

Spatial Transcriptomics of Schizophrenia Insular Cortex Reveals Blood-Brain Barrier Hyperglycolysis and Increased Parenchymal Mitochondrial Respiration

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Abstract

Background

The blood-brain barrier (BBB), the brain's obligate energy gateway, has to our knowledge not been examined by spatially resolved profiling in any psychiatric disorder. Metabolic dysregulation is implicated in schizophrenia spectrum disorders (SSD), yet prior post-mortem evidence derives largely from bulk dorsolateral prefrontal cortex (DLPFC) tissue, in which vascular and parenchymal signals are mixed. The insular cortex, a salience-network hub implicated in SSD, has received little molecular attention.

Methods

We used the GeoMx Digital Spatial Profiler with the Human Whole Transcriptome Atlas to profile post-mortem insular cortex from 8 donors with schizophrenia or schizoaffective disorder and 8 matched controls (Netherlands Brain Bank-Psychiatry). Under neuropathological guidance, three CD31-positive (BBB) and three CD31-negative (parenchymal) areas of interest (AOIs) were delineated per donor in cortical layers III to VI, yielding 96 profiles of which 95 passed quality control.

Results

Endothelial enrichment of the BBB AOIs was confirmed by five canonical markers. Unsupervised clustering separated BBB from parenchyma, and within the BBB SSD samples formed a distinct subcluster. Gene set enrichment analysis identified three glucose metabolism pathways enriched in SSD BBB and seven mitochondrial energy metabolism pathways enriched in SSD parenchyma. At gene level, reported as exploratory because no gene survived correction for multiple testing, the endothelial glycolytic regulator *PFKFB3* was increased in SSD BBB despite reduced expression of the transporters *GLUT1* and *MCT1*, suggesting a compensatory downregulation of glucose and lactate transporters associated with heightened glycolytic flux. Additionally, the mitochondrial gene *COX7A1* was increased in parenchyma.

Conclusions

The BBB may contribute to the metabolic pathophysiology of SSD. Expression of energy metabolism pathways was increased in both compartments, opposite in direction to the reductions reported in DLPFC, suggesting that metabolic changes in SSD may be region-specific rather than generalised, and that bulk tissue analysis averages out a distinct BBB signal.

Background

Within the central nervous system, neurons need a suitable microenvironment to safeguard synaptic communication. The blood-brain barrier (BBB), a highly evolved structure consisting of brain endothelial cells (ECs), pericytes, a basal membrane, and astrocyte end-feet [1, 84], separates the blood from the brain tissue proper (the parenchyma). The BBB tightly and dynamically regulates which cells and substances enter and exit the brain, and secretes signalling factors to safeguard homeostasis. The BBB thus functions as a dynamic physical, transport, metabolic and immunological barrier. As such, the BBB is a crucial regulator of the neuronal microenvironment. The concept of the neurovascular unit describes how neurons and astrocytes signal their homeostatic needs to the adjacent BBB, e.g., by increasing the supply of energy substrate when neuronal activity is high [43, 73]. Therefore, the BBB is a functionally heterogeneous structure depending on its location in the vascular tree (e.g., arteriole, venule, capillary), its anatomic brain location (e.g., thalamus versus prefrontal cortex), but also based on the dynamic needs of the tissue it supplies (e.g., high vs low neuronal activity) [43, 73]. Since the largest surface area of blood vessels in the brain consists of capillaries, the BBB in the brain capillary bed is quantitatively the most important for brain homeostasis [43].

In the last decade, converging evidence points towards immune and metabolic aberrations as contributing factors to schizophrenia-spectrum disorders (SSD) [25, 31]. As the metabolic and inflammatory gatekeeper to the brain, malfunction of the BBB could explain these findings. Importantly, involvement of the BBB in SSD could have clinical implications since the BBB is therapeutically easily accessible through the blood. The hypothesis that the BBB could be implicated in schizophrenia is only recently being investigated [68, 69].

The BBB is small and closely integrated with the brain parenchyma, and has therefore been very difficult to study in isolation. Imaging techniques (e.g., fMRI and MRS) do not offer sufficient resolution to study the BBB at the (sub)cellular level. Post-mortem brain research can provide the molecular resolution required [42, 52], but thus far depended on tissue homogenisation to allow for whole transcriptome analysis, rendering a separation between the BBB and brain parenchyma impossible. Recently, novel techniques have been developed allowing for whole transcriptome analysis while retaining crucial spatial information [49, 53], therefore theoretically allowing for the omics-scale analysis of the BBB.

However, at present, spatially resolved transcriptomics has predominantly been used in cancer research (3,937 of 7,523 PubMed records) and only very limitedly in psychiatry (182 records; searched August 2026) [40, 70]. Characteristically, tumour tissue exhibits large transcriptional changes compared to healthy tissue and can be easily morphologically separated. In contrast, the molecular biology of psychiatric disorders is heterogeneous, characterised by small transcriptional changes in many genes in morphologically normal appearing tissue [2, 68]. A caveat to spatial transcriptomics of the BBB is the limited number of BBB cells within the maximum field of analysis compared to for instance tumour tissue. Additionally, for any post-mortem human brain study, the usual confounding factors including post-mortem delay, fixation time, agonal changes, cause of death, comorbidities etc. need to be taken into account [15, 41, 46, 79, 80]. To further complicate this research field, psychiatric diagnoses are syndromal, with overlapping diagnostic criteria; diagnoses as such do not (yet) reflect clear-cut

differences in underlying pathophysiology [72]. Furthermore, there is a general shortage of donated brains from psychiatric patients [19], especially of SSD patients, often leading to added issues regarding tissue quality and sample quantity. Altogether, the technical and practical feasibility of using spatial transcriptomics to study SSD in general, and the BBB in particular, remains to be determined.

The aim of the current study was to characterize the BBB and surrounding brain parenchyma transcriptome in post-mortem insular cortex from donors with SSD compared to non-affected controls, using spatially resolved whole transcriptome analysis. We focused on the insular cortex tissue because, despite its clear involvement in SSD [20, 56, 61, 75, 81, 85], this region remains molecularly understudied [56, 85].

Methods

Institutional review board approval

The study was approved by the Erasmus MC Institutional Review Board (METC) under the number MEC-2023-0373. The Review Board confirmed that the rules laid down in the Medical Research Involving Human Subjects Act (also known by its Dutch abbreviation WMO), do not apply to this research proposal.

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) post-mortem brain samples of the insular cortex were selected for this study. The clinical inclusion criteria included a main diagnosis of schizophrenia or schizoaffective disorder for the SSD cohort and the absence of a psychiatric diagnosis for the control cohort, with an age range of 18–85 years. Notably, SSD samples were matched to controls as closely as possible for sex, age, and overall cause of death (e.g., cancer, cardiovascular, respiratory) to create a homogeneous sample set. Exclusion criteria included intracerebral pathology such as malignancy, cerebrovascular accidents affecting the insular cortex, neuro-inflammatory diseases, and neurodegeneration (Braak stage > 2). Additionally, to minimise the confounding effects of post-mortem delay (PMD) and low pH on gene expression analyses [6, 46, 47], we included samples with a PMD < 24 hours and a post-mortem cerebrospinal fluid (CSF) pH ≥ 6 .

For spatial transcriptomics analysis, additional technical inclusion criteria were applied: FFPE samples with a maximum fixation time of 8 weeks, samples stored as FFPE tissue blocks for a maximum of 15 years, and freshly cut 5 μm tissue sections prepared and stored at 4°C for a maximum of 3 days before analysis.

The Netherlands Brain Bank - Psychiatry (NBB-Psy; <https://www.brainbank.nl>) exclusively stores brain donor material with a PMD typically below 12 hours. NBB-Psy follows a standardised protocol for donor brain preparation, including a formalin fixation time of 4 weeks and the storage of extensively sampled

brain tissue in both FFPE and fresh-frozen formats. Additionally, NBB-Psy provides comprehensive, high-quality clinical information on donor medical and psychiatric history, cause of death, agonal state, and a standardised neuropathological examination. Based on these well-defined standards, we proceeded with material from NBB-Psy.

Dissection follows one standardised regional protocol, so that anatomical provenance is identical across donors rather than reconstructed afterwards. CSF is drawn from the lateral ventricles at autopsy and its pH measured in every donor [57], giving a direct index of agonal state.

Neuropathological assessment

Two independent sources of neuropathological information were available for each donor. First, every NBB-Psy donor underwent a standardised neuropathological examination of extensively sampled brain regions, reported by an NBB neuropathologist, in which the insula is examined as part of the standard putamen, pallidum and insula block. Second, the study neuropathologist (VvD) examined the sections used for profiling, both the fluorescence-stained sections and adjacent haematoxylin-eosin sections, and confirmed the absence of microscopic alterations in the tissue actually analysed. The first was used to apply the exclusion criteria set out above, the second to establish the state of the profiled tissue.

Digital spatial whole transcriptome atlas profiling

We used the GeoMx Human Whole Transcriptome Atlas [27] (WTA) on the GeoMx Digital Spatial Profiler (DSP) Platform [26] to measure our samples following the manufacturer's instructions [24, 53] (NanoString, a Bruker company; Seattle). Slide preparation was performed on the Leica Bond RXm system according to the manufacturer's instructions and as previously described by Merritt et al. [53]. Freshly cut 5 µm FFPE tissue sections were loaded onto the Leica Bond RXm system, where they underwent baking, deparaffinisation, and epitope retrieval using ER2 solution (Leica Biosystems, AR9640) for 20 minutes at 100°C. This was followed by incubation for 15 minutes at 37°C with 0.1 µg/mL proteinase K (Thermo Fisher Scientific, AM2546) and post-fixation in 10% neutral buffered formalin (NBF). The slides were then incubated overnight at 37°C with the Human Whole Transcriptome Atlas (WTA) probe panel. After washing in 50% formamide at 37°C, blocking was performed, followed by immunostaining with CD31 (Abcam, ab215912, Alexa Fluor 594) and with SYTO13 nuclear staining (Thermo Fisher Scientific, S7575). After staining, the slides were loaded and scanned on the GeoMx DSP instrument.

Defining regions of interest (ROIs) and areas of interest (AOIs)

Morphological markers (CD31 and nuclear staining) were used to select Regions of Interest (ROIs) in insular cortical layers III-VI containing most microvessels. Selection was performed under the guidance of an experienced neuropathologist (VvD), and with help by an experienced technician (RG) as follows: 1)

CD31+ (BBB): microvessels positive for both CD31 and nuclear staining; 2) CD31- (parenchyma): brain parenchyma adjacent to the BBB, negative for CD31 but positive for nuclear staining (Fig. 1a). To limit background signal from neuronal lipofuscin, which is strongly autofluorescent across channels in aged human cortex, cells showing non-specific fluorescence in channels other than CD31 and SYTO13 were excluded from the BBB AOIs. Such signal was deliberately retained in the parenchymal AOIs, where excluding it would have systematically removed the lipofuscin-rich pyramidal neurons that the parenchymal AOIs are intended to represent.

For the BBB Areas of Interest (AOIs), settings were adjusted to include cells bordering on CD31 + cells, to enrich pericytes and astrocyte end-feet in direct contact with microvascular endothelial cells, thus also capturing these BBB elements. Biological triplicates were selected for each AOI within each tissue section, resulting in 6 unique whole transcriptome profiles per sample (3 BBB AOIs and 3 parenchyma AOIs per sample). In total, this yielded 96 WTA profiles. The recommended minimum of 100 nuclei/AOI could not always be achieved in the BBB AOIs, because the number of BBB cells within the maximum field of analysis is limited. Therefore, the minimum nuclear count per AOI was adjusted to 40 nuclei. At this AOI size the manufacturer's performance guidelines report reliable detection of medium- and high-expressing genes and of enriched pathways, while detection of low-expressing genes is listed only for AOIs of approximately 250 cells [24]. Gene-level results in the BBB should therefore be read as covering the medium- and high-abundance range only. See Fig. 1a.

Sequencing

Sequencing libraries were prepared using the GeoMx seq code Kit. Amplified libraries were pooled and purified using AMPure XP beads (Beckman Coulter, A63880). Sequencing was performed on an Illumina NextSeq 2000, using a P3 50-cycle kit with a paired-end 2 × 27 bp configuration. Raw sequencing data were demultiplexed and data processing was conducted using the GeoMx NGS Pipeline.

Because GeoMx DSP hybridises UV-cleavable oligonucleotide-barcoded probes to mRNA in situ, RNA is never extracted; the barcodes rather than the RNA are collected and sequenced [53]. The RNA integrity number, which measures the quality of extracted RNA, is therefore not applicable to this workflow. Quality control instead uses built-in negative control probes to establish signal-to-noise per AOI [53]. Because the targeted probe design bypasses poly(A) capture, sensitivity is retained in degraded FFPE tissue in which conventional RNA sequencing typically fails [24].

Data analysis

The WTA profiles were compared between SSD and controls for both BBB and parenchyma AOIs. Analysis was performed based on a standardised workflow as recommended by NanoString/Bruker (<https://bioconductor.org/packages/release/bioc/html/GeomxTools.html>) but extended by USEQ (UMCUGenetics/GeoMx-DSP-analyses: Useq GeoMX DSP pipeline revision).

Differentially expressed genes were identified using a linear-mixed model (LMM) accounting for the non-independence of the biological triplicates sampled within each section, with Benjamini-Hochberg false discovery rate (FDR) correction applied across all detected genes [4]. The model was fitted separately within BBB and parenchyma, and its contrast is Control minus Disease, so that a positive estimate denotes higher expression in controls and a negative estimate higher expression in SSD. This convention applies throughout, including to the normalised enrichment scores.

A heatmap was created from genes reaching nominal significance in the LMM, limited to the first 100 for computational tractability, using unsupervised cluster analysis. LMM results also served as input for pathway analysis using Fast Gene Set Enrichment Analysis (FGSEA) against the Reactome and KEGG databases, pre-ranked on the LMM estimates across all 7,321 genes that passed quality control, with a minimum gene set size of 15 and a maximum of 500. Reactome pathways are reported throughout and are shown in Fig. 3; the KEGG results were concordant.

Bar plots of normalised enrichment scores (NES) of the significantly enriched Reactome pathways, selected from the top 15 in each direction, using Fast Gene Set Enrichment Analysis (FGSEA). A positive NES indicates enrichment of pathway genes in control relative to schizophrenia spectrum disorders (SSD), while a negative NES indicates enrichment of pathway genes in SSD relative to control. The left panel shows results of the blood-brain barrier (BBB), and the right panel shows the results for the parenchyma Areas of Interest (AOIs).

Results

Description of sample characteristics

Based on our clinical and technical criteria described above, we selected insular cortex samples from 8 donors with SSD and 8 control donors. See Table 1 for demographic, clinical and technical sample characteristics.

Table 1

Demographic, clinical and technical sample characteristics NBB, Netherlands Brain Bank; PMD, post-mortem delay; CSF, cerebrospinal fluid.

NBB code	Diagnosis	Sex	Age, y	Cause of death	PMD, h:min	Braak	CSF pH
Schizophrenia spectrum disorder (n = 8)							
2010-021	Schizophrenia	M	59	Cardiac arrest with renal insufficiency and infection	12:30	Unknown	5.93
2010-055	Schizophrenia	M	64	Pulmonary embolism	19:15	1	7.0
2010-127	Schizoaffective disorder, depressive type	F	79	Heart fibrillation with heart failure	04:45	1	6.34
2012-031	Schizoaffective disorder, depressive type	F	55	Euthanasia	09:50	0	6.82
2016-003	Schizophrenia	M	67	Euthanasia, terminal gastro-esophageal cancer	05:45	1	6.29
2018-102	Schizophrenia	F	65	Cachexia and dyspnea, metastatic breast cancer	07:10	2	6.40
2021-62	Schizoaffective disorder, bipolar type	M	59	Metastatic kidney cancer	04:25	1	6.71
2022-115	Schizophrenia	F	80	Old age, renal insufficiency	08:20	2	6.79
Control (n = 8)							
2011-072	None	F	76	Hepatic failure and colon carcinoma	07:15	2	6.87
2012-049	None	F	70	Cachexia, end-stage pancreatic carcinoma	07:35	2	6.03
2012-059	None	F	78	Bronchopneumonia, metastatic neuroendocrine pancreatic carcinoma	04:35	2	6.41
2012-104	None	M	79	Euthanasia, terminal heart failure	06:30	2	6.71
2014-043	None	F	60	Metastatic breast carcinoma	08:10	0	6.58

NBB code	Diagnosis	Sex	Age, y	Cause of death	PMD, h:min	Braak	CSF pH
Schizophrenia spectrum disorder (n = 8)							
2018-121	None	F	68	Acute infection, metastatic pancreatic cancer	03:30	0	7.37
2021-051	None	M	77	Metastatic prostate cancer	07:25	1	6.72
2021-119	None	M	73	Collapse and asystole, probable cardiac death	10:15	1	6.71

Neuropathological findings

The sections used for profiling showed no microscopic alterations on examination by the study neuropathologist. In the standardised NBB-Psy reports, neurofibrillary pathology was Braak stage 2 or lower in every donor (not determined in one SSD donor), and no donor carried intracerebral malignancy, neuro-inflammatory disease or Lewy body pathology.

Areas of interest (AOIs) and gene detection

A total of 96 AOIs were selected and sequenced across all samples, including 24 BBB (CD31+) and 24 parenchyma (CD31-) AOIs in both the SSD and the control group. Transcriptomic sequencing data from all AOIs, except one, passed the quality control (QC) step. The 40-nucleus criterion was met by all AOIs. For the BBB (CD31+) AOIs the mean nuclear count was 86 (range 47 to 171); for the parenchyma (CD31-) AOIs it was 1142 (range 220 to 5256). In total, 7,321 of the 18,677 targets remaining after probe quality control passed the identification threshold and were used for analysis.

Successful selection of BBB AOIs

To validate the accuracy of the BBB (CD31+) and parenchyma (CD31-) AOI selection, we assessed if endothelial cell specific gene expression was enriched in BBB. Five genes known to be highly and specifically expressed in endothelial cells were selected (*PECAM1*/CD31, *CLDN5*, *VWF*, *CD34*, *ENG*). Their expression levels were compared between the BBB and parenchyma AOIs, pooling all donors and both diagnostic groups. All endothelial cell genes showed a significantly higher expression in the BBB compared to parenchyma (*PECAM1* $P = 3.4 \times 10^{-6}$; *CLDN5* $P = 7.9 \times 10^{-22}$; *VWF* $P = 1.0 \times 10^{-17}$; *CD34* $P = 4.5 \times 10^{-14}$; *ENG* $P = 9.2 \times 10^{-13}$), confirming enrichment of endothelial cells within the BBB AOIs (Fig. 1b).

Differentially expressed genes in BBB (CD31+) between SSD and control

No individual gene reached FDR-corrected significance in either BBB or parenchyma. Gene-level results are therefore reported at nominal, uncorrected P and are exploratory, characterizing the gene-level signal underlying the pathway results rather than establishing individual genes; the pathway-level analyses are

corrected and carry the study's claims. Comparing the gene expression profiles of BBB AOs in SSD versus controls using LMM, we identified six differentially expressed genes (DEGs) at an Log_2 Fold Change ($|\text{Log}_2\text{FC}|$) cutoff of ≥ 1 and nominal $P \leq 0.05$. All 6 genes were downregulated in the BBB of SSD compared to controls (Supplementary Table 1, Additional file 1).

However, post-mortem brain whole transcriptome studies in psychiatry employ a less stringent $|\text{Log}_2\text{FC}|$ cutoff value (≥ 0.25) [2, 68] to account for the more subtle transcriptional differences in psychiatric diseases as compared to diseases like cancer. At a $|\text{Log}_2\text{FC}| \geq 0.25$, we identify 236 DEGs in the BBB AOs at nominal significance, including 205 downregulated and 31 upregulated in SSD compared to control. In the BBB, the fold-change threshold excluded no gene, since all genes reaching nominal significance already exceeded it.

Limited differentially expressed genes in parenchyma (CD31-)

In parenchyma, applying the stringent $|\text{Log}_2\text{FC}|$ threshold of ≥ 1 yielded no DEGs between SSD and control. Of the 48 genes reaching nominal significance in parenchyma, adjusting the $|\text{Log}_2\text{FC}|$ cutoff to ≥ 0.25 yielded only 5 (1 downregulated and 4 upregulated in SSD compared to control). Effect sizes were therefore much smaller in parenchyma than in BBB. One DEG in parenchyma was involved in energy metabolism (*COX7A1*, increased in SSD). The mitochondrial complex I assembly factor *NDUFAF2* was likewise increased in SSD at nominal significance ($\text{Log}_2\text{FC} = -0.19$, $P = .014$), but fell below the $|\text{Log}_2\text{FC}| \geq 0.25$ threshold and is therefore not counted among the five parenchymal DEGs.

Clear clustering of BBB vs parenchyma and strong clustering of SSD vs control BBB

Genes reaching nominal significance in the LMM were displayed in a heatmap using unsupervised cluster analysis (Fig. 2). The insular cortex BBB (CD31+) and parenchyma (CD31-) AOs are clustered separately. Within the BBB cluster, a clearly separable distinction between SSD and controls can be made. Within the parenchyma cluster, no clear clustering of SSD samples was visible.

Heatmap showing the results of an unsupervised cluster analysis of the top-ranked genes by nominal (uncorrected) P . Yellow colour indicates increased gene expression, while purple colour represents reduced gene expression. The first cluster distinctly separates the blood-brain barrier (BBB) from the parenchymal Areas of Interest (AOIs). Further clustering within the BBB AOs reveals a clear distinction between schizophrenia spectrum disorders (SSD; 'disease') and control samples, whereas no such distinction is observed within the parenchyma AOs.

Distinct metabolic signature in SSD insular BBB and parenchyma: insights from pathway analysis

Pathway analysis using FGSEA in both BBB and parenchyma AOs revealed pathways differentially enriched between SSD and control at FDR-corrected significance, in contrast to the gene-level results,

which are nominal throughout. Selecting the top 15 positively and top 15 negatively enriched pathways yielded 20 such pathways in BBB and 20 in parenchyma (Fig. 3). In BBB AOs, 6 pathways were enriched in SSD, while in parenchyma 13 pathways were enriched in SSD. Notably, pathways related to energy metabolism were disproportionately represented in both BBB and parenchyma. In SSD BBB, three glucose metabolism pathways were enriched (Gluconeogenesis; Glycolysis; Glucose Metabolism); in SSD parenchyma, eight energy metabolism pathways were enriched, seven of which mitochondrial (Respiratory Electron Transport, ATP Synthesis by Chemiosmotic Coupling, and Heat Production by Uncoupling Proteins; Cristae Formation; Complex I Biogenesis; TCA Cycle and Respiratory Electron Transport; Respiratory Electron Transport; Formation of ATP by Chemiosmotic Coupling; Mitochondrial Protein Import). Given this strong emphasis on energy metabolism pathways and genes, alongside the relatively smaller effect sizes of DEGs in the parenchyma AOs, the next section will primarily explore findings related to energy metabolism within the insular cortex BBB.

Upregulation of glycolysis in SSD BBB

To further investigate glucose metabolism in the BBB, we focused on the DEGs within the 3 enriched pathways related to glucose metabolism: *Gluconeogenesis*, *Glycolysis* and *Glucose Metabolism*. These three pathways shared many overlapping genes, with *Glucose Metabolism* encompassing genes from both the *Gluconeogenesis* and *Glycolysis* pathways. Therefore, we narrowed our analysis to the *Gluconeogenesis* and *Glycolysis* pathways. In our BBB dataset, 16 genes were present in the *Gluconeogenesis* pathway and 37 genes were present in the *Glycolysis* pathway. Seven genes were common to both pathways, encoding for enzymes that can convert substrates back and forth. These 7 shared genes were overrepresented in the top upregulated DEGs of the *Gluconeogenesis* pathway, therefore disproportionately driving the Normalised Enrichment Score (NES) for that pathway. This implies the *Gluconeogenesis* pathway is enriched in the pathway list primarily based on genes shared with the *Glycolysis* pathway. Consequently, we focused exclusively on the *Glycolysis* pathway.

Since endothelial cells (ECs) exhibit a specialised form of glycolysis, known as aerobic glycolysis, where pyruvate is converted into lactate by lactate dehydrogenase (LDH, Fig. 4), we included the genes coding for lactate dehydrogenase (*LDHA* and *LDHB*) to the list of DEGs in the *Glycolysis* pathway. Notably, in the *Aerobic Glycolysis* pathway in BBB, one gene stood out from the remainder by an order of magnitude in nominal significance: *PFKFB3*, the principal driver of glycolytic flux in endothelial cells [18, 71], was upregulated in SSD BBB ($\text{Log}_2\text{FC} = -0.47$; nominal $P = 0.002$), the only gene in the pathway to reach nominal significance; the next smallest P was 0.06, and the next glycolytic enzyme, *ALDOC*, reached only $P = 0.26$. Since the expression of *PFKFB3* is regulated by various different stimuli [18, 48, 71], it is beyond the scope of this study to investigate the possible mechanisms behind the upregulation of *PFKFB3* in SSD BBB. See Fig. 5 for a graphic representation of DEGs in the *Aerobic Glycolysis* pathway in SSD BBB.

The aerobic glycolysis pathway in the blood-brain barrier (BBB) is depicted, with enzymes (represented as oval boxes) colour-coded based on their gene expression levels in SSD compared to control. Enzymes with red colouring indicate increased expression, with deeper hues representing stronger upregulation. Genes that did not pass quality control (QC) are shown in grey. Genes upregulated at nominal

significance (uncorrected $P < .05$) are outlined with a green stroke. The conversion of pyruvate to lactate by LDH (dashed grey box) is not typically considered part of the *Glycolysis* pathway, however, it plays a critical role in endothelial cells (ECs), which predominantly rely on aerobic glycolysis. Abbreviations: 1,3BPG: 1,3-bisphosphoglycerate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; DHAP: dihydroxyacetone phosphate; F1,6BP: fructose-1,6-bisphosphate; F2,6BP: fructose-2,6-bisphosphate; F6P: fructose-6-phosphate; G6P: glucose 6-phosphate; GADP: glyceraldehyde-3-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GPI: glucose-6-phosphate isomerase; HK: hexokinase; LDH: lactate dehydrogenase; NAD⁺: nicotinamide adenine dinucleotide, oxidised; NADH: nicotinamide adenine dinucleotide, reduced; PEP: phosphoenolpyruvate; PFK1: phosphofructokinase-1; *PFKFB3*: 6-phosphofructo-2-kinase/fructose-2,6 bisphosphatase 3; PGAM: phosphoglycerate mutase; PGK: phosphoglycerate kinase; PK: pyruvate kinase; TPI1: triosephosphate isomerase 1

Decreased expression of glucose and lactate transporters in SSD insular cortex BBB

To further elucidate the role of energy substrate transporters in relation to the enriched *Aerobic Glycolysis* pathway in SSD BBB, we specifically examined the gene expression levels of glucose and lactate transporters in the BBB and parenchyma AOs (Fig. 5). These transporters include 1) *SLC2A1* (GLUT1): the main glucose transporter in BBB ECs [60], also expressed at lower levels in astrocytes [35, 59]; 2) *SLC2A3* (GLUT3): the main neuronal glucose transporter [59], less abundant than GLUT1 in ECs [21] though its transcript is enhanced in brain endothelial cells relative to other brain cell types [36]; 3) *SLC16A1* (MCT1), the main lactate transporter of the BBB endothelium, also expressed at lower levels in astrocytes [5, 34], where lactate is produced by aerobic glycolysis [18, 71] (Fig. 4). Lactate derived from aerobic glycolysis in ECs is transported by MCT1 into the perivascular space and interstitium, from which pericytes and astrocytes take it up [5, 45]. Astrocytes, in turn, export lactate derived from astrocytic aerobic glycolysis into the interstitial space, mainly through MCT1 and MCT4 [5]. Finally, neurons take up lactate from the interstitial space through *SLC16A7* (MCT2), to be used as an energy substrate in mitochondria [5].

We found a decreased expression of *SLC2A1* (*GLUT1*) and of *SLC16A1* (*MCT1*) in SSD BBB at nominal significance, while no difference was detected in the expression of *SLC2A3* (*GLUT3*) compared to controls. In contrast, there was no difference in the expression of *SLC16A1* (*MCT1*) or *SLC2A3* (*GLUT3*) in parenchyma. Importantly, *SLC16A7* (MCT2) did not pass QC and pre-processing. See Supplementary Table 2 for statistics and Fig. 5 for a background on energy substrate transport and metabolism in the neurovascular unit.

Sensitivity analyses

Agonal state and tissue pH: Repeating the full analysis after excluding the single SSD donor with severe agonal changes and a CSF pH of 5.93 left the primary findings unchanged in direction and significance

(*SLC16A1* became marginal, $P = .051$).

Hypoxia: *PFKFB3* is hypoxia-inducible through HIF-1 [54], so the BBB glycolytic signal could in principle reflect pre-mortem ischaemia. Several observations argue against a hypoxia-only account, including reduced rather than increased expression of the HIF-1 target *P4HA1* [78] in SSD BBB; these are set out in the Supplementary Results.

Discussion

In this study, we conducted the first spatial profiling analysis investigating the possible role of the insular cortex BBB in the pathophysiology of SSD. To achieve this, we utilised a state-of-the-art spatially resolved transcriptomics technique (GeoMx DSP WTA) to generate whole transcriptome profiles of the BBB and the surrounding brain parenchyma in post-mortem brain tissue of SSD compared to controls. Insular cortex was selected due to its known involvement in SSD [56, 85], and because this region remains largely understudied in post-mortem SSD research. Selecting insular cortex ROIs containing only small-calibre blood vessels, combined with a nuclear marker and an EC marker, and using adjusted settings to include cells bordering on ECs (i.e., pericytes and astrocyte end-feet) technically enabled us to enrich for BBB ECs in the BBB AOIs. The enrichment was further improved by excluding areas with non-specific fluorescence in other channels from the BBB AOIs (mostly lipofuscin-induced autofluorescence). Using this approach, we were able to include a minimum of 47 nuclei per AOI for downstream WTA, providing sufficient sensitivity to robustly detect differentially expressed genes with medium and high expression levels, as well as to perform reliable pathway analysis [24]. The study's robustness was further enhanced by analysing 3 biological replicates per AOI in each sample. In spite of practical and technical challenges (e.g., the globally limited availability of post-mortem brain tissue from SSD patients with a low post-mortem interval, storage duration < 15 years, as well as the relatively low number of cells within the BBB AOIs), we found this technique and setup are effective to study the BBB in SSD.

Using a commonly accepted Log_2FC cut-off of ≥ 0.25 for post-mortem psychiatric studies [2], we identified 236 DEGs in the BBB and 5 in the parenchyma comparing SSD to controls, all at nominal significance. Cluster analysis showed a clear separation between both BBB and parenchyma, and SSD and control BBB, indicating a distinct BBB transcriptional phenotype in SSD. No such separation was observed in the parenchyma, suggesting that the BBB is the primary site of SSD-associated transcriptional divergence. Results from differential expression and pathway analysis (FGSEA) suggest increased glucose metabolism (glycolysis) in the BBB alongside enhanced mitochondrial energy metabolism in the insular cortex parenchyma of SSD patients. Alterations in brain energy metabolism (both glucose and mitochondrial metabolism) are considered an important pathophysiological model for SSD [31]. To contextualize these findings, we first provide an overview of energy substrate transport and metabolism in the BBB and in brain parenchyma (Fig. 5).

The adult human brain predominantly relies on glucose as an energy source [59]. Glucose is transported from the brain capillary lumen into BBB ECs mainly by Glucose Transporter-1 (GLUT1; gene: *SLC2A1*).

GLUT1 is an insulin-independent glucose transporter that facilitates glucose transport along its gradient across the cell membrane [21], making intracellular glucose levels in ECs independent of insulin signalling. *GLUT1* is highly and selectively expressed in BBB ECs, with much lower expression in glial cells and little to no expression in neurons [35, 59].

ECs exhibit a distinct metabolic phenotype: they are highly glycolytic even in the presence of ample oxygen (the 'Warburg effect' or 'aerobic glycolysis'), while normally glycolysis is inhibited under high oxygen conditions due to a shift towards the more ATP-yielding mitochondrial oxidative phosphorylation (the 'Pasteur effect') [44]. In aerobic glycolysis, pyruvate is converted into lactate by LDH [21]. Up to 85% of total cellular EC ATP is generated through aerobic glycolysis. Their glucose consumption rate is remarkably high, rivalling that of cancer cells [18, 71]. Mitochondria occupy a modest share of EC cytoplasmic volume and primarily serve as biosynthetic hubs rather than for ATP production [71]. Furthermore, when glucose levels are elevated, ECs further upregulate aerobic glycolysis while suppressing oxidative phosphorylation, a phenomenon known as the 'Crabtree effect' [3, 44].

Similar to ECs, astrocytes primarily rely on aerobic glycolysis rather than oxidative phosphorylation for energy production [9]. This metabolic shift in BBB ECs and astrocytes helps preserve key energy substrates (e.g., lactate) and oxygen for neurons, which depend on the high-ATP-yielding oxidative phosphorylation to sustain their function [32, 44, 71]. Due to their reliance on mitochondrial respiration, neurons are particularly vulnerable to disruptions in mitochondrial function in all of its facets ('mitochondrial dysfunction'). For instance, an impaired ability to buffer oxygen radicals generated by oxidative phosphorylation can lead to oxidative stress, contributing to neurodegeneration [16].

Lactate derived from aerobic glycolysis in BBB ECs and astrocytes is transported through MCT1 (ECs and astrocytes) and MCT4 (astrocytes) into neurons (MCT2) for use in oxidative phosphorylation [5, 45]. Residual glucose is transported through GLUT1 (ECs and astrocytes) into neurons (GLUT3).

Neurons coordinate their energetic needs with astrocytes and ECs through a process called 'neurovascular coupling', the mechanisms of which remain largely unexplored [43]. During heightened neuronal activity, neurons locally increase blood flow to ensure an adequate supply of nutrients and oxygen [43]. BBB ECs play a central role in neurovascular coupling by sensing neural activity through e.g., potassium channels, GABA_A and NMDA receptors [43]. Furthermore, neurons can induce the Crabtree effect in surrounding astrocytes, thus ensuring transport of sufficient bioenergetic substrates needed to sustain their function [23, 30]. As such, metabolism within the BBB appears to be at least partially regulated by the metabolic needs of downstream neurons. Interestingly, this mechanism is reminiscent of tumour ECs, which exhibit a hyperglycolytic phenotype in order to fuel tumour growth [50].

Glycolytic flux in ECs, astrocytes and neurons is mainly regulated through PFKFB3, an enzyme converting F6P into F2,6BP. In turn, F2,6BP is a potent allosteric activator of phosphofructokinase-1 (PFK1), the rate-limiting enzyme of glycolysis (Figs. 4 and 5) [71]. Both ECs and astrocytes express high levels of PFKFB3 to sustain glycolytic flux [8, 18]. PFKFB3 is a major inducer of glycolysis in both astrocytes [8] and ECs [18, 50], particularly during high bioenergetic demand, such as in hypoxia, physiological angiogenesis

and tumour angiogenesis. In contrast, neurons maintain a low glycolytic flux because PFKFB3 protein is absent from them despite comparable transcript levels, owing to continuous proteasomal degradation by APC/C-Cdh1 [32].

This background on energy substrate transport and metabolism in the NVU provides context for our findings. We observed an enrichment in *Glycolysis* pathway genes, an increase in *PFKFB3* expression and a non-significant increase in LDH genes expression in SSD BBB compared to controls. These findings suggest an upregulation of aerobic glycolysis in SSD BBB via *PFKFB3* induction. Additionally, we found a decrease in *GLUT1* and *MCT1* expression in SSD BBB, without a compensatory upregulation of *GLUT3*. Given the concurrent increase in aerobic glycolysis gene expression, this transporter downregulation may reflect a response to high substrate availability in the BBB (glucose, lactate). Some studies suggest that BBB *GLUT1* is downregulated in hyperglycaemia [63], though this remains debated, as ECs have been reported not to downregulate *GLUT1* at high extracellular glucose [21]. Ample bioenergetic substrate availability is also supported by our finding of increased expression of mitochondrial energy metabolism pathway genes and an increase in *COX7A1* expression in SSD insular cortex parenchyma. An impairment of the EC/astrocyte-to-neuron glucose and lactate shuttle as an alternative explanation to reduced levels of BBB *GLUT1* and *MCT1* would lead to decreased mitochondrial respiration in the parenchyma, reflected in decreased expression of mitochondrial energy metabolism genes, which we did not observe. To our knowledge, decreased *GLUT1* expression in SSD BBB has not been previously described. Sullivan et al. [77] found reduced *GLUT1* and *GLUT3* expression, increased *MCT1* and decreased glycolytic enzyme expression (HK and PFK1) in laser-capture microdissected (LCM) pyramidal neurons (but not astrocytes) in the dorsolateral prefrontal cortex (DLPFC) in SSD. However, they did not investigate the BBB or the insular cortex [77].

While speculative at this stage, our gene expression findings could point towards increased mitochondrial energy metabolism in the insular cortex parenchyma in SSD, supplied by a heightened glycolytic flux in the BBB induced by upregulated expression of *PFKFB3*. Alternatively, elevated BBB glycolysis may actively drive mitochondrial energy metabolism in the insular cortex in SSD. Notably, fMRI studies have previously reported insular cortex hyperactivity in SSD patients in response to neutral stimuli [20].

Recent hypotheses propose that cerebral hypermetabolism induced by (transient) hyperglycolysis could contribute to bipolar mania [12]. Since SSD and bipolar disorders have overlapping etiologies [13], a hypermetabolic state in both the BBB and insular cortex parenchyma may represent a disease-driving mechanism in SSD. If so, targeting hyperglycolysis in the BBB could offer a novel therapeutic strategy to restore brain parenchymal metabolic balance in SSD. Additional explanations for our findings could include mitochondrial dysfunction in SSD insular parenchyma, potentially leading to oxidative stress and neuronal dysfunction (in line with the 'oxidative stress' hypothesis of SSD) [7]. Increased expression of oxidative phosphorylation genes is not by itself evidence of healthy mitochondria: the same transcript pattern is expected when impaired mitochondria are being compensated for. Both readings predict greater electron leak and therefore more reactive oxygen species, which is what the SSD literature on

redox dysregulation describes [17]. We found no enrichment of oxidative stress response pathways in the parenchyma, so our data cannot distinguish increased mitochondrial capacity from compensation for impaired mitochondria.

Whether the observed increase in aerobic glycolysis and decrease in *GLUT1* and *MCT1* expression in the SSD insular cortex BBB is part of the primary disease process, or a consequence of long-term antipsychotic (AP) treatment remains speculative. Frequently used APs are known to elevate fasting blood glucose level [10], though the extent varies between different APs [66]. There is some evidence that APs can influence the expression of glycolytic enzymes, but findings are inconsistent [33]. In differentiated human neuron-like cells, risperidone produced coordinate downregulation of oxidative phosphorylation and glycolysis gene sets, whereas amisulpride, aripiprazole and clozapine did not significantly alter these gene sets, although aripiprazole reduced mitochondrial respiration dose-dependently without transcriptional change [62]. This variability is likely due to differences in model systems used (both in vivo and in vitro), with many studies focussing on neurons, which as explained have inherently different energy metabolism properties than BBB ECs and astrocytes [14, 39, 55, 74]. Since *PFKFB3* expression in ECs can be induced by various different stimuli, such as circulating growth factors, hormones, hypoxia [11, 18, 48, 71], the current study is not designed to determine the precise mechanism underlying its increased expression.

Alterations in glycolysis and mitochondrial energy metabolism have been previously reported in post-mortem SSD brain transcriptomic and proteomic studies [31]. However, the majority of these studies have found a reduced expression of genes and proteins involved in glycolysis and mitochondrial energy metabolism [22, 31, 38, 67]. Combined with an intrinsic association of SSD with dysregulated glucose metabolism, such as a genetic predisposition to insulin resistance, hyperglycaemia and type 2 diabetes, these findings gave rise to the hypothesis that a cerebral bioenergetic deficiency may play a causative role in the pathophysiology of SSD [28, 31, 65]. Our gene expression findings of increased BBB aerobic glycolysis and increased parenchyma mitochondrial metabolism in SSD insular cortex are in apparent contrast with these prior findings. A key reason for this discrepancy may be the predominant focus of prior research on the DLPFC, while other brain regions remain largely understudied. Although both the DLPFC and insular cortex are implicated in SSD, their functions are incomparable, playing roles in e.g., executive functioning versus interoception and self-awareness, respectively [56, 76, 85]. Furthermore, studies indicate considerable regional variation in BBB function [43], meaning molecular findings in one brain region cannot be extrapolated to another. Indeed, our results align with the only other post-mortem study on the insular cortex in SSD: Pennington et al. [64] used LCM to analyse insular cortex layer 2 (i.e., containing a mixture of neurons, glial cells, BBB cells) and found increased protein levels of a glycolytic enzyme (ALDOC, fold change 1.32 and 1.31 across two spots) and of a mitochondrial ATPase subunit (ATP5C1, 1.93 and 1.16). That study flagged both proteins as predicted by covariates, ALDOC by age and antipsychotic status and ATP5C1 by brain pH, so the convergence is supportive rather than confirmatory. Notably, their study used tissue from the Stanley Foundation Brain Consortium, the same tissue repository used in other studies reporting decreased glycolysis and mitochondrial energy metabolism in DLPFC. These contrasting findings in different brain regions derived from material from the same donor

cohort suggest that SSD may involve region-specific metabolic alterations rather than a generalised bioenergetic deficiency as previously proposed [31].

Another possible explanation is that post-mortem transcriptomic and proteomic studies in SSD rely on tissue from a limited number of brain banks, primarily the Stanley Foundation Brain Collection, which may restrict the generalizability of findings. Furthermore, these studies often involve material with a long PMD and low tissue pH, both of which can significantly impact mitochondrial gene expression [6, 47, 82]. In our sample set, only one SSD sample exhibited significant agonal changes and a CSF pH < 6 (5.93). All other samples had a pH > 6 and did not display post-agonal changes on neuropathological examination. To ensure this sample did not unduly influence our finding, we repeated the entire data analysis excluding it. The results remained unchanged, confirming the robustness of our conclusion (data not shown).

The cohort itself deserves a word, because in post-mortem psychiatry tissue quality usually constrains possible research questions. A rapid autopsy programme delivering post-mortem delays typically below 12 hours [57], one fixation protocol of bounded duration, uniform storage capped at 15 years, one standardised dissection so that every donor contributes the identical anatomical block, CSF pH measured at autopsy in every case, a standardised neuropathological report, and lifetime clinical and medication documentation, are conditions that the dedicated psychiatric brain bank NBB-Psy can supply and that most collections cannot. Because the Netherlands is a small, densely populated and logistically well-connected country, rapid autopsy is possible of all donors at one location using a consistent protocol [58]. Probe hybridisation in situ targets short sequences and is therefore robust to the partial RNA degradation characteristic of FFPE material [83], but post-mortem delay, fixation time and storage duration still shape the signal recovered. An AOI of a few dozen endothelial nuclei leaves little margin for that variation, which is considerable in most psychiatric post-mortem collections.

A crucial distinction of our study is the use of a spatially resolved approach, allowing us to analyse the BBB and brain parenchyma separately. Most prior studies relied on homogenised brain tissue, containing a mixture of e.g., neurons, glial cells and BBB cells. Because of the distinct metabolic phenotype of these cell types (Fig. 5), analysing bulk tissue may obscure critical metabolic differences. Some studies attempted to enrich specific cells using LCM, primarily focusing on DLPFC neurons [77]. One small study applied LCM to isolate microvascular BBB from DLPFC of SSD donors and controls for transcriptomic analysis, reporting a hypo-inflammatory BBB phenotype in SSD but no metabolic alterations, with no individual gene surviving correction for multiple testing [29]. Differences in study design, including the number of studied samples, region (DLPFC), storage (frozen), the use of LCM to enrich for BBB, cDNA conversion and microarray for transcriptomic analysis, and a longer PMD may explain the lack of metabolic findings in that study compared to ours. Another study employed single-nucleus RNA sequencing on sorted cell types, including ECs, from dissociated SSD midbrain tissue compared to controls [68], and reported 14 differentially expressed genes across all BBB cell types, of which a single one was endothelial. However, this near-absence of endothelial findings may stem from the use of a non-spatially resolved technique, the limited number of microvascular ECs sequenced, and the selection of

midbrain, an anatomically complex region with diverse nuclei and functions. Notably, that study did identify DEGs in ependymal cells (which form the blood-cerebrospinal fluid barrier, not the BBB) and pericytes. Taken together, these methodological differences likely explain the discrepancies between previous studies and our findings.

To our knowledge, our study is the first to apply spatial WTA to SSD insular cortex tissue, enabling the separate analysis of the BBB and parenchyma. While the inclusion of 16 samples for spatial biology studies is substantial [37, 51], our findings cannot be generalised to the entire SSD spectrum. In that context, we consider the relatively small sample size as one of the potential limitations of this study. In addition, solely profiling the insular cortex restricts the detection of potentially contrasting metabolic signatures in other brain regions. A further limitation is that no individual gene survived correction for multiple testing in BBB or parenchyma, which is expected at 8 donors per group against transcriptome-wide testing, so that all gene-level results are exploratory and the study's claims rest on the pathway-level analyses. The nominally significant genes are not scattered at random: *PFKFB3*, the strongest of them, is the key regulator of glycolytic flux in the Glycolysis pathway that FGSEA flagged independently at corrected significance. No independent validation was performed. Finally, transcript abundance in fixed post-mortem tissue does not necessarily reflect metabolic flux, and the functional interpretations offered above are inferences from expression rather than measurements of actual metabolic activity.

Conclusions

Using spatial transcriptomics (GeoMx DSP WTA), we identified a hyperglycolytic transcriptional phenotype in the BBB and increased mitochondrial energy metabolism in the surrounding parenchyma in insular cortex tissue of SSD patients. These findings challenge the assumption of generalised bioenergetic alterations in SSD, indicating that metabolic dysregulation may be region- and compartment-specific. Our results highlight the need for a more comprehensive 'brain mapping' approach examining multiple brain regions from the same donor. Additionally, the distinct metabolic profiles of the BBB and brain parenchyma emphasise the importance of spatial multi-omics in post-mortem psychiatric research, and raise the possibility that BBB function could represent a novel therapeutic target in SSD warranting functional follow-up.

Abbreviations

ADP: adenosine diphosphate; AOI: area of interest; ATP: adenosine triphosphate; BBB: blood-brain barrier; COX7A1: cytochrome c oxidase subunit 7A1; CSF: cerebrospinal fluid; DEG: differentially expressed gene; DLPFC: dorsolateral prefrontal cortex; DSP: Digital Spatial Profiler; EC: endothelial cell; F1,6BP: fructose-1,6-bisphosphate; F2,6BP: fructose-2,6-bisphosphate; F6P: fructose-6-phosphate; FDR: false discovery rate; FFPE: formalin-fixed paraffin-embedded; FGSEA: fast gene set enrichment analysis; GEO: Gene Expression Omnibus; GLUT1: glucose transporter 1; GLUT3: glucose transporter 3; LDH: lactate dehydrogenase; LMM: linear mixed model; MCT1: monocarboxylate transporter 1; MCT2: monocarboxylate transporter 2; NAD⁺: nicotinamide adenine dinucleotide, oxidised; NADH: nicotinamide

adenine dinucleotide, reduced; NBB-Psy: Netherlands Brain Bank - Psychiatry; NBF: neutral buffered formalin; NES: normalised enrichment score; NVU: neurovascular unit; PFK1: phosphofructokinase-1; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PMD: post-mortem delay; QC: quality control; ROI: region of interest; ROS: reactive oxygen species; SSD: schizophrenia spectrum disorder; WMO: Medical Research Involving Human Subjects Act; WTA: Whole Transcriptome Atlas.

Declarations

Ethics approval and consent to participate

Approved by the Erasmus MC Institutional Review Board (MEC-2023-0373). The Review Board confirmed that the Medical Research Involving Human Subjects Act (WMO) does not apply. All brain donors provided written informed consent during life for brain donation and for the use of tissue and clinical data for research purposes, in accordance with the 1964 Declaration of Helsinki and its later amendments.

Consent for publication

Not applicable.

Availability of data and materials

The data generated in this study will be made available in the Gene Expression Omnibus (GEO) database following the peer review process.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KH and DAMM conceived and designed the study. All authors acquired, analysed or interpreted the data. KH, DAMM and NJMvB drafted the manuscript. TT, MC, IN and RG performed the statistical analysis.

VvD, RG and IN provided administrative, technical or material support. KH and DAMM obtained funding. DAMM, NJMvB and KH supervised the study. All authors critically revised the manuscript for important intellectual content, and all authors read and approved the final manuscript. KH and DAMM contributed equally and share first and last authorship. TT and VvD share second authorship.

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Authors' information

Not applicable.

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Figures

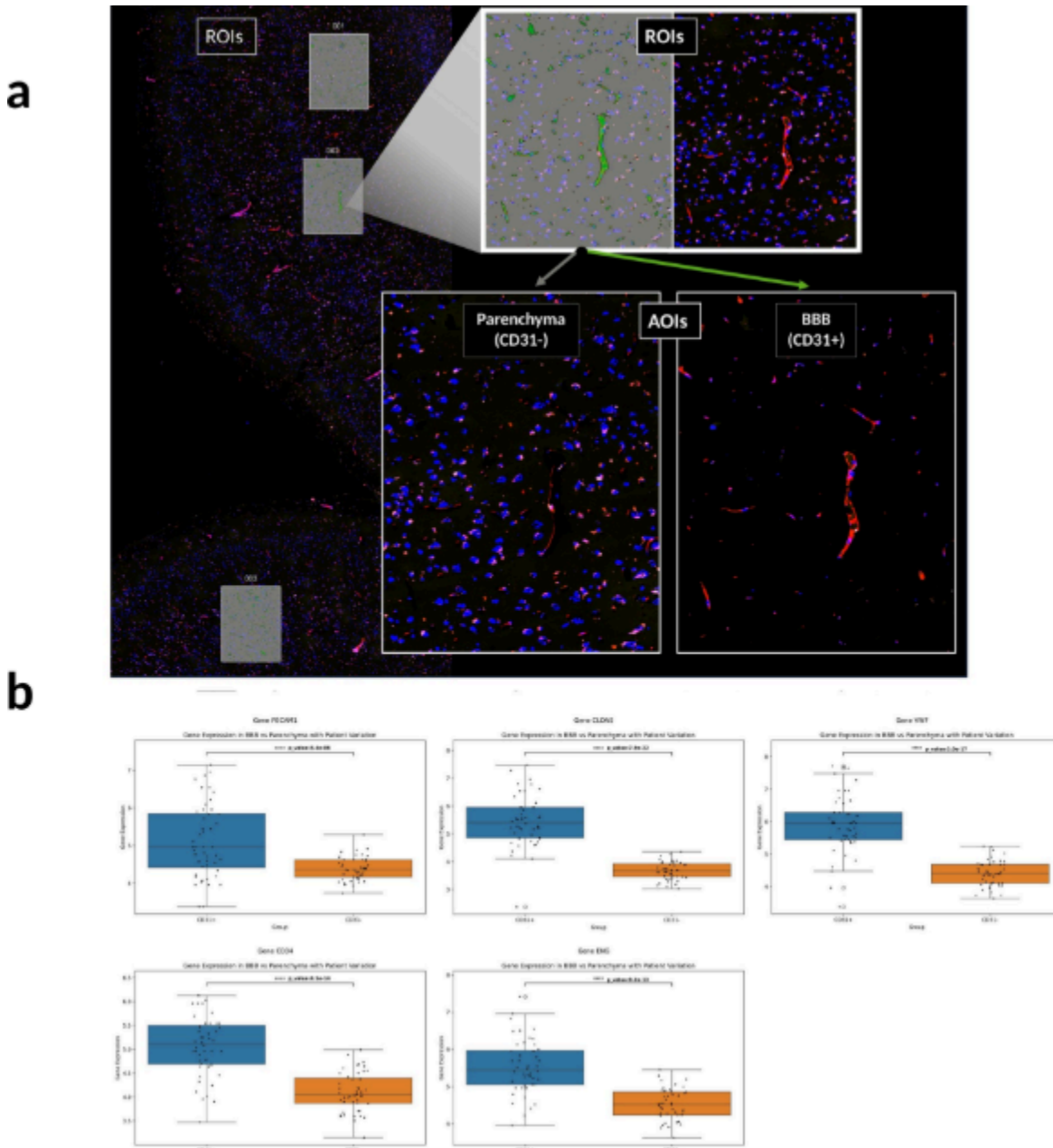


Figure 1

Spatial delineation of BBB and parenchyma in the insular cortex for transcriptomic profiling

(a) Representative image illustrating the selection of Regions of Interest (ROIs) and Areas of Interest (AOIs) in human insular cortex tissue. The left panel (low magnification) shows three selected ROIs (rectangles 001, 002, and 003). The enlarged view of ROI 002 (right bottom panel, high magnification) demonstrates AOI segmentation based on CD31 staining, distinguishing CD31⁺ blood-brain barrier (BBB) regions from CD31⁻ parenchymal areas. To minimise background signal from neuronal lipofuscin, cells with non-specific fluorescence in other channels were excluded from the BBB AOIs, whereas such signal was retained in the parenchymal AOIs to avoid omitting lipofuscin-rich neurons. (b) Boxplots of normalised expression of five canonical endothelial cell markers, *PECAM1* (CD31), *CLDN5*, *VWF*, *CD34*

and *ENG*, in CD31⁺ (BBB) and CD31⁻ (parenchymal) AOIs, with individual AOI values overlaid. Annotated P values are uncorrected.

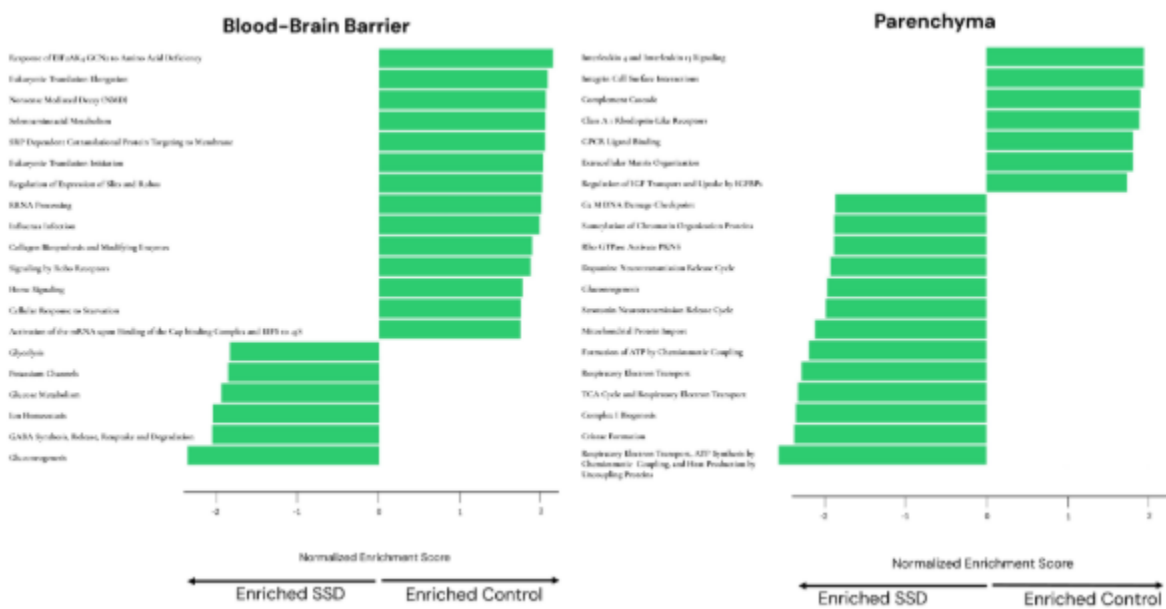


Figure 2

Normalised Enrichment Scores of Significantly Enriched Pathways (FGSEA)

Bar plots of normalised enrichment scores (NES) of the significantly enriched Reactome pathways, selected from the top 15 in each direction, using Fast Gene Set Enrichment Analysis (FGSEA). A positive NES indicates enrichment of pathway genes in control relative to schizophrenia spectrum disorders (SSD), while a negative NES indicates enrichment of pathway genes in SSD relative to control. The left panel shows results of the blood-brain barrier (BBB), and the right panel shows the results for the parenchyma Areas of Interest (AOIs).

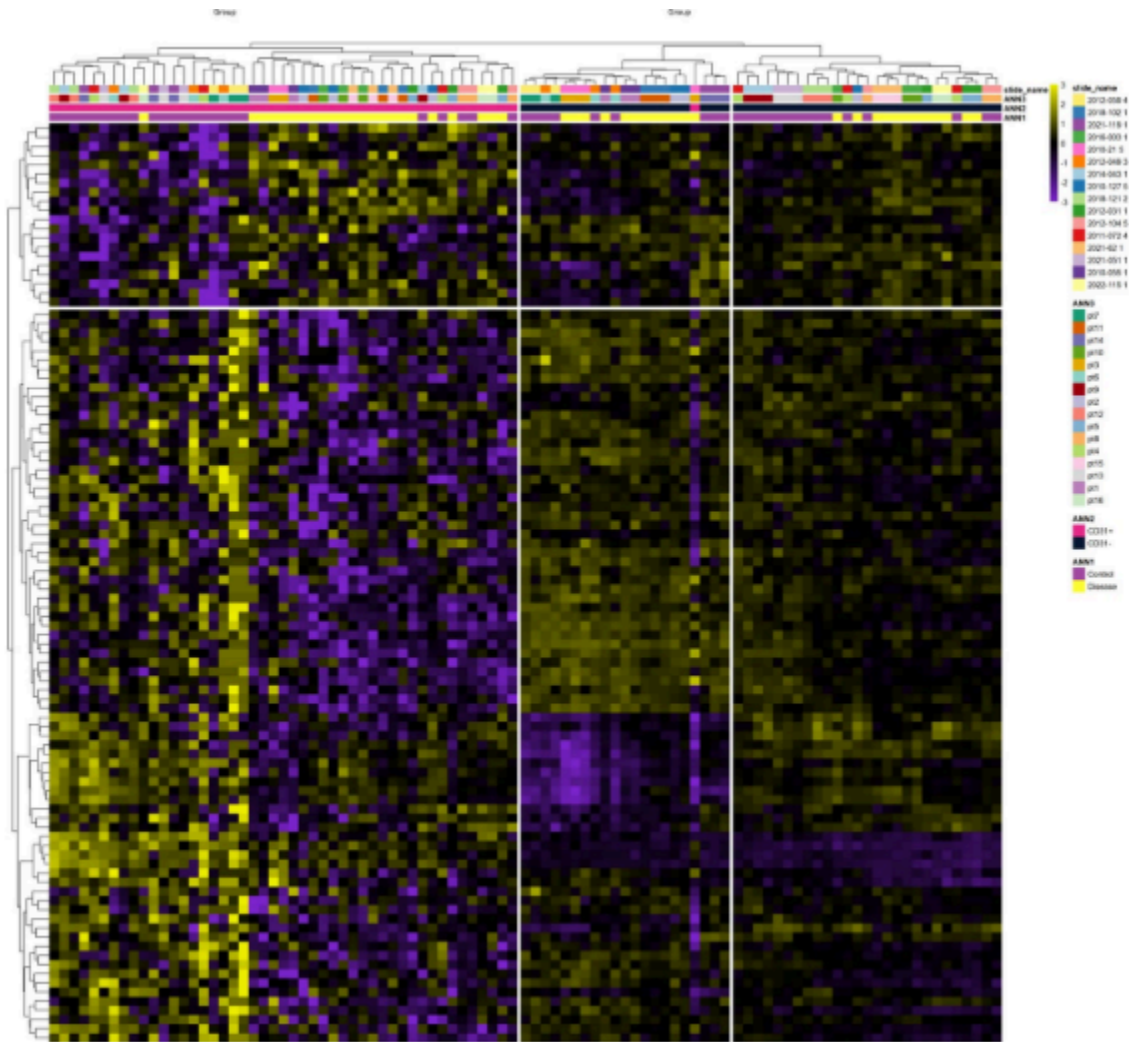


Figure 3

Unsupervised Cluster Analysis Reveals Distinct Transcriptional Profiles in BBB vs. Parenchymal AOIs

Heatmap showing the results of an unsupervised cluster analysis of the top-ranked genes by nominal (uncorrected) P. Yellow colour indicates increased gene expression, while purple colour represents reduced gene expression. The first cluster distinctly separates the blood-brain barrier (BBB) from the parenchymal Areas of Interest (AOIs). Further clustering within the BBB AOIs reveals a clear distinction between schizophrenia spectrum disorders (SSD; 'disease') and control samples, whereas no such distinction is observed within the parenchyma AOIs.

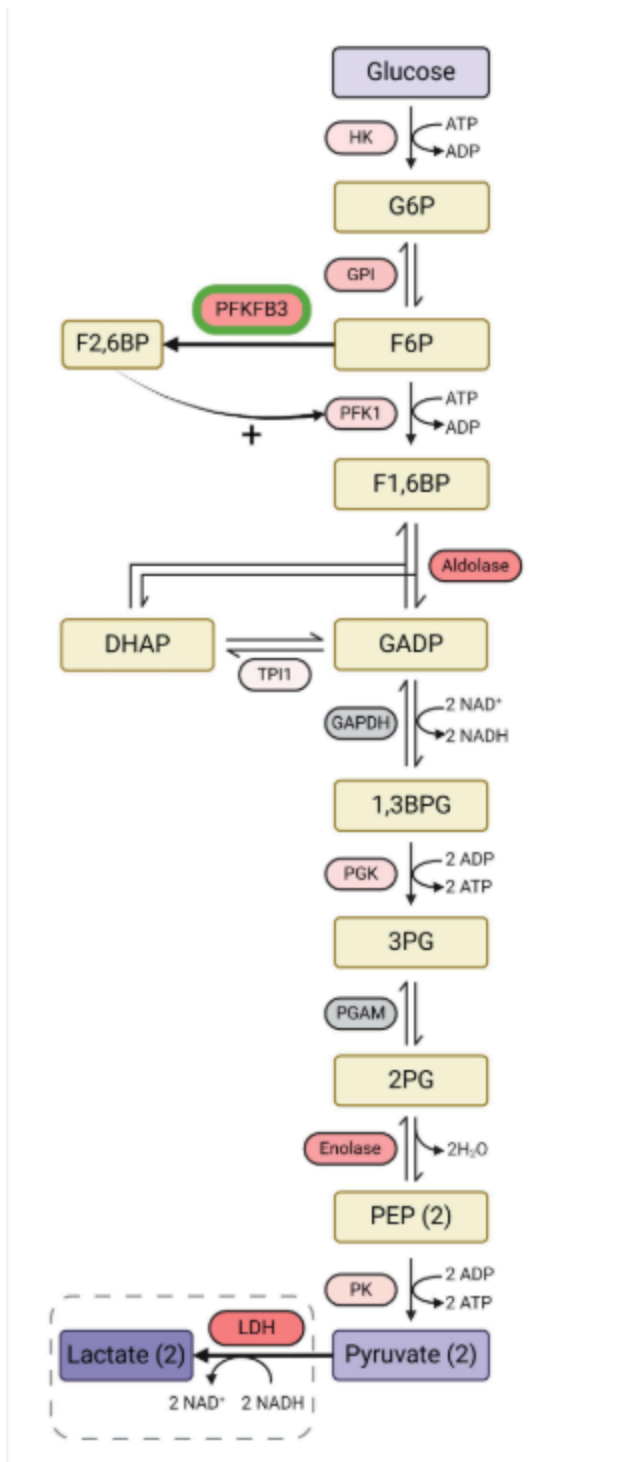


Figure 4

Aerobic Glycolysis Pathway in BBB

The aerobic glycolysis pathway in the blood-brain barrier (BBB) is depicted, with enzymes (represented as oval boxes) colour-coded based on their gene expression levels in SSD compared to control. Enzymes with red colouring indicate increased expression, with deeper hues representing stronger upregulation. Genes that did not pass quality control (QC) are shown in grey. Genes upregulated at nominal significance (uncorrected $P < .05$) are outlined with a green stroke. The conversion of pyruvate to lactate

by LDH (dashed grey box) is not typically considered part of the *Glycolysis* pathway, however, it plays a critical role in endothelial cells (ECs), which predominantly rely on aerobic glycolysis. Abbreviations: 1,3BPG: 1,3-bisphosphoglycerate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; DHAP: dihydroxyacetone phosphate; F1,6BP: fructose-1,6-bisphosphate; F2,6BP: fructose-2,6-bisphosphate; F6P: fructose-6-phosphate; G6P: glucose 6-phosphate; GADP: glyceraldehyde-3-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GPI: glucose-6-phosphate isomerase; HK: hexokinase; LDH: lactate dehydrogenase; NAD⁺: nicotinamide adenine dinucleotide, oxidised; NADH: nicotinamide adenine dinucleotide, reduced; PEP: phosphoenolpyruvate; PFK1: phosphofructokinase-1; *PFKFB3*: 6-phosphofructo-2-kinase/fructose-2,6 bisphosphatase 3; PGAM: phosphoglycerate mutase; PGK: phosphoglycerate kinase; PK: pyruvate kinase; TPI1: triosephosphate isomerase 1

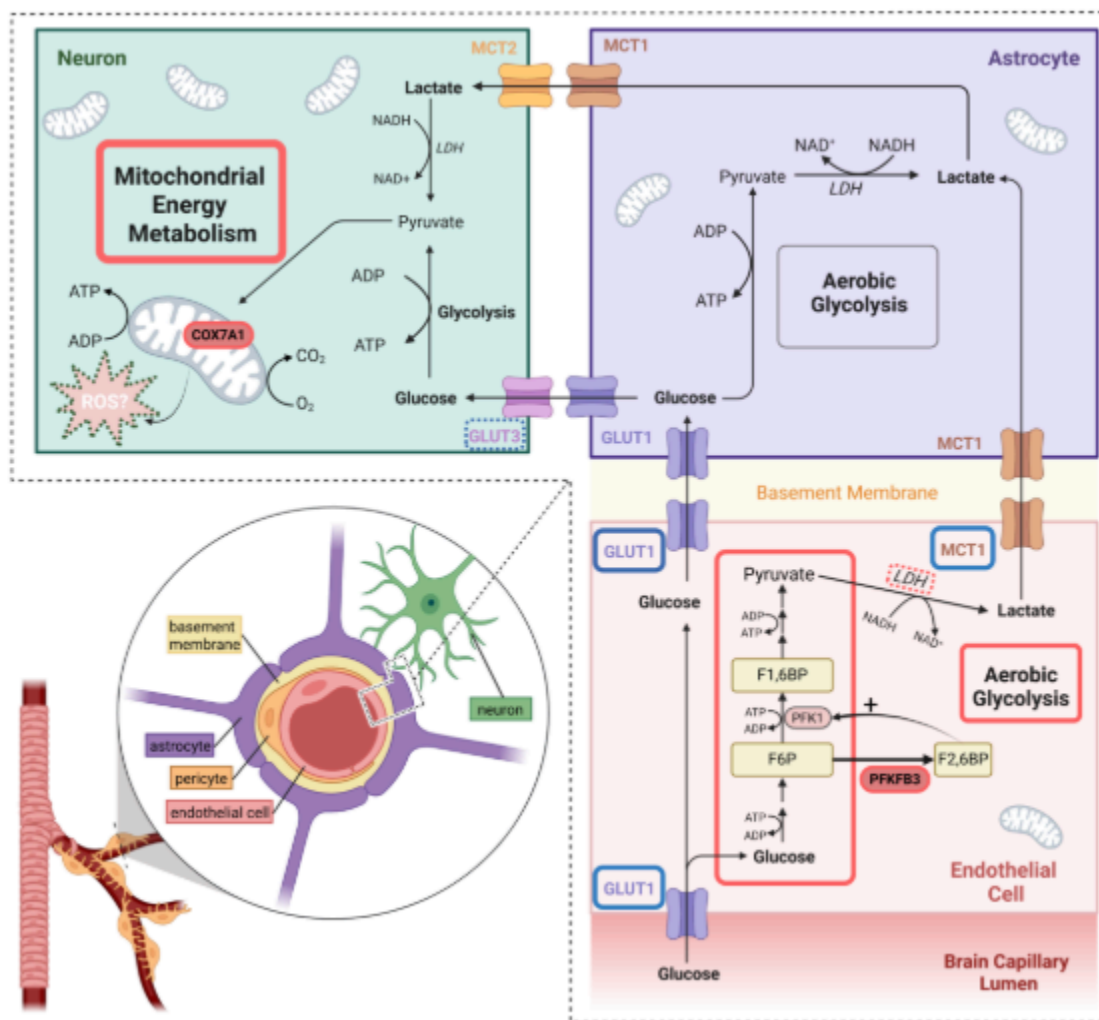


Figure 5

Energy Substrate Transport and Metabolism in the Neurovascular Unit Lower left: Diagram illustrating the blood-brain barrier (BBB) composed of endothelial cells (ECs), a basement membrane, pericytes and astrocyte endfeet. Together with adjacent neurons, the BBB forms the neurovascular unit (NVU). Lower

right and top: Schematic representation of energy substrate transport and metabolism within the NVU, highlighting key differences between specific cell types. Glucose is transported along its concentration gradient from the brain capillary lumen into ECs by GLUT1, an ATP- and insulin-independent glucose transporter. ECs predominantly rely on aerobic glycolysis for ATP production, generating lactate. Surplus glucose in ECs is transported to the basement membrane and into astrocytes via GLUT1, where it can be metabolised through (aerobic) glycolysis or be transported into the brain interstitial space and into neurons via GLUT3. Lactate derived from EC aerobic glycolysis is transported towards the basement membrane via MCT1. Astrocytes also transport lactate through MCT1. Pericytes depend on EC-derived lactate for energy [15, 44], while astrocytes primarily utilise glycolysis for ATP production, with the capacity to increase aerobic glycolysis when neuronal activity is high [23]. Excess lactate from astrocytes is transported into the interstitial space through MCT1 and MCT4, and into neurons by MCT2. This dynamic shuttling of metabolites ensures a stable energy supply to neurons, balancing glycolysis for rapid, low-yield ATP and mitochondrial respiration for slower, high-yield ATP production. Red and blue boxes: In SSD insular cortex BBB, we found upregulation of glycolysis (Red box, lower right) and a (non-significant) upregulation of LDH. These findings suggest enhanced aerobic glycolysis in BBB ECs in SSD. The reduced expression of *GLUT1* and *MCT1* (Blue boxes) point to a downregulation of these transporters in response to high substrate availability (glucose and lactate) in the BBB. Finally, *PFKFB3* expression was significantly increased in SSD BBB. Since *PFKFB3* is the main driver of glycolytic flux in ECs [18], the increased *PFKFB3* expression may drive the upregulation of glycolysis in SSD BBB. In SSD insular cortex parenchyma, upregulation of mitochondrial energy production pathways (Red box, upper left) was observed, with no difference in *GLUT3* ($P = .91$). These findings may reflect increased substrate availability for neuronal energy production. Mitochondrial respiration, however, generates Reactive Oxygen Species (ROS) [76], which can contribute to oxidative stress. Although oxidative stress is implicated in the pathophysiology of SSD [17], we did not detect significant changes in the oxidative stress response in the parenchyma of this cohort. All gene-level changes shown are nominal and exploratory; no gene reached FDR-corrected significance.

Supplementary Files

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