**Ward hierarchical clustering.** Both hyper-glutamatergic (C) and hypo-glutamatergic (D) patients form homogeneous groups regardless of genotype. There is some overlap with controls, but mostly patients cluster together and have a markedly decreased expression of most metabolites. OPLS-DA score plots showing separations of controls compared to **A)** **hyper-glutamatergic** and **B)** **hypo-glutamatergic patients.** Although control samples showed high intergroup variability, both OPLS-DA models showed good separation between the groups (R2Y(cum) > 0.9 ,Q2Y(cum) > 0.7, RMSEE < 0.2). **C-D) VIP scores.** Both diseases show similar metabolic alterations in relation to controls.

**Figure 2. Identification of significantly altered metabolites and their impact on group classification A-B)** OPLS-DA score plots showing separations of controls compared to **A)** **hyper-glutamatergic** and **B)** **hypo-glutamatergic patients.** **C) hyper-glutamatergic** had 18 metabolites with VIP score > 1 **D)** **hypo-glutamatergic** patients ha 17. **E-F) Integrated results of UVA and MVA analysis. E) Rett** had a subset of metabolites that were only identified by UVA **F) hypo-glutamatergic patients.** For both groups there was a small number of metabolites identified as altered by both univariate and multivariate analyses. Both diseases showed similar metabolic alterations, though RTT (**C**) had a higher number of significantly altered metabolites. Interestingly, RTT patients showed some metabolites that were significantly altered but did not contribute to the classification of the OPLS-DA model. **G-H)** **Hierarchical clustering using only the selected metabolites** showed perfect separation between patients and controls for both groups. **G) Hyper-glutamatergic patients** showed a few more metabolites that had increased concentrations when compared to controls than did **H) hypo-glutamatergic patients**, but overall the altered metabolites were decreased in both groups of patients

**Figure 3. Pathways affected by selected metabolites.** Altered metabolites for both RTT and hypo-glutamatergic patients had the highest impact in galactose metabolism, amino sugar and nucleotide sugar metabolism, and glycerolipid metabolism. The pathways that were most altered were ABC transporters, galactose metabolism, and tryptophan metabolism.

**Figure 4.** **Alterations in amino acid metabolism. A)** Summary of tryptophan metabolism pathways. **B) Alterations in tryptophan metabolism.** Of the metabolites involved in tryptophan metabolism that were analyzed, only 5-Hydroxyindole-3-acetic acid was significantly decreased in both disease groups, while kynurenine was significantly decreased in RTT patients and non-significantly decreased in hypo-glutamatergic patients. Tryptophan, N-Acetyl-5-hydroxytryptamine, anthranilic acid, and kynurenic acid showed a slight decrease that was not statistically significant in both RTT and hypo-glutamatergic patients. 3-Hydroxyanthranilic acid did not vary between either group and the controls.

**Figure 5.** **Tryptophan and BCAAs transport across the blood-brain barrier. A)** **Alterations in LNAAs in CSF samples**: Valine and leucine were decreased in patients compared to controls (statistically significant for hyper-glutamatergic samples), while both groups had non-significantly altered levels of isoleucine and threonine. **B,C) Analysis of the expression of *SLC7A5* (LAT1) in brain samples from Rett mouse models.** Three different brain samples were analyzed for each group (Rett and controls) in two independent experiments. **B)** Representative blot of the expression of SLC7A5 is shown, where tubulin has been used as a loading control. **C)** Quantification with ImageJ of all the experiments; \*\* p-vale < 0.001

We performed multi-factor ANOVA to determine if the alterations in BCAAamino acid concentrations were significant when accounting for the effect of all BCAA alterations.