Metabolomics sample preparation, analysis, and processing

Cortex tissue samples were analyzed via liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS and LC-HRMS/MS) using previously described methods. Briefly, harvested cortex tissue samples were thawed on ice and lysed in 500 µL ice-cold lysis buffer (1:1:2, acetonitrile: methanol: ammonium bicarbonate 0.1M, pH 8.0) followed by probe tip sonication and normalized via protein amount (100 µg) based on a bicinchoninic acid protein assay (Thermo Fisher Scientific, Waltham, MA). Deproteinization of individual samples was performed by addition of 800 µL of ice-cold methanol following the addition of isotopically labeled standards (phenylalanine-D8, biotin-D2 and lauryl carnitine-D3) to determine sample process variability. Precipitated proteins were centrifuged at 10,000 rpm for 10 min after overnight incubation at -80°C. Metabolite extracts were dried in vacuo and further purified via liquid-liquid extraction (LLE) method with tert-Butyl methyl ether (MTBE). The hydrophilic fraction of each sample extract was transferred into a new Eppendorf tube, dried in vacuo, and stored at -80°C until further use.

Prior to mass spectrometry analysis, individual extracts were reconstituted in 80 µl acetonitrile/water (80:20, v/v) containing isotopically labeled standards, tryptophan-D3, inosine-4N15, valine-D8, and carnitine-D9, and centrifuged for 5 min at 10,000 rpm to remove insoluble material. A pooled quality control (QC) sample was prepared by pooling equal volumes of individual samples following reconstitution. The QC sample allowed for column conditioning (eight injections), retention time alignment, and assessment of mass spectrometry instrument reproducibility throughout the sample set.

LC-HRMS and LC-HRMS/MS analyses were performed on a high-resolution Q-Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Vanquish UHPLC binary system (Thermo Fisher Scientific, Bremen, Germany) in positive and negative ion modes. Extracts (5µL injection volume) were separated on an ACQUITY UPLC BEH Amide HILIC 1.7µm, 2.1 × 100 mm column (Waters Corporation, Milford, MA) held at 30°C using the LC method as previously described.

Full MS analyses were acquired over the mass-to-charge ratio (m/z) range of 70-1,050 in positive ion mode. The full mass scan was acquired at 120,000 resolutions with a scan rate of 3.5 Hz and an automatic gain control (AGC) target of 1x106. A maximum ion injection time of 100 ms and MS/MS spectra were collected at 15,000 resolutions, with an AGC target of 2x105 ions and a maximum ion injection time of 100 ms.

Progenesis QI v.3.0 (Non-linear Dynamics, Newcastle, UK) was used to review, process and normalize the mass spectrometry data. The pooled QC sample was used to align all MS and MS/MS sample runs. Unique ions (retention time and m/z pairs) were de-adducted and de-isotoped to generate unique "features" (retention time and m/z pairs). Data were normalized to all features detected, and further curated by applying QA practices to the data. Specifically, compounds or metabolites with spectral features > 30% coefficient of variation (CV) in the pooled QC samples were removed. Sample process and instrument variability were also assessed using the normalized measurements of the isotopically labeled standards to determine sample and batch acceptance. QA metrics for sample process variability and

instrument variability were ≤ 25% CV and ≤ 10% CV, respectively. Accurate mass measurements (< 5 ppm error), isotope distribution similarity, and fragmentation spectrum matching (when applicable) were used to determine tentative, putative, and validated (Level 1-3) annotations of amino acids and metabolites associated with glutamate synthesis and transport. Compounds were searched using Human Metabolome Database (HMDB) and a highly curated in-house library available in the Center for Innovative Technology at Vanderbilt University.