EXPLORING THE EFFECTS OF *DE NOVO* MUTATIONS IN SCHIZOPHRENIA USING INDUCED PLURIPOTENT STEM CELLS

Ph.D. thesis

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1. Introduction

Schizophrenia (SCZ) is a chronic disorder characterized by hallucinations, delusions and cognitive symptoms. Despite recent efforts, the pathophysiology of the disorder remains unclear, and current therapies are insufficient in many cases. SCZ is highly heritable, and previous research has shown that in a subset of cases *de novo* mutations (DNMs) in coding regions of genes contribute significantly to genetic risk.

Human induced pluripotent stem cells (hiPSCs) are an innovative tool to investigate cellular level pathophysiological processes by obtaining neurons that harbor the genetic architecture of the SCZ patient.

The investigation of DNMs in SCZ using hiPSCs and CRISPR-based genome editing provides a promising avenue for understanding the genetic and molecular underpinnings of the disorder. By focusing on specific high-impact mutations, we can gain insights into the neurodevelopmental processes and synaptic functions disrupted in SCZ, contributing to better understanding of the disorder and the development of more targeted and effective treatments.

Before my participation in the studies, SCZ patients carrying DNMs were selected from a cohort, the patient and isogenic control or parental control cell lines were created and established in the laboratory.

2. Objectives

We aimed to determine whether a given mutation plays a role in the development of SCZ in patients, to what extent, and through which mechanisms. Specifically, we seek to understand the molecular changes these mutations cause at the cellular level.

To address these broad questions, we formulated the following specific research objectives:

1. **Neuronal Differentiation of hiPSC lines from SCZ samples:**

- Can pluripotent stem cells derived from SCZ patients develop into hippocampal neuronal progenitors and functional dentate gyrus granule cells (DGGCs)?
- Are there observable differences in the differentiation, growth and morphology of neural progenitor cells (NPCs) and neurons derived from SCZ patients?

2. **Analysis of transcriptomic profiles and protein expression of NPCs and neurons:**
   - Are there any changes in protein expression and transcriptomic profiles in SCZ samples compared to healthy and isogenic controls?
   - Does RNA sequencing reveal differences in mRNA profiles of NPCs and DGGCs derived from SCZ samples compared to their controls?
3. **Functional Assessment of NPCs and Neurons:**
   - Can NPCs respond to different external chemical stimuli, e.g. glutamate? Is this response different in SCZ samples compared to controls?
   - Do neurons derived from SCZ patients generate functional action potentials and calcium transients? Do they respond to the addition of neurotransmitters?
   - Is there a difference in the magnitude of the response?

3. Methods

3.1. Differentiation of hippocampal neural progenitor cells and dentate gyrus granule cells

A directed neuronal differentiation protocol was used developed by Rusty Gage's laboratory in Salt Institute, USA. Patient hiPSCs were exposed to culture medium containing specific morphogens that urge the cells to differentiate into hippocampal NPCs and later neurons resembling dentate gyrus granule cells.

Evidence shows that in mammals the dentate gyrus in the hippocampus is a region where neurogenesis is continued throughout adulthood. The impairment of neurogenesis might contribute to SCZ pathogenesis.
Immunocytochemistry was used to characterize the cells. At neuronal progenitor stage SOX2 and NESTIN markers were stained, and PROX1 and MAP2 were stained to identify DGGCs.

3.2. RNA sequencing

We used bulk RNA sequencing of samples obtained from SCZ patients and controls at different stages of neuronal differentiation to assess transcriptional differences between them. Differential gene expression analysis was done using DeSeq2 and Sleuth. Gene ontology analysis was used to obtain further information about differentially expressed genes.

3.3. Functional measurements

Multi-electrode array measurements were used to assess maturity of neurons by measuring extracellular electric potentials. Calcium imaging was used to detect spontaneous calcium transients and detect response to glutamate stimulation.

4. Results

These studies investigate the biological impact of two DNMs found in SCZ patients. Previously, de novo mutations in schizophrenic patients were identified, and two of them were selected for further experiments.

4.1 Investigation of the effects of *ZMYND11* mutation

In the first project, a patient with a *de novo* heterozygous single nucleotide variant causing a missense mutation in the *ZMYND11* gene is presented.

4.1.1. *Morphological characterization and immunophenotyping of hiPSC-derived hippocampal NPC and DGGC cultures*

We created isogenic hiPSC lines, where the *ZMYND11* mutation was either corrected in patient-derived cells or introduced into control cells. These lines were differentiated into hippocampal NPCs and DGGCs. The mutation did not affect the viability, growth capacity and visible morphological properties of the cells and cell cultures. They all expressed NPC and DGGC specific markers similarly.

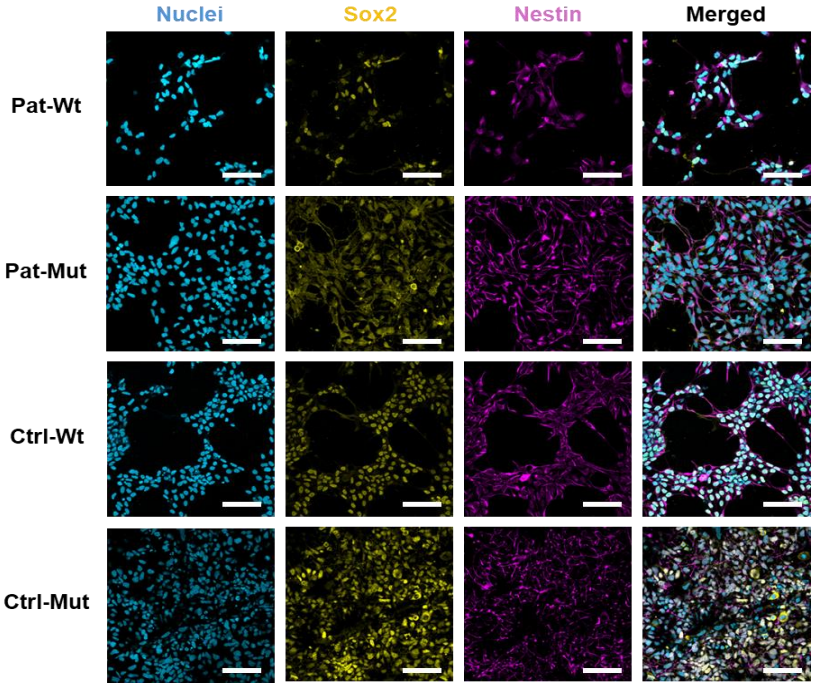

Figure "9". Characterization of genome-edited human induced pluripotent stem cell (hiPSC)-derived hippocampal neural progenitor cells (NPCs). Representative images display immunofluorescence stainings of NPC cultures for NPC markers SOX2 (yellow) and Nestin (purple), conducted in at least two parallel experiments. Scale bars are 100 μm.

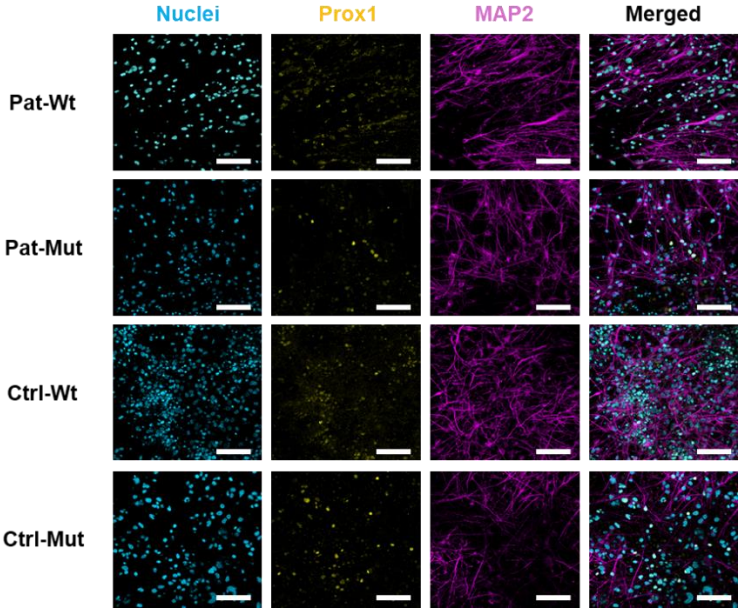

Figure "11". Characterization of genome-edited hiPSC-derived neural cultures. A) Representative images demonstrate immunofluorescence staining for neural markers PROX1 (yellow) and MAP2 (purple) in neural cultures at 5 weeks of neural differentiation. Scale bars are 100 µm.

ZMYND11 localization was changed in the mutant hiPSC. We observed cytoplasmatic localization of the protein.

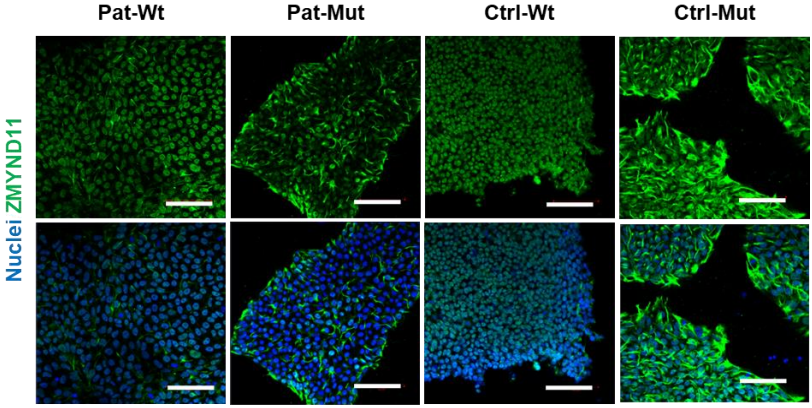

Figure "'12". Intracellular localization of ZMYND11 protein in hiPCSs. Immunostaining was used to study the cellular localization of ZMYND11 (green).

Mutant cell lines exhibit increased cytoplasmic and decreased nuclear staining. Nuclei were counterstained with DAPI (blue). Scale bars represent 100 µm.

4.1.2. *Transcriptomic Evaluation of Hippocampal NPCs and DGGCs*

Transcriptomic profiling of the differentiated cells revealed significant changes in gene expression. In the mutant lines, there was a notable upregulation of genes associated with neuronal differentiation and a downregulation of genes involved in cell adhesion. This suggests that the ZMYND11 mutation alters neuronal differentiation.

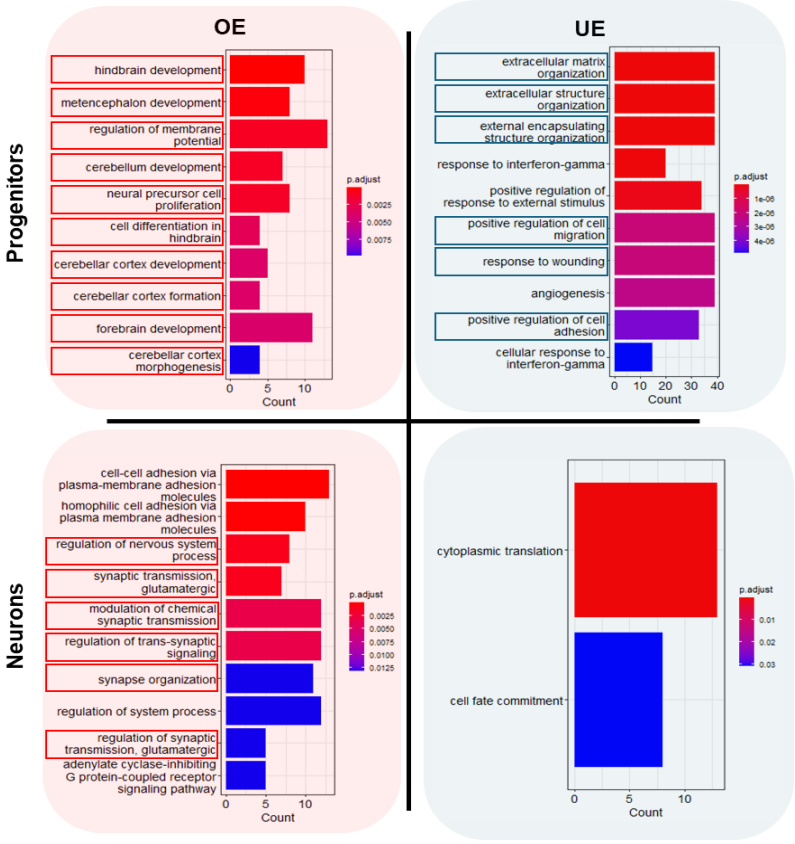

Figure "18". Gene Ontology analysis of NPCs and neurons. Bar plots present the results of Gene Ontology Enrichment Analysis, with enriched categories on the

vertical axis and gene counts for each category on the horizontal axis. Each bar is color-coded based on statistical significance for enrichment.

4.1.3. Functional measurements

Multi-electrode array measurements showed spiking and bursting activity characteristic of neuronal activity, showing that the in vitro neuronal differentiation resulted in functional neurons.

Calcium imaging was used to Functionally, the mutant neurons exhibited decreased reactivity to glutamate, as demonstrated by calcium-imaging experiments. This reduced glutamate response was quantified and found to be significantly lower in mutant cells compared to wild-type controls.

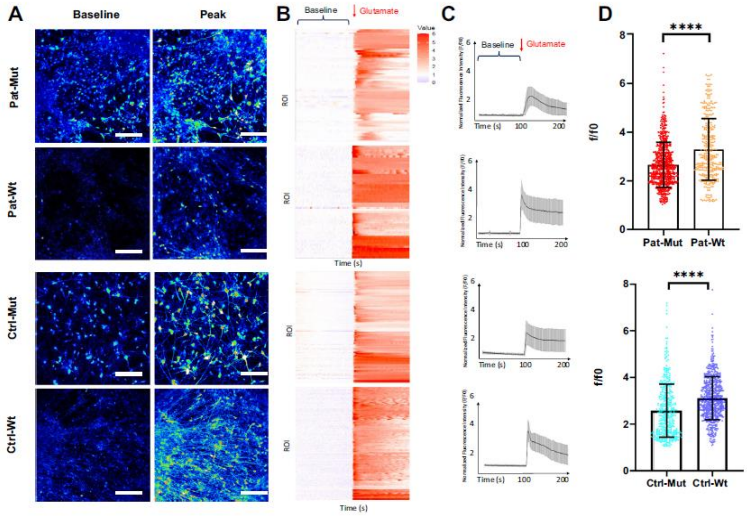

Figure "22". Demonstration of calcium-imaging analysis of the response to glutamate in dentate gyrus granule cell cultures. A) Confocal microscopy images show neural cultures stained with Fluo-4-AM calcium dye, displaying baseline fluorescence and peak signal intensity. Scale bars: 100 μm. B) Heatmaps illustrate fluorescence intensity changes over time for regions of interest (ROIs).C) Average normalized fluorescence intensity (F/F0) during recording. D) Quantification of glutamate reaction involves 400–600 cells from 3 to 5 experiments, with a significant difference indicated by Mann-Whitney U test ($p < 0.0001$)

4.2 Investigation of the effects of *KHSRP* mutation

In the second project involving a patient with 3 DNMs (*KHSRP*, *LRRC7 and KIRD1L2*) was presented. In this project we decided to use a different experimental approach: we compared the cells of the patient to the cells of both their parents.

4.2.1. Transcriptomic differences in KHSRP and LRRC7 mutant NPCs

Bulk RNA sequencing revealed transcriptional differences in the NPCs. GO and pathway analyses indicated that the differentially expressed genes were enriched in pathways critical to neuron formation, axon development, neurogenesis, Wnt signaling, and Calcium signaling.

4.2.2. Functional phenotypes found in the KHSRP mutant by Calcium imaging

Further phenotypic analysis using calcium imaging techniques showed significant functional differences in the NPCs' activity. The NPCs derived from the SCZ patient demonstrated a significantly weaker response to glutamate compared to those from the unaffected parents.

4.3. Synthesis of the results.

The findings from both projects show that these DNMs impact neurodevelopmental processes and synaptic function in NPCs and DGGCs. Both mutations led to significant changes in gene expression and neuronal activity, suggesting a common pathway through which these genetic alterations might contribute to the pathophysiology of SCZ. By integrating results from more projects, we may get a broader perspective of the molecular mechanisms underlying SCZ.

Figure "24" below summarizes our hypothesis of possible cause and effect cascade, how a DNM at the DNA level may have slightly disrupted the delicate equilibrium of neurodevelopment. This disruption, along with many other factors, may contribute to a clinically observable imbalance of mental function that we call SCZ.

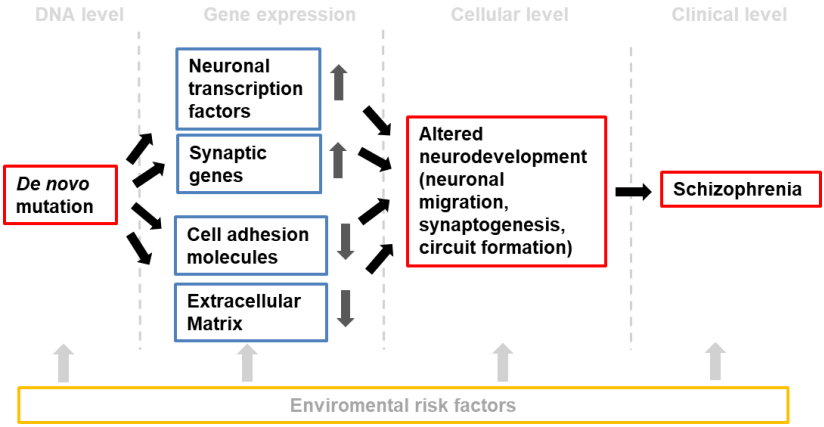

Figure "24". Schematic summary of possible cause-effect relationships at different biological levels.

5. Conclusions

- **1. Neuronal Differentiation of hiPSC lines from SCZ samples**:
    - hiPSCs derived from SCZ patients can develop into hippocampal neuronal progenitors and functional DGGCs.
    - There were no significant observable differences in the differentiation, growth, and morphology of neural progenitor cells (NPCs) and neurons derived from SCZ patients.
- **2. Analysis of transcriptomic profiles and protein expression of NPCs and neurons:**
    - RNA sequencing revealed significant changes in transcriptomic signatures of ZMYND11 mutant progenitors and neurons
    - During neuronal differentiation, ZMYND11 protein becomes mostly cytoplasmatic.

- ZMYND11 mutant hiPSCs show altered localization of ZMYND11 protein

- **3. Functional Assessment of NPCs and Neurons:**
    - NPCs can respond to glutamate. KHSRP, LRRC7 and KIR2DL1 mutant NPCs show decreased reaction to glutamate compared to parental cells.
    - Neurons derived from ZMYND11 mutant SCZ patients generate functional action potentials and calcium transients. They respond to the addition of glutamate.
    - We observed reduced reaction of ZMYND11 mutant neurons compared to isogenic controls.

6. Bibliography of the candidate's publications

Publications related to the thesis:

1.
Tordai, Csongor; Hathy, Edit; Gyergyák, Hella; Vincze, Katalin; Baradits, Máté; Koller, Júlia; Póti, Ádám; Jezsó, Bálint; Homolya, László; Molnár, Mária Judit et al.
Probing the biological consequences of a previously undescribed de novo mutation of ZMYND11 in a schizophrenia patient by CRISPR genome editing and induced pluripotent stem cell based in vitro disease-modeling
SCHIZOPHRENIA RESEARCH (2024)
IF: 3,6

2.
Hathy, Edit; Szabó, Eszter; Varga, Nóra; Erdei, Zsuzsa; Tordai, Csongor; Czehlár, Boróka; Baradits, Máté; Jezsó, Bálint; Koller, Júlia; Nagy, László et al.
Investigation of de novo mutations in a schizophrenia case-parent trio by induced pluripotent stem cell-based in vitro

disease modeling: convergence of schizophrenia- and autism-related cellular phenotypes
STEM CELL RESEARCH & THERAPY (2020)
IF: 7,1

Publications not related to the thesis:
Bálint, Jezsó; Sára, Kálmán; Kiara, Gitta Farkas; Edit, Hathy; Katalin, Vincze; Dzsenifer, Kovács-Schoblocher; Julianna, Lilienberg; Csongor, Tordai; Zsófia, Nemoda; Homolya, László et al.
Haloperidol, Olanzapine, and Risperidone Induce Morphological Changes in an In Vitro Model of Human Hippocampal Neurogenesis
BIOMOLECULES (2024)
IF: 4,8

∑IF: 15,5