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Extracellular matrix dysfunction and synaptic alterations in schizophrenia

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Schizophrenia is a complex disorder with strong evidence implicating neurodevelopmental abnormalities in its pathophysiology. Olfactory neuroepithelial cells from patients provide a unique platform for studying neurodevelopmental processes. These cells can be cultured into neurospheres and differentiated into neurons, allowing the study of patient-specific alterations related to the disorder. In this study, we utilized olfactory neuroepithelial cells from patients with schizophrenia and controls to explore putative neurodevelopmental dysregulations. RNA-sequencing of neurospheres transcriptome revealed significant alterations in extracellular matrix-related gene expression, suggesting extracellular matrix dysregulation as an underlying contributor to schizophrenia etiopathogenesis. Upon differentiation of olfactory neuroepithelial cells into neurons, transcriptomic analysis identified a significant downregulation of genes involved in synaptic organization and extracellular matrix interactions. To validate these findings, we quantified the protein levels of these genes in olfactory neuroepithelium-derived neurons and in postmortem dorsolateral prefrontal cortex tissue from schizophrenia patients and matched controls. Consistent with our transcriptomic data, schizophrenia subjects exhibited decreased levels of L1CAM, SCG2, and NPTXR proteins. Furthermore, we identified a correlation between the protein levels of L1CAM and NPTXR in the brains of individuals with schizophrenia, a relationship that was absent in control subjects. Our findings provide robust evidence for extracellular matrix and synaptic dysregulation in schizophrenia, linking neurodevelopmental disruptions to molecular alterations in both patient-derived cellular models and postmortem brain. These results underscore the utility of olfactory neuroepithelium cells as a model for studying the neurodevelopmental basis of schizophrenia. They also highlight extracellular matrix-related pathways as potential targets for future search of biomarkers and therapeutic development.

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INTRODUCTION

Schizophrenia is a chronic and disabling illness that typically begins in adolescence and worsens over time, reducing life expectancy by approximately 14.5 years [1]. Several hypotheses regarding etiopathogenesis of schizophrenia have been suggested, but none has been consistently confirmed. Thus, schizophrenia is a complex condition influenced by a combination of genetic, biological, and environmental factors. Genetic factors are estimated to contribute to roughly 80% of the risk associated with this disorder [2, 3]. Moreover, accumulating evidence from epidemiologic, clinical, and basic neuroscience research suggests that schizophrenia is primarily a neurodevelopmental disorder [4]. Abnormalities during critical periods of brain development, including altered neuronal migration, impaired synaptogenesis, and disrupted maturation of neural circuits, are thought to contribute to the emergence of schizophrenia symptoms [5, 6]. These neurodevelopmental disruptions are often linked to both genetic predispositions and environmental factors, which together shape the disorder's onset and progression.

Investigating the molecular underpinnings of neurodevelopment in schizophrenia has been challenging due to the inaccessibility to brain tissue of living subjects. However, patient-derived olfactory neuroepithelial cells (ONECs) offer a unique and innovative model for studying neurodevelopmental processes in brain disorders [7–9]. ONECs, obtained non-invasively from the nasal cavity, are neural progenitor cells capable of forming neurospheres (NSFs) and differentiating into neurons [7, 10]. These cells retain patient-specific genetic and epigenetic information, providing a valuable system for examining cellular and molecular changes associated with schizophrenia [11]. Moreover, their neurodevelopmental properties allow direct investigation of processes such as cell proliferation, migration, and differentiation, which are critical events implicated in the pathophysiology of the disorder [12].

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With this background, the aim of this study was to investigate the potential neurodevelopmental abnormalities present in schizophrenia. ONECs were cultured to form NSFs and their growth characteristics, self-renewal potential and gene expression profile were evaluated. By integrating transcriptomic analyses of NSFs and ONEC-derived neurons with protein quantification in both cells and postmortem brain tissue, we aimed to identify neurodevelopmental disruptions and their implications for schizophrenia etiopathogenesis.

Our findings revealed substantial extracellular matrix (ECM) and synaptic proteins dysregulation in schizophrenia, supporting the hypothesis that ECM abnormalities may contribute to the synaptic and neurodevelopmental disruptions underlying this neuropsychiatric disorder.

Moreover, our findings highlight the utility of ONECs as a model for understanding the neurodevelopmental origins of schizophrenia and underscore their potential in identifying biomarkers and novel therapeutic targets for the disorder.

METHODS

Subjects and samples

Three different cohorts were used in this study (Table 1). Each cohort was strategically used to address specific experimental objectives, ensuring a comprehensive investigation of neurodevelopmental and molecular alterations in schizophrenia.

Cohort 1 consisted of schizophrenia patients and matched controls whose olfactory neuroepithelial cells (ONECs) were cultured to form neurospheres (NSFs). This cohort enabled the examination of cellular growth characteristics, neurodevelopmental processes such as proliferation and maintenance as well as transcriptome evaluation.

Cohort 2 comprised schizophrenia patients and matched controls whose ONECs were differentiated into neurons. This cohort was used to evaluate molecular and transcriptomic changes associated with synaptic organization and neurodevelopment at the neuronal level.

Cohort 3 included postmortem dorsolateral prefrontal cortex (DLPFC) samples from schizophrenia subjects and matched controls. This cohort allowed us to validate findings from the patient-derived cell models by quantifying protein levels of key genes differentially expressed in the disorder.

This multi-cohort approach enabled the integration of findings across complementary models, providing robust evidence for the neurodevelopmental and synaptic alterations associated with schizophrenia.

Olfactory neuroepithelium samples. Subjects who met inclusion criteria for schizophrenia based on a Structured Clinical Interview (according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders, DSM-5 [13]) were included into the study as cases. For controls, inclusion criteria were: age 18–60 and no neurological or psychiatric diagnoses. Controls were recruited regarding sex and age matching criteria. Exclusion criteria for all subjects included meeting criteria for any severe mental disorder different from schizophrenia, history of severe congenital, medical, or neurological illnesses, current medical conditions affecting the nasal region (such as rhinitis or bleeding), or the consumption of any substance of abuse. General information regarding the demographic characteristics of the experimental groups can be found in Supplementary Table 1 and Supplementary Table 2.

The olfactory epithelium was obtained by nasal exfoliation as previously described [7] by trained sanitary staff. All participants provided the written consent. The corresponding Human Research Ethics Committee (Comité de Ética en la Investigación (CEI) OSI Ezkeraldea-Enkarterri-Cruces code CEI E22 /27), approved the entire procedure.

Postmortem brain samples. Human brain samples were collected during autopsies conducted at the Basque Institute of Legal Medicine, Bilbao, adhering to the research and ethical guidelines for postmortem brain studies. A retrospective review of medical diagnoses and treatments was carried out for each case using data from examiners and records from hospitals and mental health centers. Based on previous works [14], 18–19 subjects would be needed in each group to detect a difference in effect size of 1.11, assuming a significance level of 5% and a power of 90%.

Brain samples from 20 individuals diagnosed antemortem with schizophrenia, according to DSM-5 criteria, were paired with samples from 20 control subjects in a matched study design. Control subjects were selected based on the absence of neuropsychiatric disorders or drug abuse and were matched to the schizophrenia group based on sex, age, and postmortem interval (PMI, the time between death and tissue dissection/freezing). A blood toxicology screening was conducted for all subjects to detect the presence of antipsychotics, other drugs, and ethanol, with analyses performed at the National Institute of Toxicology in Madrid, Spain.

Toxicological assessments in plasma samples detected antipsychotic drugs in 10 schizophrenia cases (AP+), whereas 10 were antipsychotic-free (AP−) at the time of death. Demographic characteristics and PMI values did not significantly differ between schizophrenia and control groups, nor between AP+ and AP− subjects (Table 1). Samples of DLPFC were dissected at autopsy (0.5–1 g tissue) following standard procedures [15] and immediately stored at −80 °C until assay. A full description of the demographic characteristics of the cohort can be found in Supplementary Table 3.

Neurospheres culture

Neurospheres were obtained following already stablished methods [7]. Briefly, the samples obtained by nasal exfoliation underwent a manual disaggregation followed by a centrifugation and the pellet resuspended specific medium. When the neurospheres reached approximately 100–150 µm in diameter, subsequent passages were initiated to evaluate their ability to reassemble and generate new neurospheres. The number of neurospheres formed per 100 seeded cells was quantified (Fluidlab R-300, Anvajo, Germany) and their diameters were manually measured under a bright-field microscope using a calibrated scale. Further details are provided in the Supplementary Methods.

Neuron-enriched culture

Neuron-enriched cultures were obtained from olfactory neuroepithelium adherent cultures as previously described [7]. Briefly, samples from the olfactory neuroepithelium obtained by nasal exfoliation were manually disaggregated and cultured in supplemented medium. At passage 5, cells expressing the neuronal protein PSA-NCAM in their surface were selected by magnetic sorting. Cells were then cultured for 30 days in differentiation medium (see Supplementary Methods).

Table 1. Demographic characteristics of the three cohorts used in the study.

	Cohort 1		Cohort 2		Cohort 3		
	Schizophrenia	Control	Schizophrenia	Control	Schizophrenia		Control
	($n = 5$)	($n = 5$)	($n = 10$)	($n = 10$)	AP+ ($n = 10$)	AP− ($n = 10$)	($n = 20$)
Age (years)	30.2 ± 2.1	30.4 ± 1.6	31 ± 2.2	31.3 ± 2.3	41.8 ± 4.0	40.1 ± 3.6	41.3 ± 2.7
Sex(M/F)	4 M/1 F	4 M/1 F	5 M/5 F	5 M/5 F	6 M/4 F	8 M/2 F	14 M/6 F
PMI (hours)	---	---	---	---	19.1 ± 3.6	15.2 ± 4.1	22.9 ± 3.8
Antipsychotic treatment	+	−	+	−	+	−	−

Antipsychotic-free (AP−), antipsychotic-treated (AP+). Mean ± S.E.M. PMI: postmortem interval. No differences between groups in any of the parameters.

Gene expression analysis

The gene expression analysis was conducted using neurosphere samples from 5 controls and 5 schizophrenia patients (cohort 1) and neuron samples from 10 controls and 10 schizophrenia patients (cohort 2). RNA from neurospheres and neuron-enriched cultures was extracted and libraries prepared according to the "NEBNext Ultra Directional RNA Library Prep kit for Illumina" (New England Biolabs, Massachusetts, USA) instructions and sequenced using a "NextSeq™ 500 High Output Kit" in a 1×75 single read sequencing run on a NextSeq500 sequencer. Differential expression analysis was carried out using the CUFFDIFF tool. Further details are provided in the Supplementary Methods.

Pathway and process enrichment analysis

Metascape [16] was used to analyse the enrichment in specific gene ontologies for each comparison. For each given gene list, pathway and process enrichment analysis were carried out with the following ontology sources: KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways, CORUM, WikiPathways, and PANTHER Pathway (see Supplementary Methods).

Western blots

Western blot was performed as previously described [17] with minor modifications. Specific antibodies against SCG2, L1CAM, NPTXR, PTN, NUF2 and β-actin or GAPDH were used (Supplementary Table 4). Membrane immunodensity signal was then detected following addition of ECL WB substrate. The immunodensity value of the target proteins was normalized by the corresponding value of β-actin or GAPDH and an external reference sample included in every gel (see Supplementary Methods).

Statistical analyses

Data were analyzed using GraphPad Prism™ 11 (GraphPad Software, San Diego, California, USA) and InVivoStat. Results are expressed as means ± standard error of the mean (SEM) and individual values. Before statistical analyses, data were inspected for outliers using Grubb's test ($Z > 1.96$). Two-group comparisons were performed using unpaired Student's t-tests, while multiple-group comparisons were analyzed using two-way ANOVA, followed by Bonferroni's or Benjamini-Hochberg's post hoc analyses. Statistical significance was set at $p < 0.05$. A detailed description of the statistical analyses can be found in the Supplementary Material section.

RESULTS

SZ-derived neurospheres display altered proliferation rate and growth

First, we generated neurospheres from the ONECs of both schizophrenia patients and their age and sex-matched controls (Fig. 1a). After nasal exfoliation, neurospheres were obtained by dissociating olfactory neuroepithelium cells grown in proliferation medium. Neurospheres were grown for approximately 15 days before passaging, when were then dissociated into single cells and re-seeded (passage 1, P1) for 15 days before performing another passage. The self-renewal capacity (proliferation) and size of neurospheres were evaluated.

SZ-derived cells formed less primary neurospheres at passage 0 (P0) compared to controls; however, this difference was not statistically significant (Fig. 1b; Supplementary Table 5). However, neurosphere self-renewal capacity was strongly dysregulated in SZ-cells as was demonstrated by the number of newly formed neurospheres of the first-generation (P1) (Fig. 1c). The two-way ANOVA analyses results (Supplementary Table 5) revealed a significant interaction between time (days after seeding) and the disorder, indicating that from P1 onwards, the self-renewal capacity of cells was significantly lower in SZ compared to controls.

When the size of these neurospheres was evaluated, a similar pattern of growth was observed in P0 and P1, with a diameter mean of 52.90 ± 1.61 µm for controls and 50.30 ± 1.95 µm for SZ (Fig. 1d, e). However, at passage 2 (day 45), SZ-neurospheres were significantly smaller (−28.20%) compared to controls (63.20 ± 4.44 µm for controls and 45.40 ± 1.12 µm for SZ). This reduction increased progressively in the subsequent passages. At passage 3 (day 60, Fig. 1d), SZ-neurospheres showed a further size decrease (−46.60%) (193.0 ± 21.17 µm for controls and 103.0 ± 4.43 µm for SZ). At passage 4 (day 75, see Fig. 1d), the reduction persisted (−53.82%) (206.6 ± 7.21 µm for controls and 95.40 ± 1.63 µm for SZ), and finally, at passage 5 (day 90, Fig. 1d) SZ-neurospheres size reached the most substantial decrease (−56.41%) observed across all passages (207.40 ± 8.41 µm for controls and 90.40 ± 0.87 µm for SZ) (see Supplementary Table 5 for detailed statistics).

Extracellular matrix-related genes expression dysregulation as the main feature in SZ-derived neurospheres

To gain further insight into the deficits in proliferative and self-renewal capacity that we observed in the SZ-neurospheres, RNA sequencing assays were performed. The results showed that the two populations were clearly differentiated based on their gene expression profile.

RNAseq gene expression profiling identified 293 differentially expressed genes in the neurospheres of SZ-patients, including genes implicated in ECM organization, cell adhesion and development (Fig. 2a and Supplementary Table 6). Enriched ontology clusters analysis identified a dysregulation of pathways involved in extracellular matrix organization (R-HSA-1474244; GO0030198), ECM proteoglycans (R-HSA-3000178), collagen formation (R-HSA-1474290), the NABA matrisome associated (M5885; an ensemble of genes encoding extracellular matrix and extracellular matrix-associated proteins), and nervous system development (R-HSA-9675108) (Fig. 2b), among others.

To further capturing the relationships between the pathways, these were rendered as a network plot, where similar terms are connected by edges. Each node represents an enriched term and is colored first by its cluster ID (Fig. 2c) and then by its p-value (Fig. 2d).

Differentiated neurons from SZ-derived olfactory neuroepithelium showed extracellular matrix-related and synaptic pathology genes expression dysregulation

The second approach of this study involved the evaluation of neurons derived from the olfactory neuroepithelium to corroborate the findings obtained from the neurosphere assay. The primary objective was to determine whether alterations in the extracellular matrix (ECM) components observed in the neurospheres model, were also present in these neuron-derived cultures. By assessing these alterations, the study aimed to further understand how ECM dysregulation might contribute to the neurodevelopmental abnormalities characteristic of this disorder. To achieve this, RNA sequencing analysis was conducted on neurons derived from the olfactory epithelium of 10 patients with schizophrenia, and 10 controls (Supplementary Table 2). For this, neurons from adherent cultures from the olfactory epithelium were isolated and cultured for 30 days, following our previously stablished protocol [7], and after that, RNA-seq assays were carried out (Fig. 3a).

RNAseq gene expression profiling identified 50 differentially expressed genes in the neurons of patients with SZ, including 24 genes downregulated and 26 genes upregulated (Fig. 3b, c and Supplementary Table 7).

Enriched ontology clusters analysis identified a dysregulation of pathways involved in regulation of cell proliferation (GO:0008285) and developmental growth (GO:0048639), signaling by nuclear receptors (R-HAS-9006931), modulation of chemical synaptic transmission (GO:0050804) and NABA matrisome associated (M5885) (Fig. 3d and see Supplementary Fig. S3 in Supplementary Material).

To further exploring these findings, we chose to validate the expression of key proteins whose coding genes were significantly dysregulated. These proteins were SCG2 (secretogranin II), L1CAM (L1 cell adhesion molecule), and NPTXR (neuronal pentraxin receptor) (downregulated genes) and PTN (pleiotrophin) and

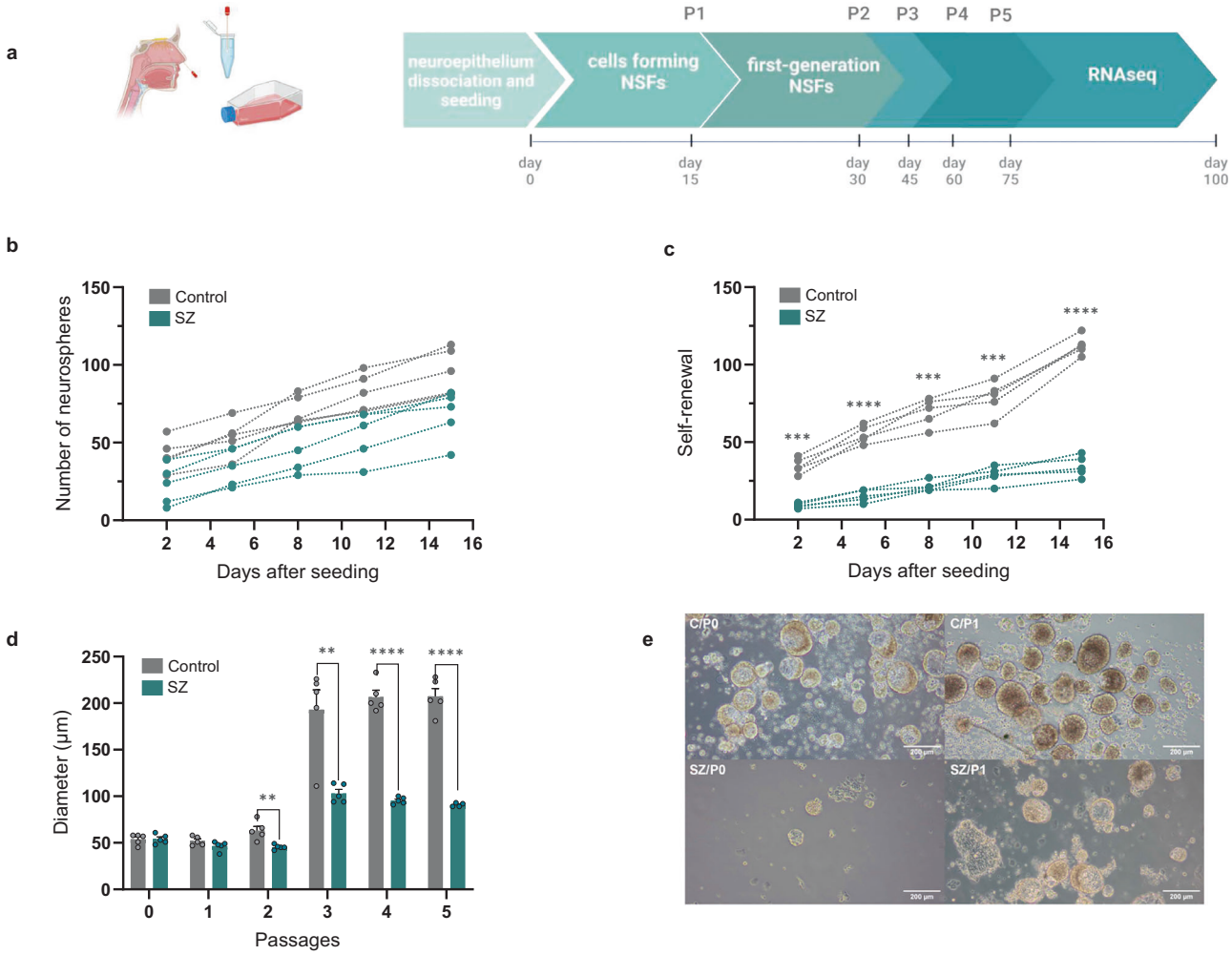


Fig. 1 Reduced proliferation and growth in SZ-derived neurospheres. Proliferation and growth of olfactory neuroepithelium derived neurospheres from schizophrenia patients and their matched controls **a** Experimental scheme showing the production of neurospheres and relative experiment timeline **b** Individual points showing the number of primary neurospheres (P0) obtained by plating 100 cells/subject from the olfactory tissue from SZ subjects ($n = 5$) and controls ($n = 5$) **c** Neurosphere self-renewal capacity evaluated by the number of newly formed neurospheres (bars represent mean ± SEM) of the first-generation (P1)/plated cells * 100 obtained from the dissected tissue from SZ subjects ($n = 5$) and their matched controls ($n = 5$) **d** Graphical representation showing the diameter of neurospheres (mean ± SEM and individual points) from passage P0–P5 obtained from the dissected tissue from SZ subjects ($n = 5$) and their matched controls ($n = 5$) **e** Representative images of neurospheres from SZ subjects (lower panel) and their controls (upper panel) at passage 0 (P0) and passage 1 (P1). Scale bars = 200 µm. Asterisks indicate significant differences. $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$ as assessed by two-way ANOVA followed by Šídák's multiple comparisons test.

NUF2 (NUF2 Component of NDC80 Kinetochore Complex) (upregulated genes). Genes *SCG2, NPTXR, L1CAM*, and *PTN* are involved in key processes that have been proposed to be dysregulated in schizophrenia, particularly those linked to neurodevelopment, synaptic transmission, and extracellular matrix remodeling. *NUF2* is involved in cellular mitosis and was chosen because it was the most overexpressed gene in the RNA-seq assays.

A downregulation in the protein levels of SCG2 (−45%; $p < 0.05$), L1CAM (−54,8%; $p < 0.05$) and NPTXR (−46%; $p < 0.05$) was found in the SZ patients-derived cells (Fig. 3e). On the contrary, an increased protein expression that did not reach statistical significance was also observed for PTN. Immunodensity of NUF2 (Fig. 3e) was similar in both groups.

SCG2, L1CAM and NPTXR are downregulated in human postmortem DLPC in SZ

For enhancing the translational relevance of the study and bridging the gap between in vitro findings and the brain pathology, we chose to validate the identified proteins in brain DLPFC tissue from subjects with schizophrenia. This approach not only ensures translational relevance but also allows us to explore whether the observed molecular dysregulation in olfactory neuroepithelial cells reflects similar patterns in the brain tissue of individuals with schizophrenia.

We found a downregulation of SCG2 (−23%; $p < 0.005$) in the DLPFC in schizophrenia, as compared to sex-, age-, and PMI -matched controls (Fig. 4a,b). This downregulation was observed in both AP− (−29%; $p < 0.05$), and AP+ (−19% $p < 0.05$) subgroups. Comparison between AP− and AP+ cases did not yield statistically significant differences (Supplementary Fig. S4). These data suggest that schizophrenia is associated with lower cortical expression of SCG2 protein, and antipsychotic medication has no effect on SCG2 levels in these subjects

Cortical L1CAM amounts were significantly lower in schizophrenia samples (−29%; $p < 0.0001$), as compared to sex-, age-, and PMI-matched control samples (Fig. 4a,b). Lower L1CAM immunoreactivity was observed in both AP− (−30%; $p < 0.005$)

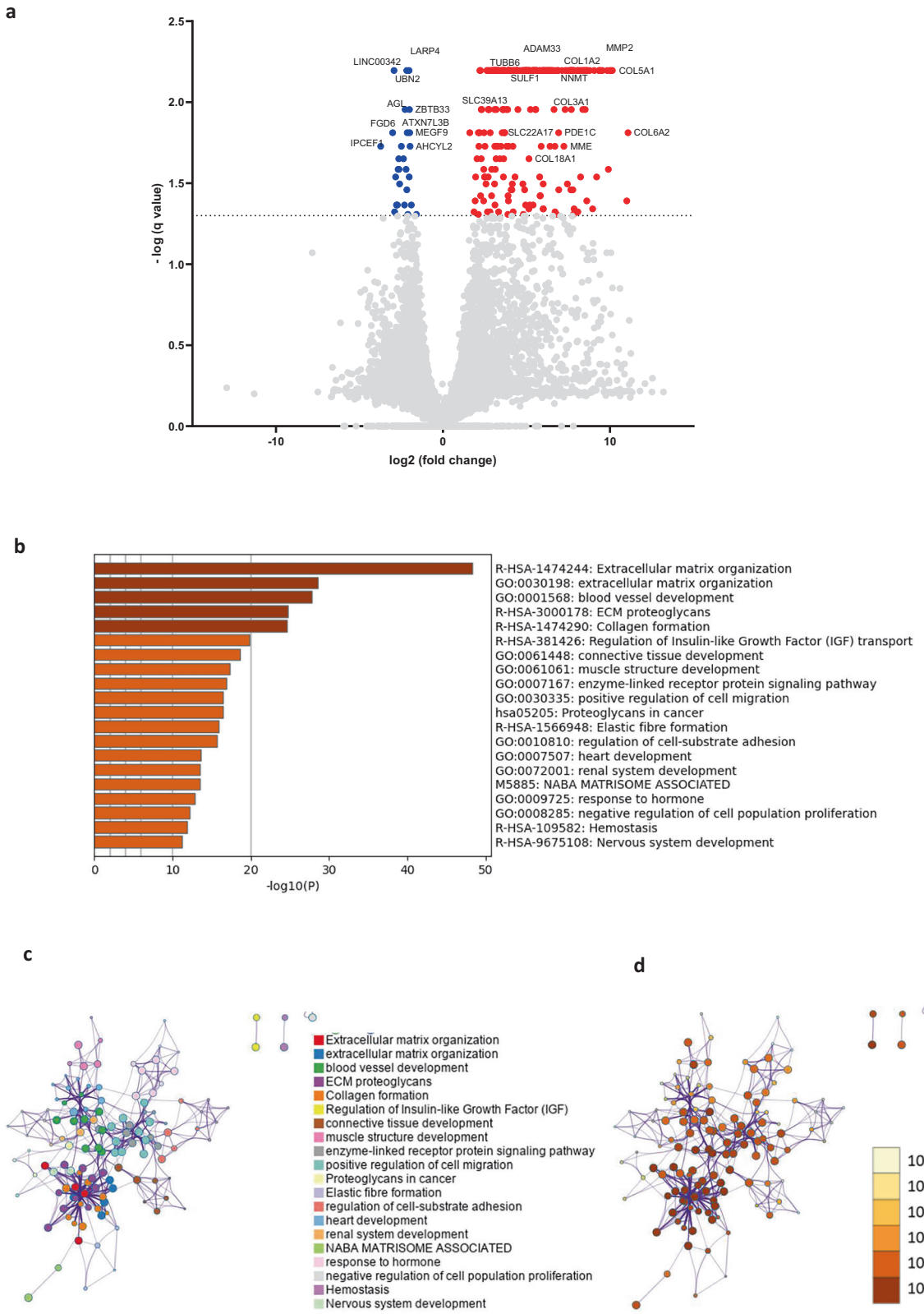
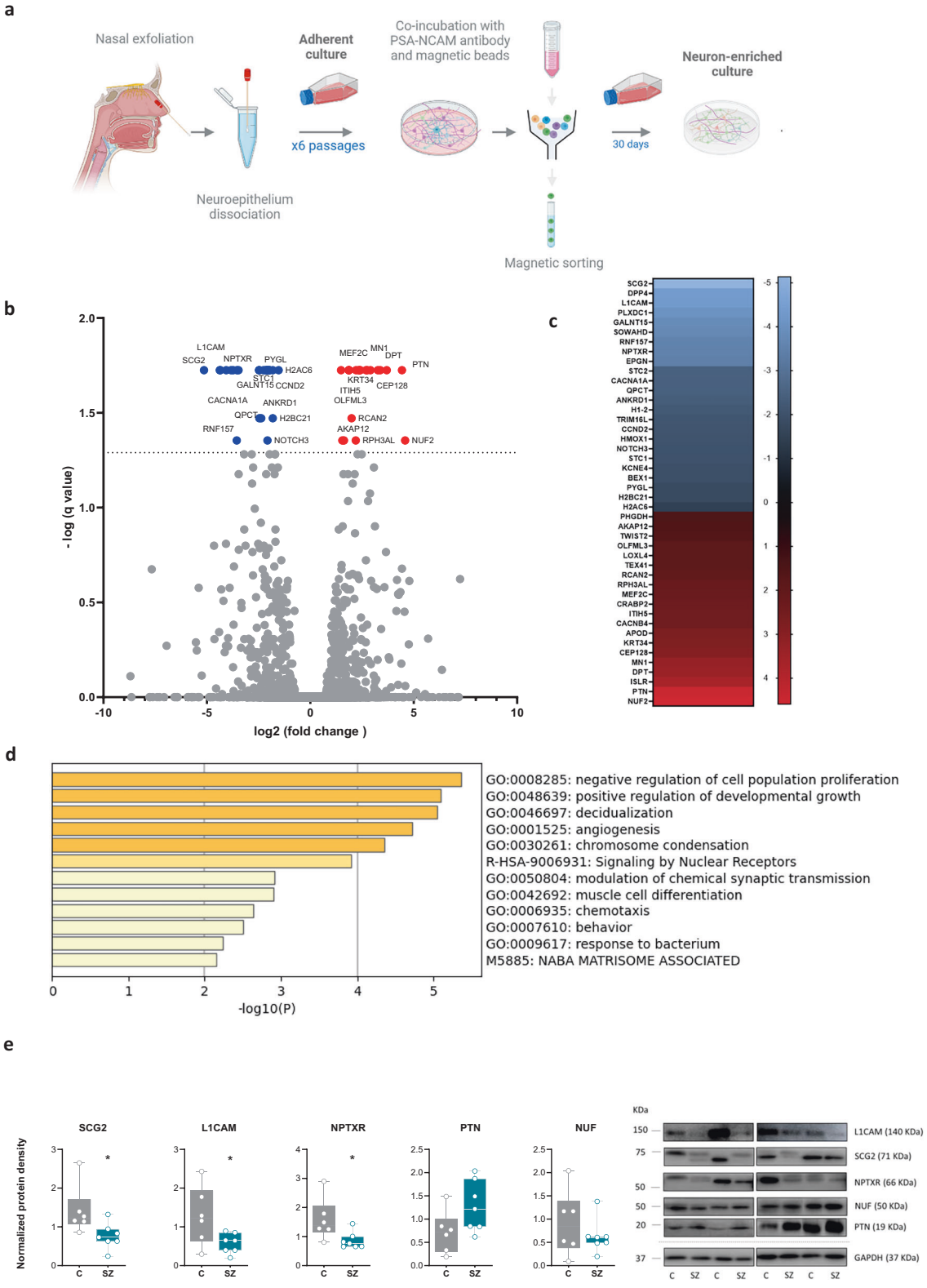


Fig. 2 ECM gene dysregulation in SZ-derived neurospheres. RNAseq assay of olfactory neuroepithelium derived neurospheres from schizophrenia patients and their matched controls **a** Volcano plot representing the differential gene expression in neurospheres SZ ($n = 5$) vs. non-SZ controls ($n = 5$). On the x-axis is log2 (fold change) of genes in subjects with SZ compared to controls, points to the right of 0 represent genes that are increased, and points to the left of 0 genes that are decreased in SZ compared to controls. Statistical significance is displayed on the y-axis (-log (q value)), and $p < 0.05$ are labeled in blue (downregulated) and red (upregulated). Selected genes with both high fold change and significance are labeled **b** Significant GO terms identified in patients with SZ. Visualization of the network of enriched terms: **c** colored by cluster ID, and **d** colored by p-value.



and AP+ (−25% $p < 0.05$) schizophrenia subgroups. Direct comparison between AP− and AP+ cases did not yield statistically significant differences (see Supplementary Fig. S4 in Supplementary Material). These data suggest that schizophrenia is associated with lower cortical expression of L1CAM protein, and antipsychotic medication has no detectable effect on L1CAM levels.

We also found a downregulation of NPTXR (−24%; $p < 0.05$) in the DLPFC samples of schizophrenia subjects, as compared to matched controls (Fig. 4a,b). Subgroup analyses did not detect significant differences between AP− or AP+ and their respective controls or between both of them (Supplementary Fig. S4). These results may be due to the number of individuals in each group,

Fig. 3 Dysregulation of ECM and synaptic pathways in SZ-derived neurons. RNAseq assay of olfactory neuroepithelium derived neurons from schizophrenia patients and their matched controls **a** Experimental scheme showing the production of neurons from adherent cultures from the olfactory neuroepithelium **b** Volcano plot representing the gene differential expression in SZ-neurons ($n = 10$) vs. non-SZ controls ($n = 10$). On the x-axis is log2 (fold change) of genes in subjects with SZ compared to controls, points to the right of 0 represent genes that are increased, and points to the left of 0 genes that are decreased in SZ compared to controls. Statistical significance is displayed on the y-axis, and $p < 0.05$ are labeled in blue and red. Selected genes with both high fold change and significance are labeled **c** Heatmap of the significant genes. Genes above zero represent genes that are increased, and genes below zero genes that are decreased in SZ compared to controls. **d** Significant GO terms identified in patients with SZ **e** Box plots representing normalized SCG2, L1CAM, NPTXR, PTN and NUF2 immunodensities in SZ-neurons ($n = 7$) vs. controls ($n = 6$). Unpaired t-tests detected significant differences ($^*p < 0.05$) in SZ compared to controls for SCG2 ($t1,11 = 2.382$), L1CAM ($t1,11 = 2.328$) and NPTXR ($t1,11 = 2.479$). Representative SCG2, L1CAM, NPTXR, PTN, NUF2 and GAPDH immunoblots depicting samples from controls (C) and schizophrenia (SZ) samples. Molecular mass (in kDa) of the most proximal prestained protein marker (Bio-Rad) to the target proteins is indicated on the left.

since a decrease in protein expression is also observed but does not reach statistical significance. Finally, cortical immunodensities of PTN and NUF2 (Fig. 4a,b) were similar in all groups.

The effect of potentially confounding variables (age, PMI, brain pH) on proteins immunodensities was also evaluated by a multivariate analyses. This analyses detected possible effects of PMI on brain L1CAM amounts in all subjects (Fig. 4c). However, this effect disappeared when both populations were separated. Complementary ANCOVAs adjusting for PMI discarded potential effects of PMI on the reported results. In addition, we found a statistical correlation between the expression levels of different proteins in SZ. Pearson's correlation analyses revealed that lower level values of L1CAM were associated with lower amounts of NPTXR protein (but not others) in SZ samples (Pearson´s $r = 0.447$; $p = 0.0483$) but not in controls (Pearson´s $r = 0.351$; $p = 0.1410$) (Fig. 4c).

DISCUSSION

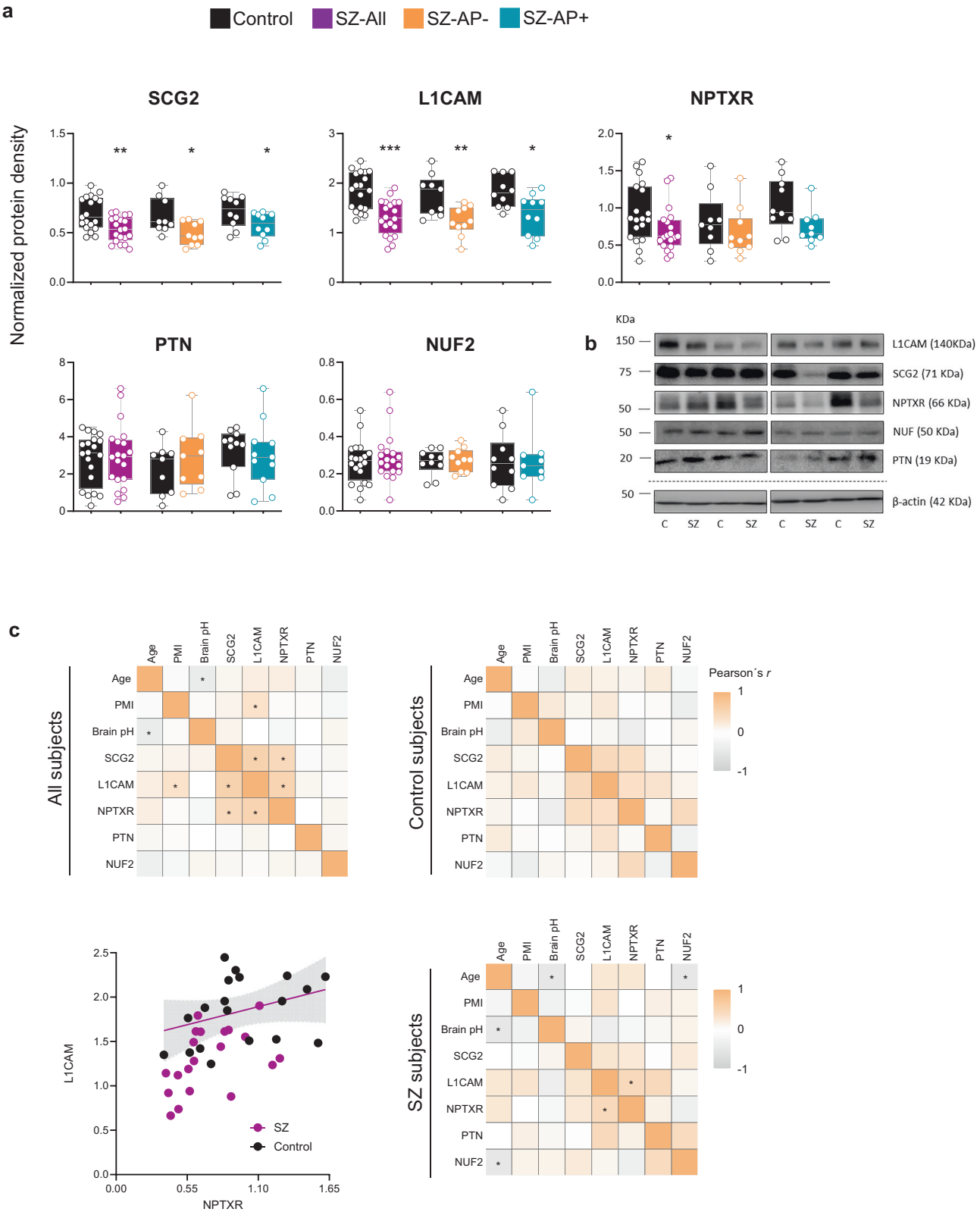
This study aimed to investigate neurodevelopmental alterations in schizophrenia using a combination of assays in cells from the olfactory neuroepithelium and in postmortem brain tissue from subjects with schizophrenia. Our results provided striking evidence of a dysregulation in genes and proteins involved in key processes linked to the ECM organization, neurodevelopment and synaptic transmission. This dysregulation was evident in both patient-derived cell models (neurospheres and neuron-derived cultures from the olfactory epithelium) and in postmortem SZ-brain (DLPFC). It supports the potential of these in vitro models to show pathophysiological features relevant to schizophrenia.

Neurospheres are valuable tools for modeling early neurodevelopmental processes and brain disorders [12]. The reduced proliferation capacity and smaller size of neurospheres observed in SZ-patients point to differences in neural stem cell (NSC) behavior in schizophrenia, that may be related to brain development and functionality [18]. The reduced self-renewal capacity of NSCs in SZ-patients indicates that NSCs may be unable to sustain the proliferative demands of neurodevelopment, potentially leading to cell number deficits in the brain. Such deficits could underlie some of the structural abnormalities observed in schizophrenia, including cortical thinning and reduced gray matter volume [19]. The smaller size of SZ-derived neurospheres had been previously described in hiPSC lines [20], and may reflect a reduced proliferation and possibly impaired differentiation. Neurosphere size typically correlates with NSC viability and the potential to differentiate into multiple neuronal and glial lineages [21]. A smaller neurosphere size suggests that even if NSCs from schizophrenia patients were able to differentiate, they would generate fewer cells, impacting overall neuronal network density and connectivity. This could contribute to the reduced synaptic connectivity seen in the disorder, which is thought to underlie cognitive and functional impairments in schizophrenia [22]. These findings align with the neurodevelopmental hypothesis of schizophrenia, which suggests that disruptions in brain development, particularly during early gestation, increase susceptibility to the disorder [23].

The RNA sequencing analysis identified significant upregulation of genes encoding ECM components and remodeling proteins, including COL1A2, COL3A1, COL5A1, COL6A2, COL18A1, MMP2, and ADAM33. These genes regulate cell adhesion, migration, and structural integrity, and their dysregulation may alter the biophysical properties of the stem cell niche, impairing NSC expansion and leading to the observed deficits in self-renewal [24]. Conversely, a downregulation of genes involved in cytoskeletal organization and cell adhesion was found, such as FGD6 and MEGF9 [25], which are essential for maintaining cell-cell interactions and neurosphere stability. The differential expression of genes related to proliferation control and transcriptional regulation, such as LARP4, LINC003, UBN2, AGL, and ZBTB33, further suggests that altered gene expression dynamics may contribute to defective neurogenesis [24]. Given that ECM integrity and stem cell niche signaling play a crucial role in NSC maintenance and fate decisions, our findings suggest that the dysregulation of ECM-related and proliferation-associated genes underlies the impaired self-renewal and neurosphere formation capacity observed in schizophrenia-derived cultures.

The ECM is a complex and dynamic network of cell-secreted macromolecules with different physical and biochemical properties that provides structural support and biochemical signals to cells, tissues and organs [26]. The mammalian ECM matrisome consists of more than 300 distinct proteins, which surround cells and provide structural support [26]. ECM represents an essential player in NSCs physiology, since it can directly or indirectly modulate the maintenance, proliferation, self-renewal and differentiation of NSCs [27] and neural progenitor cells (NPCs) [28]. It also governs the development of cellular morphology including axonal and dendritic elongation regulating neuronal connectivity and cortical folding [29]. The disturbed expression of ECM-related molecules observed in SZ-neurospheres could be, therefore, associated with the defect in the ability to form new neurospheres in vitro [28, 30].

The second approach of this study involved the evaluation of neurons derived from the olfactory neuroepithelium to corroborate and expand the findings obtained from the neurospheres. The primary objective was to determine whether alterations in ECM components observed in the neurosphere model, were also present in these neuron-enriched cultures. Enriched ontology clusters analysis identified, again, a dysregulation of ECM (NABA Matrisome Associated), and in pathways involved the regulation of cell proliferation and developmental growth, signaling by nuclear receptors and modulation of chemical synaptic transmission. In the brain, the ECM appears in a diffuse form that surrounds the synapses and regulates their functioning. It is also present as condensed structures, the perineuronal nets, which play important roles in the physiology and the regulation of the synaptic input of the cells surrounded by them, or the perinodal ECM, which regulates conduction speed of action potentials [31, 32]. Consequently, alterations in the composition the ECM may disrupt or



modify all of these physiological functions in which it is implicated.

Gene ontology enrichment analysis identified not only the ECM-specific NABA Matrisome pathway but also alterations in pathways related to neuronal growth, synapse organization, and cytoskeletal dynamics, all of which are influenced by ECM homeostasis. While not all differentially expressed genes are ECM-specific, their functional interactions with ECM components suggest a broader dysregulation of the extracellular microenvironment, ultimately affecting neuronal connectivity and integrity.

Fig. 4 Reduced SCG2, L1CAM and NPTXR protein expression in SZ-postmortem brain. Immunodensities of target proteins in schizophrenia brain samples **a** Box plots representing normalized SCG2, L1CAM, NPTXR, PTN and NUF2 immunodensities in the DLPFC of age-, sex- and PMI-matched pairs of schizophrenia (SZ) cases and controls, either altogether (SZ; $n = 20$) or stratified by the absence (AP−; $n = 10$) or presence (AP +; $n = 10$) of antipsychotic drugs in the blood sample of the SZ subject pair at the time of death. Unpaired t-tests detected significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) between the diagnostic groups for SCG2 (SZ, t1,37 = 3.422; AP−, t1,17 = 2.63; AP +, t1,18 = 2.163), L1CAM (SZ, t1,37 = 4.609; AP−, t1,17 = 3.24; AP +, t1,18 = 2.829), and NPTXR (SZ, t1,37 = 2.455) **b** Representative SCG2, L1CAM, NPTXR, PTN, NUF2 and β-actin immunoblots depicting samples from controls (C) and schizophrenia (SZ) samples. Molecular mass (in kDa) of the most proximal prestained protein marker (Bio-Rad) to the target proteins is indicated on the left **c** Heatmaps representing Pearson's r-coefficients of the pairwise associations between the potentially confounding variables of the study and the studied proteins immunodensities in postmortem samples of the DLPFC from subjects with schizophrenia (SZ) and controls, combined altogether or segregated by diagnosis. Color scale on the r-values is shown on the top-right corner. *$p < 0.05$. Scatterplot depicting association between the immunodensity values of L1CAM and NPTXR in the DLPFC of SZ subjects.

In SZ, we found 50 differentially expressed genes, some of them playing roles in pathways critical for neural development, synaptic function, and neuronal survival, and could have complex interactions in neurons [33–37]. We chose to explore the proteins SCG2, L1CAM and NPTXR due to their significant downregulation in SZ-neurons. Each of them represents a critical piece of a larger puzzle involving ECM interaction, neural development and synaptic transmission pathways [38–42]. Additionally, we selected the two most upregulated genes in SZ, PTN and NUF2 for further validation.

We decided to validate our findings in human postmortem brain tissue to ensure the relevance of the gene expression changes observed in the patient-derived cells. Moreover, we wanted to show that the identified changes in proteins were not specific to cell culture but relevant in the context of the brain pathology.

The findings regarding the downregulation of SCG2, L1CAM, and NPTXR proteins in the DLPFC from schizophrenia subjects, independently of antipsychotic treatment, further support the hypothesis of altered neurodevelopmental and synaptic processes in schizophrenia. The consistent downregulation of SCG2 across both treated and untreated schizophrenia subgroups suggests that this alteration is likely intrinsic to the disorder's pathology rather than a secondary effect of antipsychotic medication. SCG2 plays a vital role in neuropeptide processing and neurotransmitter release and is implicated in the modulation of synaptic plasticity [39]. In line with our results, a recent work by Lin and colleagues [43] proposes that SCG2 mediates schizophrenia-like behaviors after traumatic brain injury. Reduced expression of SCG2 could therefore contribute to the deficits in synaptic function and plasticity that have been widely documented in schizophrenia, potentially disrupting cognitive processes dependent on the DLPFC, such as working memory and executive function. Moreover, there is also evidence that SCG2 dysregulation can interfere with neurite extension and synaptogenesis [40].

Similarly, the marked reduction in cortical L1CAM expression in schizophrenia highlights a potential disruption in cell adhesion and neural network formation within the cortex. L1CAM is a crucial mediator of axon guidance, cell migration, and synapse formation [41]. Lower L1CAM immunoreactivity in the DLPFC, observed in both schizophrenia subgroups, may underlie some of the structural abnormalities in cortical circuitry associated with schizophrenia, including decreased cortical thickness and altered white matter integrity [44]. Given the role of L1CAM in fostering stable synaptic connections [45], its downregulation may contribute to the impaired connectivity and synaptic plasticity reported in schizophrenia, reinforcing the theory that disruptions in cell adhesion molecules and the extracellular matrix are integral to the disorder's neuropathology [44]. Furthermore, the observation of L1CAM downregulation in both treated and untreated schizophrenia subgroups suggests that this alteration is intrinsic to the disease [46, 47] rather than an effect of antipsychotic exposure. In fact, it has been described an association between polymorphisms of L1CAM gene and schizophrenia [46]. It is also interesting to mention that L1CAM interacts with different components of the ECM, including those of perineuronal nets [41].

The downregulation of NPTXR in the DLPFC supports this notion of synaptic and network dysfunction in schizophrenia. NPTXR is involved in synapse formation, maintenance, and remodeling, acting as a receptor for secreted neuronal pentraxins that mediate synaptic clustering and the internalization of AMPA receptors [42]. Interestingly, these functions of pentraxins are mediated by ECM remodeling [48]. Impaired AMPA receptor function and synaptic plasticity are strongly associated with cognitive deficits and altered neuronal communication [49], both of which are core features of schizophrenia. Dysfunctional NPTXR-mediated synaptic processes could thus contribute to these symptoms. Although subgroup analyses did not show statistically significant differences between treated and untreated groups, a trend toward reduced NPTXR levels in both groups suggests that this alteration may still be relevant to the disorder's underlying pathology. The lack of statistical significance might stem from limited sample sizes, as trends indicating a reduction in NPTXR were apparent but did not reach significance.

Globally, our findings indicate that alterations found in protein expression are not significantly modified by antipsychotic treatment, suggesting that these alterations may represent a core pathophysiological feature of schizophrenia rather than a secondary consequence of medication exposure.

Antipsychotics are highly effective in reducing positive symptoms, however, negative symptoms and cognitive impairments remain largely unresponsive to these treatments. In this context, ECM directly influences synaptic plasticity and long-term potentiation (LTP), both of which are essential for learning and memory. Persistent ECM dysfunction, even in the presence of antipsychotics, could explain why cognitive deficits and negative symptoms persist despite pharmacological intervention.

Additionally, our study reveals a novel and significant positive correlation between L1CAM and NPTXR protein levels in the DLPFC of individuals with SZ, a pattern absent in controls. This correlation in SZ suggests an altered regulatory relationship between L1CAM and NPTXR specific to the disorder. It may reflect an adaptive response possibly as an attempt to preserve synaptic connectivity or structural integrity in the brain. In controls, L1CAM and NPTXR levels appear to fluctuate independently, implying a regulated dissociation of function under non-pathological conditions. This finding identifies a previously uncharacterized relationship between L1CAM and NPTXR in schizophrenia and presents a promising molecular target for understanding the mechanistic underpinnings of brain dysfunction in this disorder. In this context, NPTXR is critical for synaptic plasticity and the stability of neuronal connections [42], while L1CAM supports neuronal adhesion, migration, and guidance [50]. Moreover, matrix metalloproteases (MMPs) regulate neuronal development and plasticity by catalyzing the shedding of NPTXR [38] and L1CAM [33, 51], among other proteins and components of the ECM. With reduced levels of L1CAM and NPTXR, the functional impact of MMP activity may shift, further destabilizing synaptic connections and potentially contributing to cognitive deficits and other symptoms characteristic of schizophrenia. The interaction of L1CAM and NPTXR with matricellular components and their modulation of MMP activity

may result in reorganizations of the ECM, which have been described in psychotic patients and patients with schizophrenia, particularly in their DLPFC [52, 53]. This interplay between NPTXR, L1CAM, and MMPs could provide a novel perspective on the molecular disruptions underlying the synaptic pathology of schizophrenia. Moreover, SCG2 contribute to the formation and release of neuropeptides and peptide hormones and may influence ECM dynamics by modifying extracellular signaling molecules.

The lack of significant differences between SZ and control groups in DLPFC for PTN and NUF2 protein levels, indicates that their overexpression in vitro may not directly translate to changes in the cortical protein levels in brain pathology. PTN is a growth factor implicated in neurogenesis [54] and NUF2 is essential for proper chromosome alignment during cell division [55]. These genes may be differentially regulated in isolated neuronal cultures compared to the more complex, in vivo cortical environment where regulatory mechanisms could counterbalance their expression levels.

In summary, the consistent downregulation of SCG2, L1CAM, and NPTXR in both, neurons derived from the olfactory neuroepithelium and postmortem DLPFC, underscores the robustness of these findings and their relevance to schizophrenia pathophysiology. Moreover, this downregulation provides further evidence of disrupted neurodevelopmental and synaptic processes in the disorder and the implication of the ECM in these alterations. In the ECM context, one hypothesis could be that SCG2-derived peptides affect L1CAM and NPTXR expression or function indirectly by modulating neurotransmitter signaling that feeds back into ECM-associated pathways. Additionally, L1CAM's interactions may facilitate synaptic remodeling by recruiting NPTXR to synaptic sites, which then regulates receptor trafficking.

Cell models are invaluable tools for studying the pathophysiological mechanisms underlying schizophrenia; however, they present notable limitations when studying ECM alterations associated with the disorder. These models often lack the complexity and dynamic interactions present in the three-dimensional architecture of the brain ECM, which plays a crucial role in synaptic plasticity, cell signaling, and neural connectivity. Additionally, in vitro cell models may not fully replicate the biochemical composition and structural remodeling of the ECM observed in schizophrenia. Thus, while these models provide insights into cellular and molecular processes, they may oversimplify the complexity of ECM-related changes in schizophrenia. Importantly, the strong concordance between patient-derived olfactory models and postmortem brain tissue supports their utility in identifying reliable biomarkers for schizophrenia. Such biomarkers could significantly advance early diagnosis, monitoring, and the development of targeted therapeutics. Additionally, research into therapeutic interventions targeting ECM or their counterparts, cell adhesion molecules and/or synaptic receptors may hold promise for addressing the underlying synaptic and connectivity deficits in schizophrenia.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

LU, L-FC and PU-L designed the experiments. MR, OM, and RS recruited the donors and collected the samples. PU-L, EC-V, RB-B, EO, IH, RD-A performed the experiments. LU, L-FC, JJM, JN, RD-A and PU-L, analyzed the data, and LU, LF-C and PU-L wrote the manuscript. All the co-authors gave their final approval of this manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All methods were performed in accordance with the research and ethical guidelines for human and postmortem studies. All subjects gave their written consent before sample extraction. Informed consent was obtained from all individual participants included in the study. The corresponding Human Research Ethics Committee from University Cruces Hospital, Comité de Ética en la Investigación (CEI) OSI Ezkeraldea-Enkarterri-Cruces approved the entire procedure with the code CEI E22 /27.

ADDITIONAL INFORMATION

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