Metabolomics

Previously frozen striatal tissue was lysed in 400µL ice-cold lysing buffer (1:1:2, ACN:MeOH:Ammonium Bicarbonate (0.1M, pH 8.0) (LC-MS grade). Individual samples were sonicated using a probe tip sonicator, 10 pulses, at 30% power and cooled down on ice between samples. A BCA protein assay was used to determine the protein concentration for each individual sample, and adjusted to a total amount of protein of 200µg in 200 µL of lysis buffer. Isotopically labeled standards, Creatinine-D3 and Lysine-D4, were added to each sample to assess sample processing steps (metabolite extraction and reconstitution). Following lysis and standard addition, protein precipitation was performed by adding 800µL of ice-cold methanol (4x by volume). Samples were incubated at -80°C overnight. Following incubation, samples were centrifuged at 10,000 rpm for 10 min to eliminate proteins. The supernatants containing metabolites were dried via speed-vacuum.

Dried metabolite extracts were stored frozen at -80°C until ready to use. Prior to mass spectrometry analysis, extracts were reconstituted in 100 µl of acetonitrile/ water (80:20, v/v) and centrifuged for 5 min at 15K rpm to remove insoluble material. Quality control samples were prepared by pooling equal volumes of each sample. Isotopically labeled standards, Valine-D8 and Inosine-4N15, were added to each sample to determine MS instrument reproducibility.

Global, untargeted mass spectrometry analyses was 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) using an optimized parallel reaction monitoring (PRM) method. This method permits monitoring and detection of all metabolites as well as specific molecules (precursor ions) added to an inclusion list. In the same run (injection), associated fragment ions (MS2 scan) of individual ions are also detected. Particular to this study, parameters consisted of an MS1 (precursor ion) scan at 60,000 resolution with an automatic gain control (AGC) value of 1e6, max injection time (IT) of 100 ms, and scan range from *m/z* 70–1050 recorded as profile data. Following the MS1 scan, the method allows for up to six targeted MS2 scans at a resolution of 15,000 and with an AGC value of 1e5, a max injection time of 50 ms, a 1.3 *m/z* isolation window, with stepped collision energy of 20 and 40, and was performed and recorded as profile data. Lastly, the method was set with the following PRM parameters: a timed inclusion list containing the target precursor *m/z* value, charge, and a 2 min retention time window. These values were determined from prior analyses of synthetic standards.

After reconstitution, metabolite extracts (5uL injection volume) were separated on a SeQuant ZIC-HILIC 3.5-µm, 2.1 mm × 100 mm column (Millipore Corporation, Darmstadt, Germany) held at 40°C. Liquid chromatography was performed at a 200 µl min^{-1} using solvent A (5mM Ammonium formate in 90% water, 10% acetonitrile) and solvent B (5mM Ammonium formate in 90% acetonitrile, 10% water) with the following gradient: 90% B for 2 min, 90-40% B over 16 min, 40% B held 2 min, and 40-90% B over 10 min, 90% B held 10 min (gradient length 40 min).

Data Processing

Ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) raw data were imported, processed, normalized and reviewed using Progenesis QI v.2.1 (Non-linear Dynamics, Newcastle, UK). Sample runs were aligned against a quality control pool reference run, and peak picking was performed on individual aligned runs to create an aggregate data set. Unique ions (retention time and m/z pairs) were grouped (a sum of the abundancies of unique ions) using both adduct and isotope deconvolutions to generate unique compounds (retention time and m/z pairs) representative of unannotated metabolites. Data were normalized to all compounds using Progenesis QI and normalized data was utilized for relative quantitation. Significance was assessed using p-values and fold changes calculated from normalized compound abundance data. Tentative and putative annotations were determined by using accurate mass measurements (< 5 ppm error), isotope distribution similarity, and fragmentation spectrum matching (when applicable) by searching the Human Metabolome Database (HMDB), METLIN, NIST, and an internal curated library[20–22].

Metaboanalyst 4.0 (www.metaboanalyst.ca/) was used to perform pathway and metabolite enrichment analyses from annotated compounds with statistical significance ($p<0.05$) after Mn exposure in each genotype. Briefly, MetaboAnalyst assigns a pathway impact factor which reflects the location of an enriched compound within that particular biologic pathway and its impact on the downstream targets. Negative log p-values were calculated based upon the number of enriched compounds detected by UPLC-MS/MS within a particular metabolic pathway compared to the total number of compounds known to be present in that pathway.

Statistics

The statistical analyses for striatal Mn, body weights, mRNAs, ARG2 and GLT1 protein were conducted using a 2-way ANOVA with post hoc comparisons after determining either a main effect of either genotype or Mn exposure or a statistically significant ($p<0.05$) interaction between genotype and Mn exposure (GraphPad Prism 6.0). ARG2 and GLT1 protein levels were quantified by normalizing all samples to the mean of all WT-0 (WT-vehicle) samples within the same protein membrane. Data are represented as the mean $\pm$ standard error of the mean (sem).