**METABOLOMICS ANALYSIS:**

Samples were sent to the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat, where a targeted metabolomics analysis was conducted by liquid chromatography with tandem mass spectrometry (LC-MS-MS) for tryptophan related metabolites and acylcarnitines. Gas chromatography–mass spectrometry (GC-MS) was performed for organic acids and sugar metabolites related to energy metabolism and the TCA cycle. A total of 82 metabolites were detected, and the results were presented as micromolar (μM) concentrations.

**LC-MS/MS:** CSF (50 μL) samples were diluted in methanol (200 μL, 100%) and the set of labelled internal standards (DL-kynurenine, DL-tryptophan, 5-hydroxyindole-3-acetic acid, L-citrulline, 2-picolinic acid, nicotinic acid, kynurenic acid, indole-3-acetic acid (Sigma Aldrich); serotonin hydrochloride, kynurenine-d4 trifluoroacetic acid salt, D-tryptophan-d5, 5-hydroxyindole-3-acetic acid-d2 (5-HIAA-d2), serotonin-d4 hydrochloride, 2-picolinic-d4 acid, nicotinic acid-d4, 3-hydroxyanthranilic acid, 3-hydroxyanthranilic acid-d3, kynurenic acid-d5, indole-2,4,5,6,7-d5-3-acetic-α,α-d2 acid, 3-indolepropionic-d2 acid, n-acetyl-5-hydroxytryptamine (Toronto Research Chemicals); trigonelline hydrochloride (TCI); anthranilic acid (Glentham life sciences); hydrocortisone, androstenedione, testosterone, progesterone (Sigma Aldrich and Toronto Research Chemicals). Following centrifugation (10 min, 15000 rpm, 4ºC), the supernatant was dried in a SpeedVac concentrator and reconstituted in methanol (50 μL, 100%).

The equipment consisted on an UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series (Agilent Technologies) and a Kinetex 2.6 μm Polar C18, 100 Å, 150 x 2.1 mm (Phenomenex) analytical column. Chromatographic separation was performed in mode negative electrospray ionization (ESI) at 20ºC with an injection volume of 1 μL. Mobile phase A was formic acid (0.1%) and mobile phase B was methanol:formic acid (10:1, v/v).

**GC-MS:** CSF (50 μL) samples were diluted in water:methanol (200 μL, 8:2, v/v) and the set of labelled internal standards (Succinic-d4 acid, myristic-d27 acid, d-glucose 13C6 and L-Methionine-(carboxy-13C,methyl-d3) (Sigma Aldrich)). Following centrifugation (5 min, 15000 rpm, 4ºC), the supernatants were dried in a SpeedVac concentrator at 45ºC and reconstituted in methoxyamine (30 μL, 100%). Samples were incubated at 37ºC for 90 min. and then silylated with 45 μL of MSTFA + 1 % TMCS at room temperature for 60 min.

The separation was performed on a GC-QTOF 7200 and a HP5-MS UI capillary column (30 m x 250 μm I.D., 0.25 μm film thickness), both from Agilent Technologies. Helium (>99.999%) was used as the carrier gas with a constant flow 1.1 mL/min. Initial oven temperature was set at 60ºC, then increased by 10ºC/min. to 320ºC and held constant for 10 min. Samples were injected in split mode 1:20 at injection temperature 250ºC. Compounds were detected through MS in electron ionization (70 eV) and full-scan monitoring mode (m/z 50–600) mode with an acquisition rate of 5 spectra/s. Ion source temperature was 250ºC and quadrupole temperature was 200ºC.

Organic acids were identified and semi-quantified using the spectra library Fiehn-pct-2013 and their pure analytical standards (Pyruvic acid, lactic acid, glycolic acid, 3-hydroxybutyric acid, glycerol, succinic acid, glyceric acid, fumaric acid, malic acid, d-threitol, threonic acid, α-ketoglutaric acid, arabitol, glycerol-1-phosphate, 3-phosphoglyceric acid, citric acid, d-mannitol, myo-inositol, glucose-6-phosphate, d-sucrose and α-tocopherol (Sigma Aldrich)).

**STATISTICAL ANALYSIS**

The goal of the analysis was to compare the metabolic profiles of patients to those of healthy controls. All statistical tests were conducted in R version 4.3.0. Metabolic profiles of patients were compared to those of health controls, first or categorized on the glutamatergic behavior of the disease (hypoglutamatergic diseases vs hyperglutamatergic/hypoGABAergic).

The metabolite concentrations were log2-transformed in order to account for the expected high variability and noise found in metabolomics data. Because metabolomics deals with The statistical methods chosen for this study have been recently applied in several studies dealing with metabolomics and lipidomics data (Chen et al., 2020; Iurova et al., 2022; Wang et al., 2022; Zandl-Lang et al., 2022, Zhang et al., 2022).

Multivariate statistical methods were applied on the transformed data in order to account for the small sample size and the inherent multicollinearity of the data. Unsupervised principal component analysis (PCA) was performed in order to obtain an overall view of the variation between samples and their separation into clusters. Ward hierarchical clustering with Euclidian distance combined with a heatmap (pheatmap version 1.0.12) was used to compare the log-transformed concentrations of all the analyzed metabolites in patients and controls. The supervised classification model orthogonal partial least-squares discriminant analysis (OPLS-DA) from the ropls package (version 1.32.0) was used as to identify metabolites responsible for group separation. This method has the advantage that it removes sources of variation unrelated to group labels, so it is particularly useful when dealing with heterogeneous data like metabolite concentrations (Trygg, J. & Wold, S. 2002). The models were evaluated by goodness of fit (R2Y), goodness of prediction (Q2Y), and the root mean square error of estimation (RMSEE). 7-fold cross-validation was performed to mitigate the risk of overfitting caused by the high dimensionality of the data. Metabolites were selected based on their variable importance in projection (VIP) scores (VIP > 1). Permutation testing (n=1000) was used to determine the statistical significance of the results (p<0.05).

Univariate statistical analysis was carried out.by performing Mann–Whitney U tests to find differences in individual metabolite concentrations between patients and controls. The non-parametric test was chosen because the smallsizedid not contain enough information to determine whether its underlying distribution was Gaussian. Correction for the false discovery rate (FDR) was applied in order to account for the issue of multiple testing (p<0.05).

Pathway analysis was performed for metabolites with p<0.05 or VIP score > 1 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the KEGGREST package (version 1.40.0). Pathways were identified as important when two or more of their metabolite components were found to be altered between the disease groups and the healthy controls. The results were plotted after excluding pathways that are ubiquitous or unrelated to synaptic metabolism. Special attention was given to the tryptophan metabolism pathway, as well as to the amino acid substrates of Large Neutral Amino Acid Transporter (LAT1).