Materials and Methods

Cell harvest and metabolite extraction:

Optima grade LC-MS solvents for the mass spectrometry analyses were obtained from Thermo Fisher Scientific (Fair Lawn, NJ). Two separate metabolomics analyses using slightly different methods were performed with different SM and PM iPSC lines.

For method #1 (RPLC pos/ HILIC pos) day 11 neural dopaminergic precursor cells (floor plate cells of one well of a 6 well plate) were harvested into 500 µl of ice-cold methanol, flash frozen and then stored at -80ºC. To extract the metabolites, the 500 µl methanol cell suspensions were thawed and 100 µl of H_2O added. Then the samples were frozen on dry ice for 3 min, defrosted in ice over a 10 min period, and sonicated with 10 pulses using a probe sonicator at 30% power. The freeze-thaw-sonication sequence was repeated three times. The proteins were precipitated by placing the lysates at -80°C overnight and then pelleted by centrifugation at 15,000 rpm for 15 minutes. Cleared supernatants containing the metabolites were placed in clean Eppendorf tubes, dried in a vacuum concentrator and stored frozen at -80°C. For reverse phase liquid chromatography (RPLC)-positive (pos) mode mass spectrometry analysis the dried extracts were reconstituted in 60 µl of RPLC buffer (acetonitrile/water with 0.1% formic acid, 2:98, v/v). Samples were vortexed rigorously to solubilize the metabolites, cleared by centrifugation for 5 min at 15,000 rpm, and the supernatants were injected twice (5µl/injection) randomly. Quality control samples were prepared by combining equal volumes (10 µl) of each sample. After RPLC mass spectrometry, the remaining samples were dried down *in vacuo*, and the metabolites reconstituted in 40 µl of HILIC buffer (acetonitrile/water, 90:10, v/v) and 5 µl of each sample was injected twice in random sequence to perform hydrophilic interaction liquid chromatography (HILIC)-positive (pos) mode mass-spectrometry.

In method #2 (HILIC pos/neg) the cells were washed three times with 2.5 ml of an ammonium formate buffer (50 mM, pH 6.8), scraped into the same buffer, centrifugated at 200 x

for 5 min, the cell pellets flash frozen in liquid nitrogen and stored at -80^{o}C. To extract the metabolites, cell pellets were lysed in 200 µl ice-cold lysis buffer (1:1:2, Acetonitrile:MeOH:Ammonium Bicarbonate 0.1M, pH 8.0, LC-MS grade) and sonicated once as described above. The protein concentration was determined (BCA assay, Thermo Fisher Scientific) and adjusted to 1 mg/ml. Isotopically labeled standard molecules, Phenylalanine-D8 and Biotin-D2 were added to the 200 µl cell lysates, the protein precipitated by addition of 800 µl of ice-cold methanol and stored at -80°C overnight. Upon thawing, the precipitated proteins were pelleted by centrifugation at 9300 x g for 10 min, the supernatants transferred into two clean Eppendorf tubes, dried down *in vacuo* and stored at -80°C. To perform HILIC-positive mode and HILIC-negative (neg) mode mass spectrometry, each sample was reconstituted in 60 µl of HILIC reconstitution buffer (acetonitrile/water, 90:10, v/v) and 5 µl of each sample was injected once for positive mode and 8 µl for negative mode. During the final reconstitution, isotopically labeled standard molecules, Tryptophan-D3, Carnitine-D9, Valine-D8, and Inosine-4N15, were added to each sample, and quality control sample was prepared by pooling equal volumes from each individual sample.

Mass spectrometry and data acquisition

UPLC-IM-MS and data-independent acquisition (MS^E) were performed on a Waters Synapt G2 HDMS (Milford, MA, USA) mass spectrometer equipped with a Waters nanoACQUITY UPLC system and autosampler (Milford, MA, USA). Metabolites were separated on a reverse phase 1 mm × 100 mm HSS T3 C18 column packed with 1.8-µm particles (Waters, Milford, MA, USA) held at 45°C. Liquid chromatography was performed using a 30-min gradient at a flow rate of 75 µl min^{-1} using solvent A (0.1% formic acid in H_2O) and solvent B (0.1% formic acid in acetonitrile). A 1 min wash period (99% solvent A) was performed prior to any gradient changes. After 1 min, solvent B increased to 60% over 10 min and up to 99% over another 10 min. The column was re-equilibrated to 99% solvent A for 5 min after each run. IM-MS^E analyses were run in resolution mode, with a capillary voltage of 2.75 kV, source temperature at 100°C, sample cone voltage at 30 V, extraction cone voltage at 5 V, source gas flow of 400 ml min^{-1}, desolvation gas temperature of 325°C, He cell flow of 180 ml min^{-1}, and an IM gas flow of 90 ml min^{-1}. The data were acquired in positive ion mode from 50 to 2000 Da with a 1 sec scan time; Leucine enkephalin was used as the lock mass (*m/z* 556.2771 in ES+ mode) at a concentration of 2 ng ml^{-1} infused at a flow rate of 7 µl min^{-1}. All analytes were analyzed using MS^E with an energy ramp from 10 to 40 eV.

High resolution (HR) MS and data-dependent acquisition 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 and autosampler (Thermo Fisher Scientific, Germany).

For HILIC analysis both positive and negative mode, metabolite extracts 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 (5 mM Ammonium formate in 90% water, 10% acetonitrile) and solvent B (5 mM Ammonium formate in 90% acetonitrile, 10% water) with the following gradient: 95% B for 2 min, 95-40% B

over 16 min, 40% B held 2 min, and 40-95% B over 15 min, 95% B held 10 min (gradient length 45 min).

Full MS analyses were acquired over a mass range of m/z 70-1050 using electrospray ionization both positive and negative mode. Full mass scan was used at a resolution of 120,000 with a scan rate of 3.5 Hz. The automatic gain control (AGC) target was set at 1×10^6 ions, and maximum ion injection time was at 100 ms. Source ionization parameters were optimized with the spray voltage at 3.0 kV, and other parameters were as follows: transfer temperature at 280°C; S-Lens level at 40; heater temperature at 325°C; Sheath gas at 40, Aux gas at 10, and sweep gas flow at 1. Tandem mass spectra were acquired using a data dependent scanning mode in which one full MS scan (m/z 70-1050) was followed by 2, 4 or 6 MS/MS scans. MS/MS scans were acquired in profile mode using an isolation width of 1.3 *m/z*, stepped collision energy (NCE 20, 40), and a dynamic exclusion of 4 s. MS/MS spectra were collected at a resolution of 15,000, with an automatic gain control (AGC) target set at 2×10^5 ions, and maximum ion injection time of 100 ms. The retention times and peak areas of the isotopically labeled standards were used to assess data quality.

Metabolite data processing and analysis

The acquired UPLC-IM-MS^E raw data and LC-HR MS/MS raw data were imported, processed, normalized and reviewed using Progenesis QI v.2.1 (Non-linear Dynamics, Newcastle, UK). All MS and MS/MS sample runs for one particular analysis (RPLC or HILIC, positive or negative mode) were aligned against a quality control (pooled) 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 using both de-adduction and de-isotoping to generate unique "features" (retention time and m/z pairs) representative of unannotated metabolites. Data were normalized to all features using Progenesis QI. Compounds with <30% coefficient of variance (%CV) were retained for further analysis. *P*-values were calculated by Progenesis QI using variance stabilized measurements achieved through log normalization, and metabolites with a *p*-value ≤ 0.1 (method #1) and p-value ≤ 0.05 (method #2) calculated by a one-way analysis of variance (ANOVA) test were considered significant.

Tentative and putative annotations were determined within Progenesis QI software using accurate mass measurements (<5 ppm error), isotope distribution similarity, and fragmentation spectrum matching database searches against Human Metabolome Database (HMDB) [130], METLIN [131] the National Institute of Standards and Technology (NIST) database [132] and an in-house library. Annotations from both RPLC and HILIC analyses were performed for all significant compounds (*p*-value ≤ 0.1 or 0.05). Annotated metabolites were further analyzed by pathway overrepresentation analysis using MetaboAnalyst 4.0 [133]. In these experiments, the level system for metabolite identification confidence was utilized. Briefly, many annotations were considered to be tentative (level 3, L3) when a top candidate cannot be prioritized [134], but they still represent families of molecules representative for the data acquired. The annotations considered putative (level 2, L2) and validated (level 1, L1) are for molecules with a fragmentation spectrum matching one of the databases or a standard molecule from the in-house library.