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ORIGINAL ARTICLE

# Proteomic and genomic evidence implicates the postsynaptic density in schizophrenia

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The postsynaptic density (PSD) contains a complex set of proteins of known relevance to neuropsychiatric disorders, and schizophrenia specifically. We enriched for this anatomical structure, in the anterior cingulate cortex, of 20 schizophrenia samples and 20 controls from the Stanley Medical Research Institute, and used unbiased shotgun proteomics incorporating label-free quantitation to identify differentially expressed proteins. Quantitative investigation of the PSD revealed more than 700 protein identifications and 143 differentially expressed proteins. Prominent among these were altered expression of proteins involved in clathrin-mediated endocytosis (CME) (Dynamin-1, adaptor protein 2) and *N*-methyl-D-aspartate (NMDA)-interacting proteins such as CYFIP2, SYNPO, SHANK3, ESYT and MAPK3 (all $P < 0.0015$). Pathway analysis of the differentially expressed proteins implicated the cellular processes of endocytosis, long-term potentiation and calcium signaling. Both single-gene and gene-set enrichment analyses in genome-wide association data from the largest schizophrenia sample to date of 13 689 cases and 18 226 controls show significant association of *HIST1H1E* and *MAPK3*, and enrichment of our PSD proteome. Taken together, our data provide robust evidence implicating PSD-associated proteins and genes in schizophrenia, and suggest that within the PSD, NMDA-interacting and endocytosis-related proteins contribute to disease pathophysiology.

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## INTRODUCTION

The postsynaptic density (PSD) is a highly organized structure attached to the postsynaptic neuronal terminal comprised of a complex network of cytoskeletal scaffolding and signaling proteins. These proteins facilitate the movement of receptor and signaling complexes. The PSD is critical to normal neurotransmission, but is also critical to adaptive behaviors such as learning and memory.[1] It has been strongly implicated in neuropsychiatric disorders such as schizophrenia through its roles in synaptic plasticity[2–4] and cognitive function,[5] and known constituents of the PSD have been implicated in schizophrenia at both genetic[6,7] and protein expression[5,8,9] levels. However, the protein expression of the PSD has not yet been compared between schizophrenia and control subjects.

Mass spectrometry (MS)-based proteomic methods have the ability to reliably identify and quantify several thousands of disease-associated protein changes derived from complex anatomic structures. However, the reliable quantitation of low abundance proteins remains a challenge and there has been a shift toward the use of prefractionation enrichment methods combined with sensitive label-free MS-based proteomic techniques.[8,10] This approach when used to target samples enriched for specific anatomical structures, such as the membrane microdomain[8] and myelin[11] has yielded novel insights, and studies targeting the PSD in rodents have provided equally important insights into synaptic and dendritic function.[1,12] Critically, the approach has been shown to be valid for the PSD in post-mortem human brain tissue,[12] and until now has not been applied to post-mortem schizophrenia samples.

Synaptic plasticity within the PSD occurs through the modulation of signaling mechanisms such as those involving synaptic neurotransmitters (*N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate receptors), many of which are known candidates for schizophrenia.[13–16] NMDA receptor hypofunction is particularly strongly implicated in schizophrenia on the basis of NMDA receptor antagonists, mutants with altered NMDA NR1 subunit expression[14] and an anti-NMDA receptor encephalitis,[17] all recapitulating aspects of schizophrenia.[18,19] Explanations for the proposed NMDA receptor hypofunction are varied[18–21] and include altered membrane trafficking and recycling[22–24] such as that mediated by altered CME.[25–27] CME is dependent on a set of proteins referred to as the CME 'interactome',[24] which includes proteins such as clathrin and adaptor protein 2 (AP2), amphiphysin and Dynamin, which have been shown to be dysregulated in schizophrenia.[22,23,28]

In the current investigations, we enriched for the PSD in schizophrenia and control human brain samples, and undertook a label-free liquid chromatography-MS (LC-MS/MS) investigation to characterize disease-associated protein expression. First, we investigated the differentially expressed proteins. Second, we hypothesized that the PSD in schizophrenia compared with controls would be associated firstly with altered expression of proteins involved in the core CME interactome,[24] and secondly with altered expression

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of NMDA-interacting proteins. We also hypothesized that the expression of these candidates would not be influenced by antipsychotic medication used to treat psychosis. We tested this using an animal model of chronic exposure to antipsychotic drug treatment. Finally, we sought to validate our proteomic findings by checking for gene-based associations with schizophrenia, and also gene-set enrichment of the PSD, as characterized by our study and an independent study, in the largest schizophrenia genome-wide association study to date.[29]

## MATERIALS AND METHODS

### Samples

*Human samples*. Human post-mortem brain tissue of the supragenual (BA24) anterior cingulate cortex (ACC) was obtained from the Stanley Medical Research Institute's (SMRI) Array Collection (http://www.stanleyresearch.org). The series consists of 105 subjects, including 35 schizophrenia, 35 bipolar disorder and 35 control cases. Information on prescribed psychotropic medication is provided by the SMRI.

A subset of 20 samples was selected to match as closely as possible for age and tissue pH.[30,31] Supplementary Table 1 provides detailed demographic information on these subjects. To obtain enough tissue for the enrichment protocol, two samples were subpooled based on the Euclidean distance. Investigators were blind to group identity until completion of the data analysis. Ethical approval (application No. REC080) was granted by the Royal College of Surgeons in Ireland Research Ethics Committee.

*Haloperidol-treated rats*. To assess the effects of psychotropic medication on the expression of candidate proteins, cortex tissue was harvested from rats chronically treated with haloperidol[32] (for further details see Supplementary Methods).

### Comparing the PSD in ACC in schizophrenia and controls

The enrichment for the PSD was undertaken using methods established previously,[33,34] and recently validated for post-mortem brain material.[12] The method involves differential sucrose centrifugation and further fractionations by Triton X-100 extraction first at pH 6, and then at pH 8, leading to the separation of a synaptosomal membrane fraction, including the synaptic vesicle and presynaptic fraction, as well as the PSD fraction (see Supplementary Methods and Results and Supplementary Figures 1 and 2 for details of PSD enrichment and validation).

### Synaptophysin in whole ACC

To account for the possibility that differences in the PSD protein expression between our disease and control samples may reflect a primary change in synaptic density, we quantified the expression of the synaptic marker synaptophysin (Dako Diagnostics, Dublin, Ireland; 1:2000) in whole tissue lysates of the ACC from the same series that was enriched for the PSD using western blotting according to standard methods (see Supplementary Methods).

### Statistical analysis

We chose, *a priori*, the covariates age, post-mortem interval, refrigerator interval and brain pH to be of interest as possible confounders of label-free quantification intensities. Analysis of covariance was performed on the normalized data for each protein, with age, post-mortem interval, refrigerator interval and brain pH included as covariates. Estimated differences between schizophrenia and controls were then obtained using linear contrasts and exponentiated (power of 2) to obtain fold changes. Significance testing was performed at the 5% level using analysis of covariance. A false discovery rate[35] of 5% was used to flag those protein identifications statistically significant after adjustment for multiple comparisons.

To assess the effect of antipsychotic medication (life-time dose), a *post hoc* analysis of the effect in the patient group alone was performed using uni- and multivariate linear regression.

The management of data and statistical analyses were carried out with SAS version 9.1 statistical software (SAS Institute Inc., Cary, NC, USA) and R version 9.1 statistical software (R Foundation for Statistical Computing, Vienna, Austria).

### Classification of findings

DAVID NIH was used for pathway analysis according to Kyoto Encyclopedia of Genes and Genomes (KEGG; http://david.abcc.ncifcrf.gov/) using two lists, (1) the total PSD protein list identified by us ($n = 727$) and (2) the PSD proteins differentially expressed between schizophrenia and control ($n = 143$).

### Validation of differentially expressed proteins

We selected proteins for validation based on their degree of differential expression, the rank of the fold change differences in expression and biological relevance. As our hypothesis focused on CME- and NMDA-associated proteins we also targeted these proteins where antibodies were available and working. Western blotting and dot blots were performed as per standard protocols. For further details on the procedure and specificity of the antibodies used for this study see Supplementary Methods.

### Gene-based association with schizophrenia

Genome-wide SNP association results were available from the Psychiatric Genetic Consortium on the largest schizophrenia study to date.[29] A gene-based test was performed using VEGAS software (http://gump.qimr.edu.au/VEGAS) by considering the *P*-values of all SNPs ($n = 9\,898\,078$) within 17 769 unique autosomal genes.[36] Based on PSD differential protein expression, 133 genes were available for testing. Genes were considered significant, if they surpassed a Bonferroni level of correction ($P = 0.05/133$ genes, $P \leqslant 0.00037$).

### Gene-set enrichment analysis

Two proteomic profiles of the PSD and three relevant pathways were tested for enrichment association with schizophrenia. First, our experimentally derived PSD proteome, and second, an independent PSD proteomic profile obtained by Bayes *et al*.,[5] and also investigated in Kirov *et al*.,[6] was tested. Three relevant pathways were also included: long-term potentiation and endocytosis, and clathrin-mediated endocytosis. The enrichment of the gene sets in schizophrenia were tested using a competitive test of enrichment, GSEA v2.0.[37,38] Gene sets meeting the recommended discovery criteria of an uncorrected *P*-value $< 0.05$ and a false discovery rate (FDR)-corrected *q*-value $< 0.25$ were considered significant.

### Pathway analysis on gene expression data from SMRI

Available gene expression data from previous schizophrenia studies in the SMRI collection was tested for enrichment in KEGG pathways (http://www.genome.jp/kegg/pathway.html) using WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/).[39,40] Further details are available in Supplementary Methods.

## RESULTS

### Identification of PSD proteins dysregulated in schizophrenia

*LC-MS/MS and analysis*. Using 20 samples (10 schizophrenia and 10 controls; see Supplementary Table 1 for details), a total of 734 proteins were identified with 1% FDR by MS, after data input to the MaxQuant bioinformatics software (http://www.maxquant.org/). Exclusion of proteins with less than five sample results available and further statistical analysis using analysis of covariance (correcting for age, brain pH, post-mortem interval and refrigerator interval) left 727 proteins. *Post hoc* analysis investigating the effect of freezer time showed that it was not significantly different between groups and we did not include this variable as a potential confounder. One hundred and forty-three proteins were differentially expressed in schizophrenia compared with controls ($P \leqslant 0.05$). Twenty-five proteins were significant after correcting for multiple testing with FDR $< 0.05$ (see Table 1 and Supplementary Table 3). We compared our list of identified proteins with the previous literature defining the PSD proteome and identified up to 98% of the proteome described by Kirov *et al*.[6] Similarly, we identified 29% of the CME interactome as characterized by Schmid

**Table 1.** Differentially expressed protein identifications of the supragenual (BA24) human anterior cingulate PSD after ANCOVA correcting for age, pH, PMI, RI

| *Protein no.* | *Fold-change SCZ vs CTRL* | P-*value* | *Gene name* | *Description* |
|---|---|---|---|---|
| 735 | 4.00362 | 0.0001[a] | *H3F3B* | *Histone H3* |
| 37 | 3.07717 | 0.0001[a] | *MAPRE2* | *Microtubule-associated protein RP/EB family member 2* |
| 430 | 3.70687 | 0.0001[a] | *ARF5* | *ADP-ribosylation factor 5* |
| 631 | 3.80387 | 0.0001[a] | *DNM1* | *Dynamin-1* |
| 251 | 3.64426 | 0.0001[a] | *ESYT1* | *Extended synaptotagmin-1* |
| 66 | −2.91545 | 0.0001[a] | *VAPB* | *Vesicle-associated membrane protein-associated protein B/C* |
| 198 | −3.47558 | 0.0001[a] | *MAPK3* | *ERT2; extracellular signal-regulated kinase 1; mitogen-activated protein kinase 3* |
| 642 | 2.86980 | 0.0001[a] | *HNRNPA3* | *Heterogeneous nuclear ribonucleoprotein A3* |
| 386 | −3.26401 | 0.0002[a] | *CALM2* | *Calmodulin 2 (phosphorylase kinase, delta)* |
| 528 | 3.03883 | 0.0002[a] | *SRP14* | *Signal recognition particle 14 kDa protein* |
| 125 | −3.05401 | 0.0002[a] | *IDH2* | *Isocitrate dehydrogenase 2 (NADP+), mitochondrial variant* |
| 456 | −2.74307 | 0.0003[a] | *MTHFD1* | *Methylenetetrahydrofolate dehydrogenase* |
| 225 | −3.02061 | 0.0003[a] | *MYH11* | *Myosin heavy chain 11 smooth muscle isoform* |
| 432 | −2.80119 | 0.0004[a] | *HNRNPK* | *Heterogeneous nuclear ribonucleoprotein K* |
| 315 | −2.89687 | 0.0004[a] | *SYNPO* | *Synaptopodin* |
| 344 | 2.66194 | 0.0004[a] | *ACSL3* | *Long-chain fatty acid-CoA ligase 3* |
| 216 | −2.89885 | 0.0005[a] | *SHANK3* | *SH3 and multiple ankyrin repeat domains protein 3* |
| 712 | −2.62447 | 0.0005[a] | *PLP1* | *Proteolipid protein 1* |
| 326 | 3.20294 | 0.0006[a] | *CCT6A* | *Chaperonin subunit 6A* |
| 670 | 2.66518 | 0.0007[a] | *HNRNPU* | *Heterogeneous nuclear ribonucleoprotein U* |
| 698 | −2.93645 | 0.0012[a] | *CYFIP2* | *Cytoplasmic FMR1-interacting protein 2* |
| 280 | −3.09081 | 0.0013[a] | *USP5* | *Ubiquitin-specific-processing protease 5* |
| 688 | 2.70386 | 0.0014[a] | *IARS* | *Isoleucyl tRNA synthetase, cytoplasmic* |
| 191 | −2.97297 | 0.0017[a] | *MT-CO2* | *Cytochrome c oxidase polypeptide II* |
| 542 | −2.77184 | 0.0017[a] | *CAMK2G* | *Calcium/CaM kinase II gamma* |
| 551 | 2.81998 | 0.002 | *SLC1A2* | *Sodium-dependent glutamate/aspartate transporter 2* |
| 677 | −2.55366 | 0.0021 | *IMMT* | *Mitochondrial inner membrane protein; Mitofilin* |
| 276 | −2.93937 | 0.0025 | *RASGRF2* | *Ras guanine nucleotide exchange factor 2* |
| 457 | −2.75849 | 0.0026 | *GJA1* | *Connexin-43; Gap junction alpha-1 protein* |
| 582 | 2.72572 | 0.0026 | *AP1B1* | *Adapter-related protein complex 1 subunit beta-1* |
| 252 | −2.62022 | 0.003 | *SLC25A3* | *Solute carrier family 25 member 3* |
| 408 | 2.74150 | 0.0031 | *C1orf167* | *Uncharacterized protein C1orf167* |
| 477 | 2.64864 | 0.0031 | *PRPS1* | *Phosphoribosyl pyrophosphate synthase isoform I* |
| 325 | 2.23449 | 0.0033 | *HNRNPC* | *Heterogeneous nuclear ribonucleoproteins C1/C2* |
| 531 | −2.55586 | 0.0035 | *SRPRB* | *Signal recognition particle receptor subunit beta* |
| 437 | −2.37216 | 0.0037 | *VDAC1* | *Voltage-dependent anion-selective channel protein 1* |
| 708 | 2.55681 | 0.0037 | *LRPPRC* | *Leucine-rich PPR motif-containing protein, mitochondrial* |
| 165 | −2.62971 | 0.0038 | *EEF1A2* | *Eukaryotic translation elongation factor 1 alpha 2 variant* |
| 164 | 2.68801 | 0.004 | *FHL1* | *Four and a half LIM domains protein 1* |
| 365 | −2.47067 | 0.0041 | *ATP8A1* | *ATPase class I type 8A member 1* |
| 323 | 2.61191 | 0.0043 | *S100A6* | *Calcyclin; S100 calcium-binding protein A6* |
| 175 | −2.65006 | 0.0044 | *CHCHD3* | *Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial* |
| 422 | −2.31852 | 0.0044 | *BAIAP2* | *Brain-specific angiogenesis inhibitor 1-associated protein 2* |
| 178 | −2.24268 | 0.0047 | *GAP43* | *Axonal membrane protein GAP43* |
| 368 | −2.70099 | 0.0047 | *SEPT7* | *CDC10 protein homolog;Septin-7* |
| 50 | −2.58377 | 0.0048 | *STK39* | *Serine/threonine-protein kinase 39* |
| 297 | −2.79787 | 0.0048 | *HSPB1* | *Heat-shock protein beta-1* |
| 629 | −2.72986 | 0.0048 | *SAMM50* | *Sorting and assembly machinery component 50 homolog* |
| 291 | −2.74689 | 0.0051 | *ATP6V1G2* | *V-type proton ATPase subunit G* |
| 480 | −2.63691 | 0.0052 | *SLC25A11* | *Solute carrier family 25 member 11* |
| 42 | −2.40531 | 0.0057 | *MTCH2* | *Mitochondrial carrier homolog 2* |
| 20 | −2.46228 | 0.006 | *INA* | *Alpha-internexin; neurofilament 5* |
| 556 | 2.42229 | 0.0063 | *SLC12A5* | *Neuronal K–Cl cotransporter; solute carrier family 12 member 5* |
| 710 | 2.52615 | 0.0067 | *AP2B1* | *Adapter-related protein complex 2 beta subunit; clathrin assembly protein* |
| 416 | 2.26490 | 0.0068 | *ARFGAP1* | *ADP-ribosylation factor GTPase-activating protein 1* |
| 465 | −2.67500 | 0.0068 | *HPCA* | *Neuron-specific calcium-binding protein hippocalcin* |
| 93 | 2.38619 | 0.0075 | *RPLP1* | *Acidic ribosomal phosphoprotein P1* |
| 101 | −2.52642 | 0.008 | *NCKIPSD* | *NCK-interacting protein with SH3 domain* |
| 554 | −2.48187 | 0.0082 | *SYN1* | *Synapsin-1* |
| 121 | 2.44413 | 0.0084 | *CCT5* | *T-complex protein 1 subunit epsilon* |
| 431 | 2.42210 | 0.0086 | *ARF6* | *ADP-ribosylation factor 6* |
| 464 | −2.53933 | 0.0086 | *H3F3B* | *Histone H3/f* |
| 361 | −2.25324 | 0.0088 | *CDS2* | *CDP-DAG synthase 2* |
| 572 | 2.34332 | 0.0088 | *AMPH* | *Amphiphysin I* |
| 57 | −2.38846 | 0.0092 | *SPTBN1* | *Spectrin, non-erythroid beta chain 1* |
| 402 | −2.23052 | 0.0098 | *UNC13A* | *Munc13-1;Protein unc-13 homolog A* |
| 588 | −2.30616 | 0.01 | *PFKL* | *Phosphofructokinase 1; phosphohexokinase* |
| 256 | −2.37587 | 0.0105 | *METTL7A* | *Methyltransferase-like protein 7A; protein AAM-B* |
| 641 | −2.36285 | 0.011 | *CCDC64* | *Putative uncharacterized protein CCDC64* |
| 94 | −2.51713 | 0.0117 | *RPLP2* | *60S acidic ribosomal protein P2* |
| 267 | 2.53732 | 0.0118 | *RAB3A* | *Ras-related protein Rab-3A* |
| 550 | −2.46890 | 0.0123 | *ABI2* | *Abelson interactor 2* |
| 25 | −2.35683 | 0.0129 | *ANXA7* | *Annexin A7* |
| 73 | 2.25259 | 0.013 | *SEC22B* | *ER–Golgi SNARE of 24 kDa; vesicle-trafficking protein SEC22b* |
| 268 | −2.38405 | 0.0134 | *CDK5* | *Cell division protein kinase 5; cyclin-dependent kinase 5* |
| 483 | 2.40666 | 0.0136 | *NDUFS5* | *NADH dehydrogenase (ubiquinone) iron-sulfur protein 5* |
| 524 | −2.42537 | 0.0138 | *EPB49* | *Dematin;Erythrocyte membrane protein band 4.9* |
| 468 | −2.35737 | 0.0143 | *CAMK2B* | *Calcium/calmodulin-dependent protein kinase type II subunit beta* |

**Table 1.** (Continued)

| Protein no. | Fold-change SCZ vs CTRL | P-value | Gene name | Description |
|---|---|---|---|---|
| 316 | 2.29510 | 0.0148 | *CAP2* | *Adenylyl cyclase-associated protein 2* |
| 171 | 2.48614 | 0.0159 | *RAP1A* | *Ras-related protein Rap-1A* |
| 82 | −2.28829 | 0.0162 | *TUBA4A* | *Tubulin alpha-4A chain* |
| 250 | 2.01540 | 0.0166 | *RAB11FIP5* | *Gamma-SNAP-associated factor 1;Rab11 family-interacting protein 5* |
| 594 | −2.31959 | 0.0169 | *MYL6* | *Myosin light polypeptide 6* |
| 729 | −2.07220 | 0.0169 | *CCNYL2* | *Cyclin-Y-like protein 2* |
| 429 | 2.32575 | 0.0174 | *DDX3X* | *ATP-dependent RNA helicase DDX3X* |
| 34 | −2.37663 | 0.0175 | *HSPA5* | *Heat-shock 70 kDa protein 5* |
| 596 | −1.96813 | 0.0175 | *HSPA6* | *Heat-shock 70 kDa protein 6* |
| 467 | 2.33849 | 0.0182 | *RPL30* | *60S ribosomal protein L30* |
| 407 | −2.30978 | 0.0185 | *HEPACAM* | *Hepatocyte cell adhesion molecule* |
| 734 | −2.34938 | 0.0189 | *ABR* | *Active breakpoint cluster region-related protein* |
| 557 | −2.37333 | 0.0193 | *CTNND2* | *Catenin delta-2; Neurojungin* |
| 567 | 2.21010 | 0.0202 | *NIPSNAP1* | *Protein NipSnap homolog 1* |
| 553 | 2.33308 | 0.021 | *RAB3B* | *Ras-related protein Rab-3B* |
| 292 | 2.29065 | 0.022 | *RPL19* | *60S ribosomal protein L19; ribosomal protein L19* |
| 54 | 2.40712 | 0.0223 | *ACTR2* | *Actin-related protein 2* |
| 249 | 1.95457 | 0.0225 | *PHYHIP* | *Phytanoyl-CoA hydroxylase-interacting protein* |
| 387 | −2.38921 | 0.023 | *GNAS* | *Adenylate cyclase-stimulating G alpha protein* |
| 447 | −2.23522 | 0.023 | *HIST1H1E* | *Histone H1e* |
| 388 | 2.26114 | 0.0231 | *OGDH* | *Oxoglutarate (alpha-ketoglutarate) dehydrogenase* |
| 62 | 2.23015 | 0.0236 | *LIN7A* | *Mammalian lin-seven protein 1homolog A* |
| 253 | −2.34967 | 0.0236 | *AP2M1* | *Adapter-related protein complex 2 mu subunit 1; clathrin coat assembly protein AP50* |
| 699 | −2.09630 | 0.024 | *ATP6V0A1* | *ATPase, H+ transporting, lysosomal V0 subunit a1* |
| 46 | −2.29613 | 0.0242 | *HSPA8* | *Heat-shock 70 kDa protein 8; heat-shock cognate 71 kDa protein* |
| 658 | −2.05586 | 0.0248 | *DYNC1H1* | *Cytoplasmic dynein 1 heavy chain 1, cytosolic* |
| 104 | 2.38722 | 0.0257 | *PIP4K2A* | *1-Phosphatidylinositol-5-phosphate 4-kinase 2-alpha* |
| 345 | −2.25753 | 0.0263 | *SSBP1* | *Single-stranded DNA-binding protein 1, mitochondrial* |
| 16 | −2.02864 | 0.0283 | *PRDX1* | *Peroxiredoxin-1* |
| 481 | −2.23086 | 0.0283 | *CMPK1* | *Cytidine monophosphate kinase* |
| 373 | 2.14547 | 0.0287 | *INPP4A* | *Inositol polyphosphate 4-phosphatase type I* |
| 359 | 2.28931 | 0.0295 | *FARSA* | *Phenylalanine-tRNA ligase alpha chain* |
| 47 | 2.24831 | 0.03 | *NDUFA9* | *NADH-ubiquinone oxidoreductase 39 kDa subunit* |
| 667 | 2.07162 | 0.03 | *DSTN* | *Actin-depolymerizing factor;Destrin* |
| 363 | −2.19689 | 0.0302 | *RPH3A* | *Exophilin-1; Rabphilin-3A* |
| 346 | −2.26581 | 0.0303 | *CNTN1* | *Contactin-1; glycoprotein gp135; neural cell surface protein F3* |
| 502 | −2.05784 | 0.0309 | *NEFL* | *68 kDa neurofilament protein; neurofilament light polypeptide* |
| 711 | −2.21453 | 0.0317 | *FLOT2* | *Epidermal surface antigen; flotillin-2* |
| 192 | −2.13834 | 0.0321 | *MAPRE3* | *EB1 protein family member 3; end-binding protein 3* |
| 414 | −2.12419 | 0.0327 | *CAMK2D* | *Calcium/calmodulin-dependent protein kinase type II subunit delta* |
| 274 | −1.96613 | 0.034 | *VDAC2* | *Voltage-dependent anion-selective channel protein 2* |
| 100 | 2.01986 | 0.0351 | *NAPA* | *N-ethylmaleimide-sensitive factor attachment protein alpha* |
| 393 | −1.94159 | 0.0353 | *PFN2* | *Profilin; profilin-2* |
| 348 | 2.12900 | 0.0359 | *ACAT1* | *Acetoacetyl-CoA thiolase* |
| 53 | 2.25121 | 0.0364 | *LONP1* | *Lon protease homolog, mitochondrial* |
| 317 | 2.07305 | 0.0367 | *PRDX2* | *Peroxiredoxin-2* |
| 724 | −2.05974 | 0.0367 | *LOC646048* | |
| 130 | 2.22281 | 0.037 | *COMT* | *Catechol O-methyltransferase* |
| 10 | 1.99637 | 0.0374 | *FAM171A1* | *Astroprincin* |
| 525 | 2.09658 | 0.0376 | *TUBB8* | *Tubulin beta-8 chain* |
| 352 | −1.91869 | 0.0379 | *GRIA2* | *Glutamate receptor ionotropic, AMPA 2* |
| 12 | 2.23315 | 0.0383 | *MYO5A* | *Dilute myosin heavy chain, non-muscle* |
| 43 | −1.95370 | 0.0387 | *MAP2* | *Microtubule-associated protein 2* |
| 131 | −2.17296 | 0.0389 | *ACAA1* | *Acetyl-CoA acyltransferase* |
| 220 | −2.13601 | 0.0394 | *HSD17B4* | *3-Hydroxyacyl-CoA dehydrogenase* |
| 622 | 2.05212 | 0.0394 | *ESYT2* | *Extended synaptotagmin-2* |
| 488 | 2.12592 | 0.0404 | *DCTN2* | *Dynactin subunit 2* |
| 296 | 1.85028 | 0.0411 | *EIF4A2* | *Eukaryotic initiation factor 4A-II* |
| 655 | −2.22416 | 0.0411 | *SEPT9* | *Septin epsilon; MAFK protein* |
| 587 | −1.88108 | 0.0415 | *ANXA5* | *Annexin A5* |
| 444 | 2.22174 | 0.0416 | *DARS* | *Aspartate-tRNA ligase; aspartyl-tRNA synthetase, cytoplasmic* |
| 200 | −2.03549 | 0.0421 | *HK1* | *Brain form hexokinase* |
| 140 | −2.20479 | 0.0453 | *ADRBK1* | *Beta-adrenergic receptor kinase 1; G-protein-coupled receptor kinase 2* |
| 568 | −2.15717 | 0.0466 | *CAMKV* | *CaM kinase-like vesicle-associated protein* |
| 661 | 1.90503 | 0.0481 | *KIAA0528* | *Uncharacterized protein KIAA0528* |

Abbreviations: ANCOVA, analysis of covariance; CaM, calmodulin-dependent protein kinase; CTRL, control; PMI, postmortem interval; PSD, postsynaptic density; RI, refrigerator interval; SCZ, schizophrenia. [a]Marks proteins testing significant after a false discovery rate of 5% was used to flag those protein identifications statistically significant after adjustment for multiple comparisons.

and McMahon[24] and http://www.endocytosis.org/Interactomes, and 32% as characterized by Schubert *et al*.[25]

## Validation of proteomic findings

Differential expression of four proteins was confirmed using an alternative method: AP2B1, DNM1, MAPK3 and SYNPO. Extensive validation to confirm the MS findings was undertaken. See Figure 1a for the details of this validation work.

### *Validation using human samples*

AP2B1: *AP2B1*: Using western blotting on samples from the ACC, increased expression in the disease group was confirmed (*t*-test,

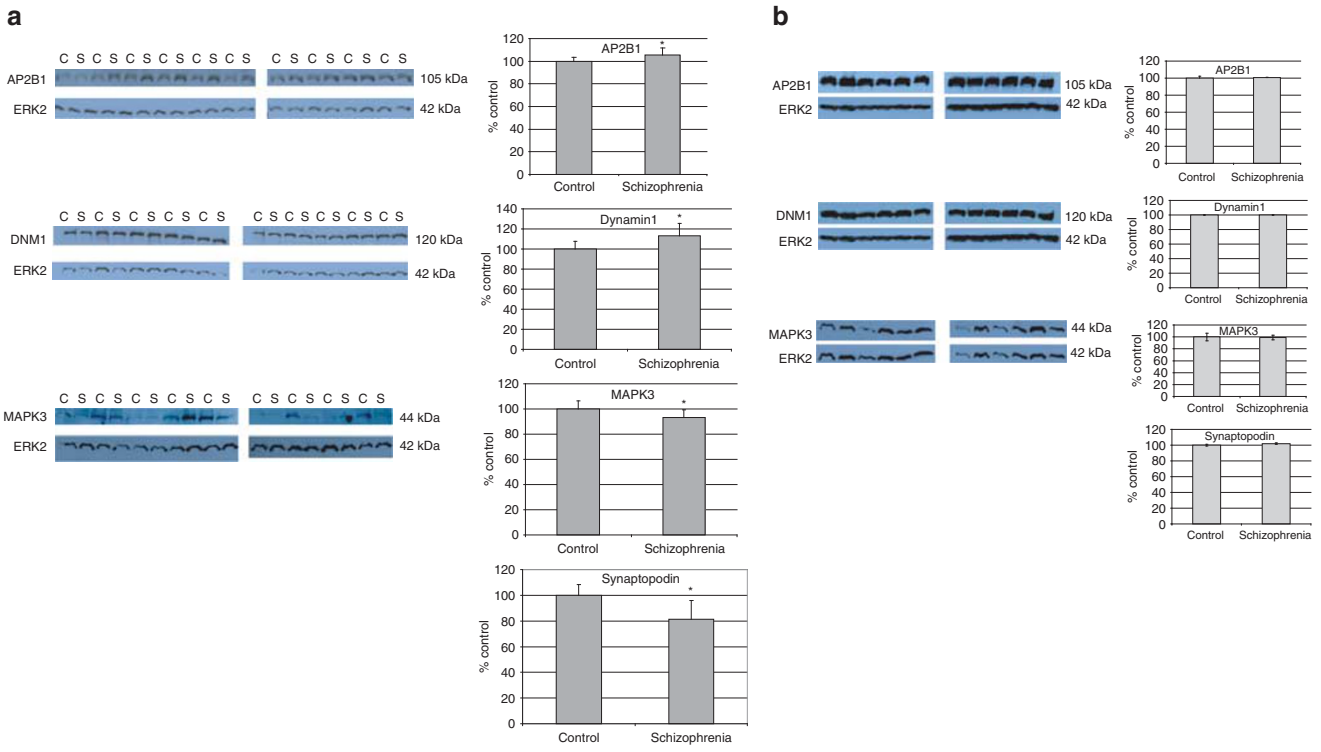


**Figure 1.** Validation of differentially expressed proteins. *$P < 0.05$. (**a**) Protein expression changes were determined in the 20 subpooled cases of the SMRI Array Collection using western blotting and dot blots. The means of three independent experiments are presented. Error bars indicate s.d. Western blots were prepared using lysates of subpools of ACC samples from patients with schizophrenia (S), and control subjects (C). Immunoblots were incubated with antibodies that specifically recognize Dynamin (DNM1) at 120 kDa, AP2B1 at 105 kDa, MAPK3 at 44 kDa and ERK2, used as a loading control, at 42 kDa. The images show a typical blot and the corresponding graphs represent the signal intensity of the designated antibody measured by densitometry and corrected by the signal intensity of ERK2. The mean of three independent experiments is presented. Error bars indicate s.d. ERK2 showed no significant differences between disease and control ($P = 0.7$). Synaptopodin dot blot were developed with diaminobenzidine, measured by densitometry. The mean of three independent experiments is presented. Error bars indicate s.d. In keeping with our LC-MS/MS experiments, DNM1 and AP2B1 expression were increased and MAPK3 and SYNPO were found to be reduced. (**b**) Protein expression changes determined by western blot for (**a**) AP2B1, (**b**) Dynamin and (**c**) MAPK3, and (**d**) by dot blots for SYNPO in rats chronically treated with haloperidol. The images show a typical blot and the graphs represent the signal intensity of the respective antibody measured by densitometry and corrected by the signal intensity of ERK2. ERK2 showed no significant differences between haloperidol and control ($P = 0.9$). The mean of three independent experiments ± s.d. is presented. No significant alterations in protein expression between treated and vehicle-treated animals were observed for all proteins.

$P = 0.03$). Results from LC-MS/MS showed an increased expression in schizophrenia (2.59-fold; $P = 0.007$).

DNM1: *DNM1*: Using western blotting on samples from the ACC, increased expression in the disease group was confirmed (*t*-test, $P = 0.01$). Results from LC-MS/MS showed an increased expression in schizophrenia (3.8-fold; $P = 0.001$).

MAPK3: *MAPK3*: Using western blotting on samples from the ACC, reduced expression in the disease group was confirmed (*t*-test, $P = 0.03$). Results from LC-MS/MS showed a decreased expression in schizophrenia (−3.5-fold; $P = 0.0001$).

SYNPO: *SYNPO*: Dot blots from samples showed significant reductions in protein levels for the disease group (*t*-test, $P = 0.02$). Results from LC-MS/MS showed a reduced expression in schizophrenia (−2.9-fold; $P = 0.0004$).

### Pathway analysis of dysregulated proteins

The top three pathways of the 143 differentially expressed PSD proteins identified by KEGG involved endocytosis, long-term potentiation and the calcium signaling pathway (see Table 2); the top pathways for the 25 FDR significant findings were identified as long-term potentiation and the neurotrophin signaling pathway. Using String to visualize functional protein association networks (Supplementary Figure 3) showed a cluster of inter-related differentially expressed proteins, consisting of proteins that have been linked to NMDA receptor-related proteins, endocytosis-related from KEGG and CME (from published work by Schmid and McMahon *et al.*[24] and Wieffer *et al.*[41]), long-term potentiation, the calcium signaling pathway and from gene-based findings.

### Pathway analysis of gene expression studies in schizophrenia

Pathway analysis was performed on previous gene expression studies on SMRI schizophrenia samples to investigate prior evidence for our findings here. The most informative analysis was combining all studies together for a total of 236 genes. Supplementary Table 5 shows the top-ten regulated pathways. The results for all studies are also available in Supplementary Information. In keeping with our proteomic and genomic findings implicating MAPK3, there is significant evidence for the involvement in metabolic pathways, including the MAPK signaling pathway

### Synaptic density in whole tissue

There was no evidence of a primary change in synaptic density between disease and control as measured by synaptophysin expression in schizophrenia and control ACC samples ($P > 0.2$; see Supplementary Figure 4).

### Exploring the effects of antipsychotic drugs on the PSD proteome in rats and human

We explored the effect of antipsychotic medication on the PSD using a rat model of antipsychotic drug treatment. First, we verified successful enrichment of the PSD using prefractionation in rat tissue using western blotting and fraction specific markers (see Supplementary Figure 5). A total of 1471 proteins were identified with 1% FDR by MS, after data input to the MaxQuant bioinformatics software. Comparison with our human PSD findings revealed 100% overlap. Twenty-six proteins in the treatment group were found to be statistically differentially expressed compared with controls, but none included any of our identified candidate proteins (see Supplementary Table 5). Western blot analysis demonstrated that AP2B1, DNM1, SYNPO and MAPK3 were not significantly altered in rats treated with haloperidol (Figure 1b). This suggests that our findings are not related to prescribed medication.

In the schizophrenia group alone, a *post hoc* analysis of the effect of antipsychotic drugs (life-time dose) was performed using multiple linear regression. Only five proteins were identified (*P*-value $< 0.05$) as associated with antipsychotic medication dose (PTPRZ1, GAP43, MGST1, ATP6V1A and GRIN1). Of these, only two (GRIN1 and SRP54) remained after adjusting for other confounder variables (age, RI, PMI and pH) and neither were found to be differentially expressed in schizophrenia compared with controls.

**Table 2.** Top three significant pathways identified among the 143 differentially expressed proteins (*t*-test; $P < 0.05$) between schizophrenia and controls

| *KEGG pathway* | *O* | *C/E/P-value* |
|---|---|---|
| Endocytosis | ARF6, ARFGAP1, RAB11FIP5, AP2B1, AP2M1, ADRBK1, DNM1, HSPA6, HSPA8 | C = 201<br>E = 0.65<br>P = 2.24e − 08 |
| Long-term potentiation | RAP1A, CAMK2B, CAMK2D, CAMK2G, CALM2, GRIA2, MAPK3 | C = 70<br>E = 0.23<br>P = 3.30e − 09 |
| Calcium signaling pathway | GNAS, CAMK2B, CAMK2D, CAMK2G, CALM2, VDAC1, VDAC2 | C = 177<br>E = 0.57<br>P = 1.98e − 06 |

Pathways were identified according to Kyoto Encyclopedia of Genes and Genomes (KEGG) and proteins assigned to each pathway are listed. C is the number of reference proteins in the category/pathway, O is the number of proteins in the data set and also in the category. E is the expected number in the category and P the adjusted *P*-value is corrected for testing multiple categories in a group of functional gene-set categories by the method recommended by the authors (Benjamini and Hochberg[24]).

### Genomic associations with schizophrenia

We tested the genetic association of our proteomic findings in two regards. First, we tested the association of 133 PSD genes that were differentially expressed in our study in a large schizophrenia genome-wide association study. Two genes surpass our gene-based Bonferroni significance threshold *HIST1H1E* ($P = 0.000019$) and *MAPK3* ($P = 0.000186$) (see Supplementary Figure 6 and Supplementary Tables 7 and 8 for results). Second, we hypothesized that the PSD proteome, as we determined experimentally and by an independent sample, is important in schizophrenia. Table 3 shows significant enrichment of PSD gene sets in schizophrenia, as determined in this proteomic study ($n = 983$ genes, $P = 0.012$, FDR = 0.18), and an independent proteomic study[6] ($n = 661$ genes, $P = 0.0093$, FDR = 0.18). There is an overlap of 409 genes between the two lists (enrichment of this gene set was also tested, $P = 0.03$, FDR = 0.15). The set of genes related to long-term potentiation and endocytosis show borderline significance of enrichment ($P = 0.07$, FDR $< 0.25$), but the CME pathway did not show enrichment with schizophrenia ($P > 0.05$, FDR $> 0.25$).

To check the robustness of our gene-set enrichment analysis to the contribution of the most significant gene, the top genes were removed from the gene sets and an enrichment analysis was performed. Both proteomic profiles of the PSD remained significantly associated with schizophrenia (PSD profile here (without *HIST1H1E),* $P = 0.018$) and PSD independent (without *NDUFA13*, $P = 0.016$).

To determine that the enrichment of the PSD was specific to schizophrenia, we tested its enrichment in comparably large genome-wide association scans of bipolar disorder,[42] Crohn's disease[43] and type 2 diabetes,[44] using the same method as detailed above. The PSD gene sets were not associated with bipolar disorder, Crohn's disease or type 2 diabetes (see Table 3). However, there is a hint of an enrichment of the long-term potentiation pathway with bipolar disorder ($P = 0.081$, FDR = 0.20). However, the borderline association of the LTP gene-set pathway with schizophrenia and bipolar disorder, this association was

**Table 3.** Gene-set enrichment analysis of PSD proteomic profile in psychiatric disorders and non-psychiatric disorders

| *Gene-set* | *Gene-set size* | *Schizophrenia* | | | *Bipolar disorder* | | | *Type 2 diabetes* | | | *Crohn's disease* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *NES* | P-*value* | *FDR* | *NES* | P-*value* | *FDR* | *NES* | P-*value* | *FDR* | *NES* | P-*value* | *FDR* |
| *PSD proteomic profiles* | | | | | | | | | | | | | |
| PSD profile | 983 | 1.09 | **0.012** | **0.18** | 1.05 | 0.12 | 0.65 | 0.90 | 1 | 1 | 0.96 | 0.73 | 1 |
| PSD independent | 661 | 1.11 | **0.0093** | **0.18** | 0.99 | 0.59 | 0.71 | 0.87 | 1 | 1 | 0.95 | 0.85 | 0.93 |
| *Relevant pathways* | | | | | | | | | | | | | |
| Long-term potentiation | 66 | 1.20 | 0.072 | **0.18** | 1.18 | 0.081 | **0.20** | 1.00 | 0.49 | 1 | 0.97 | 0.58 | 1 |
| Endocytosis | 180 | 1.12 | 0.077 | **0.24** | 0.88 | 0.94 | 0.91 | 1.10 | 0.11 | 0.58 | 0.96 | 0.79 | 1 |
| Clathrin-mediated endocytosis | 51 | 0.89 | 0.77 | 0.88 | 1.01 | 0.49 | 0.78 | 0.68 | 0.99 | 1.00 | 0.86 | 0.80 | 0.91 |

Abbreviations: FDR, false discovery rate; NES, normalized enrichment score; PSD, postsynaptic density. Gene-set enrichment analysis was performed to test the association of the gene sets of proteomic profiling of the postsynaptic density with schizophrenia, bipolar disorder, type 2 diabetes and Crohn's disease. Gene-set size is the number of autosomal genes that map to the relevant proteins. NES is the enrichment score for the gene set after it has been normalized across analyzed gene sets. The enrichment score for the gene set is the degree to which this gene set is over-represented at the top or bottom of the ranked list of genes in the expression data set. The *P*-value is the nominal *P*-value and represents the statistical significance of the enrichment score. The FDR *Q*-values are adjusted for gene-set size and multiple hypotheses testing, while the *P*-value is not. Gene sets of an uncorrected *P*-value $< 0.05$, or FDR-corrected *Q*-value of $< 0.25$ are considered significant and are highlighted in bold. The results are $n = 5000$ permutations and the mean of three runs.

attenuated when the top gene *CACNA1C*[45] was removed from the gene set.

## DISCUSSION

Our study has combined state-of-the-art proteomic and genomic methods to study the PSD in schizophrenia and provides robust complementary data implicating this multiprotein complex. First, we specifically enriched for the PSD fraction of the ACC in schizophrenia and control samples and used MS-based proteomic methods to characterise differential expression of PSD proteins in schizophrenia. The most notable proteomic changes involved proteins with roles in endocytosis and long-term potentiation, including proteins interacting with the NMDA receptor. Second, in a genomic analysis we tested and affirmed the hypothesis that certain PSD schizophrenia risk genes, and the PSD-associated genome itself, are associated with schizophrenia. Taken together, these findings provide powerful evidence implicating the PSD in schizophrenia.

The PSD is an electron-dense multiprotein complex under the postsynaptic membrane which is readily identified by electron microscopy.[34,46] It has been characterised previously in the rodent and human cortex using proteomic methods[1,5,47,48] and it contains many neuroreceptors such as NMDA, AMPA and metabotropic glutamate receptors that influence long-term potentiation[49] and synaptic plasticity, and which are implicated in schizophrenia.[6,50,51] However, to our knowledge no previous study has enriched for the PSD in schizophrenia and assessed its differential expression compared with controls. The validity of the enrichment approach has been demonstrated previously by Hahn *et al*.[12] and in the current study we have built on this work by extending the study to post-mortem ACC tissue in schizophrenia and control.

We previously observed differential expression of proteins involved in CME in the cortex[52,53] and in the hippocampus[28] in schizophrenia, and based on these findings, we developed the hypothesis that abnormal CME alters NMDA receptor recycling leading to NMDA receptor hypofunction in schizophrenia.[25] In keeping with this hypothesis, we observed reduced expression of two proteins centrally involved in CME, namely Dynamin-1 and AP2. Using western blotting, we confirmed altered expression of these proteins. Changes in the expression of numerous other proteins involved in CME, such as amphiphysin, ARF6, ARFGAP1, AP2M1 and heat-shock proteins HSPA6 and HSPA8, were also observed. These findings confirm the role of membrane trafficking and CME in schizophrenia and are keeping with recent studies implicating endosomal and membrane trafficking more broadly in schizophrenia.[13–15]

We also observed altered expression of proteins with known roles in NMDA function. Specifically, we observed differential expression of MAPK3, SHANK3, SYNPO, MYL6, CYFIP2, VDAC, ATP6V0A1, CAMK2B, PRDX1 and ESYT. We confirmed the expression of MAPK3 and SYNPO using western blotting and dot blots, respectively. Further, pathway analysis of previous schizophrenia gene expression studies from the SMRI samples provides support for our findings by implicating metabolic pathways, including the MAPK signaling pathway. Taken together, our findings are in keeping with a recent genomic study of schizophrenia, which found an excess of copy number variants in schizophrenia with functions in the postsynaptic membrane.[6] In particular, our observations of differential protein expression of HSPB1, CYFIP2, RPH3A and MAPK3 are supportive of Kirov *et al*.'s[6] genomic findings. These findings are in keeping with the results of the pathway analysis undertaken on the 25 proteins differentially expressed following FDR, which implicated long-term potentiation. Long-term potentiation is dependent on NMDA receptor function and is critical to synaptic plasticity,[49] which is considered altered in schizophrenia.[50,51,54,55] However, as there is obviously a trade-off between false discovery rate and sensitivity/false-negative rates,[35] we also undertook pathway analysis of all proteins shown to be differentially expressed at unadjusted levels of significance. This analysis implicated endocytosis, long-term potentiation and also the calcium signalling pathway.

To substantiate our results, we used the largest available genome-wide association study of schizophrenia to test the gene association from our proteomic results and we highlight genes of particular interest: *HIST1H1E* and *MAPK3*. There are several lines of evidence from the literature to suggest roles for *HIST1H1E* and *MAPK3* in schizophrenia. First, common genetic variants from the histone and major histocompatibility complex gene cluster on chromosome 6p21–6p22 region are the most replicated genome-wide genetic association with schizophrenia;[29,56,57] second, a *de novo* loss-of-function *HIST1H1E* frameshift mutation was detected in a schizophrenia proband by exome sequencing,[58] and furthermore a gene expression study suggests that *HIST1H1E* may have predictive value for the treatment of depression.[58,59] Two independent copy number variant studies of schizophrenia have implicated *de novo* mutations in *MAPK3*[6,60] and another in psychosis.[61] Indeed, MAPK3 has critical roles in many cellular processes, including translational regulation, dendritic organization, long-term potentiation and synaptogenesis,[62] which are implicated in schizophrenia. It is expressed in the developing and adult human brain and MAPK3-deficient mice display abnormal behavioral patterns.[63]

Gene-set enrichment analyses have already suggested important biologic pathways in psychiatry[64,65] and related neurocognitive traits.[66–68] We have contributed to these findings by suggesting the importance of the PSD to schizophrenia susceptibility from our own experimental proteomic profile of the PSD, and an independent proteomic profile. Our findings are further strengthened in the specificity of the enrichment association with schizophrenia, and not bipolar disorder, Crohn's disease or type 2 diabetes. Our data suggest that genetic variation in the PSD proteome could be one of the potential sources of differential protein expression observed here, and warrants further experimental investigations. Further evidence for the association of PSD genetic variation with schizophrenia was recently published in genome-wide studies employing exome sequencing approaches,[69,70] and copy number variation surveys.[6,70]

Our study has several strengths and weaknesses that should be considered. Post-mortem studies have well-known confounds such as chronic exposure to neuroleptics, alcohol, tissue pH, and post-mortem delay can confound these studies. We designed our study so that these variables were matched closely across groups and where this was not possible our analysis accounted for these differences. To account for the potential effect of chronic exposure to neuroleptics in our schizophrenia cases, we studied the PSD proteome, using the same methods, of mice chronically exposed to the neuroleptic haloperidol. Few changes were identified following this treatment, and none involved the proteins we showed to be differentially expressed in schizophrenia. It should also be acknowledged that the PSD enrichment method we used required a great deal of tissue and that we were obliged to pool the precious cortical material from the 20 cases and 20 controls into 10 matched pairs per group. Future studies should attempt validation using larger and independent samples and to study bipolar disorder, as well as other cortical regions. It should also be appreciated that our understanding of the broader protein content of the PSD is based on proteomic studies of enriched samples and that the methods used can vary leading to different PSD proteomes. For example, we had identified 98% of the more stringently defined PSD proteome defined by Kirov *et al*.,[6] but only 53% as identified by Grant and co-workers (http://www.g2conline.org). The differences probably relate to the different methods and the presence of multiple isoforms of some proteins identified by Bayes *et al*.[5] However, our PSD enrichment method is relatively

standard[12,33] and this along with our sensitive LC-MS/MS method yielded a PSD proteome, which is very similar to that described previously.[1,5,6] Finally, it is possible that the PSD protein changes that we observed reflect a more generalised reduction of synaptic connections. For this reason, we assessed the protein expression of presynaptic marker synaptophysin in whole tissue lysates. We demonstrated no differential expression of synaptophysin in the ACC in schizophrenia, indicating that synaptic density changes are unlikely to be the basis of our findings.[71]

While our gene-based association test with schizophrenia was based on the largest available genome-wide meta-analysis using a widely regarded method, there are certain limitations to our approach including the omission of non-autosomal genes, the impact of non-causal SNPs to dilute association,[56] the potential of the genetic data not to tag a causative genetic variant missing genetic association and our lack of knowledge on the underlying genetic architecture of psychiatric illness. It is however a valuable complex trait genetics tool and we wait with anticipation for the next meta-analysis by the Psychiatric Genetics Consortium to replicate our findings.

In conclusion, in the first study of its kind, we have identified and confirmed protein changes within the PSD in schizophrenia. Genomic analysis supports the importance of the PSD in schizophrenia and in particular points to a role for PSD-associated NMDA-interacting proteins. Taken together, these findings provide novel insight into the contribution of the PSD to schizophrenia and suggest mechanisms, involving endocytosis, LTP and NMDA receptor function, that are responsible for this contribution.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)