INTRODUCTION

Schizophrenia has been conceptualized as a disease of dysfunctional synaptic plasticity and aberrant cortical–subcortical connectivity, with a multigenic etiopathogenesis and a complex biological architecture, which likely involves different biological pathways. Genome Wide Association Studies (GWAS) have confirmed a large pool of genetic variants associated with the disorder and that genetic risk identified by such variants converges onto a relatively small number of biologically meaningful trajectories or pathways. Interestingly, about 30% of genomic variation associated with schizophrenia by GWAS is meaningful trajectories or pathways. Interestingly, about 30% of genomic variation associated with schizophrenia by GWAS is significantly affected by variations in neuroactive amino acid levels and glutamate-related synaptic elements. Furthermore, a Machine Learning hypothesis-free unveiled other discriminative clusters of molecules, one in the DLPFC and another in the hippocampus. Overall, while confirming a key role of glutamatergic synapse in the molecular pathophysiology of schizophrenia, we reported molecular signatures encompassing elements of the glutamatergic synapse able to discriminate patients with schizophrenia and normal individuals.

Schizophrenia is a disorder of synaptic plasticity and aberrant connectivity in which a major dysfunction in glutamate synapse has been suggested. However, a multi-level approach tackling diverse clusters of interacting molecules of the glutamate signaling in schizophrenia is still lacking. We investigated in the post-mortem dorsolateral prefrontal cortex (DLPFC) and hippocampus of schizophrenia patients and non-psychiatric controls, the levels of neuroactive \(\alpha\)- and \(\beta\)-amino acids (\(L\)-glutamate, \(D\)-serine, glycine, \(L\)-aspartate, \(D\)-aspartate) by HPLC. Moreover, by quantitative RT-PCR and western blotting we analyzed, respectively, the mRNA and protein levels of pre- and post-synaptic key molecules involved in the glutamatergic synapse functioning, including glutamate receptors (NMDA, AMPA, metabotropic), their interacting scaffolding proteins (PSD-95, Homer1b/c), plasma membrane and vesicular glutamate transporters (EAAT1, EAAT2, VGlut1, VGlut2), enzymes involved either in glutamate-dependent GABA neurotransmitter synthesis (GAD65 and 67), or in post-synaptic NMDA receptor-mediated signaling (CAMKII) and the pre-synaptic marker Synapsin-1. Univariable analyses revealed that none of the investigated molecules was differently represented in the post-mortem DLPFC and hippocampus of schizophrenia patients, compared with controls. Nonetheless, multivariable hypothesis-driven analyses revealed that the presence of schizophrenia was significantly affected by variations in neuroactive amino acid levels and glutamate-related synaptic elements. Similarly, it stands unclear whether variation of one single element, rather than the coordinated variation of levels of many elements of the glutamatergic synapse is critical to the onset of the disorder.

To shed new light on such cryptic areas of our understanding for schizophrenia, here we performed a multimodal research of post-mortem dorsolateral PFC (DLPFC) and hippocampus of schizophrenia patients and non-psychiatric individuals on which we used high-performance liquid chromatography (HPLC) analysis to measure levels of glutamate and related amino acids with neurotransmitter/neuromodulatory activity, including \(\alpha\)-amino acids, whose role as endogenous NMDAR modulators is emerging
significant in schizophrenia pathophysiology. Moreover, in the same brain areas, by quantitative RT-PCR (qRT-PCR) and western blotting analyses we investigated, respectively, mRNA and protein levels of pre- and post-synaptic key molecules, including glutamate receptors (NMDAR, AMPAR, metabotropic), their interacting scaffolding proteins (PSD-95, Homer1b/c), plasma membrane and vesicular glutamate transporters (EAAT1, EAAT2, VGLUT1, VGLUT2), enzymes involved either in glutamate-dependent synaptic function, including glutamate receptors (NMDAR, AMPAR, metabotropic), and protein levels of pre- and post-synaptic key molecules, including glutamate receptors (NMDAR, AMPAR, metabotropic), and the pre-synaptic α subunit of NMDARs (GluN1, GluN2A, GluN2B), mGluR1 (GRM1), mGluR2/3 (GRM2/3), mGluR5 (GRM5), the post-synaptic density proteins, Homer1b/c (Homer1b/c), and the pre-synaptic marker Synapsin-1 (SYN1).

We used a multiset approach to analyze data: (1) a univariable statistical analysis to detect possible significant differences between patients and controls in the levels of molecular and neurochemical elements previously mentioned; (2) a hypothesis-driven multivariable statistical analysis, and (3) a hypothesis-free Machine Learning analysis. The latter allowed us to (a) detect pathways of molecules able to discriminate schizophrenia cases from controls on the basis of the joint distribution of their levels in the DLPFC and the hippocampus; (b) identify interactions among elements of the synapse that defined molecular signatures of schizophrenia cases and controls.

RESULTS

HPLC analysis of neuroactive d- and l-amino acids levels in the post-mortem DLPFC and hippocampus of schizophrenia and control subjects

Compelling evidence supports the hypothesis that deficient glutamatergic activity contributes in schizophrenia etiology and pathophysiology. Here, we measured by HPLC the levels of the amino acids L-Glu, L-Asp, D-Asp, D-Ser, L-Ser, Gly, known to stimulate and modulate the activity of NMDARs and, their precursors, L-Gln, L-Asn, and L-Ser in the DLPFC and hippocampus of schizophrenia patients and non-psychiatric controls. Before proceeding with statistical comparisons of amino acid levels between schizophrenia and controls, we assessed whether the two groups were imbalanced with respect to the following clinical variables (confounders): gender, age at decease, post-mortem interval (PMI) and samples’ pH. No statistically significant differences were found in gender (number of males (%)): CTRL = 16 (80%), SCZ = 12 (60%), $\chi^2 = 1.071$, df = 1, p = 0.301 from Chi-Square test and pH (median [IQR]: CTRL = 6.54 [6.49–6.63], SCZ = 6.50 [6.42–6.56], t = 1.070, df = 26, p = 0.285 from two-sample t-test on log values), while significant differences were found in age (median [IQR]: CTRL = 73.5 [66.00–80.3] years, SCZ = 72.5 [39.5–61.3] years, t = 4.819, df = 38, p < 0.001 from two-sample t-test) and PMI (median [IQR]: CTRL = 12.9 [11.8–16.3] years, SCZ = 15.3 [12.5–24.6] years, t = −2.426, df = 38, p = 0.020 from two-sample t-test on log values) (Table 1).

| Characteristics | Control | Schizophrenia | Statistic | p value |
|-----------------|---------|---------------|-----------|---------|
| Subjects (total number) | 20      | 20            | −         | −       |
| Gender (M/F) | 16/4    | 12/8          | χ² = 1.071 (df = 1) | 0.301* |
| Age (years, median [IQR]) | 73.50 [66.00–80.25] | 52.50 [39.50–61.25] | t = 4.819 (df = 38) | <0.001b |
| PMI (hours, median [IQR]) | 12.90 [11.80–16.32] | 15.25 [12.52–24.58] | t = −2.426 (df = 38) | 0.020c |
| pH (median, [IQR]) | 6.54 [6.49–6.63] | 6.50 [6.42–6.56] | t = 0.708 (df = 26) | 0.485c |
| RIN (median, [IQR]) | 6.05 [5.50–7.12] | 6.75 [6.05–7.08] | t = −0.036 (df = 38) | 0.971b |

Continuous variables are reported as median along with IQR. *p-values calculated using ANCOVA models, including both gender and age as confounders. In the DLPFC, this analysis revealed significantly higher levels in D-Asp and Gly in schizophrenia patients, compared to controls (adjusted means [95% CI] for D-Asp: CTRL = 159.2 [135.2–183.3], SCZ = 213.2 [189.2–237.3] nmol/g tissue, F(1,36) = 8.167, p = 0.007; Gly: CTRL = 1103.8 [813.4–1394.3], SCZ = 1652.1 [1361.6–1942.5] nmol/g tissue, F(1,36) = 5.780, p = 0.021; Fig. 1f, Table 2). Moreover, we found a borderline significantly higher levels in D-Ser in schizophrenia patients, compared to controls (adjusted means [95% CI]: CTRL = 160.8 [133.5–188.1], SCZ = 204.2 [176.9–231.5] nmol/g tissue, F(1,36) = 4.097, p = 0.050; Fig. 1g, Table 2). On the other hand, no significant differences were found in D-Asp, L-Asp, L-Ser, L-Glu, and L-Gln levels, as well as in D-Asp/total Asp, D-Ser/total Ser and L-Gln/ L-Glu ratios (Fig. 1c-e,h,i,k-m, Table 2). Also in the hippocampus, we found a significantly higher levels in L-Asn in schizophrenia patients, compared to controls (adjusted means [95% CI]: CTRL = 163.1 [137.0–189.2], SCZ = 210.4 [183.5–237.4] nmol/g tissue, F(1,36) = 3.519, p = 0.029; Fig. 1q), while no alterations in other amino acids were detected (Fig. 1n–p–r–x, Table 2).

However, after the correction of p values for multiple testing, no statistically significant differences were found in any of the molecules analyzed in both brain regions between schizophrenia and non-psychiatric subjects (Fig. 1, Table 2).

Analysis of the expression of glutamatergic synapse-related genes and proteins in the post-mortem DLPFC and hippocampus of schizophrenia and control subjects

GWASs have identified several genes encoding proteins implicated in glutamatergic functioning as risk genes for schizophrenia. Based on these findings, we evaluated the mRNA and protein expression of genes implicated in glutamatergic signaling at both pre-synaptic and post-synaptic level in the post-mortem DLPFC and hippocampus of the same schizophrenia and non-psychiatric subjects analyzed for amino acids content. Through qRT-PCR and western blotting we analyzed, respectively, the mRNA (name indicated below in brackets) and protein levels of the subunits of the NMDARs, GluN1 (GRIN1), GluN2A (GRIN2A), GluN2B (GRIN2B), and of the AMPARs, GluA1 (GRIA1), GluA2/3 (GRIA2/3) and Glu4 (GRIA4), the metabotropic glutamate receptors, mGluR1 (GRM1), mGluR2/3 (GRM2/3), mGlur5 (GRM5), the post-synaptic density proteins, Homer1b/c (Homer1b/c) and PSD-95 (DG1), the glutamate decarboxylase isoforms, GAD65 (GAD1) and GAD67 (GAD2), the excitatory amino acids transporters, EAAT1 (SLC1A3) and EAAT2 (SLC1A2), the vesicular glutamate transporter.
transporters, VGluT1 (SLC17A7) and VGluT2 (SLC17A6), the synaptic vesicle membrane protein, Synapsin-1 (SYN1), the calcium/calmodulin-dependent protein kinase II alpha, CaMKIIα (CAMK2A), and its phosphorylated form, Thr286-P-CaMKIIα.

As regards to transcripts, we found that GRIA2 and Homer1 levels resulted significantly lower in the hippocampus of schizophrenia patients, compared with non-psychiatric controls from ANCOVA models (adjusted means [95% CI]; GRIA2: CTRL = 1.1 [0.7–1.8], SCZ = 0.5 [0.3–0.7] arbitrary units, F(1,36) = 6.226, p = 0.017; Homer1: CTRL = 1.3 [0.9–1.8], SCZ = 0.7 [0.5–0.9] arbitrary units, F(1,36) = 6.787, p = 0.013; Fig. 2e, f, Supplementary Table 4), while no significant alterations were reported in the levels of the other hippocampal and cortical mRNAs detected (Fig. 2, Supplementary Table 4).

As regards to proteins, we observed a significantly higher levels in EAAT1, and lower levels in GluN2A and EAAT2 in the DLPFC of schizophrenia patients, compared with non-psychiatric controls (adjusted means [95% CI]; EAAT1: CTRL = 87.8 [59.2–116.3], SCZ = 140.4 [109.7–171.0]% of control, F(1,32) = 5.123, p = 0.031; GluN2A: CTRL = 117.3 [96.2–138.4], SCZ = 76.0 [54.2–97.8]% of control, F(1,35) = 5.965, p = 0.020; EAAT2: CTRL = 97.0 [76.4–117.5], SCZ = 62.6 [42.0–83.2]% of control, F(1,36) = 4.524, p = 0.04; Fig. 3o,c,p, Supplementary Fig. 1, Supplementary Table 5). EAAT2 levels were significantly lower also in the hippocampus.
| Amino acid | DLPFC | SCZ | Statistics | HIP | SCZ | Statistics |
|------------|-------|-----|------------|-----|-----|------------|
|            | CTRL  | No. | Mean (95% CI) | No. | Mean (95% CI) | No. | F(df1,df2), p-value raw | p-value adjusted |
| D-aspartate | 14.6 (11.3–17.9) | 20 | 11.1 (7.8–14.4) | 20 | 1,36 = 1.846; p = 0.183 | 1,000 | 8.2 (5.5–10.8) | 15 | 5.8 (3.2–8.5) | 15 | F(1,26) = 1.317; p = 0.262 |
| L-aspartate | 1647.3 (1321.1–1973.5) | 20 | 2012.4 (1686.2–2338.6) | 20 | 1,36 = 2.034; p = 0.162 | 1,000 | 2027.9 (1680.0–2375.7) | 15 | 2572.5 (2224.6–2920.3) | 15 | F(1,26) = 4.101; p = 0.053 |
| D-aspartate/total aspartate | 0.8 (0.6–1.1) | 20 | 0.6 (0.4–0.8) | 20 | 1,36 = 1.631; p = 0.210 | 1,000 | 0.4 (0.3–0.6) | 15 | 0.2 (0.1–0.4) | 15 | F(1,26) = 3.453; p = 0.075 |
| D-serine | 160.8 (133.5–188.1) | 20 | 204.2 (176.9–231.5) | 20 | 1,36 = 4.097; p = 0.050 | 0.555 | 199.2 (165.3–233.0) | 20 | 185.2 (151.3–219.1) | 20 | F(1,36) = 0.276; p = 0.603 |
| L-serine | 901.6 (690.5–1112.8) | 20 | 1170.8 (959.6–1381.9) | 20 | 1,36 = 2.637; p = 0.113 | 1,000 | 2038.2 (1674.4–2402.0) | 20 | 2480.0 (2116.2–2843.8) | 20 | F(1,36) = 2.393; p = 0.131 |
| D-serine/total serine | 16.4 (14.0–18.9) | 20 | 15.4 (13.0–17.9) | 20 | 1,36 = 0.281; p = 0.600 | 1,000 | 9.6 (7.9–11.2) | 20 | 7.0 (5.3–8.6) | 20 | F(1,36) = 3.964; p = 0.059 |
| L-glutamate | 6424.3 (4889.5–7959.2) | 20 | 7374.9 (5840.0–8909.7) | 20 | 1,36 = 0.622; p = 0.435 | 1,000 | 3499.5 (2599.9–4399.2) | 20 | 4081.3 (3151.9–5010.7) | 19 | F(1,35) = 0.658; p = 0.423 |
| L-glutamine | 3996.3 (2531.3–5461.3) | 20 | 5606.4 (4141.4–7071.4) | 20 | 1,36 = 1.961; p = 0.170 | 1,000 | 2625.8 (1700.0–3551.6) | 20 | 3395.5 (2439.1–4351.9) | 19 | F(1,35) = 1.087; p = 0.304 |
| L-glutamine/L-glutamate | 63.7 (43.1–84.3) | 20 | 81.8 (61.2–102.4) | 20 | 1,36 = 1.247; p = 0.271 | 1,000 | 72.3 (54.5–90.2) | 20 | 87.7 (69.3–106.2) | 19 | F(1,35) = 1.174; p = 0.286 |
| L-asparagine | 159.2 (135.2–183.3) | 20 | 213.2 (189.2–237.3) | 20 | 1,36 = 8.167; p = 0.007 | 0.078 | 163.1 (137.0–189.2) | 20 | 210.4 (183.5–237.4) | 19 | F(1,35) = 5.169; p = 0.029 |
| Glycine | 1103.8 (813.4–1394.3) | 20 | 1652.1 (1361.6–1942.5) | 20 | 1,36 = 5.780; p = 0.021 | 0.236 | 1034.7 (783.9–1285.5) | 20 | 1387.1 (1127.9–1646.2) | 19 | F(1,35) = 3.104; p = 0.095 |

All the amino acids were detected in a single run by HPLC. Results are reported as age and post-mortem interval adjusted means along with their 95% confidence interval (CI).

DLPFC dorsolateral prefrontal cortex, HIP hippocampus, CTRL control subjects, SCZ patients with schizophrenia, No. = number of subjects with non-missing values for each considered variable.

*To test the difference of adjusted means between the two groups, p-values (raw) were computed from ANCOVA models which include the presence of SCZ as the main grouping variable and age and post-mortem interval as confounders.

#Adjusted p-values correspond to the raw p-values corrected for multiple testing following the Bonferroni method. F(df1,df2) is the quantile of the F-distribution with df1 and df2 degrees of freedom corresponding to main grouping variable effect.
Fig. 2  Glutamatergic synapse-related gene expression in the post-mortem dorsolateral prefrontal cortex and hippocampus of control subjects and patients with schizophrenia. mRNA expression levels of a, a’ GRIN1, b, b’ GRIN2A, c, c’ GRIN2B, d, d’ GRIA1, e, e’ GRIA2, f, f’ GRIA3, g, g’ GRIA4, h, h’ GRM1, i, i’ GRM2, j, j’ GRM3, k, k’ GRM5, l, l’ Homer1, m, m’ DLG4, n, n’ GAD1, o, o’ GAD2, p, p’ SLC1A3, q, q’ SLC1A2, r, r’ SLC17A7, and s, s’ SLC17A6, t, t’ CAMK2A, and u, u’ SYN1 in the a–u dorsolateral prefrontal cortex and a’–u’ hippocampus homogenates of control (CTRL) and schizophrenia (SCZ) patients. mRNA expression was normalized to the mean of two housekeeping genes, β-actin and cyclophilin (PPIA), and expressed as arbitrary units. The number of examined samples is reported in Supplementary Table 4, for each considered mRNA.
of schizophrenia patients, while a significantly higher levels in mGluR1 were found in this brain region, compared to controls (adjusted means [95% CI]; EAAT2: CTRL = 97.1 [73.3–120.8], SCZ = 46.4 [21.8–70.9]% of control, F(1,35) = 7.078, p = 0.012; mGluR1: CTRL = 88.1 [49.0–123.1], SCZ = 158.4 [119.3–197.5]% of control, F(1,24) = 5.435, p = 0.028; (Fig. 3p,h, Supplementary Fig. 2, Supplementary Table 5). All other cortical and hippocampal proteins were comparable between diagnoses (Fig. 3, Supplementary Figs. 1, 2, Supplementary Table 5).

However, also in this case, after the correction of p values for multiple testing, no statistically significant differences were found in any of the analyzed transcripts or proteins between
schizophrenia and non-psychiatric subjects in both brain regions (Figs. 2, 3, Supplementary Tables 4, 5).

Linear combinations of multiple molecules of the glutamatergic synaptic components are predictive of schizophrenia in the post-mortem DLPFC

Here, we generated arbitrary (hypothesis-driven) multivariable logistic models, defined on the basis of the functional interaction among different neurochemical and molecular elements of the glutamatergic synapse and tested whether linear combination of these molecules could discriminate between control and schizophrenia group, in both the DLPFC and hippocampus (Table 3, Supplementary Table 6).

As already shown, subject’s age at deceased and PMI were strongly predictive of the presence of schizophrenia. When both included in a multivariable logistic regression (reference model), increasing age resulted associated with a lower disease probability (OR = 0.88, 95% CI: 0.80–0.94, p = 0.003) whereas increasing PMI was associated with a higher disease probability (OR = 1.22, 95% CI: 1.02–1.54, p = 0.048). Both covariates discriminated schizophrenia patients from controls with a very high predictive accuracy (AUC = 0.90, 95% CI: 0.80–0.98) and therefore these were necessarily accounted as strongest confounders for the analyses of multivariable logistic models.

Significant associations were found using molecules from the DLPFC only. Indeed, higher levels of both GluN1 and D-Ser levels resulted still associated to higher disease probability (both OR ≥ 1.00) and significantly outperformed the reference model, which included age and PMI only (χ² = 8.515, df = 2, p = 0.014 from deviance test). Moreover, the linear combination of L-Glu, mGluR2/3, mGluR5 and EAAT2 (χ² = 11.065, df = 4, p = 0.026 from deviance test), as well as GluA1 and PSD-95 (χ² = 7.945, df = 2, p = 0.019 from deviance test) significantly outperformed the reference model, evidencing their contribution for the improvement in statistical association.

Machine Learning analysis finds pathways of molecules of the glutamatergic synapse that are predictive of schizophrenia in the post-mortem DLPFC and hippocampus

Results from iterative Random Forests (iRFs) at the last iteration are reported both in the DLPFC (Fig. 4) and hippocampus (Fig. 5). A graphical plot of the Brier Scores achieved by iRFs in the OOB data at different parameter values (i.e. during the “tuning phase”) is reported in Supplementary Fig. 3 whereas results of a finer grid search for the optimal number of iterations and regularization factor, among iRFs with 100,000 trees, is reported in Supplementary Table 7. As for DLPFC, iRF achieved a relatively small prediction error (Brier Score = 0.186) and a very high discriminative power (AUC = 0.85, 95% CI: 0.71–0.95). The molecules that mostly contributed to discriminate schizophrenia from controls were EAAT2, CAMK2A, Synapsin-1, L-Asn, GRIA2, GRIA4, and SLC17A7 whereas the ones that barely contributed to the discrimination were: Glik, mGluR5, CaMkIIa, VGluT1, and EAAT2 (Fig. 5a). The pathway of the most stable interactions was represented in Fig. 5b. The most recurrent interactions were found with respect to EAAT2 levels. As shown by ALE plots (Fig. 5c), higher EAAT2 levels (% of control) were associated to a relevant linear decrease in schizophrenia probability whereas the ones associated to a non-linear relationship were detected with respect to the rest of the molecules. Moreover, PDP (Fig. 5d) suggested that subjects with lower levels of EAAT2 in conjunction with lower levels of SLC17A7 or Synapsin-1 were more likely to achieve higher discrimination of schizophrenia.

Moreover, Classification And Regression Tree (CART) showed that in the DLPFC, subjects with D-Ser > 185 nmol/g achieved 85% chance of having the schizophrenia whereas those with D-Ser ≤ 185 nmol/g were only 21% more likely to have the disease (Supplementary Fig. 4). In the hippocampus, subjects with CAMK2A expression < 0.79 and (at the same time) with GRIA2 expression < 0.64 achieved 95% chance of having the schizophrenia whereas those with low levels of both CARTs was AUC = 0.73 (95% CI: 0.60–0.85) and AUC = 0.92 (95% CI: 0.83–0.99) for DLPFC and hippocampus data, respectively.
Table 3. Results from multivariable logistic regression models which include a linear combination of multiple molecules of synaptic components (chosen a priori) as main covariates along with age and post-mortem interval (PMI) as confounders.

| Cluster | DLPFC | HIP |
|---------|-------|-----|
|         | N.SCZ/total | Variable | OR (95% CI) | p-value | Deviance test statistic; p-value* | N.SCZ/total | Variable | OR (95% CI) | p-value | Deviance test statistic; p-value* |
| GluN1 + D-serine | 20/40 | Age | 0.84 (0.71–0.93) | 0.006 | $\chi^2 = 8.515$ (df = 2); $p = 0.014$ | 20/40 | Age | 0.86 (0.74–0.93) | 0.005 | $\chi^2 = 2.764$ (df = 2); $p = 0.251$ |
| | | PMI | 1.35 (1.08–1.68) | 0.025 | | | | | | |
| | | GluN1 | 1.00 (0.99–1.02) | 0.543 | | | | | | |
| | | D-serine | 1.03 (1.01–1.07) | 0.028 | | | | | | |
| L-glutamate + mGluR2/3 + mGluR5 + EAAT2 | 19/39 | Age | 0.83 (0.65–0.93) | 0.019 | $\chi^2 = 11.065$ (df = 4); $p = 0.026$ | 18/38 | Age | 0.89 (0.79–0.97) | 0.021 | $\chi^2 = 6.857$ (df = 4); $p = 0.144$ |
| | | PMI | 1.25 (0.94–2.02) | 0.212 | | | | | | |
| | | L-glutamate | 1.00 (1.00–1.00) | 0.808 | | | | | | |
| | | mGluR2/3 | 1.01 (0.99–1.05) | 0.224 | | | | | | |
| | | mGluR5 | 0.97 (0.92–1.00) | 0.087 | | | | | | |
| | | EAAT2 | 0.95 (0.89–0.99) | 0.075 | | | | | | |
| GluA1 + PSD-95 | 17/35 | Age | 0.77 (0.57–0.90) | 0.013 | $\chi^2 = 7.945$ (df = 2); $p = 0.019$ | 17/34 | Age | 0.87 (0.75–0.94) | 0.008 | $\chi^2 = 4.040$ (df = 2); $p = 0.017$ |
| | | PMI | 1.29 (0.96–2.05) | 0.147 | | | | | | |
| | | GluA1 | 0.96 (0.91–1.00) | 0.112 | | | | | | |
| | | PSD-95 | 0.97 (0.92–1.01) | 0.196 | | | | | | |
| L-glutamate + GluN2A + GluN2B | 19/39 | Age | 0.78 (0.59–0.90) | 0.017 | $\chi^2 = 7.781$ (df = 3); $p = 0.051$ | 16/35 | Age | 0.86 (0.73–0.94) | 0.013 | $\chi^2 = 4.252$ (df = 3); $p = 0.236$ |
| | | PMI | 1.31 (1.02–1.87) | 0.061 | | | | | | |
| | | L-glutamate | 1.00 (1.00–1.00) | 0.210 | | | | | | |
| | | GluN2A | 0.95 (0.90–0.99) | 0.058 | | | | | | |
| | | GluN2B | 1.02 (0.99–1.07) | 0.244 | | | | | | |
| GluN2A + GluN2B | 19/39 | Age | 0.81 (0.66–0.91) | 0.008 | $\chi^2 = 5.847$ (df = 2); $p = 0.054$ | 17/36 | Age | 0.85 (0.72–0.93) | 0.010 | $\chi^2 = 3.473$ (df = 2); $p = 0.176$ |
| | | PMI | 1.26 (1.01–1.73) | 0.076 | | | | | | |
| | | GluN2A | 0.96 (0.92–0.99) | 0.050 | | | | | | |
| | | GluN2B | 1.02 (0.99–1.05) | 0.233 | | | | | | |
| PSD95 + GluN2A + GluN2B | 16/34 | Age | 0.75 (0.51–0.89) | 0.022 | $\chi^2 = 7.327$ (df = 3); $p = 0.062$ | 14/31 | Age | 0.84 (0.69–0.93) | 0.015 | $\chi^2 = 2.289$ (df = 3); $p = 0.151$ |
| | | PMI | 1.44 (1.05–2.73) | 0.083 | | | | | | |
| | | PSD-95 | 0.98 (0.93–1.02) | 0.296 | | | | | | |
| | | GluN2A | 0.95 (0.87–1.00) | 0.117 | | | | | | |
| | | GluN2B | 1.03 (0.97–1.09) | 0.299 | | | | | | |
| Synapsin-1 + VGluT1 + VGluT2 | 17/36 | Age | 0.83 (0.65–0.93) | 0.020 | $\chi^2 = 7.030$ (df = 3); $p = 0.071$ | 20/40 | Age | 0.89 (0.80–0.95) | 0.004 | $\chi^2 = 0.913$ (df = 3); $p = 0.822$ |
| | | PMI | 1.19 (0.88–1.79) | 0.295 | | | | | | |
| | | Synapsin-1 | 0.97 (0.91–1.01) | 0.350 | | | | | | |
| | | VGluT1 | 1.02 (0.99–1.07) | 0.266 | | | | | | |
| | | VGluT2 | 0.97 (0.91–1.01) | 0.156 | | | | | | |
| GluN1 + Glycine | 20/40 | Age | 0.88 (0.79–0.95) | 0.009 | $\chi^2 = 4.859$ (df = 2); $p = 0.088$ | 19/39 | Age | 0.87 (0.76–0.96) | 0.017 | $\chi^2 = 5.134$ (df = 2); $p = 0.077$ |
| | | PMI | 1.26 (1.04–1.64) | 0.038 | | | | | | |
| | | GluN1 | 1.00 (0.98–1.01) | 0.690 | | | | | | |
Table 3 continued

| Cluster | DLPFC | | | | HIP | | | |
|---------|-------|--------|-----------------|--------|-------|--------|-----------------|--------|
|         | N.SCZ/ total | Variable | OR (95% CI) | p-value | Deviance test statistic; p-value | N.SCZ/ total | Variable | OR (95% CI) | p-value | Deviance test statistic; p-value |
|         | Glycine | 1.00 (1.00–1.00) | 0.071 | \( \chi^2 = 6.697 \) (df = 3); \( p = 0.001 \) | Glycine | 1.00 (1.00–1.00) | 0.154 | \( \chi^2 = 5.766 \) (df = 3); \( p = 0.012 \) |
|         | Age | 0.83 (0.67–0.93) | 0.013 | | Age | 0.85 (0.67–0.95) | 0.039 | \( \chi^2 = 5.766 \) (df = 3); \( p = 0.012 \) |
|         | PMI | 1.46 (1.08–2.44) | 0.053 | | PMI | 1.34 (1.03–2.16) | 0.092 | |
|         | EAAT2 | 0.97 (0.92–1.01) | 0.190 | | EAAT2 | 0.96 (0.92–0.99) | 0.053 | |
|         | PSD-95 | 0.97 (0.91–1.01) | 0.164 | | PSD-95 | 1.00 (0.97–1.02) | 0.848 | |
|         | Homer 1b/c | 0.99 (0.94–1.04) | 0.677 | | Homer 1b/c | 1.03 (0.99–1.10) | 0.249 | |
|         | GluN2A | 0.98 (0.95–1.00) | 0.087 | | GluN2A | 1.00 (0.98–1.02) | 0.827 | |
|         | CAMKII | 1.02 (0.99–1.06) | 0.212 | | CAMKII | 1.00 (1.00–1.03) | 0.528 | |

Separate models were performed for each defined cluster in the post-mortem dorsolateral prefrontal cortex and hippocampus, respectively. Only the most associated clusters in the post-mortem dorsolateral prefrontal cortex were reported (for the rest of the table please see the Supplementary Table 6). Logistic regression was used to model the probability of having the schizophrenia disease (i.e. outcome), conditioned to the values of the independent variables (i.e. exposures) included into the model as a linear combination. The OR quantifies how many times the risk (i.e. odds) of the disease is higher per one unit increase of each independent variable. OR > 1 means greater odds of association with the exposure and outcome; OR = 1 means there is no association between exposure and outcome and OR < 1 means there is a lower odds of association between the exposure and outcome.

DLPFC dorsolateral prefrontal cortex; HIP hippocampus; CTRL controls; SCZ patients with schizophrenia; OR odds ratio; CI confidence interval; N.SCZ/total number of SCZ patients (numerator) and total subjects (denominator) with no missing data for all the variables included in the model (i.e. complete case analysis), df degrees of freedom.

This statistic is based on the model’s residual deviance, which assess the extent to which the likelihood of the “full” model (i.e. which includes age, PMI and pattern-related covariates) exceeds the likelihood of the “reference” model (i.e. which includes age and PMI confounders only). It follows a Chi-square (\( \chi^2 \)) distribution with degrees of freedom equal to the number of parameters in the model (i.e. age, PMI and covariate patterns) minus two (i.e. the number of confounders: age and PMI). When statistically significant (\( p < 0.05 \)), this statistic suggests that the full model, which included both confounders and the covariates of interest outperforms the “reference” model, which included confounders only.
DISCUSSION

Despite the recognized role of the glutamate system in the molecular pathophysiology of schizophrenia\textsuperscript{5,30}, studies in post-mortem brains analyzing multiple clusters of molecules related to the glutamate signaling are missing. In the present work, we exploited this strategy, measuring both mRNA and protein...
expression of fundamental molecules at the glutamatergic synapse, as well as neuroactive amino acids levels in the post-mortem DLPFC and hippocampus of schizophrenia patients and non-psychiatric controls.

Paradoxically, univariate analyses revealed that, after the adjustment for multiple comparisons, none of the molecules we investigated was significantly different between schizophrenia patients and controls in the post-mortem DLFC and hippocampus. Nonetheless, when shifting the approach from a univariate perspective to multivariate hypothesis-driven and hypothesis-free strategies, we discovered that the odds of belonging to the schizophrenia instead of the control group were significantly affected by variations in amino acids and glutamate-related synaptic elements. The hypothesis-driven strategy revealed three molecular patterns including: (1) the GluN1 subunit of the NMDAR and its ligand, D-Ser, the major NMDAR co-agonist in the forebrain; (2) L-Glu, the metabotropic receptors, mGluR2/3, mGluR5, along with the glutamate transporter EAAT2; (3) the scaffolding protein PSD-95 and the AMPAR subunit GluA1. On the other hand, the hypothesis-free strategy evidenced two robust and stable molecule pathways, one in the DLPFC and one in the hippocampus, whose levels were correlated within stable statistical interactions and predictive of each individual classification as a schizophrenia patient or control.

In detail, the cluster including the GluN1 subunit of NMDARs, prevalently distributed at the post-synaptic portion of the synapse, and its respective ligand, D-Ser, is of interest to both the pathophysiology and the treatment of schizophrenia. First of all, a reduction of GluN1 in the post-mortem PFC of patients with schizophrenia has been reported in different cohorts and has been suggested to modify NMDAR stoichiometry, therefore being responsible for the endogenous NMDAR deficit reported in schizophrenia. On the other hand, multiple lines of evidence indicate D-Ser as a major modulator of NMDAR function with potential therapeutic effects and in vivo significant implication as an auditory and cognitive enhancer. Moreover, different studies pointed to D-Ser as a potential biomarker given its reduced levels in the serum and CSF of schizophrenia patients, compared to controls. However, other investigations and meta-analysis study reported no alterations in D-Ser levels in the blood and CSF, as well as in the post-mortem brain of schizophrenia patients, as also found in the present work. Consistent with the relevance of this issue, other investigations call to clarify such controversial results.

By adopting the same approach as above, we also identified a cluster of post-synaptic proteins, namely the ionotropic AMPAR GluA1 subunit and PSD-95, which have been both implicated in schizophrenia and are synergically involved in the post-synaptic glutamate signaling along with synaptic neuroplasticity rearrangements of relevance to schizophrenia pathophysiology. Indeed, this cluster of molecules is highly representative of the molecular machinery responsible for the architecture and functional modulation of the post-synaptic density in schizophrenia patients. Specifically, the PSD-95 is an integral part of the post-synaptic density and have attracted interest in schizophrenia pathophysiology based on GWAS. Moreover, PSD-95 is involved in the targeting, clustering, and dynamic retention of AMPARs to post-synaptic densities. Therefore, our results confirmed previous reports that changes in glutamate receptors may not be the only molecular event responsible for glutamate signaling perturbation in schizophrenia since also alterations at the post-synaptic level downstream receptor activation may contribute to the emergence of schizophrenia pathophysiology.

Another key cluster of molecules discriminating schizophrenia patients and controls included L-Glu, mGluR2/3, mGluR5, along with EAAT2. Such a cluster captures a critical portion of molecular variation at both pre- and post-synaptic side of the glutamatergic synapse. Indeed, mGluR5 is mainly localized at post-synaptic level, where it is implicated in excitatory events mediating neural plasticity and cognitive processes. Importantly, mGluR5 has been linked to schizophrenia pathophysiology and regarded as a potential novel target for antipsychotic therapy with modulator agents. On the other hand, mGluR2 and mGluR3 are found in various combinations of pre-synaptic, post-synaptic and glial localizations. Moreover, GRM3 gene, encoding mGluR3, has been pinpointed as putative harbor for schizophrenia risk variants by structural and functional GWAS. This association was confirmed by a comprehensive meta-analysis including 11,000 subjects. EAAT2 is expressed predominantly in astroglial cells and is regarded as the main glutamate transporter, responsible for the vast majority of glutamate clearance at the glutamate synapse level. Interestingly, in agreement with our data that include mGluR2/3 and EAAT2 in a cluster discriminating schizophrenia and control subjects, previous studies identified reduced EAAT2 expression in the PFC of subjects with high-risk GRM3 haplotype associated with schizophrenia and highlighted multiple EAAT2 interactome-associated biological pathways alteration in the disorder. Finally, in line with the strong tendency to reduction of EAAT2 in both the DLPFC and hippocampus of schizophrenia patients, compared with controls, other studies have previously revealed significant decrease in EAAT2 expression in the post-mortem DLPFC and parahippocampal regions of schizophrenia subjects.

Interestingly, when we used the Machine Learning hypothesis-free analysis, we identified in both DLPFC and hippocampus stable molecule pathways that discriminate schizophrenia patients from non-psychiatric controls, which could not be conceived using the hypothesis-driven approach. Indeed, the latter only allowed for assessing the association between the weighted linear combination of some molecule levels and the presence of the disease, although excluding the possibility to formulate any a priori assumption about the specific molecular patterns underlying such combination. Although possible associations between VGLUT2, EAAT2, GAD67, D-Ser, and PSD-95 levels and the presence of the disease in the DLPFC were originally assessed in the hypothesis-driven approach, strongest interactions between D-Ser with D-Asp/total Asp levels, as well as between GAD67 with VGLUT2 levels, along with a marginal effect of GRIAT1 and GRM5 mRNA
expressions, were found using only the hypothesis-free approach. Importantly, the Machine Learning algorithm also provided helpful insights into the molecular signatures of schizophrenia at the hippocampus level. Indeed, in such a brain region, surprising strong and stable pairwise interactions of the EAAT2 levels with \textit{SLC17A7} and \textit{GRIA2} transcripts, \textit{Synapsin-1} and \textit{CAMK2A} were detected. Altogether, these results underline that different pattern of multiple interacting proteins both at glutamate pre-synaptic and post-synaptic level could account for discriminating patients from control subjects.
Our study has some weakness. First, our samples of post-mortem brains from patients’ group had significantly longer PMI compared with healthy individuals. Moreover, patients are on average younger than controls. However, this apparent discrepancy is in line with literature reporting a reduced life expectation in patients with schizophrenia,

In conclusion, our results indicate that changes in the overall landscape of glutamate synapse more than alteration in single molecules underpin the pathophysiology of schizophrenia. This observation suggests, in turn, that future pharmacological strategies aiming to reduce symptoms of schizophrenia may be directed towards large interactomes operating within such a synapse, more than targeting one single molecule.

HPLC analysis

Post-mortem brain samples were homogenized in 1:10 (w/v) 0.2 M trichloroacetic acid. The samples were sonicated (3 cycles, 10 s each) and centrifuged at 13,000 x g for 20 min. Precipitated protein pellets were stored at −80 °C for protein quantification.

Our study has some weakness. First, our samples of post-mortem brains from patients’ group had significantly longer PMI compared with healthy individuals. Moreover, patients are on average younger than controls. However, this apparent discrepancy is in line with literature reporting a reduced life expectation in patients with schizophrenia (DLPFC and hippocampus samples from post-mortem brains of non-psychiatric controls and schizophrenia patients (n = 20) were obtained from The Human Brain and Spinal Fluid Resource Center (Los Angeles Healthcare Center, Los Angeles, CA, USA). All tissue collection and processing were carried out under the regulations and licenses of the Human Tissue Authority and in accordance with the Human Tissue Act of 2004. Clinical diagnosis of schizophrenia was made according to DSMIII-R criteria. Frozen tissues were pulverized in liquid nitrogen and stored at −80 °C for subsequent processing.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from post-mortem tissues using RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). A total of 1 μg of total RNA of each sample was reverse transcribed with Quantitect Reverse Transcription (Qiagen, Hilden, Germany) using oligo-dT and random primers according to the manufacturer’s instructions. Quantitative RT-PCR with Real Time ready catalog Assays (Roche Diagnostics) and LightCycler® 480 Probe Master (Roche Diagnostics) was performed on a Light Cycler 480 Real Time PCR thermocycler with 96-well format (Roche Diagnostics). All measurements from each subject were performed in duplicate. The following protocol was used: 10 s for initial denaturation at 95 °C followed by 40 cycles consisting of 10 s at 94 °C for denaturation, 10 s at 60 °C for annealing, and 6 s for elongation at 72 °C temperature.

The primers used for GRIN1, GRIN2A, GRIN2B, GRIA1, GRIA2, GRIA3, GRM1, GRM2, GRM3, GRM5, Homer1, DLG4, GAD1, GAD2, SLC1A3, SLC1A2, SLC1A7, SLC1A6, CAMK2A, SYN1 mRNA amplification are listed in Supplementary Table 1. mRNA expression levels were normalized to the mean of two housekeeping genes: β-actin (ACTB) and cyclophilin (PPIA). mRNA expression was calculated using the geometric mean of the two reference genes selected and the relative quantification method (2−ΔΔCt).
Western blotting

Frozen, powdered samples from post-mortem DLPFC and hippocampus tissues were sonicated in 1% SDS and boiled for 10 min. Aliquots (2 μl) of the homogenate were used for protein determination using a Bio-Rad Protein Assay kit. Equal amounts of total proteins (30 μg) for each sample were loaded on pre-cast 4-20% gradient gel (BioRad Laboratories). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare) using Trans-Blot Turbo System. Membranes were immuno-blotted overnight using the following primary antibodies: GluN1, GluN2A, GluN2B, GluA1, GluA2/3, GluA4, mGluR1, mGluR2/3, mGluR5, Homer1b/c, PSD-95, GAD65, GAD67, EAAT1, EAAT2, VGLUT1, VGLUT2, Synapsin-1, CaMKIIa, Thr-286-P-CaMKIIa (antibodies specimens are listed Supplementary Table 2). Blots were then incubated with α-rabbit or α-mouse horseradish peroxidase conjugated secondary antibodies. Immunoreactivity was detected by enhanced chemiluminescence (ECL) (GE-Healthcare) and quantified by Quantity One software (Bio-Rad). Optical density values were normalized to GAPDH for variations in loading and transfer. Normalized values were then averaged and used for statistical comparisons.

All blots derive from the same experiment and were processed in parallel.

Statistical methods

Data are reported as medians, along with interquartile range (first-third quartiles—IQR), and as absolute and relative frequency (percentages) for categorical variables. Continuous and categorical variables, respectively. The normality assumption was assessed by the Shapiro-Wilk test. For continuous variables with right-skewed distribution, statistical analyses were performed using their log-transformed values. Comparisons of clinical characteristics (age at deceased, gender, PMI, pH) between schizophrenia patients and controls were performed using two-tailed two-sample t-test or Chi-Square statistic with Yates’s correction for continuous and categorical variables, respectively. Age and PMI—adjusted comparisons between schizophrenia patients and controls were performed by ANCOVA models and p-values were also corrected for multiple testing, following the Bonferroni method. For t-tests and ANCOVAs, t-values and F-values along with degrees of freedom were also provided, respectively. Furthermore, to assess whether a linear combination of multiple molecules of the synaptic components was predictive of the presence of schizophrenia, a multivariable logistic model, which included both the molecules as main effects and the strongest confounders (i.e., age, PMI) as covariates, was performed and compared to the one which included confounders only by the deviance test. Results were reported as odds ratio (OR), along with their 95% confidence interval (CI). Unknown patterns of multiple molecules of the synaptic components were detected by the Iterative Random Forest (IRF) algorithm18 using a complete dataset with imputed values. Imputation was performed by the Multivariate imputation by chained equations (MICE) algorithm19 with 10 chains of multiple imputations and 50 iterations per chain, using a random forest of 10 trees per each iteration (see the paragraph: “Handling missing values” in the Supplemental Statistical Methods section of the Supplemental Information for further details). IRF is an ensemble of machine learning (model-agnostic) method for classification and regression that operates by constructing a multitude of decision trees. IRF is a generalization of a Random Forest (RF) and is commonly used to train a feature-weighted ensemble of decision trees to detect stable and high-order interactions20. As well as in a RF, each decision tree in the IRF is built on a bootstrap sample from the original dataset. The portion of the bootstrap dataset not used for the building of each tree is called Out Of Bag (OOB) data and is employed to get both an unbiased estimate of the RF prediction error (i.e. the Brier Score) and an estimate of a “variable importance” (VIMP). The predicted individual probability of having the disease is computed as the average of all probabilities over all trees in the forest estimated in OOB data for that individual and the Brier Score is computed as the mean squared difference between such predicted probabilities and the actual outcomes. The Brier Score varies from 0 (i.e. RF is perfectly calibrated) to 1 (i.e. RF is perfectly miscalibrated). To address the between groups imbalances (i.e. adjusting the analysis by subjects’ age and PMIs) in the IRFs, new individual weights were estimated following the Inverse Probability Weighting method21 (as a first step) and then such Inverse Probability Weights (IPWs) were supplied to IRFs (as a second step). Because of this, observations with higher IPWs are selected more frequently into each bootstrap sample, which will be used to build each decision tree of the forest, with respect to those with lower IPWs (see the paragraph: “Handling imbalance data between patients and controls in the IRF algorithms” in the Supplemental Statistical

Methods section of the Supplemental Information for further details). In the IRF algorithm, a RF will be iteratively performed K times. At the first iteration, a subsample of candidate variables (i.e. features) will be randomly selected at each split of a decision tree. On the basis of the variable importance and a regularization factor, new weights will be assigned to each variable so that, at the next iteration, variables with higher weights will be selected with higher probability than the others. Therefore, at the last iteration, the IRF will include regularized trees and decision rules extracted from such feature-weighted RF are mapped21. This mapping allows to identify prevalent interactions in the RF through a computation-ally efficient algorithm (i.e. generalized Random Intersection Trees—RIT–algorithm22) that searches for high-order interactions in binary data. This mapping step eventually assesses the stability of recovered interactions with respect to the bootstrap perturbation of the data. The proportion of times (out of B bootstrap samples) an interaction appears as an output of the RIT defines a “stability score” (i.e. 0 = totally instable interaction, 1 = totally stable interaction). The following parameters must be set to enable the IRF training, some of them were fixed in advance whereas some others were determined after a “tuning phase”: (1) the number of random forest iterations: from 1 to 10 iterations were evaluated during the tuning phase; (2) the number of the trees included into the random forest (within each iteration): from 50 to 100,000 trees were evaluated during the tuning phase; (3) the choice of the variable regularization factor, where possible fixed values were: 1.0 (no regularization), 0.9 (weak regularization), 0.8 (moderate regularization), <0.8 (strong regularization) and were evaluated during the tuning phase; (4) the number of randomly chosen features that possibly split at each node of the tree: this parameter was fixed to seven features; (5) the number of outer-layer bootstrap samples: this parameter was fixed to 30; (6) the node splitting criterion: Gini impurity measure; (7) the minimal node size: it was fixed that the final leaves of each tree in the forest must include at least five subjects. The “tuning phase” consists in a grid search of the optimal parameters combination that minimize the Brier Score achieved by IRF in the OOB data (see the paragraph: “Sensitivity of IRFs algorithms to tuning parameters” in the Supplemental Statistical Methods section of the Supplemental Information for further details). Accumulated local effects (ALE) and partial dependence plots (PDP) were performed to better quantify changes in disease probabilities at different variable values and detect the direction of the ”most stable” interactions found by the RIT, respectively. To define a single classification rule, a tree-growing algorithm that recursively splits data into subgroups (i.e. Classification And Regression Tree) was eventually performed and the choice of tree size was determined on the basis of 10-fold cross validation of the prediction error. The discriminatory ability of both models and machine-learning algorithms was assessed by the Area Under the ROC Curve (AUC), along with its 95% CI computed with 1000 stratified bootstrap replicates. Further details about statistical analysis can be found in Supplemental Information. A p-value < 0.05 was considered for statistical significance. Statistical analyses and plots were performed using R foundation for statistical computing, Vienna, Austria (version 4.04).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS
A.D.R., T.N., M.G., A.D.M. and D.P. performed experiments; A.F. undertook the statistical analyses; M.C. and A.B. participated in revising and editing the final manuscript; F.E., A.R. and A.d.B. wrote the manuscript, participated in revising and editing the final manuscript; A.U. conceived the work, wrote the manuscript, participated in revising and editing the final manuscript. A.D.R. and A.F. share co-first authorship; T.N., M.G., A.D.M., and D.P. contributed equally to this work.

COMPETING INTERESTS
The authors declare no competing interests.

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