Multi-scale investigation of cellular and molecular patterns in schizophrenia

PhD thesis

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1. Introduction

Schizophrenia (SCH) is a devastating, complex neuropsychiatric disorder with a global prevalence of ~0.5–1%. It manifests through heterogenous symptoms traditionally classified into three domains: **positive** (such as hallucinations, delusions), **negative** (such as social withdrawal and loss of motivation), and **cognitive** (such as deficits in attention and memory). Today, SCH is thought to arise from **genetic–environmental interactions** that disrupt **neurodevelopment**, leading to abnormal brain connectivity and chemistry. Despite extensive research, its etiology remains elusive, highlighting the need for a change of perspective in understanding this disorder.

SCH **symptomatology** is thought to be associated with **disrupted frontal cortical activity, dopamine dysfunction, and excitatory–inhibitory imbalance**. **Cortical interneuron dysfunction** has been proposed as a key mechanism deeply associated with negative and cognitive symptoms.

Motivated, goal-directed behaviour is mediated by functionally segregated, dynamically interacting **cortico–basal ganglio–cortical circuits**: associative, limbic, and motor fronto–striatal networks. The basal ganglia (BG), comprising the caudate nucleus (CN), putamen, globus pallidus, and – from a functional aspect – the subthalamic nucleus and substantia nigra, participate in crucial functions such as motor control, learning, and value-based decision-making. Although the structure and neurochemical organization of the BG have been found highly conserved across vertebrates, striking differences have also been elucidated recently regarding its cellular composition and cell type-specific properties, suggesting it may also nurture unexpected hubs for evolutional innovation.

Disruptions in the fronto-striatal loops have been implicated in the pathophysiology of SCH. Thus, we chose to investigate the CN and the dorsolateral prefrontal cortex (DLPFC), representing

important hubs of **the associative fronto-striatal loop essential for goal-directed behaviour and executive function.**

As the primary input structure of the fronto–striatal circuitry, the striatum receives, selects, integrates, and relays information from cortical areas, and is therefore crucial for many areas of motor and cognitive function. Although local interneurons are heavily outnumbered by projection neurons in the striatum, they are essential modulators of striatal activity and output. Based on the recent **transcriptomic profiling of the human striatum**, the most abundant striatal interneurons belong to the **calretinin/tachykinin 3** (*CALB2*/*TAC3*)-expressing, and the parathyroid hormone-like hormone (*PTHLH*)-expressing subtypes. Morphologically, the calretinin protein (CR)-expressing interneurons can be classified into "small", "medium", and "large" classes, the latter largely overlapping with large cholinergic neurons – suggesting that the transcriptomically defined *CALB2/TAC3* population, which is not cholinergic, largely corresponds to the "small" and "medium" CR neurons.

This *CALB2*/*TAC3* population is of particular interest: it has been recently described as a newly emerged, **primate-specific cell type.** Furthermore, previous histological data suggests that striatal CR interneurons showed significant expansion during primate brain evolution. Together, these data indicate that non-cholinergic CR neurons may have been "upgraded" during evolution to provide more refined control necessary for more complex behaviours and **therefore may represent a cell type key to understanding human behaviour – and striatum-related disorders as well.** Regarding SCH, structural changes of the BG have been reported with no clear consensus, and lower striatal cholinergic neuron density has been also described. Furthermore, recent observations of patient-derived ventral forebrain organoid models revealed early developmental dysfunction in striatal neurons. However, although the striatum

is recognized as a key hub of dysfunction in SCH, comprehensive histological and transcriptomic investigations are lacking.

Another key feature of SCH is **working memory (WM) impairment,** making the **DLPFC**, a critical WM hub, a primary focus of SCH research. The DLPFC projects heavily to the CN via the associative fronto-striatal loop. A line of studies measuring the density and mRNA content of cortical interneurons reported alterations in SCH primarily involving parvalbumin (PV), calbindin (CB), and somatostatin (SST) interneurons – although these results were often conflicting. The few post-mortem studies involving cortical CR interneurons have found no significant alterations in SCH, although these were often limited by technical factors and low sample numbers. **In summary, the accumulating evidence indicates that GABAergic interneurons may be present in physiological density in SCH, with a subset of them potentially being dysfunctional – but not uniformly in every patient.**

Importantly, SCH seems to be characterized by **network-level functional disturbances** across brain regions, affecting many, if not all, cell types. This makes SCH challenging to evaluate with classical histological methods due to technical limitations. **Single cell or nucleus RNA sequencing** (scRNAseq/snRNAseq) enables the **simultaneous quantification of gene expression in individual cells or nuclei across many samples** – thus, it can identify the most vulnerable cell types, clarify potential disease mechanisms, and reveal therapeutic targets, offering a unique tool to investigate complex disorders.

2. Objectives

The aim of this doctoral research was to reveal SCH–associated cellular and molecular patterns in the human CN and the DLPFC.

The main objectives were the following:

A) **Performing quantitative histological assessment of CR interneuron density in the human CN in SCH.**

B**) Performing quantitative histological assessment of excitatory neuron, CR, and PV interneuron density in the human DLPFC in SCH.**

C) **Identifying cell type–specific gene expression differences between control and SCH samples with snRNAseq in the DLPFC.**

3. Methods

3.1. Materials

Formalin-fixed, paraffin-embedded (FFPE) and fresh frozen (FF) tissue samples from the Brodmann area 9 and the precommissural striatum containing the CN were obtained from multiple brain banks. FF samples were used for snRNAseq, while 6 µm FFPE sections were prepared for immunohistochemistry (IHC) and RNAscope. FF cortical grey matter was micro-dissected under a stereotactic microscope, from cases with a post-mortem interval (PMI) less than 24 hours to ensure RNA integrity. Material was collected from donors from whom (or from their next of kin) written informed consent had been obtained and the experiments were approved by the Research Ethics Committee of the Hungarian Medical Research Council (38711-1/2019/EKU). Cases were matched as closely as possible for PMI, age, and sex to minimize biological variance.

3.2. Immunohistochemistry

Adjacent CN sections were stained for CR, Iba1, and TMEM119. Iba1 and TMEM119 markers were used to visualize microglia and reveal potential patterns of neuroinflammation. Adjacent DLPFC sections were stained for CR, PV, SMI-31.1, and cresyl violet (Nissl). SMI-31.1 is a neuronal marker which labels the neurofilament medium and heavy chains and preferentially stains pyramidal neurons, making it useful for estimating the total density of cortical pyramidal cells. Cresyl violet staining was used to estimate total neuronal density and to identify cortical layers.

Tissue sections were deparaffinized, rehydrated, and treated with H_2O_2 to block endogenous peroxidase activity, followed by heat treatment for antigen retrieval. Primary antibodies were applied in tris-buffered saline containing Triton X-100 (pH=7.6) and incubated for 1 hour at room temperature, followed by

horseradish peroxidase-linked secondary antibody incubation for another hour. Staining was developed with 3,3'-diaminobenzidine. Nuclei were counterstained with haematoxylin. Then ssections were dehydrated and coverslipped. Negative controls did not produce specific staining.

3.3. Single nucleus RNA sequencing

FF DLPFC tissue from 9 SCH and 14 CTR cases was processed for snRNAseq by M. Y. Batiuk and colleagues (University of Copenhagen, Khodosevich Group). Nuclei were isolated via a protocol optimized by M. Y. B. – briefly, tissue was homogenized in homogenization buffer; homogenate was then filtered and centrifuged. Then, nuclei were further purified with iodixanol gradient ultracentrifugation. Neuronal nuclei were then sorted with fluorescence-activated nucleus sorting (FANS) using the neuron-specific NeuN staining. Library preparation was carried out according to 10X Genomics guidelines. Sequencing was performed on an Illumina NovaSeq 6000 platform.

3.4. RNAscope combined with immunohistochemistry

RNAscope immune co-detection experiments were carried out according to manufacturers' protocols, including tissue pre-treatment, antigen retrieval, probe hybridization, and fluorescent signal amplification. Sections were incubated with anti-CR primary antibody, followed by Alexa Fluor 488 secondary antibody. DAPI staining was applied for visualizing nuclei, and slides were mounted with Fluoromount-G for imaging. In the case of the CR–*CALB2* experiment, autofluorescence was quenched with TrueBlack Plus treatment.

3.5. Image analysis

Stained sections were digitized with whole-slide scanners (Aperio ScanScope AT Turbo, Leica Biosystems; Pannoramic Flash Desk DX, 3DHistech). The Aperio ImageScope and the 3DHistech SlideViewer softwares were used for annotation. The whole CN was outlined and all immunoreactive neurons were marked. In the DLPFC, regions of interests (ROIs) were placed, layers were outlined, and immunoreactive neurons were annotated in layers separately. Cell densities were calculated based on cell counts and layer areas. Sections stained with microglial markers were analysed with an Aperio Positive Pixel Count algorithm.

Fluorescent slides were digitized by an LSM780 confocal laser scanning microscope. The 20× and 63× objectives were used to produce tile scans containing all cortical layers within a 1 mm-wide column. ROI placement and evaluation was carried out similarly as described above.

The sections produced in the CR–*CALB2* RNAscope experiment were scanned with a fluorescent whole-slide scanner (Pannoramic MIDI II, 3DHistech). At least two ROIs per section were outlined in SlideViewer (1-mm-wide columns only containing supragranular layers, not separated). Neurons were classified into five categories based on the level of protein and mRNA expression. Only cells with visible nuclei were analysed.

3.6. Density heatmaps

Intact (i.e., devoid of rips and bubbles) cortical grey matter areas (0.25–1 cm^2) were delineated on the scanned sections, and immunoreactive cells were annotated. Over 45,000 CR neurons were mapped across 18 cm^2 of cortical grey matter. Cell coordinates were loaded into a spatial informatics software (QGIS v2.18.3) and maps were generated with standard parameters. The heatmaps were merged with their respective tissue images using GIMP (v2.10.22) for better spatial context.

3.7. Bioinformatics analysis

Raw sequencing data was processed by our research partners at the University of Copenhagen (Khodosevich Group) and at Harvard University (Kharchenko Lab). Downstream analysis involved filtering doublets and poor-quality nuclei, normalization, integration, clustering, and Uniform Manifold Approximation and Projection (UMAP) embedding. Cell type-specific differentially expressed genes (DEGs) were identified, and their functional relevance was investigated by Gene Ontology pathway analysis.

For unpublished snRNAseq figures, I re-analysed the raw data for visualization purposes. After filtering, normalization, integration, dimensionality reduction (PCA), clustering and UMAP-embedding, the main cell types were identified using known marker genes to facilitate understanding and to explore the potential subclusters expressing *CALB2* (encoding CR).

3.8. Statistical analyses

In the case of CN data, SPSS (v22.0) was used. Unpaired, two-tailed Student's t-tests were used to test whether the mean of age, PMI, and cross-sectional areas of the CN were different between groups. Fisher's exact test was used to compare the distribution of sex between the groups. General linear models were fitted on the data, including cell density as dependent variable, diagnosis and cell type and explanatory variables, and PMI, age, and sex as potential confounders.

In the case of DLPFC data, the R software was used (v4.3.1.). Linear mixed models (LMM) were applied with post-hoc multiple comparisons followed by Bonferroni correction. Cell types were investigated in separate models. Cell density was set as the dependent variable, sample ID was included as random effect, layer was a within-subject factor, while diagnosis was a between-subject factor. PMI, age, and sex were included as confounders.

The CR-*CALB2* RNAscope experiment was analysed similarly, however, contrast matrices were applied to directly test the biologically relevant questions without producing unnecessary comparisons.

My own work included sectioning, immunohistochemical staining, RNAscope (the CR-*CALB2* experiment, at the Department of Pharmacology and Pharmacotherapy, Semmelweis University, in collaboration with Dr. Zoltan V. Varga), whole-slide scanning, confocal microscopy, manual cell counting, statistical analyses, and participation in writing the manuscripts. Unpublished, extra snRNAseq figures presented here are my own work based on the original, published dataset.

4. Results

4.1 Quantitative histological assessment of neuron density in SCH

4.1.1. Calretinin interneurons in the caudate nucleus

Analysing striatal sections from 6 SCH and 6 CTR cases revealed significantly lower total density of CR neurons in the CN (based on altogether 21,443 annotated neurons, p=0.018; GLM). The densities of small, medium, and large CR interneurons were all relatively lower in SCH; however**, statistical testing only revealed the density of small CR interneurons as significantly lower** (p=0.013; n=6/group; GLM). This was not confounded by PMI, age, or gender (p>0.05; n=6/group; GLM). The cross-sectional CN areas did not differ significantly between groups.

4.1.2. Striatal microglial activity in SCH

To determine whether the observed lower CR neuron density may be associated with increased neuroinflammation, we immunohistochemically visualized microglia.

We found no significant differences in immunoreactive area fractions between groups (**CTR/Iba1**: 5.04% ± 0.42%, **SCH/Iba1**: 3.66% ± 0.87%, p=0.164; **CTR/TMEM119**: 3.32% ± 0.46%, **SCH/ TMEM119:** 2.96% ± 0.72%, p=0.659; n=6/group; t-test).

4.1.3. Calretinin and parvalbumin interneurons in the DLPFC

Analysing DLPFC sections from 10 SCH and 10 CTR samples revealed **significantly lower density of CR neurons in cortical layer 2** (based on altogether 5254 neurons; p=0.0028; n= 10/group; LMM followed by post-hoc multiple comparison). Notably, only a subset of SCH cases demonstrated lower density, which was not associated with PMI or other known confounding factors. PV neuron density was also markedly

lower in cortical layer 2 in SCH; however, this result was confounded by PMI ($p=8.3\times10^{-7}$; n=10/group; LMM followed by post-hoc multiple comparison). Therefore, we excluded PV interneurons from further analyses.

Mapping CR neuron distribution on a larger scale confirmed inhomogeneous CR cell body distribution, suggesting a "patchy" distribution pattern in SCH with frequent, visually striking low-density patches. Similar patterns were also visible in some CTR cases, although generally to a lesser extent. Although these observations are not corroborated by statistical analyses, the intriguing patterns may suggest disrupted upper-layer structure of CR interneurons, which could be the result of developmental disturbances.

4.1.4. Excitatory neuron and total neuron density in the DLPFC

Annotating all neuronal elements in a subset of cases stained with cresyl violet revealed **no overall difference in total neuron density between the groups** (based on altogether 45,446 neurons, p=0.875; n=6/group; LMM). Measuring the overall density of **excitatory neurons** stained by SMI-31.1 in the whole cohort, we found **no significant difference** between the two groups (based on altogether 58,964 neurons, p=0.721; n=10/group; LMM).

4.2 Gene expression differences between CTR and SCH DLPFC

4.2.1. Cortical interneuron diversity

To assess cell type-specific gene expression differences in SCH, 9 SCH and 14 CTR DLPFC samples were processed for snRNAseq by our collaborating partners at the University of Copenhagen. 209,053 nuclei were retained after quality control (81,817 SCH and 127,236 control nuclei).

Main excitatory (EX) cell types were identified by markers such as *CUX2* (supragranular EX), *RORB* (layer 4 EX), *FEZF2*, and *THEMIS* (infragranular EX). Main inhibitory cell types (IN) were identified by the expression of markers such as *PVALB, SST, VIP, RELN, PAX6, COL15A1,* and *CALB2*. With the highest resolution, 15 EX and 20 IN clusters were identified, which aligned well with reference databases provided by the Allen Brain Institute.

4.2.2. Differentially expressed genes

While many cell types exhibited significant DEGs, only a fraction of these remained strongly significant after adjusting p-values for multiple testing.

Notably, some DEGs were shared between IN and EX cell types, such as *FP236383.1*, *C5orf17*, and *HES4*, the latter involved in early development. *C5orf63*, *CRYAB*, and *H1FX* were enriched in EX subtypes. Inhibitory subtypes had more subtype-specific DEGs – such as *ADARB2*, upregulated in several ***SST*** clusters despite being normally absent or only very lowly expressed in medial ganglionic eminence-derived neurons. Ionotropic kainite glutamate receptors 3 and 4 (*GRIK3*; *GRIK4*), which play key roles in mediating excitatory neurotransmission crucial for both development and cognitive functioning, were upregulated in the ***PVALB-CRH*** cluster. In the ***SST-NPY*** cluster, *HSP1A* (linked to proteostasis and stress protection) was upregulated, while *THS7DA*, *EYA4*, and *CDH9* (involved in developmental processes and synaptic transmission) were downregulated. ***VIP*** clusters – largely corresponding to "CR interneurons" – showed downregulation of *SEMA3C* and *TUBA4A* (involved in cortical interneuron development and cytoskeleton organization, respectively) while the expression of heat shock protein gene *HSPB1* was upregulated. **Only a small fraction of DEGs overlapped with those associated with antipsychotic**

treatment based on previous experiments involving human and macaque bulk RNA-seq.

A hypothesis-driven approach was also applied to test clinically relevant transcripts. Notably, *OXTR* (oxytocin receptor) was downregulated in SCH in the ***SST_NPY*** subtype (p=0.0168; n=9 SCH/14 CTR; Wald test). The chandelier ***PVALB*** cluster showed downregulated *GABRA1* and *GRIN2A* (p=0.00573, p=0.0446; n=9 SCH/14 CTR; Wald test, respectively), and several ***SST*** subtypes also demonstrated lower *GABRA1* levels. *CHRFAM7A*, encoding an intriguing, human-specific fusion protein associated with several psychiatric disorders, was markedly downregulated in both ***ID2*** and ***VIP*** subtypes in SCH. RNAscope confirmed a 77% reduction in average *CHRFAM7A* expression in L2 CR-expressing neurons, although it was very lowly expressed, making evaluation challenging.

4.2.3. Gene Ontology pathway analysis

To assess the functional relevance of significant DEGs, Gene Ontology pathway analysis was performed. The most widely **downregulated pathways were related to mitochondrial function, translation, protein localization, and transmembrane transport.** Translation-associated pathways were downregulated in ***VIP*** subclusters. Furthermore, genes associated with synapse organization were specifically downregulated in SCH in the ***SST_NPY*** cell cluster.

Conversely, **upregulated pathways were rather associated with neuronal transmission, synaptic signaling, and developmental processes,** affecting mainly excitatory cell types (***L5-6_FEZF2_TLE4***; ***L2_CUX2-LAMP5_PDGFD***) and specific GABAergic clusters (***ID2_LAMP5_NOS1***; ***PVALB_SST***; ***ID2_NCKAP5***). These findings depict a complex picture, suggesting that transcriptional disturbances play a central role in the mechanisms and symptoms of SCH, highlighting the most vulnerable cell types and paving the way

for future, more targeted experiments. However, these results must be interpreted with caution, keeping in mind the possible confounding factors such as post mortem changes and the complex and wide-ranging effects of decade-long antipsychotic treatment.

4.2.4. Calretinin mRNA and protein expression in the DLFPC

While in our **histological** analysis we observed significantly **lower layer 2 CR interneuron density** in the DLPFC, snRNAseq showed **no significant difference in *CALB2* expression** between groups. This discrepancy suggested that CR neurons may be present at physiological density in SCH, with a subset of them failing to produce detectable amounts of CR protein. To address this, we simultaneously visualized CR protein and *CALB2* mRNA in a subset of 5 CTR and 5 SCH cases. As hypothesized, **several SCH cases showed lower proportion of neurons expressing high levels of both the protein and the mRNA** (adjusted $p<0.0001$; n=5/group; LMM) and **higher proportions of neurons expressing only the mRNA but not the protein** (adjusted $p<0.05$, n=5/group; LMM). We plan to expand the sample size to n=10/group, and conduct layer-specific analysis to gain more robust results.

5. Conclusions

The presented results suggest that both cortical and striatal CR interneurons may be functionally disturbed in SCH.

A) In the CN, small CR-expressing interneurons were present in significantly lower density in SCH. Regardless of the exact mechanism behind this observation, this can affect local calcium homeostasis, synaptic signalling, and local inhibition, which can consequently impact striatal activity and output, and thus may directly contribute to the disrupted fronto-striatal function in SCH.

B) In the DLPFC, CR interneurons were present in significantly lower density in layer 2 in SCH – however, only in ~50% of SCH cases. This, in line with other studies, highlights the importance of within-group heterogeneity in SCH. Since CR neurons have been shown to demonstrate disturbed maturation and integration into the circuitry in patient-derived cerebral organoids, it is imperative to further study their potential developmental disturbance in SCH. Our preliminary results suggest that these neurons are present in SCH, but a subset of them fail to express detectable levels of CR protein, contributing to disrupted calcium homeostasis, disturbed inhibition, and thus, excitatory-inhibitory imbalance.

C) Single nucleus RNA sequencing confirmed that many, primarily upper-layer residing neuron subtypes are disturbed in the DLPFC in SCH, with energy metabolism-related pathways primarily downregulated in GABAergic interneurons and neurotransmission-related pathways primarily upregulated in excitatory cell types.

Together, our results suggest that CR protein expression is affected in SCH in both cortical and subcortical elements of the associative cortico-striatal network.

6. Bibliography of the candidate's publications

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