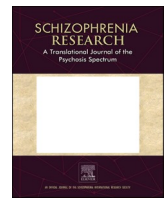




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Probing the biological consequences of a previously undescribed de novo mutation of ZMYND11 in a schizophrenia patient by CRISPR genome editing and induced pluripotent stem cell based in vitro disease-modeling

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ABSTRACT

Background: Schizophrenia (SCZ) is a severe neuropsychiatric disorder of complex, poorly understood etiology, associated with both genetic and environmental factors. De novo mutations (DNMs) represent a new source of genetic variation in SCZ, however, in most cases their biological significance remains unclear. We sought to investigate molecular disease pathways connected to DNMs in SCZ by combining human induced pluripotent stem cell (hiPSC) based disease modeling and CRISPR-based genome editing.

Methods: We selected a SCZ case-parent trio with the case individual carrying a potentially disease causing 1495C > T nonsense DNM in the zinc finger MYND domain-containing protein 11 (ZMYND11), a gene implicated in biological processes relevant for SCZ. In the patient-derived hiPSC line the mutation was corrected using CRISPR, while monoallelic or biallelic frameshift mutations were introduced into a control hiPSC line. Isogenic cell lines were differentiated into hippocampal neuronal progenitor cells (NPCs) and functionally active dentate gyrus granule cells (DGGCs). Immunofluorescence microscopy and RNA sequencing were used to test for morphological and transcriptomic differences at NPC and DGGC stages. Functionality of neurons was investigated using calcium-imaging and multi-electrode array measurements.

Results: Morphology in the mutant hippocampal NPCs and neurons was preserved, however, we detected significant transcriptomic and functional alterations. RNA sequencing showed massive upregulation of neuronal differentiation genes, and downregulation of cell adhesion genes. Decreased reactivity to glutamate was demonstrated by calcium-imaging.

Conclusions: Our findings lend support to the involvement of glutamatergic dysregulation in the pathogenesis of SCZ. This approach represents a powerful model system for precision psychiatry and pharmacological research.

1. Introduction

De novo mutations (DNMs) have been implicated in schizophrenia (SCZ), a chronic psychiatric disorder affecting 1 % of the adult population worldwide, characterized by hallucinations, delusions,

disorganized behavior and speech, decreased motivations, and impairments of neurocognition (Kahn et al., 2015; Jauhar et al., 2022). These symptoms often lead to impairment of school and work performance and community functioning despite pharmacological and psychosocial treatment efforts. The genetics and neurobiology of SCZ have been

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extensively studied unraveling molecular disease mechanisms and specific brain alterations; however, our overall knowledge remains fragmented. Recent genetic research has indicated both common genetic variants, i.e. single nucleotide polymorphisms (SNPs), and rare genetic variants, i.e. single nucleotide variants (SNVs) in the etiopathogenesis of SCZ (Rethelyi et al., 2013; Legge et al., 2021; Nakamura and Takata, 2023). While SNPs are predominantly localized in regulatory regions, exert small effects and act in a polygenic manner, SNVs have large effects on disease risk, are localized in protein-coding regions and arise predominantly from one generation to the next one, resulting in DNMs. The enrichment of rare variants found in SCZ has been described across various ancestries in evolutionarily constrained genes, including SETD1A, CUL1, XPO7, TRIO, CACNA1G, SP4, RB1CC1, AKAP11. These genes are mostly implicated in glutamatergic synapse formation and regulation, however several other rare variants have been reported (Singh et al., 2022; Liu et al., 2023). Although exome sequencing in SCZ patients, i.e. testing of rare variants in protein coding genes is not part of the clinical protocol as of now, it has been suggested that it might confer clinical insight for the etiology and treatment of individual patients (Balakrishna and Curtis, 2020).

Both human induced pluripotent stem cell (hiPSC) based in vitro disease modeling and clustered regularly interspaced short palindromic repeats (CRISPR) genome editing methodologies have been successfully applied for SCZ research. hiPSC-derived neuronal progenitor cells (NPCs), neurons and organoids generated from SCZ patients show various molecular alterations compared to their healthy control counterparts, depending on the applied differentiation protocol. The field of hiPSC-based disease modeling in SCZ was founded by the seminal paper of Brennand et al. (Brennand et al., 2011) that has been followed by a series of studies looking at different aspects of SCZ-related in vitro phenotypes. Briefly, alterations in SCZ patient-derived NPCs and neurons supported by independent studies include decreased synaptic connectivity and plasticity in glutamatergic synapses, impaired Wnt-signaling, increased oxidative stress and altered mitochondrial function, and in some studies accelerated differentiation (Moslem et al., 2019; Rasanen et al., 2022). Although most studies applied cortical differentiation protocols (Srikanth et al., 2015; Flaherty et al., 2017; Nakazawa et al., 2017; Xu et al., 2018; Kathuria et al., 2019), in vitro models recapitulating hippocampal development and adult neurogenesis have also demonstrated significant alterations (Brennand et al., 2011; Sarkar et al., 2018; Yu et al., 2014). In 3D organoids SCZ has been associated with transcriptomic deficiencies of synapse development, the MAPK-ERK signaling pathway, and genes identified previously by GWAS (Kathuria et al., 2020).

CRISPR genome editing represents an unprecedented opportunity to correct or introduce putative disease-causing mutations in several biological model systems, including hiPSC models. The generated genome-edited isogenic cell lines offer a unique method to examine differential molecular signatures between isogenic cell lines, thus focusing on alterations associated solely with the examined genetic variant, and no other sources of genetic variation. The possibilities of this approach are curtailed by specific limitations, such as off-target hits, and effects of transfection and single-cell cloning (Michael Deans and Brennand, 2021; Brennand, 2022). Using CRISPR Schrodte et al. have showed the utility of manipulating SNPs that have been described as quantitative trait loci in genes *FURIN*, *SNAP91*, *TSNARE1* and *CLCN3*. Modified neurons demonstrated transcriptomic alterations converging on pre- and post-synaptic machinery (Schrodte et al., 2019).

Zinc finger MYND domain-containing protein 11 (ZMYND11, also called BS69) gene is coded at 10p15.1, and entails 15 exons. It is expressed in almost all neuronal and non-neuronal cell types, with highest levels of mRNA expression in the thyroid gland, cerebellum, spinal cord, hippocampus, and frontal cortex (Yu et al., 2009; Varsome, 2024; GeneCards, 2024). ZMYND11 is a chromatin reader that specifically recognizes and binds histone H3.3K36 tri-methylated (H3.3K36me3) (Wen et al., 2014a; Wen et al., 2014b). The protein

contains PHD, Bromo, PWWP and MYND domains and several ZnF motifs, co-localizes with highly expressed genes, and functions as a transcription co-repressor. ZMYND11 also induces intron retention (IR) during mRNA maturation (Guo et al., 2014; Lan and Shi, 2015). It is localized predominantly to the nucleus, but it has been indicated in the cytoplasm as well (GeneCards, 2024).

In neurons, ZMYND11 is a repressor of neuronal differentiation, while silencing of ZMYND11 resulted in accelerated neuronal differentiation (Yu et al., 2009). Recently, aberrant splicing of Zmynd11 has been demonstrated in the brain and spinal cord of mice overexpressing a mutant form of TDP-43, an important gene implicated in ALS and FTD (Narayanan et al., 2023). Genetic mutations in ZMYND11 have been shown in tumorigenesis (Wang et al., 2020), intellectual disability with or without epilepsy (Yates et al., 2020; Huynh et al., 2021; Oates et al., 2021), and in one patient, SCZ (Balakrishna and Curtis, 2020), although the biological mechanisms causing these phenotypes remain elusive.

In summary, we have used CRISPR-genome editing and hiPSC-based in vitro disease modeling to investigate the biological function of a previously undescribed DNM in the ZMYND11 gene. The rationale for this approach was to correct a potentially disease-causing DNM in a hiPSC line derived from a schizophrenia patient, and to introduce similar mutations in wild-type control hiPSCs, thereby generating two pairs of isogenic lines for differentiation experiments and downstream analyses. The specific aim was to probe the biological consequences of a previously undescribed DNM in ZMYND11 and interpret these in the context of the existing body of evidence linking SCZ with neuronal alterations. Using transcriptomics, calcium-imaging and multielectrode-array (MEA) experiments, we show profound transcriptomic and functional differences between the isogenic lines at NPC and neuronal stages. Our results are suggestive of an important biological role of ZMYND11 in controlling the speed of neuronal differentiation, and potentially detrimental effects of the investigated DNM.

2. Material and methods

2.1. Patient selection and exome sequencing

We used cell lines derived from a SCZ patient (Pat-Mut), and an unrelated healthy control (XCL-1/Ctrl-Wt) for all experiments (Table 1). The patient diagnosed with SCZ was selected from 16 case-parent trios, based on de novo mutations (DNMs) identified during exome-sequencing (Pulay et al., 2016). The ExomeSeq analysis was carried out after 100 bp paired ended sequencing that was run on the Illumina HiScan(TM)SQ platform and resulted in 50 M reads on average per sample. The identified DNMs were validated by Sanger sequencing. The SCZ patient (Pat-Mut) is a carrier of nonsense DNM in gene ZMYND11

Table 1

Demographic and clinical data of the investigated case-parent trio and the unrelated healthy control individual.

Subject	Sex	Age	Medical history	Reprogramming method	Code
Father	M	58	No psychiatric treatment or other major somatic disorder.	n. a.	n. a.
Mother	F	48	No psychiatric treatment or other major somatic disorder.	n. a.	n. a.
Proband (son)	M	26	Diagnosed with schizophrenia at the age of 21 years.	Sendai virus reprogramming from blood PBMCs.	Pat-Mut
Unrelated healthy control	M	New-born	No psychiatric treatment or other major somatic disorder.	Episomal reprogramming from cord umbilical blood.	XCL1/Ctrl-Wt

(R399X). The DNM has not been reported previously to the ClinVar database (ClinVar, 2023), however the same mutation was found in the Varsome database (Kopanos et al., 2019).

2.2. Generation of case and isogenic hiPSC cell lines

2.2.1. Sample collection from the selected patient and reprogramming

Informed written consent was obtained from the case-parent trio members prior to collecting blood samples. The Human Reproduction Committee of the Hungarian Health Science Council (ETT HRB-approval number: 33873-3/2014-EHR) approved both the reprogramming and the study. Peripheral mononuclear cells (PBMCs) were separated and used for reprogramming by Sendai virus (Fusaki et al., 2009) (Thermo Fisher Cytotune 2.0, Waltham, USA). Reprogramming and virus clearance monitoring were carried out as described previously (Hathy et al., 2020). The XCL1 male control hiPSC line was created from CD34⁺ cord blood cells through episomal reprogramming (XCell Sciences, Novato, USA).

2.2.2. Generation of isogenic hiPSC pairs using CRISPR genome editing

In the healthy control hiPSC line XCL1 we used non-homologous end joining (NHEJ) to introduce a frameshift mutation in the genomic region of the identified DNM. Conversely, in hiPSC line Pat-Mut the investigated ZMYND11 mutation was corrected using CRISPR homology-directed repair (HDR). For NHEJ 3 gRNAs were designed and applied using established CRISPR protocols. 200,000 cells were electroporated with 2 plasmids (1.5 µg pgRNA + 1.1 µg pCas9) using LONZA 4D-Nucleofector X UnitCB-150 program and LONZAP3 Primary Cell 4D-Nucleofector® X Kit S (32 RCT) (V4XP-3032). 48 h after transfection, the mCherry expressing cells were sorted and single-cell clones were established and expanded in mTeSR medium on Matrigel coated plates. The gRNA demonstrating best genome editing efficiency was taken forward, and T7 assay was used for clone selection. Mono- and biallelic frameshift mutations in exon 10 were identified using Sanger sequencing. Unique R399X DNM sequence in Exon 10 was used to design an appropriate sgRNA for targeting ZMYND11 (Supplementary Fig. 1). For HDR we used ssODN with 45 nucleotide homologous arms and the Cas9-cutting site in the middle. Corrected lines were identified using the inverse T7 assay, i.e. clones not showing heteroduplex formation were selected, furthermore qPCR high resolution melting analysis (HRMA), complemented with Sanger sequencing. The genome editing experiments resulted in 5 hiPSC lines, described in Supplementary Table 1. For subsequent neuronal differentiation analyses we only used two isogenic hiPSC line pairs.

2.3. Differentiation of hippocampal NPCs and dentate gyrus granule cells

Next, neuronal progenitor cells (NPCs) and dentate gyrus granule cells (DGCCs) were generated from the hiPSC lines Pat-Wt, Pat-Mut, Ctrl-Wt and Ctrl-Mut via embryoid body formation as previously described (Yu et al., 2014; Hathy et al., 2020). For further details see Supplementary methods file.

2.4. Immunostaining and flow-cytometry

For characterization of hiPSC lines Oct4 and Nanog transcription factor expression and SSEA4 surface marker expression were tested by ICC and flow cytometry, respectively, according to established protocols (Erdei et al., 2014). Immunofluorescence staining of NPCs and DGCCs for SOX2, Nestin, PROX1, and MAP2, respectively were conducted as described previously (Hathy et al., 2020). ZMYND11 was immunostained by BS69 antibody (Thermo Fischer Scientific Waltham USA, PA527899). We used a Zeiss LSM 900 confocal laser scanning microscope and the ZEN 3.1 software for visualization.

2.5. Calcium-imaging and multielectrode array measurements

Before all calcium measurements, hippocampal NPCs were seeded for 2 days onto 8-well confocal chambers previously coated with poly-ornithine/laminin and differentiated for 5 weeks. The cytoplasmic calcium signals were evaluated using Fluo-4 AM calcium indicator dye as described previously (Pentek et al., 2016; Hathy et al., 2020). Baseline was recorded for 3–5 min before application of 50 µM glutamate (Sigma, MO, USA). Cells were selected manually, marked as regions of interest (ROI) (100–300/recording), and mean gray values were extracted using ImageJ. The small number of cells that showed glial morphology were excluded from the analysis. Quantification of the response was done by dividing the maximum fluorescence intensity after application by the median fluorescence intensity of the baseline period for each cell. Statistical comparison was done comparing each isogenic pair using Mann-Whitney U test, as the single cell reactions were not normally distributed.

Extracellular electrophysiological recordings were obtained using the MEA2100-System, which was controlled by the MEASuite software package (Multi Channel Systems MCS GmbH, Reutlingen, Germany) with a 10 kHz sampling rate. NPCs from each clone (3 biological replicates) were plated onto 60-6well MEA200/30iRTi poly-ornithine coated chips, which are 6-well MEA chips with nine 30 µm diameter Titanium electrodes in each well. Neuronal differentiation followed the previously described protocol. After week 5 spontaneous activity was recorded. Recordings were carried out at 37° at a sampling rate of 10 kHz. The raw recordings were filtered using a bandpass filter of 300–3000 Hz. Representative single electrodes were manually selected for the demonstration of neuronal activity.

2.6. RNA sequencing

RNA sequencing experiments were carried out at the hippocampal NPC and DGCC stage. Total RNA was isolated using TRIzol™ reagent following the manufacturer's instructions (Thermo Fisher Scientific, MA, USA). Three biological replicates were used from each cell line. In the case of NPCs, RNA was isolated at 3 different passages between p7–12 after reaching ca. 70 % confluency. Neurons were harvested after 6 weeks of differentiation. Initial seeding density was 24,000 cells/cm². The Illumina NovaSeq technology was used for NPCs (2 × 150 bp paired-end mode, 36.5–55.3 million reads per sample), the Illumina NextSeq technology (1 × 75 bp single-end mode, 17.0–32.9 million reads per sample) for neurons.

2.7. Statistical and data analysis

Data analysis was performed using publicly available software tools and R packages. We first performed quality control of FASTQ files using FASTQC (Andrews, 2010), then aligned the reads to the human reference genome (hg38) using hisat2 (Kim et al., 2019). Gene expression levels were quantified using FeatureCounts (Liao et al., 2014), and differential expression analysis was performed using the DESeq2 R package (version: 1.36.0) (Love et al., 2014) for each isogenic pair. Given the inherent biological differences between the two RNA sequencing experiments, batch correction was deemed impractical. Batch correction methods posed a risk of obscuring significant biological changes between neurons and NPCs. Consequently, we chose to exclusively compare isogenic pairs within each batch.

After size factor normalization, a negative binomial distribution model was used to compute statistical significance, with a Wald test and Benjamin-Hochberg correction for multiple testing. We excluded genes that had <10 counts overall. Log2 fold change (l2fc) > 1 or l2fc < −1 and adjusted p-value < 0.05 were used as the criteria for selecting differentially expressed genes. To validate our results and obtain an independent list of DE genes, we also used a nonalignment-based pipeline using kallisto (Bray et al., 2016) and sleuth (Pimentel et al., 2017).

The hisat2-DESeq2 list was further filtered using the statistically significant results (qval < 0.05) of the kallisto-sleuth pipeline, where log2 fold change was not taken into consideration. Gene ontology (GO) and disease ontology (DO) analysis were performed using the ClusterProfiler R package (version: 4.4.4) (Yu et al., 2012), which applies a hypergeometric test to determine enrichment of gene sets and pathways. SynGO analysis was performed using the publicly available online analysis tool (Koopmans et al., 2019). Clustered heatmaps were generated using the “pheatmap” R package and default hierarchical clustering methods.

3. Results

3.1. Characterization of a de novo ZMYND11 mutation in a SCZ patient

Using next generation exome sequencing and Sanger-sequencing as validation, we were able to identify a 10:293374C > T mutation in a 26 years old male SCZ patient, characterized by predominant positive symptoms and some degree of negative symptoms. Importantly, the selected patient had no diagnosis of intellectual disability. The mutation was not detectable in parents. This DNM is a nonsense mutation, resulting in a premature stop codon and R399X amino acid change (Fig. 1). The Varsome human genomic variant search engine mutation prediction algorithm (Kopanos et al., 2019) indicated the ZMYND11 R399X mutation as deleterious by resulting in a stop codon in the nuclear localization signal of the protein affecting an evolutionarily conserved region (Fig. 1 and Table 2). The mutation affects 36 out of the 39 reported alternative transcripts.

3.2. Establishment of the patient-derived hiPSC cell line

PBMCs of the identified SCZ patient carrying the DNM point mutation were reprogrammed using the Sendai virus method, and a stable hiPSC line was generated (Pat-Mut). The cell line showed all the properties of pluripotency (Fig. 2A and B and Supplementary Fig. 2A).

3.3. Generation and characterization of isogenic hiPSC lines generated by CRISPR genome editing

Sanger sequencing of the CRISPR-edited hiPSC clones confirmed the correction of the point mutation in the patient-derived hiPSC line, and monoallelic or biallelic frame shift mutations in the control line (Fig. 3, Supplementary Table 1). Genome editing did not change the pluripotent properties of hiPSC lines (Fig. 3A). The transcription factors Oct4 and Nanog demonstrated nuclear localization (Fig. 2B). When we examined the localization of ZMYND11 by ICC, we found that the nuclear/cytoplasmic ratio of the protein in the mutant lines changed, with higher protein expression in the cytoplasm than in the case of the control lines (Fig. 2C). Investigation of 13–15 metaphase cells in all hiPSC lines revealed normal karyotypes (Supplementary Fig. 2B).

Table 2

Description of the identified DNM in the schizophrenia proband.

Genomic position	10:293374C > T
Gene	ZMYND11
Variation type	Nonsense
Amino acid change	R399X
Conservation of nucleotides	High
Conservation of amino acid	High
Varsome pathogenicity ranking	Likely pathogenic

3.4. Neuronal differentiation of hiPSCs into hippocampal NPCs and DGCCs

Next, heterozygous mutants relevant to the investigated patient were examined in detail; the homozygous KO hiPSC line was excluded from further experiments. Neural progenitors were successfully generated from all four hiPSC lines (Pat-Wt, Pat-Mut, Ctrl-Wt and Ctrl-Mut) based on the previously reported differentiation protocol (Yu et al., 2014). The morphology (Supplementary Fig. 3) and growth properties of hippocampal NPCs were similar and they expressed Nestin and SOX2 markers typical of progenitors (Fig. 4A). Surprisingly, the ZMYND11 protein was mostly found in the cytoplasm and a significant part of it was accumulated in neurites, in all examined cell lines, regardless of genetic differences (Fig. 4B).

Dentate gyrus granule cells (DGCCs) were differentiated from all hippocampal NPCs (Supplementary Fig. 4), and demonstrated PROX1 and MAP2 expression after 6 weeks of differentiation (Fig. 5A). ZMYND11 protein was also observed in neurons, largely in the cytoplasm and processes, similarly to NPCs (Fig. 5B). All neuronal cultures demonstrated spontaneous activity in calcium-imaging assays and spontaneous electrical potentials observed as spikes and bursting activity by multi-electrode array measurements, indicative of functionally active synapses and action potentials in neurons (Fig. 5C and D).

3.5. Transcriptomic analysis of hippocampal NPCs and DGCCs

RNA sequencing was performed in hippocampal NPCs and DGCCs to compare gene expression in ZMYND11 mutant and wild-type cells and identify key molecular pathways associated with the DNM. ZMYND11 mRNA expression levels showed approximately 20 % decreased in heterozygous mutants compared to wild types (Supplementary Fig. 5). Principal component analysis (PCA) revealed that differentiation status and genetic background were the two primary factors contributing to transcriptomic differences (Fig. 6A). Along the PC1 axis, which accounted for 59.1 % of the variance, samples were separated into neurons and progenitors. Along the PC2 axis, which accounted for 13 % of the variance, samples were separated by genetic background into control- and patient-derived groups. However, when performing PCA only for NPCs, the two PCs separated cells according to mutation status

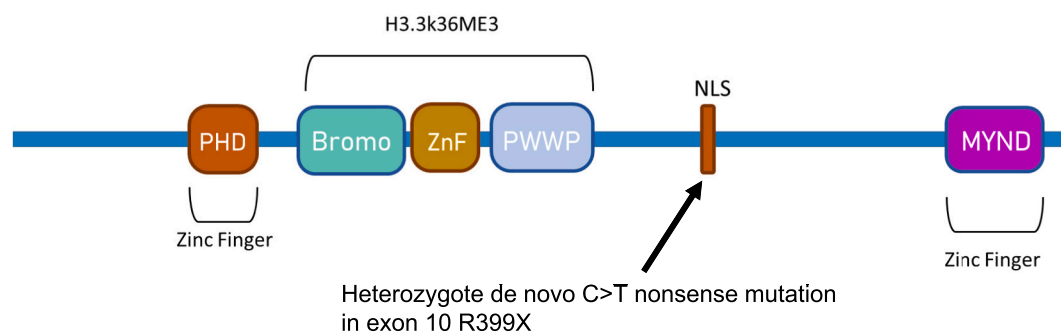


Fig. 1. Schematic diagram of the main Zmynd11 domains: the plant homeodomain (PHD) finger, followed by the bromodomain (BROMO), the CCCH-type zinc finger (ZnF) motifs, the PWWP domain (PWWP), and the MYND domain (MYND). The location of the nuclear localization signal (NLS) is also marked in the figure. Modified from Wang et al. (2014).

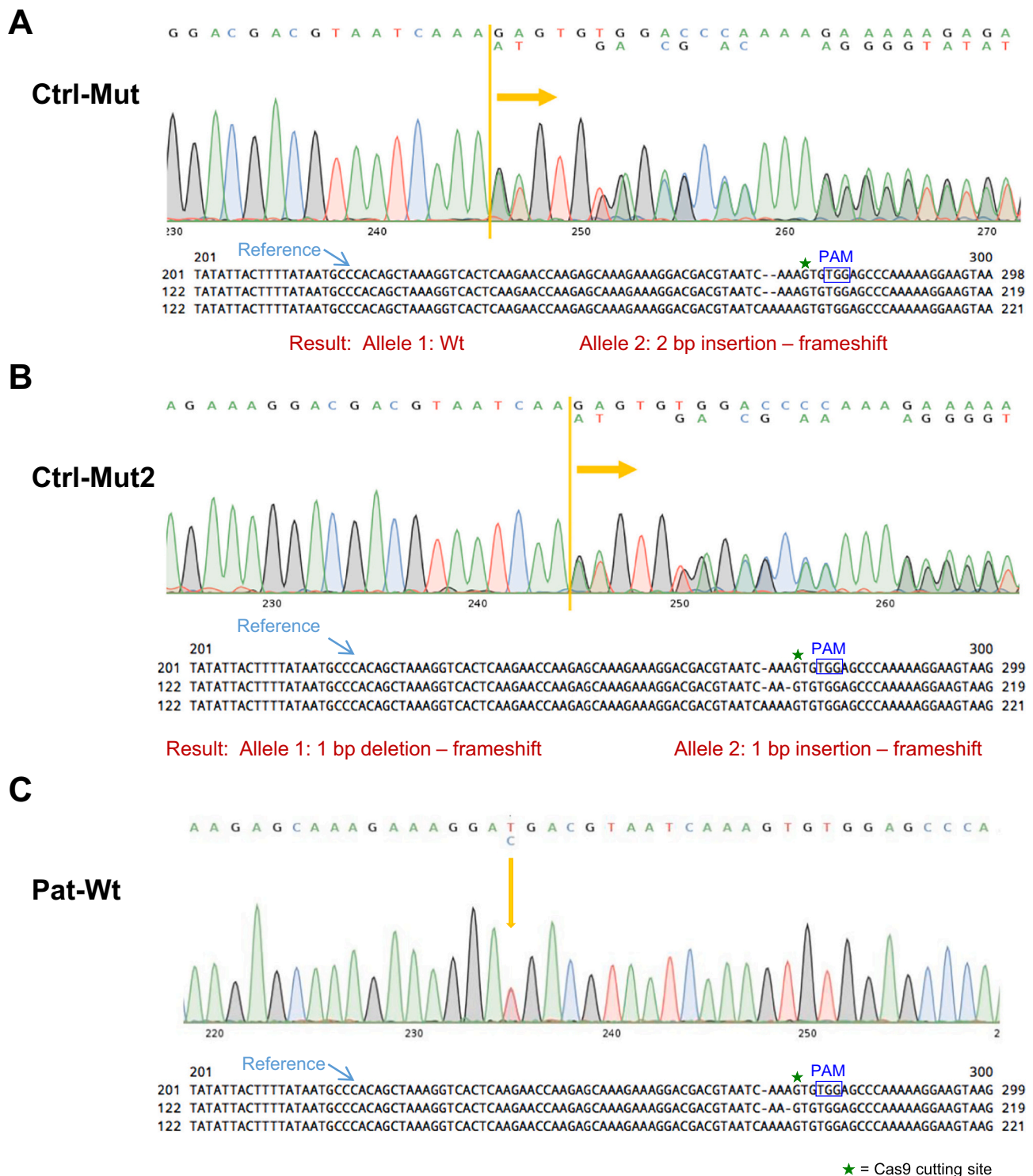


Fig. 2. Sanger sequencing data of CRISPR edited iPSC lines. A-B) The frameshift mutations in control (Ctrl-Wt/XCL-1) clones and C) point mutation in patient derived clone are indicated by yellow arrows. The Cas9 cutting site (green asterisk) and PAM sequence are also indicated in the reference sequence below the sequencing graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(PC1) and the genetic identity (PC2). The same clear separation was not reproducible in neurons (Fig. 6B and C).

Differential expression (DE) analysis was conducted on isogenic pairs: comparing mutant to wild-type cells in patient and control progenitors and neurons, respectively, resulting in four sets of DE genes (Fig. 6D). There were similar amounts of DE genes in all four comparisons, ranging from 2660 to 3636. Results were visualized using volcano

plots, to identify patterns of DE genes. (Supplementary Fig. 6). The ratio of up- and downregulated genes, and the extent of gene expression changes, as measured by log2Fold changes, was comparable across all four comparisons. Genes with the highest log2Fold change and statistical significance were different across samples (Supplementary Table 2).

To identify DE genes independent of genetic background and differentiation status, we obtained a list of DE genes that were common to

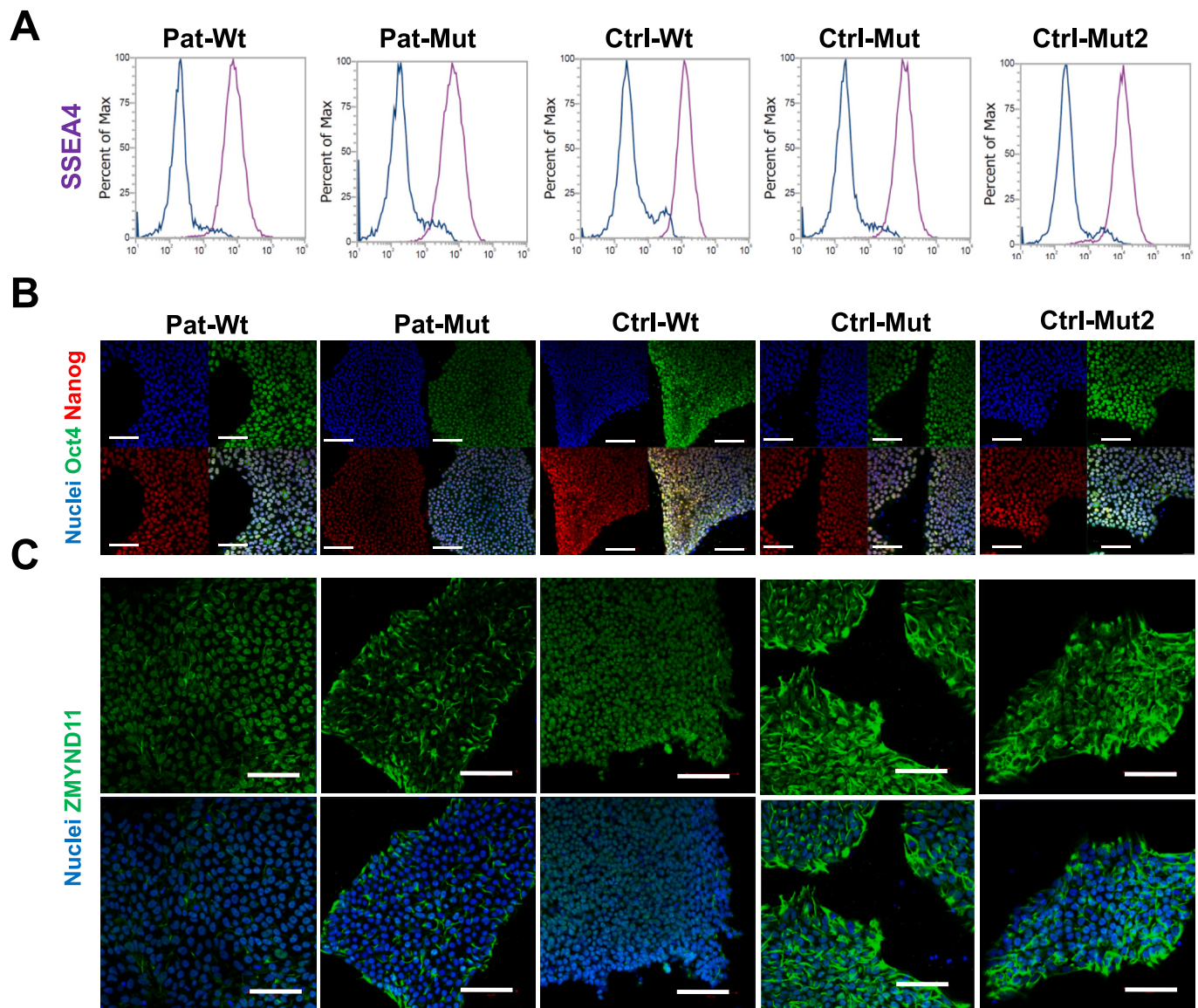


Fig. 3. Characterization of genome edited hiPSC lines. A) The SSEA4 expression levels (purple) in WT cells and genome edited clones were analyzed via flow cytometry. Isotype controls (blue) were used as a negative control on each plot. B) Immunofluorescent staining showing the Oct4 (green) and Nanog (red) expression levels in undifferentiated cells. C) Cellular localization of ZMYND11 (green) studied by immunostaining. Increased cytoplasmatic and decreased nuclear staining is visible in the mutant cell lines. The nuclei were counterstained by DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamide - blue). Scale bars are 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

both patient and control cell lines by taking the intersection of the two sets, resulting in 580 common DE genes in progenitors and 202 common DE genes in neurons. We then further refined the list by taking the intersection of these two sets, resulting in a final list of 16 DE genes that were common to both progenitors and neurons (Fig. 6E, Supplementary Fig. 7). This list includes LHX1, LHX5, KHDRBS2, SHANK1, ANO3 as overexpressed (OE), and CD74, FXYD5, RGCC, DNAJC15, BST2, CPNE8, ME3, SLC34A2, NABP1, ANXA2, TPM2 as underexpressed (UE) genes (Fig. 6E, Supplementary Fig. 7).

Gene ontology (GO) enrichment analysis was done separately for NPCs and neurons from the common DE genes in both genetic backgrounds, i.e. 580 DE genes for NPCs and 202 DE genes for neurons, as indicated in Fig. 6D by the gray frame. Analysis revealed enriched terms related to neuronal function and development among OE genes, and terms related to extracellular matrix, cell adhesion and glial function in UE genes (Fig. 6F). Enrichment analysis using the Disease Ontology (DO) database showed that genes involved in “developmental disorder of mental health” were significantly enriched in the OE genes in mutant

neurons. (Supplementary Fig. 8). Based on the enriched GO term “Synaptic transmission glutamatergic”, we further examined the expression of glutamatergic receptors and found that their expression was higher in the mutant cell line compared to the wild-type cell line in both genetic backgrounds (Fig. 7A). This pattern was not as clearly visible when investigating other neurotransmitter receptors (Supplementary Fig. 9).

Additionally, we performed SynGO analysis in the neuronal results (Fig. 7B) to identify enriched gene sets associated with synaptic function. We found enrichment of DE genes associated to the postsynaptic membrane in OE genes, and enrichment of DE genes associated to post and presynaptic ribosomes among UE genes.

Altogether, the transcriptomic result suggests that the heterozygous ZMYND11 mutation causes significant changes in the transcriptome of developing NPCs and DGGCs. Upregulation of neural differentiation and neural functional genes may be a sign of precocious or accelerated differentiation, leading to an altered cellular phenotype. We show the involvement of the synaptic machinery and the glutamatergic receptor

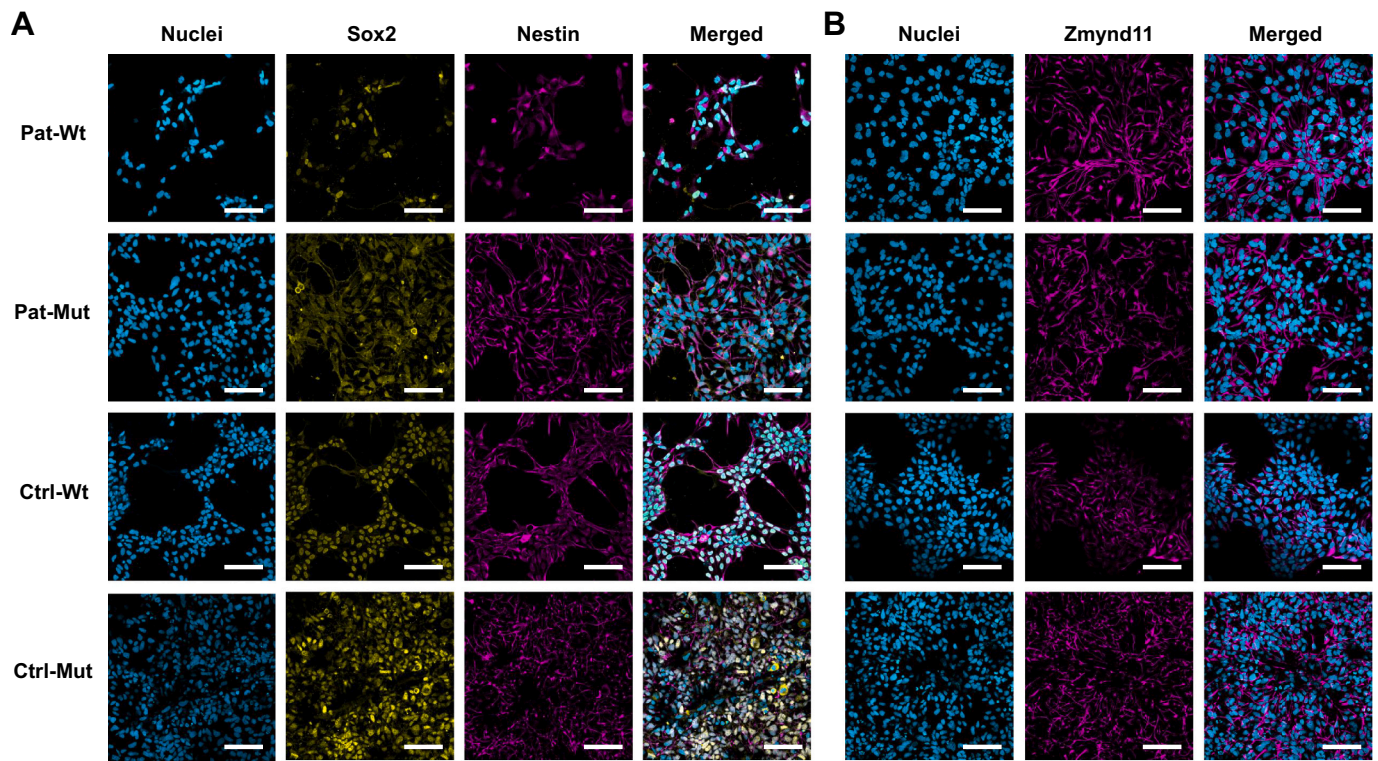


Fig. 4. Characterization of genome edited hiPSC-derived hippocampal NPCs. A) Representative images of at least two parallel immunofluorescence stainings of the NPC cultures for NPC markers: SOX2 (red) and Nestin (green). B) Cellular localization of ZMYND11 (green) studied by immunostaining. The nuclei were counterstained by DAPI. Scale bars are 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

genes further pointing to the synaptic dysfunction in SCZ.

3.6. DGGC reactivity to glutamate

Based on the transcriptomic analyses, we investigated DGGCs for reactivity to glutamate administration. Using calcium-imaging experiments baseline activity and reactions evoked by glutamate were recorded. We found no differences in spontaneous calcium transients between control and mutant neuronal cultures (Supplementary Fig. 10). After application of glutamate, we observed increased intracellular calcium concentration in both mutant and wild type neurons. Quantification of multiple experiments revealed significantly decreased reaction in the mutant cell lines compared to their wild type controls (Fig. 8).

4. Discussion

4.1. Effects of the ZMYND11 1495C > T nonsense DNM on neuronal differentiation

Here we demonstrated a potential pathogenic role in SCZ for a previously undescribed DNM in ZMYND11. Importantly, this gene has been implicated in tumorigenesis (De Braekeleer et al., 2014; Li et al., 2021) and syndromic intellectual disability, based on its biological functions and the previously identified mutations. Its role in tumor formation has been studied extensively and is connected to the loss of its co-repressor function of actively transcribed genes (Chen et al., 2019).

Only one study has demonstrated a ZMYND11 SNV in SCZ (Balakrishna and Curtis, 2020), however several DNMs have been demonstrated ZMYND11 mutations in various forms of intellectual disability with or without brain malformations (Coe et al., 2014). Interestingly, these mutations were localized mostly to the Bromo and MYND domains of the protein, and no mutation affecting the NLS region has been previously published (Fig. 9).

According to the Varsome database and personal communications

the same mutation has been found in a child with attention-deficit/hyperactivity disorder and speech delay and his unaffected mother, thus revealing itself as a transmitted SNV and its association with less severe neurodevelopmental phenotypes. We hypothesize that the previously described mutations cause more severe brain alterations and clinical manifestations, i.e. syndromic intellectual disability, due to the deficit of ZMYND11 transcriptional regulation during neurodevelopment. It is conceivable that the mutation investigated in our study has an effect on nucleocytoplasmic trafficking of the protein, thus, there is no direct deficit of ZMYND11 co-repression, however, haploinsufficiency or dominant-negative effects are possible. The involvement of nucleocytoplasmic trafficking is supported by the change of nucleocytoplasmic ratios between the wild type and mutated hiPSC lines, as shown in Fig. 2. Importantly, the hiPSC line Ctrl-Mut2 demonstrated the lowest level of nuclear localization, as expected by full truncation of the protein at the NLS.

In mouse primary cells ZMYND11 has been shown to undergo downregulation during neuronal differentiation in vitro, moreover overexpression inhibited neuronal differentiation and decreased neurite outgrowth, suggestive of an inhibitory role (Yu et al., 2009). In our model system we observed that ZMYND11 mRNA levels were lower in the mutant hippocampal NPCs and neurons, however during neuronal differentiation an overall increase of mRNA levels was seen (Supplementary Fig. 5).

Using isogenic pairs, we demonstrated that the R399X mutation has no detrimental effect on differentiation of DGGCs, both the Pat-Mut and the Ctrl-Mut hiPSC lines could differentiate into SOX2 and Nestin expressing functional hippocampal NPCs. Moreover, upon further differentiation all NPC lines reached mature neuronal stage, characterized by the expression of MAP2 and PROX1 assessed by ICC and spontaneous Ca-transients, indicative of synapse formation and neuronal activity. Interestingly, we have also shown that in hippocampal NPCs and neurons ZMYND11 is localized exclusively in the cytoplasm, suggesting that similarly to other proteins, it is sequestered from the nucleus during

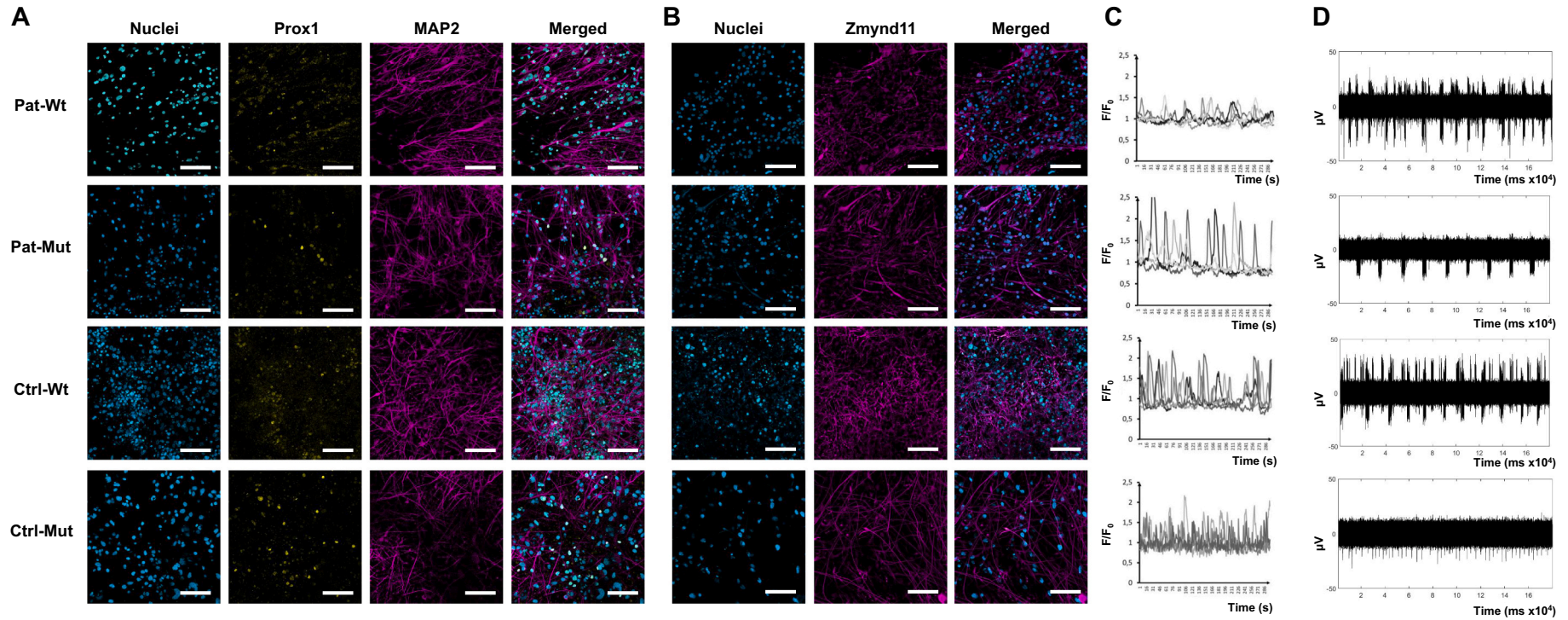
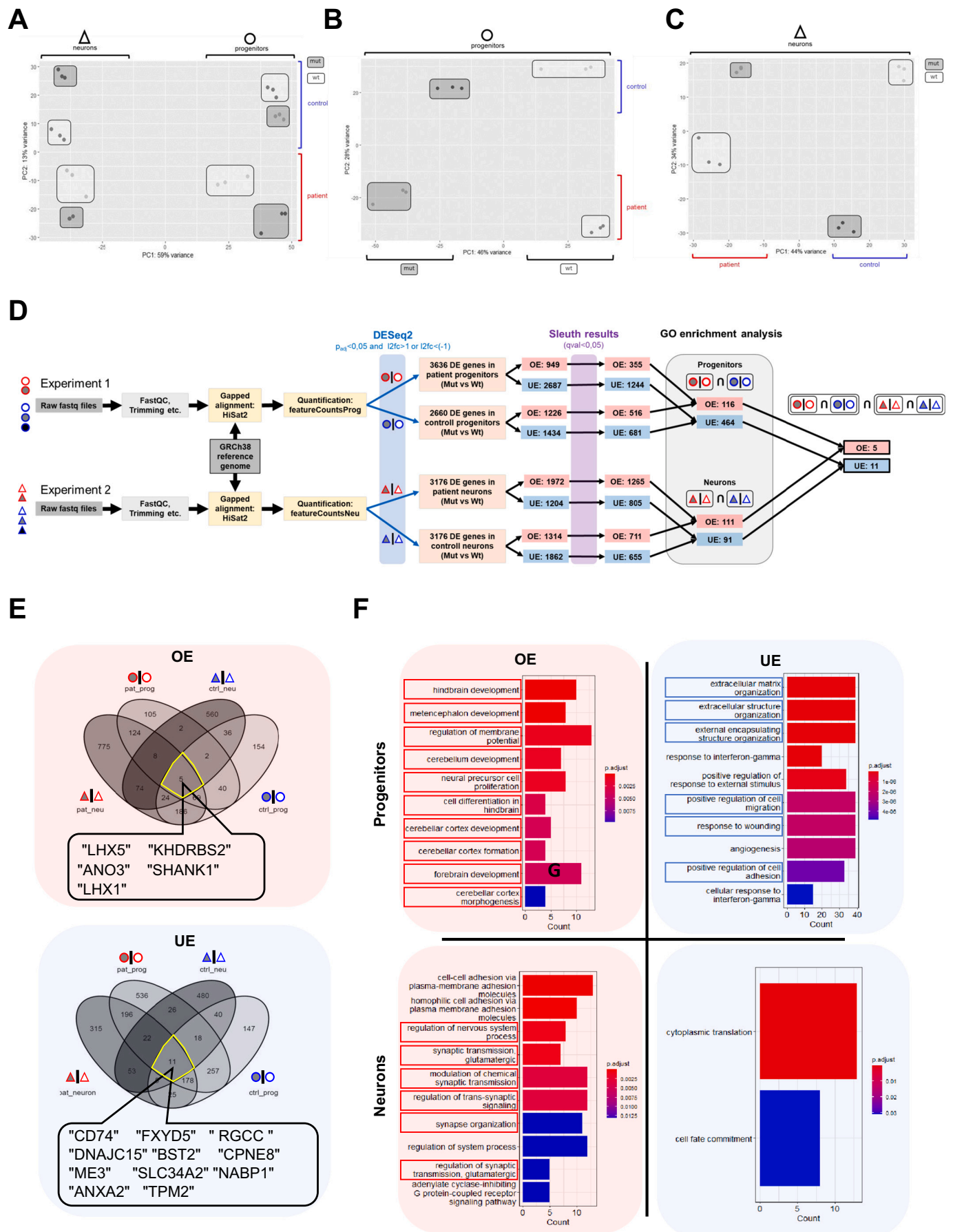


Fig. 5. Characterization of genome edited hiPSC-derived neural cultures. A) Representative images of at least two parallel immunofluorescence stainings on the neural cultures at 5 weeks of neural differentiation for neural markers: PROX1 (red) and MAP2 (green) B) Cellular localization of ZMYND11 (green) studied by immunostaining. The nuclei were counterstained by DAPI. Scale bars are 100 µm. C) Representative examples for spontaneous Ca-transients in neurons. Five manually selected cells that showed spontaneous activity during a 5 min measurement are plotted per sample. D) Representative single electrode recordings from 3 min MEA measurements showing signals characteristic to neuronal activity. Functional assays revealed spontaneous calcium-transients and synchronized electric activity detectable with MEAs in all neuronal cultures between 4 and 6 weeks of differentiation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



(caption on next page)

Fig. 6. Analysis of mRNA sequencing data in hippocampal NPCs and neurons A) Principal Component Analysis (PCA) plot of all samples showing separation based on differentiation status and genetic background. B) PCA plot of only progenitor samples. Separation is according to ZMYND11 status (PC1) and genetic background (PC2). C) PCA plot of only neuron samples. Separation is according to genetic background along PC1 axis and samples are not separated according to ZMYND11 status along PC2 axis. D) Schematic representation of the analysis pipeline used to identify differentially expressed genes, resulting in five overexpressed and eleven underexpressed targets, and highlighting the gene sets used for GO analysis later. E) Venn diagrams showing the overlap of differentially expressed genes in patient and control progenitors and neurons. Chat boxes show the genes from the innermost intersections that are differentially expressed in both genetic backgrounds and both differentiation phases. F) Bar plots showing results of Gene Ontology Enrichment Analysis, with enriched categories on the vertical axis and gene counts of each category on the horizontal axis. Each bar is color-coded by statistical significance for enrichment.

neuronal differentiation. Another protein from the same family, ZMYND8 has been shown to undergo similar nucleocytoplasmic sequestration, as a potential form of protein-level regulation in neurons (Yao et al., 2017). Next, to investigate subtle differences in gene expression levels and influencing its efficiency we took a transcriptomics approach at the NPC and neuronal stages and used RNA sequencing.

4.2. RNA sequencing results

Importantly, we used a differentiation protocol that produces hippocampal dentate gyrus granule cells, the neuronal cell type responsible for adult neurogenesis, thus shedding light on the unique contributions of this brain region to the pathophysiology of SCZ. Majority of the hiPSC-based studies in SCZ so far have been conducted using cortical neurons (Dubonyte et al., 2022). As the hippocampal formation and cortex have been shown to represent distinct gene expression patterns and functional roles in cognition and behavior, it is important to include this region as well for in vitro investigations, leading to new insights about hippocampal pathophysiology. There have been two previous SCZ studies probing hippocampal involvement (Yu et al., 2014; Han et al., 2016), but the patients involved weren't genetically characterized. In contrast, using a patient cell line with defined genetic alteration combined with isogenic controls enables us to attribute the transcriptomic changes to specific biological pathways.

Overexpression of neuronal differentiation genes at the NPC stage, and neuronal functional genes at the more mature neuronal stage is a

molecular phenotype that may be a sign of accelerated or precocious neuronal differentiation. Although our data only contains transcriptomic evidence in support for this hypothesis, others have already shown this phenotype at the cellular level using organoids (Sawada et al., 2020). Overexpression of neuronal differentiation genes and underexpression of glial function genes as a molecular signature, has already been reported in other hiPSC based models of SCZ (Narla et al., 2017). Precocious neuronal differentiation is also observed in a genetically and clinically closely related disorder, autism spectrum disorder (ASD) (Schafer et al., 2019). The convergence of hiPSC models in SCZ and ASD further supports the already established neurodevelopmental theory (Hathy et al., 2020) of both disorders.

As GO terms connected to synaptic function (e.g. “synapse organization” and “modulation of chemical synaptic transmission” etc.) were enriched among the differentially expressed genes, our study supports the hypothesis, that synaptic dysfunction is an important component of SCZ pathogenesis. Enrichment of GO term “synaptic transmission glutamatergic” more specifically points to the involvement of the glutamatergic system, as reported earlier (Coyle et al., 2012; Egerton et al., 2020). Relative overexpression of glutamate receptors is an interesting finding, as a lot of evidence points to NMDAR hypofunction as an important aspect of SCZ pathophysiology at later stages of the disorder (Coyle et al., 2012). This discrepancy between overexpressed glutamatergic genes in vitro and glutamatergic hypofunction in vivo could be explained by compensatory mechanisms, or the different time points of developmental trajectories these stages represent.

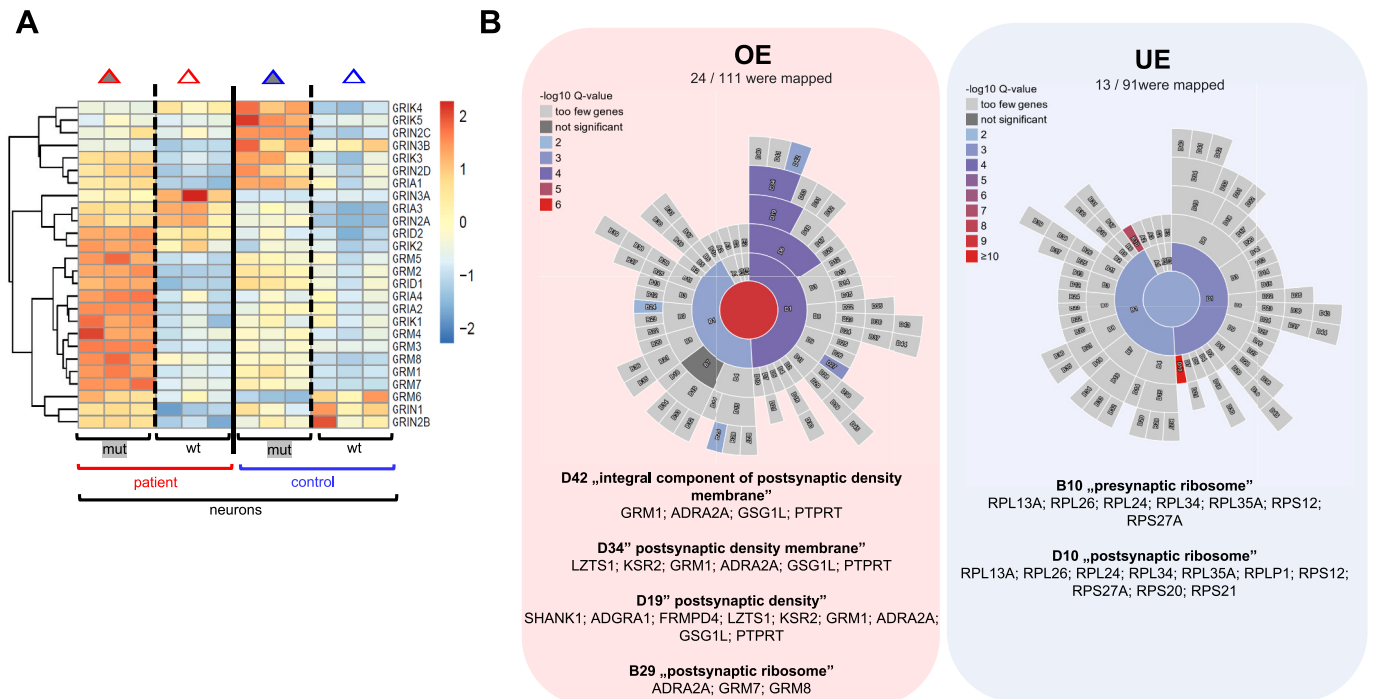


Fig. 7. Analysis of genes involved in synaptic transmission. A) A clustered heatmap showing relative expressions of glutamate receptor genes across neuronal samples. Expression values were normalized row wise. B) GO enrichment analysis of synaptic genes using SynGO Database. “Sunburst” plots show “CC” (cellular component) GO categories. Statistical significance for enrichment in our dataset is color coded. Mutant cell lines in both genetic backgrounds have higher expression of most receptors compared to their isogenic controls.

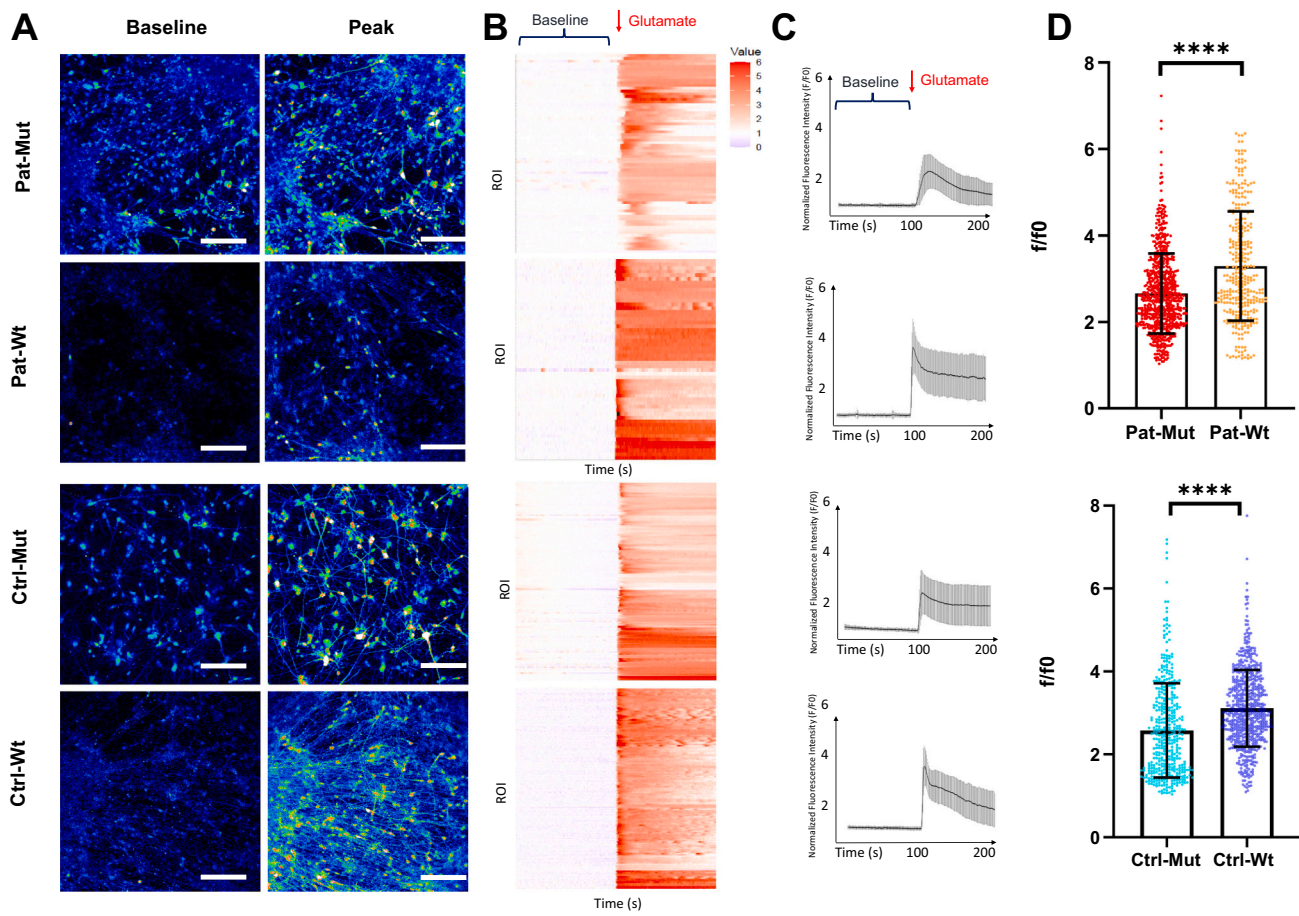


Fig. 8. Calcium-imaging analysis of DGGC reactivity to glutamate. A) Representative false color confocal microscopy images of neural cultures at 5–6 weeks of neural differentiation stained by Calcium indicator dye Fluo-4-AM. First column showing baseline fluorescence and second column showing the frame of peak signal intensity. Scale bars represent 100 μ m. B) Heatmaps of the recordings, where fluorescence intensity is color coded. Rows represent ROIs and Y-axis represents Time. C) Average normalized (F/F0) fluorescence intensity of all the ROIs during recording. D) Quantification of reaction to glutamate. Altogether 400–600 individual cells were selected from 3 to 5 independent experiments. Mann-Whitney *U* test $p < 0.0001$.

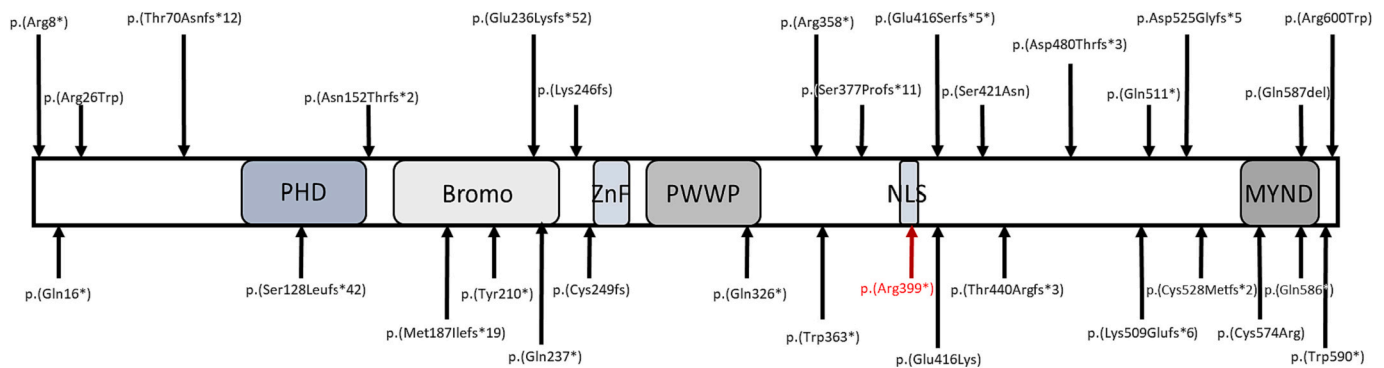


Fig. 9. Pathogenic amino acid changes in the ZMYND11 canonical protein causing syndromic intellectual disability. Figure adapted from Yates et al., 2020 (Yates et al., 2020) complemented with ClinVar data (ClinVar) and p.(Arg399*).

In the innermost intersection of DE genes (DE in both genetic backgrounds and both differentiation stages), we found genes that play a role in neuronal cells, while other genes are more challenging to interpret in the context of neuronal development. The overexpressed (OE) group contains the transcription factors LHX1 and LHX5. These play an important role in forebrain development and are necessary for formation of the hippocampus in mice (Lui et al., 2017), thus, their overexpression in the mutant cells may suggest a dysregulation of neuronal developmental pathways. Another OE gene, SHANK1 is a postsynaptic scaffold

protein in excitatory synapses that plays a role in glutamatergic synaptic transmission and ASD (Jiang and Ehlers, 2013).

ANO3 encodes a Ca-sensor transmembrane protein that has been implicated in movement disorders like Parkinson's disorder and dystonia, and has been implicated in modulation of synaptic response in the hippocampus (Huang et al., 2012; Pedemonte and Galletta, 2014). The overexpression of SHANK1 and ANO3 strengthens the link between ZMYND11 mutation and synaptic dysfunction.

Furthermore, we observed KHDRBS2 in the OE group. This is an

RNA-binding protein that has a role in alternative mRNA splicing. This suggests that dysregulation of splicing could be a potential mechanism underlying the effects of the ZMYND11 mutation. Previously this gene has also been linked to SCZ and Alzheimer's disease (Gusareva et al., 2014; Hathy et al., 2020). Further studies are warranted to investigate which splicing events are affected and how they may contribute to the observed molecular phenotype.

Among the UE genes we found SLC34A2, which is a solute carrier, with low, but detectable brain expression, and has been found to be differentially expressed in a study identifying common transcriptomic signatures of SCZ, ASD, BPD in post-mortem brain tissue (Guan et al., 2019). Tropomyosin2 (TPM2) is mainly expressed in muscle cells, but studies have shown that several tropomyosin genes are involved in the actin cytoskeleton organization and axonal growth of neurons (Li and Gao, 2003; Wang and Coluccio, 2010). The CD74 gene codes a protein that associates with HLA class II protein and regulates the antigen presentation process of the immune system. It is highly expressed in the brain by glial cells, especially microglia (proteinatlas.org, (Zeiner et al., 2015)). A previous study examining blood samples of SCZ patients found this gene significantly UE (Xu et al., 2012). Underexpression of this gene in our samples could signal a decreased glia-neuron ratio in mutant samples. Single-cell RNA sequencing would be necessary to confirm this finding. The rest of the genes in the intersection (BST2, FXD5, RGCC, ANXA2, ME3, NABP1, DNAJC15, CPNE8) are also UE in the mutant cells, but their specific roles in the context of hippocampal neurons and progenitor cells are less clear.

4.3. Decreased reactivity to glutamate as a functional phenotype

Decreased reaction to stimulation by glutamate is an important finding in the light of the increasing body of evidence supporting the glutamatergic hypothesis of SCZ in vivo (Howes and Onwordi, 2023) and in vitro (Balan et al., 2019). OE of neuronal genes in conjunction with decreased reactivity to glutamate might seem contradictory at first. However, this could be explicable by a compensatory mechanism in mutation-carrying neuronal cells. Further proteomic and functional studies are warranted to elucidate the precise mechanism.

4.4. Limitations of the study

Our findings should be interpreted in the light of its strengths and limitations. In the case of hiPSC-based disease modeling, the selection of control lines always involves compromises. The XCL1 control cell line we chose was created from the umbilical cord blood sample of a healthy newborn, therefore we have no information about whether the individual will develop any neuropsychiatric disorders. This does not affect the results of the studies revealing the role of ZMYND11, but a control chosen according to age and gender would have served as a more appropriate control. Although using CRISPR based genome editing represents a unique opportunity to understand underlying molecular disease pathways, at this stage we have very limited understanding of how the observed transcriptomic alterations in the mutation-carrying NPCs and neurons translate into functional differences, and what is the relevance of these physiological deficits for in vivo phenomena. In follow-up studies we plan to complement the molecular phenotype revealed by transcriptomic analysis with precisely measurable functional phenotypes. We only investigated one specific DNM that was observed in one specific patient; therefore, results are not generalizable to other DNMs or rare variants in SCZ. Along this line, further studies with similar methodology should address the question of the most common DNMs in SCZ, and conversely, we need further studies to establish the function and exact role of ZMYND11 in neurons and in SCZ. CRISPR-genome editing has its own methodological limitations, including off-target effects and single-cell cloning during the genome-editing process.

5. Conclusion

In summary, the methodological approach outlined in our study is amenable to the investigation of the biological significance of other DNMs in SCZ as well. Such studies could pave the way for better mechanistic insight about the genetics and neurobiology of the disorder and the identification of new molecular targets for pharmacological development. Furthermore, understanding the biological role of individual rare genetic variants will lend support for personalized medicine approaches and whole-genome sequencing in the individual treatment repertoire of SCZ.

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CRediT authorship contribution statement

Csongor Tordai: Formal analysis, Investigation, Software, Writing – original draft, Visualization. **Edit Hathy:** Conceptualization, Formal analysis, Investigation, Methodology. **Hella Gyergyák:** Formal analysis, Investigation, Methodology, Visualization. **Katalin Vincze:** Investigation. **Máté Baradits:** Investigation, Methodology, Visualization. **Júlia Koller:** Data curation, Investigation, Validation. **Ádám Póti:** Investigation, Methodology, Software, Validation. **Bálint Jezsó:** Investigation, Supervision, Visualization. **László Homolya:** Funding acquisition, Resources, Supervision. **Mária Judit Molnár:** Conceptualization, Investigation. **László Nagy:** Investigation, Methodology, Resources. **Dávid Szűts:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Validation. **Ágota Apáti:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **János M. Réthelyi:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have no competing financial interests to declare.

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Appendix A. Supplementary data

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