not conserved across species and were predicted to be benign by the Polyphen algorithm (*1*).

Family	Gene Name	Amino Acid Change
558	ATP/GTP binding protein-like 1(*AGBL1*)	R504Q
558	Integrator complex subunit 2 (*INTS2*)	R33K
558	Zinc finger, MYND-type containing 15 (*ZMYND15*)	G457S
18	Neuron Navigator 1 (*NAV1*)	A912V
18	Calcium Channel, Voltage-Dependent, L type, alpha 1S Subunit (*CACNA1S*)	E1837A
18	Chitinase 1 (*CHIT1*)	S345G
18	Protein Tyrosine Phosphatase, Non-Receptor Type 9 (*PTPN9*)	V46M
1435	HEAT repeat containing 3 (*HEATR3*)	R337K

1. Adzhubei, I. A. *et al.,* A Method and Server for Predicting Damaging Missense Mutations. *Nat Methods* **7**, 248 (2010).

Novarino et al. Table S3

Table S3: Plasma amino acids of individuals of Family 558. Plasma amino acids of blood drawn on different days and analyzed by different laboratories for unaffecteds (U) and affecteds (A). Plasma BCAA levels of the affected patients are shown in red.

Plasma amino acids February 2011 (fasting for 14 hours, analyzed at Mayo Clinic by LC-MS/MS. Values are expressed in nmol/ml).

Patient / Amino Acid	558-III-1 (U)	558-III-2 (U)	558-IV-1 (U) (23y)	558-IV-2 (A) (21y)	558-IV-3 (A) (17y)	Reference Range <18 years old	Reference Range >18 years old
Taurine	62	149	217	76	146	10-170	54-210
Asparagine	47	57	56	39	42	23-112	35-74
Serine	97	128	118	112	121	69-137	58-181
Glycine	193	373	235	227	213	127-341	151-490
Glutamine	787	767	821	579	642	264-823	205-756
Histidine	86	92	105	92	80	41-125	72-124
Threonine	135	153	107	132	156	35-226	60-225
Citruline	31	36	28	21	22	1-46	12-55
B-alanine	<25	<25	<25	<25	<25	0-7	0-12
Alanine	531	478	406	381	440	152-547	177-583
Glutamate	95	69	111	55	74	5-150	10-131
Arginine	126	98	119	75	102	10-140	15-128
Proline	297	202	219	273	200	59-369	97-329
Ornithine	59	78	60	38	54	10-163	48-195
Cystine	42	45	47	37	30	5-45	5-82
Lysine	200	200	198	169	227	48-284	116-295
Methionine	40	38	48	26	31	7-47	1042
Tyrosine	85	74	91	87	82	24-115	34-112
Phenylalanine	74	68	87	54	77	28-91	35-85
Valine	208	185	254	44	57	74-321	119-336
Leucine	133	111	191	24	33	49-216	72-201
Isoleucine	83	55	101	13	15	22-107	30-108

Plasma amino acids June 28 2011 (fasting for 14 hours, analyzed at the Baylor Research Institute by HPLC. Values are expressed in uM).

Patient / Amino Acid	558-IV-1 (U)	558-IV-2 (A)	558-IV-3 (A)	Reference Range
Phosphoserine	<1	<1	<1	0-12
Hydroxyproline	9	15	13	0-37
Histidine	83	108	78	42-116
Asparagine	43	66	53	30-78

Taurine	55	73	76	33-92
N-1-Methylhistidine	6	8	4	0-21
Serine	117	184	151	63-185
Glutamine	550	652	492	424-720
Carnosine	7	6	5	0-0
Arginine	128	193	175	20-122
Glycine	236	411	274	117-366
Anserine	<1	<1	<1	0-0
Ethanolamine	8	8	12	0-1-
Aspartic acid	23	29	27	0-10
Sarcosine	<1	<1	<1	0-0
Glutamate	82	79	78	28-112
Citrulline	29	27	24	11-51
B-alanine	3	<1	<1	0-0
Threonine	93	286	243	62-182
Alanine	463	868	793	162-572
GABA	<1	<1	<1	0-0
A-aminoadipic Acid	<1	<1	<1	0-13
Proline	260	484	340	65-380
B-Aminoisobutyric Acid	<1	<1	<1	0-0
Hydroxylysine	<1	<1	<1	0-1
A-Aminobutyric Acid	12	8	1	7-33
Cystathionine	<1	<1	1	0-0
Ornithine	56	62	63	36-118
Cystine	7	7	5	9-53
Lysine	164	319	292	122-285
Tyrosine	48	89	91	30-100
Methionine	31	42	36	14-41
Tryptophan	111	83	102	23-86
Alloisoleucine	<1	<1	<1	0-0
Phenylalanine	97	92	104	35-83
Homocystine	<1	<1	<1	0-0
Valine	256	89	86	108-295
Leucine	165	46	46	60-204
Isoleucine	88	23	26	39-119

Plasma amino acids September 21, 2011 (fasting for 14 hours, analyzed at the Baylor Research Institute by HPLC. Values are expressed in uM).

Patient / Amino Acid	558-IV-2 (A)	558-IV-3 (A)	Reference Range
Phosphoserine	<1	<1	0-12
Hydroxyproline	11	10	0-37
Histidine	107	73	42-116
Phosphoethanolamine	<1	<1	0-3
Asparagine	44	43	30-78
N-3-Methylhistidine	2	8	0-8
Taurine	69	78	33-92
N-1-Methylhistidine	4	3	0-21
Serine	147	119	63-185
Glutamine	472	467	424-720
Carnosine	<1	<1	0-0
Arginine	95	71	20-122
Glycine	283	251	117-366
Anserine	6	6	0-0
Ethanolamine	10	12	0-1-
Aspartate	11	10	0-10
Sarcosine	<1	<1	0-0
Glutamate	75	83	28-112
Citrulline	23	24	11-51
B-alanine	6	6	0-0
Threonine	171	239	62-182
Alanine	505	626	162-572
GABA	<1	<1	0-0
A-aminoadipic Acid	<1	<1	0-13
Proline	283	263	65-380
B-Aminoisobutyric Acid	<1	<1	0-0
Tryptophan	87	104	23-86
A-Aminobutyric Acid	3	<1	7-33
Cystathionine	<1	<1	0-0
Ornithine	61	95	36-118
Cystine	25	9	9-53
Lysine	242	280	122-285
Tyrosine	103	123	30-100
Methionine	34	34	14-41
Phenylalanine	82	95	35-83
Alloisoleucine	<1	<1	0-0
Homocystine	<1	<1	0-0

Valine	67	69	108-295
Leucine	42	31	60-204
Isoleucine	23	15	39-119

Table S4: Plasma amino acids of individuals of Family 18. Blood from unaffecteds (U) and affecteds (A) was obtained after 10 hours fasting and was analyzed at Bioscientia Laboratories by HPLC. Values are expressed in umol/L). Plasma BCAA levels of the affected patients are shown in red.

Patient / Amino Acid	18-III-1 (U)	18-III-2 (U)	18-IV-1 (A)	18-IV-2 (A)	Reference Range
Phosphoserine	n.d.	n.d.	n.d.	n.d.	1-30
Taurine	85	53	87	52	10-170
Phosphoethanolamine	n.d.	n.d.	n.d.	n.d.	0-69
Aspartate	5	5	7	16	1-24
Hydroxyproline	n.d.	n.d.	17	24	3-45
Theronine	115	174	145	163	35-226
Serine	113	176	152	226	69-187
Asparagine	49	60	69	64	23-112
Glutamate	37	29	37	61	5-150
Glutamine	668	766	794	784	254-823
Sarcosine	n.d.	n.d.	n.d.	n.d.	0-9
A-Aminoadipic Acid	3	3	n.d.	n.d.	0-0
Proline	239	134	172	130	59-369
Glycine	276	394	257	317	127-341
Alanine	518	333	495	392	152-547
Citrulline	42	46	39	46	1-46
A-Aminobutyric Acid	16	16	4	4	4-31
Cystine	21	17	15	14	5-45
Cystathionine	n.d.	n.d.	n.d.	1	0-3
Methionine	30	27	33	30	7-47
Ethanolamine	n.d.	n.d.	n.d.	n.d.	0-7
Lysine	193	208	259	227	48-284
Tyrosine	76	60	123	97	24-115
Phenylalanine	65	44	68	51	26-91
B-Alanine	n.d.	n.d.	n.d.	n.d.	0-7
B-Aminoisobutyric Acid	n.d.	n.d.	n.d.	n.d.	0-0
GABA	n.d.	n.d.	n.d.	n.d.	0-0
Histidine	88	71	95	102	41-125
Arginine	108	108	124	129	10-140

1-Methylhistidine	19	n.d.	11	14	0-42
Tryptophan	83	39	62	59	0-79
Carnosine	n.d.	n.d.	n.d.	n.d.	0-0
Anserine	n.d.	n.d.	n.d.	n.d.	0-0
Hydroxylysine	n.d.	n.d.	n.d.	n.d.	0-2
Ornithine	67	69	63	67	10-163
Valine	215	125	**57**	**54**	74-321
Leucine	114	61	**21**	**22**	49-216
Isoleucine	58	33	**11**	**12**	22-107

Novarino et al. Table S5

Table S5: Plasma amino acids of individuals of family 1435. Results from blood drawn on different days are reported. Plasma amino acids were quantified on bloodspot cards by FIA-MS/MS or LC-MS/MS at the Mayo Clinic. U, unaffected; A, affected. Values are expressed in nmol/ml. Plasma BCAA levels of the affected patients are shown in red.

Note: As described in the text, the Libyan revolution made it impossible to obtain dry ice shipment of plasma for amino acid analysis in Family 1435. Instead, established alternative methods of analysis were used - analysis of blood samples spotted on Guthrie cards and analyzed by FIA- or LC-MS/MS (*1*). As this analysis is mainly performed on newborns, Mayo Clinic does not have internal reference ranges for individuals in the older age group of those tested. While comparison with healthy siblings provides the most appropriate metric for this analysis, we provide below an additional comparator - published reference ranges from two distinguished sources. Note that these ranges were established using plasma samples analyzed by HPLC.

Plasma amino acids by FIA-MS/MS September 2011* (fasting status: unknown).

Patient / Amino Acid	1435-III-1 (U)	1435-IV-2 (U) (19y)	1435-IV-3 (U) (17 y)	1435-IV-4 (U) (16 y)	1435-IV-5 (U) (13 y)	1435-IV-6 (A) (9 y)	1435-IV-7 (U) (7 y)	1435-IV-8 (A) (5 y)	1435-IV-9 (U) (2 y)	Reference Range (*2*) 2-10 years	Reference Range (*2*) 10-18 years	Reference Range (*3*) 2-18 years
Glycine	n.d.	161.5	243.0	69.2	132.5	243.0	96.9	485.9	80.8	113-261	148-324	127-341
Ornithine	70.3	45.5	55.7	51.0	61.8	59.8	65.3	66.8	34.9	24-64	20-84	10-163
Arginine	1.8	1.2	1.4	1.0	1.6	1.4	1.3	1.2	0.8	38-98	45-125	10-140
Citrulline	18.8	13.7	17.9	16.3	17.6	20.3	18.7	14.4	13.3	18-50	17-49	1-46
Alanine	390.3	544.3	561.5	508.3	479.8	438.8	364.7	444.4	605.2	158-314	192-508	152-547
Serine	609.8	577.2	530.7	601.1	456.5	459.8	458.4	596.4	454.2	77-169	75-175	69-187
Glutamate	284.1	200.8	248.0	251.9	273.0	282.5	251.7	316.1	305.6	11-51	11-59	5-150
Aspartate	71.7	22.0	36.8	41.7	35.2	30.0	33.3	34.4	17.6	3-15	4-28	1-24
Threonine	135.3	304.2	176.8	230.5	154.8	163.9	163.9	223.4	125.7	48-140	72-192	35-226
Methionine	15.5	22.8	20.0	12.5	23.1	15.3	14.6	24.2	14.5	11-27	16-36	7-47
Histidine	229.8	223.0	178.3	167.7	163.2	163.6	194.8	199.1	163.3	54-106	58-106	41-125
Tyrosine	64.4	94.5	77.7	72.3	97.8	80.7	73.9	89.0	82.5	34-82	40-92	24-115
Valine	242.5	426.9	174.3	223.3	255.6	95.5	187.5	147.3	166.1	133-273	142-278	74-321
Leucine + Isoleucine	159.6	183.3	189.4	163.2	235.4	86.3	192.5	134.2	142.1	N.A.	N.A.	N.A.

* Prior to spotting on cards, these repository samples were hemolyzed after being frozen at -80 degrees Celsius and undergoing several freeze-thaw cycles.

Plasma amino acids by LC-MS/MS September 2011* (fasting status: unknown).

Patient / Amino Acid	1435-III-1 (U)	1435-IV-2 (U) (19 y)	1435-IV-3 (U) (17 y)	1435-IV-4 (U) (16 y)	1435-IV-5 (U) (13 y)	1435-IV-6 (A) (9 y)	1435-IV-7 (U) (7 y)	1435-IV-8 (A) (5 y)	1435-IV-9 (U) (2 y)	Reference Range (*2*) 2-10 years	Reference Range (*2*) 10-18 years	Reference Range (*3*) 2-18 years
Valine	190	251	224	172	235	70	132	131	167	133-273	142-278	74-321
Leucine	165	196	190	146	205	73	113	113	134	64-164	76-168	49-216
Isoleucine	85	95	98	76	119	30	62	57	80	31-83	38-94	22-107

* Prior to spotting on cards, these repository samples were hemolyzed after being frozen at -80 degrees Celsius and undergoing several freeze-thaw cycles.

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Plasma amino acids by FIA-MS/MS December 2011 (fasting for 12 hours).

Patient / Amino Acid	1435-IV-5 (U) (14 y)	1435-IV-6 (A) (10 y)	1435-IV-7 (U) (8 y)	1435-IV-8 (A) (6 y)	Reference Range (*2*) 2-10 years	Reference Range (*2*) 10-18 years	Reference Range (*3*) 2-18 years
Glycine	214.1	247.5	196.4	239.6	113-261	148-324	127-341
Ornithine	22.6	19.3	21.5	19.8	24-64	20-84	10-163
Arginine	2.9	3.0	2.5	2.0	38-98	45-125	10-140
Citrulline	17.2	17.5	21.7	15.9	18-50	17-49	1-46
HomoCitrulline	1.7	1.9	1.1	0.8	N.A.	N.A.	N.A.
Alanine	396.4	271.7	341.6	389.3	158-314	192-508	152-547
Tyrosine	60.0	71.4	62.2	63.3	34-82	40-92	24-115
Serine	151.4	168.2	165.2	150.2	77-169	75-175	69-187
Glutamate	203.6	169.8	137.0	162.4	11-51	11-59	5-150
Threonine	26.4	46.0	61.0	57.0	48-140	72-192	35-226
Aspartate	61.6	67.2	38.5	63.4	3-15	4-28	1-24
Methionine	16.2	18.2	13.3	19.6	11-27	16-36	7-47
Histidine	23.1	14.2	14.1	14.0	54-106	58-106	41-125
Phenylalanine	55.3	63.1	49.2	69.1	35-67	38-78	26-91
Valine	123.3	71.7	174.1	75.0	133-273	142-278	74-321
Leucine + Isoleucine	127.8	79.3	159.3	66.4	N.A.	N.A.	N.A.

Plasma amino acids by LC-MS/MS December 2011 (fasting for 12 hours).

Patient / Amino Acid	1435-IV-5 (U) (14 y)	1435-IV-6 (A) (10 y)	1435-IV-7 (U) (8 y)	1435-IV-8 (A) (6 y)	Reference Range (*2*) 2-10 years	Reference Range (*2*) 10-18 years	Reference Range (*3*) 2-18 years
Valine	154.0	100.0	216.0	80.0	133-273	142-278	74-321
Leucine	93.0	64.0	120.0	47.0	64-164	76-168	49-216
Isoleucine	60.0	34.0	94.0	32.0	31-83	38-94	22-107

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Novarino et al. Table S6

Table S6: Urine amino acid profile. Urine amino acids were determined for the two affecteds from Family 558 and for all the members of Family 18. Values are expressed as nmol/mg of creatinine. n.d.: not determined.

Family 558

Patient / Amino Acid	**558-IV-2** (A)	**558-IV-3** (A)
Phosphoserine	0	0
Taurine	533	1074
Aspartic acid	70	128
Serine	267	374
Hydroxyproline	94	2
Glycine	3727	2141
Glutamine	355	709
Asparagine	1	2
Histidine	538	913
Threonine	123	228
Citrulline	2	4
Sarcosine	1	0
B-alanine	5	7
Alanine	595	578
Glutamate	6	9
Carnosine	3	9
Arginine	44	65
GABA	1	2
Proline	37	4
Ornithine	2	4
Cysteine	17	28
Lysine	37	167
Methionine	10	11
Tryptophan	72	124
Tyrosine	103	189
Isoleucine	1	2
Valine	8	9
Leucine	3	7
Isoleucine	1	2

Family 18

Patient / Amino Acid	18-III-1 (U)	Reference Range	18-III-2 (U)	Reference Range	18-IV-1 (A)	Reference Range	18-IV-2 (A)	Reference Range
Phosphoserine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Taurine	56	(16-180)	117	(16-180)	70	(18-230)	108	(17-230)
Aspartic acid	n.d.	(2-7)	n.d.	(2-7)	n.d.	(1-10)	n.d.	(2-8)
Hydroxyproline	n.d.	(0-13)	n.d.	(0-13)	n.d.	(0-13)	n.d.	(0-13)
Threonine	14	(7-29)	19	(7-29)	44	(8-28)	57	(9-36)
Serine	32	(21-50)	61	(21-50)	84	(23-69)	104	(38-93)
Asparagine	13	(0-23)	20	(0-23)	43	(0-24)	41	(0-29)
Glutamic acid	3	(0-12)	3	(0-12)	3	(0-9)	3	(0-8)
Glutamine	50	(20-76)	95	(20-76)	149	(20-112)	144	(52-133)
Sarcosine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Proline	n.d.	(0-9)	1	(0-9)	1	(0-9)	1	(0-9)
Glycine	104	(43-173)	310	(43-173)	184	(64-236)	278	(91-246)
Alanine	41	(16-68)	46	(16-68)	101	(17-65)	133	(27-92)
Citrulline	1	(0-4)	1	(0-4)	1	(0-5)	1	(0-5)
Valine	4	(3-13)	3	(3-13)	3	(3-17)	4	(3-15)
Cystine	4	(3-17)	4	(3-17)	4	(4-12)	4	(4-11)
Cystathionine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Methionine	1	(2-16)	2	(2-16)	3	(3-17)	3	(5-20)
Isoleucine	2	(0-4)	1	(0-4)	n.d.	(0-6)	1	(0-5)
Leucine	4	(2-11)	4	(2-11)	3	(3-16)	3	(3-13)
Tyrosine	22	(2-23)	10	(2-23)	29	(6-26)	23	(9-35)
Phenylalanine	9	(2-19)	5	(2-19)	14	(5-20)	10	(6-26)
B-Alanine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
GABA	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Histidine	71	(26-153)	91	(26-153)	224	(43-184)	169	(61-216)
Tryptophan	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Carnosine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Anserine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Hydroxylysine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Ornithine	3	(0-5)	2	(0-5)	3	(0-6)	3	(0-7)
Lysine	8	(7-58)	10	(7-58)	32	(10-56)	17	(10-68)
Ethanolamine	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)	n.d.	(0-0)
Arginine	2	(0-5)	2	(0-5)	4	(0-6)	3	(0-7)

Novarino et al. Table S7

Table S7: Plasma organic acids are low in patients with a BCKDK nonsense mutation. Plasma organic acids of siblings of Family-558. Values are given in umol/L. Age matched control are shown as references (blood drawn after 14 hours fasting and analyzed at Baylor Research Institute).

				Age matched female controls		
Organic Acid	558-IV-1 (U)	558-IV-2 (A)	558-IV-3 (A)	16 years	17 years	18 years
Glyoxylic	0	0	0	0	0	0
Pyruvic	111	86	134	85	44	86
2-Oxoisovaleric	10	0	0	16	11	17
Acetoacetic	0	0	0	0	0	0
2-Oxo-3-methylvaleric	0	0	0	26	15	23
2-Methylacetoacetic	0	0	0	0	0	0
2-Oxoisocaproic	22	0	0	25	28	18
2-Oxoglutaric	0	0	0	0	0	0
2-Oxoadipic	0	0	0	0	0	0
4-Hydroxyphenylpyruvic	0	0	0	0	0	0
Succinylacetone	0	0	0	0	0	0
Lactic	1554	1110	1729	>2000	>2000	>2000
Glycolic	35	0	0	50	123	34
2-Hydroxybutyric	5	7	0	129	60	72
3-Hydroxypropionic	15	1	0	6	8	3
3-Hydroxyisobutyric	25	13	20	29	9	9
3-Hydroxybutyric	0	16	17	28	33	170
2-Hydroxyisovaleric	0	0	0	0	0	0
Heptanoic	0	0	0	0	0	0
2-Methyl-3-Hydroxybutyric	0	0	0	0	0	0
3-Hydroxyisovaleric	0	0	0	9	18	5
Methylmalonic	0	0	0	0	0	0
2-Hydroxyisocaproic	0	0	0	0	0	0
2-Ethyl-3Hydroxypropionic	6	0	4	10	0	8
3-Hydroxyvaleric	0	0	0	0	0	0
4-Hydroxybutyric	0	0	0	0	0	0
2-Hydroxy-3-Methylvaleric	0	0	0	0	0	0
Benzoic	5	7	2	9	23	5
4-Hydroxyisovaleric	0	0	0	0	0	0
Octanoic	0	0	0	18	0	5
2-Methyl-3-Hydroxyvaleric	0	0	0	0	0	0
Ethylmalonic	0	0	0	0	0	0
Phenylacetic	0	0	0	0	0	0
Succinic	1	1	1	4	6	11
Methylsuccinic	0	0	0	0	0	0
Glyceric	9	10	5	12	63	19
Uracil	0	0	0	0	0	0
Fumaric	2	2	2	4	0	5
5-Hydroxyhexanoic	0	0	0	0	0	0

Thymine	0	0	0	0	0	0
Glutaric	1	1	1	0	0	0
Propionylglycine	0	0	0	0	0	0
Isobutyrylglycine	0	0	0	0	0	0
Malonic	0	0	0	0	0	0
3-Methylglutaric	0	0	0	0	0	0
cis4-Decenoic	0	0	0	0	0	0
Decanoic	0	0	0	3	4	13
3-Hydroxyoctanoic	0	0	0	0	0	0
Butyrylglycine	0	0	0	0	0	0
Malic	4	2	3	5	6	23
2-Methylbutyrylglycine	0	0	0	0	0	0
Adipic	0	0	0	0	1	0
Isovalyrylglycine	0	0	0	0	0	0
5-Oxoproline	53	32	25	18	31	43
2-Hydroxyphenylacetic	0	0	0	0	0	0
3-Methyladipic	0	0	0	0	0	0
Mevalonic	0	0	0	0	0	0
3-Methylcrotonylglycine	0	0	0	0	0	0
2-Hydroxyglutaric	2	2	2	0	0	0
3-Hydroxyglutaric	0	0	0	0	0	0
Tiglylglycine	0	0	0	0	0	0
Phenyllactic	0	0	0	0	0	0
3-Hydroxy-3-methylglutaric	0	0	0	0	0	0
Pimelic	0	0	0	0	0	0
4-Hydroxycyclohexylacetic	0	0	0	0	0	0
Hexanoylglycine	0	0	0	0	0	0
4-Hydroxyphenylacetic	2	0	0	0	0	0
3-Hydroxydecanoic	0	0	0	0	0	0
Lauric	13	9	9	6	36	5
N-acetylaspartic	0	0	0	0	0	0
2-Hydroxyadipic	0	0	0	0	0	0
5-Hydroxymethyluracil	0	0	0	0	0	0
3-Hydroxyadipic	0	0	0	0	0	0
3-Methylglutaconic	0	0	0	0	0	0
Suberic	0	0	0	0	0	0
Orotic	0	0	0	0	0	0
Glutaconic	0	0	0	0	0	0
Aconitic	6	0	0	0	3	0
Azelaic	0	0	0	0	0	0
Isocitric	5	5	5	8	11	9
Citric	80	44	49	20	124	97
3-Hydroxydodecanoic	0	0	0	0	0	0
Methylcitric	0	0	0	0	0	0
Hippuric	0	0	0	0	0	0
Myristic	56	15	12	20	123	14
Sebacic	2	0	2	0	0	0
4-Hydroxyphenyllactic	4	4	4	4	3	3
Xanthine	0	0	0	0	0	0
Phenylpropionylglycine	0	0	0	0	0	0
Palmitoleic	49	26	25	29	180	40
3-Hydroxytetradecanoic	0	0	0	0	0	0
2-Hydroxydecanedioic	0	0	0	0	0	0

Palmitic	887	480	333	704	1787	401
3-Hydroxydecanedioic	0	0	0	0	0	0
N-Acetyltyrosine	0	0	0	0	0	0
3-Hydroxyhexadecanoic	0	0	0	0	0	0
Linoleic	585	497	215	372	1702	405
Oleic	628	450	289	579	990	571
Stearic	124	51	47	102	157	87
Suberylglycine	0	0	0	0	0	0

Novarino et al. Table S8

Anthropomorphic Data on Affected Individuals

Patient and Age	Head Circumference (cm)	Head Circumference (Percentile*)	Height (cm)	Height (Percentile**)	Weight (Kg)	Weight (Percentile**)
558-IV-2, 15 years	49	-3SD	N/A	N/A	N/A	N/A
558-IV-3, 11 years	51.7	25th	N/A	N/A	N/A	N/A
18-IV-I, 36 months	47	5th***	98	83rd	15	75th
18-IV-I, 69 months	52	76th	N/A	N/A	N/A	N/A
18-IV-I, 96 months	52.5	75th	130	64th	34.5	93rd
18-IV-II, 61 months	50	18th	110	57	22	85
1435-IV-6, 9 years	47.5	-2SD	N/A	N/A	N/A	N/A
1435-IV-8, 5 years	47	-3SD	N/A	N/A	N/A	N/A

*Based on United States Head Circumference Growth Reference Charts for Children 0 to 21 years of Age.(Rollins, Collins et al. 2010)

**Based on Centers for Disease Control (CDC) height and weight for age for girls (2-20 years)

*** -1 Standard Deviation according to World Health Organization head circumference for age for girls birth to 5 years and CDC Head Circumference for age (0-36 months)

Novarino et al. Table S9

Table S9: Prospective dietary records of affected individuals. Dietary Information obtained when 18-IV-1 was 8 years old. She weighed 34.5 Kg and had a height of 130 cm. Dietary Information obtained when 18-IV-2 was 5 years old. He weighed 22 Kg and had a height of 110 cm. Data were obtained by prospective monitoring and report by parents. Abbreviations: RDA is the recommended daily allowance (WHO/FAO/UNU, 2004).

	EGT 18-IV-1 Mean Three Day Record		**EGT 18-IV-2 Mean Three Day Record**	
Item	**Intake**	**RDA**	**Intake**	**RDA**
Energy (Kcal/day)	2144.60	1830	1567.56	1467
CHO (g/day)	297.28	130	223.34	130
Protein (g/day)	75.93	26.2	55.32	17.1
Fat (g/day)	76.86	ND	58.98	ND
Fiber (g/day)	8.94	25	5.41	25

Novarino et al. Table S10

Table S10: **Plasma amino acids of individuals from families 558 and 18 before and during BCAA dietary supplementation.** The amount of supplementation is in indicated as grams of BCAA supplement per patient body weight (in Kg) per day. Samples from Family 558 and 18 were quantified by HPLC and by FIA-MS/MS or LC-MS/MS, respectively (Mayo Clinic).

Patient 558-IV-2

Condition / Amino Acid	Prior to supplementation		Supplemented with 0.6 g/Kg/day		Supplemented with 1 g/Kg/day		Reference Range
	Fasting (14 hrs)	Post meal and supplement (1hr)	Fasting (14 hrs)	Post meal and supplement (1hr)	Fasting (14 hrs)	Post meal and supplement (1hr)	
Taurine	75	85	80	69	73	87	54-210
Asparagine	69	73	54	69	66	50	35-74
Serine	129	138	144	163	161	121	58-181
Glycine	293	269	292	250	281	185	151-490
Glutamine	571	568	595	631	580	569	205-756
Histidine	100	92	80	90	104	87	72-124
Threonine	181	169	173	181	145	119	60-225
Citrulline	29	24	32	23	33	44	12-55
Alanine	750	665	464	456	557	491	177-583
Glutamate	40	57	42	39	35	51	10-131
Arginine	137	150	104	137	115	152	15-128
Proline	395	398	234	337	293	276	97-329
Ornithine	47	48	47	55	42	43	48-195
Cysteine	55	51	41	53	71	55	5-82
Lysine	235	224	186	258	205	224	116-295
Methionine	32	33	31	37	39	32	10-42
Tyrosine	103	104	101	127	123	103	34-112
Phenylalanine	56	58	51	62	67	52	35-85
Valine	57	77	54	101	79	809	119-336
Leucine	20	32	24	61	29	809	72-201
Isoleucine	10	18	12	31	15	401	30-108

Patient 558-IV-3

Condition / Amino Acid	Prior to supplementation		Supplemented with 0.6 g/Kg/day		Supplemented with 1 g/Kg/day		Reference Range
	Fasting (14 hrs)	Post meal and supplement (1hr)	Fasting (14 hrs)	Post meal and supplement (1hr)	Fasting (14 hrs)	Post meal and supplement (1hr)	
Taurine	94	79	71	61	64	73	54-210
Asparagine	46	55	49	59	46	37	35-74
Serine	93	105	143	150	117	100	58-181
Glycine	164	168	220	205	153	114	151-490
Glutamine	530	531	519	573	470	442	205-756
Histidine	69	84	74	79	70	64	72-124
Threonine	137	167	161	160	105	88	60-225
Citrulline	26	24	34	22	35	34	12-55
Alanine	489	498	420	501	344	325	177-583
Glutamate	54	56	42	39	32	32	10-131
Arginine	121	144	108	138	96	108	15-128
Proline	211	264	168	245	157	153	97-329
Ornithine	53	57	69	78	43	44	48-195
Cysteine	44	40	41	45	46	42	5-82
Lysine	203	230	240	266	190	188	116-295
Methionine	22	25	31	31	28	17	10-42
Tyrosine	97	103	106	109	96	85	34-112
Phenylalanine	60	70	68	78	70	59	35-85
Valine	54	74	67	81	69	837	119-336
Leucine	18	36	26	44	26	666	72-201
Isoleucine	10	25	13	24	13	397	30-108

Patient 18-IV-1

Condition / Amino Acid	Prior to supplementation	Supplemented with 0.5 g/Kg/day	Supplemented with 0.7g /Kg/day			Reference Range
	Fasting (8 hrs)	Fasting (8 hrs)	Fasting (8 hrs)	Post meal and supplement		
				1hr	2 hrs	4 hrs	
Hydroxyproline	na	na	na	na	na	na	3-45
Threonine	51.3	37.4	36	31	33	31	35-226
Serine	202.7	132.2	240	210	245	277	69-187
Aspargine	na	na	na	na	na	na	23-112
Glutamate	116.9	128.4	117	125	109	143	5-150
Glutamine	na	na	na	na	na	na	254-823
Proline	na	na	na	na	na	na	59-369
Glycine	269.8	148.6	146	132	133	160	127-341
Alanine	436.8	329.1	399	362	396	454	152-547
Citruline	12.9	13.4	16	17	17	17	1-46
Methionine	20.6	19.7	31	22	22	18	7-47
Cyatathionine	na	na	na	na	na	na	0-3
Tyrosine	102	83.7	68	67	75	75	24-115
Phenylalanine	45.9	44.6	53	53	57	61	26-91
Homocitruline	0.6	0.8	1	1	1	1	<2
Ethanolamine	na	na	na	na	na	na	0 - 7
Ornithine	24	25.6	32	31	33	41	10-163
Lysine	na	na	na	na	na	na	48-284
Histidine	32	85.2	115	102	80	84	41- 125
Tryptophan	na	na	na	na	na	na	0-79
Arginine	13.3	3.7	12	15	13	12	10-140
Valine	35	44	71	644	443	185	74 - 321
Leucine	17	18	27	329	168	59	49 - 216
Isoleucine	11	11	17	434	168	49	22 -107

Patient 18-IV-2

Condition / Amino Acid	Prior to supplementation	Supplemented with 0.64 g/Kg/day	Supplemented with 0.8 g/Kg/day				Reference Range
	Fasting (8 hrs)	Fasting (8 hrs)	Fasting (8 hrs)	Post meal and supplement			
				1hr	2 hrs	4 hrs	
Hydroxyproline	na	na	na	na	na	na	3-45
Threonine	76	38.7	36	53	53	36	35-226
Serine	201.8	156.3	335	285	289	340	69-187
Aspargine	na	na	na	na	na	na	23-112
Glutamic acid	121.4	117.9	123	126	130	137	5-150
Glutamine	na	na	na	na	na	na	254-823
Proline	na	na	na	na	na	na	59-369
Glycine	37.5	123	143	129	132	135	127-341
Alanine	321.6	215.7	282	273	373	317	152-547
Citruline	13.8	10.3	16	16	17	17	1-46
Methionine	20.3	18.3	24	27	21	17	7-47
Cyatathionine	na	na	na	na	na	na	0-3
Tyrosine	68.1	55.5	63	92	81	43	24-115
Phenylalanine	37.6	43.2	53	67	62	41	26-91
Homocitruline	0.6	0.8	1	1	1	1	<2
Ethanolamine	na	na	na	na	na	na	0 - 7
Ornithine	22.2	19.3	31	33	35	30	10-163
Lysine	na	na	na	na	na	na	48-284
Histidine	25.8	112.9	108	102	83	83	41- 125
Tryptophan	na	na	na	na	na	na	0-79
Arginine	16.2	2.8	20	21	24	14	10-140
Valine	32	45	57	458	518	133	74 - 321
Leucine	15	23	25	270	236	29	49 - 216
Isoleucine	9	14	16	315	248	21	22 -107

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