Analysis of gene expression in two large schizophrenia cohorts identifies multiple changes associated with nerve terminal function

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ABSTRACT

Schizophrenia is a severe psychiatric disorder with a world wide prevalence of 1%. The pathophysiology of the illness is not understood, but is thought to have a strong genetic component with some environmental influences on aetiology. To gain further insight into disease mechanism, we used microarray technology to determine the expression of over 30,000 mRNA transcripts in post-mortem tissue from a brain region associated with the pathophysiology of the disease (Brodmann Area 10: anterior prefrontal cortex) in 28 schizophrenic and 23 control patients. We then compared our study (Charing Cross Hospital Prospective Collection: CCHPC) with that of an independent prefrontal cortex dataset from the Harvard Brain Bank (HBB). We report the first direct comparison between two independent studies. 51 gene expression changes have been identified that are common between the schizophrenia cohorts and 49 show the same direction of disease-associated regulation. In particular, changes were observed in gene sets associated with synaptic vesicle recycling, transmitter release and cytoskeletal dynamics. This strongly suggests multiple, small but synergistic changes in gene expression that affect nerve terminal function.

INTRODUCTION

Schizophrenia is a severe psychiatric illness with a world wide prevalence of 1%. The illness encompasses a number of domains of psychopathology: positive symptoms such as hallucinations and delusions, negative symptoms such as blunted emotional responding, lack of motivation and ideation, alongside intellectual decline, loss of insight and substantial impairment of personal and social function. Previous studies have indicated a strong genetic component of the illness (1, 2), although the degree of concordance in monozygotic twin studies suggests that environmental factors also play an important role in the disease aetiology.

While genetic studies have begun to identify putative disease genes, the pathophysiology of schizophrenia still remains poorly understood (see ref. 3). In this context, microarray analysis has been applied to post-mortem samples for several psychiatric illnesses with some success (4, 5, 6). A series of array studies have been performed on schizophrenia sample sets of variable size (7, 8, 9 10). At a superficial level of comparison, there appears to be some correlation between functional gene groups such as myelin genes (11, 12, 13, 14), metabolic genes (9, 10) and to a lesser extent, genes associated with inflammatory processes (15, 16) and some nerve terminal genes (7, 9). An issue arising from array studies, however, concerns the lack of concordance between array data and other technology platforms (10). This is compounded further by the lack of any direct comparison between published array studies. Direct comparison of microarrays has been difficult as several important factors can confound this type of analysis, including for example, the quality of analysed samples, sample size, integrity of study and analytical platform (5). In addition, a major problem is access to basic RAW signal intensity data files (.cel) for an informative comparison of published data. This has prevented the application of similar criteria for analysis across data sets and consequently the derivation of any meaningful comparison (17).

In the present study, we have analysed transcriptomic changes from a carefully curated, well documented schizophrenia cohort collected at the Charing Cross campus of Imperial College, London – the Charing Cross Hospital Prospective Collection (CCHPC). We analysed samples from the anterior prefrontal cortex (Brodmann Area 10; BA10) from 28 schizophrenic patients and compared these to 23 control samples from the same region. We analysed several publicly available studies that had similar patient and sample characteristics and selected the raw data deposited in the public domain by Glatt *et al*., 2005 (18) for the "McLean66" set from the Harvard Brain Bank (HBB) as the cohort and the quality of the available data matched our criteria. A series of published studies were eliminated because the RAW data were not available for direct analysis. Some studies were also eliminated due to experimental design (the latter representing the primary correlation with differential gene

expression). The HBB samples are dissected from BA9 (dorsolateral prefrontal cortex), a prefrontal cortical area adjacent to BA10. Reports showing schizophrenia disease pathology and cortical dysfunction associated with both BA9 and BA10 provide some of the most replicated observations in schizophrenia research and particularly dysfunction in tasks that require cognitive control (19, 20).

This is the first direct comparison between two large independent studies and furthermore, it is also the first microarray report comparing two demographically distinct cohorts – the HBB (United States) and the CCHPP (United Kingdom). To the best of our knowledge, all published studies to date have analysed samples collected within the continental US or Australia. Our data show that it is possible to detect significant and common changes in gene expression between independent cohorts. Moreover, the overlapping set comprises genes of relatively restricted role with significant enrichment in genes associated with synaptic function.

MATERIALS AND METHODS

Tissue Collection Charing Cross Hospital (CCHPC). Samples were collected as part of a prospective collection program coordinated through Imperial College London, This was a group of elderly patients whose schizophrenic illnesses had started before the introduction of antipsychotic medication, and who had progressed to long-stay psychiatric nursing facilities. Demographic, diagnostic and clinical data were ascertained from personal knowledge and scrutiny of all case notes available (Table 1). Patients were diagnosed prospectively according to DSM-III criteria by AMM supplemented by scrutiny of all case notes. All of the patients met diagnostic criteria for residual schizophrenia with pronounced negative symptoms alongside attenuated positive symptoms and intellectual dysfunction. The mean age of onset was 26 years and duration between onset and death was almost five decades, with a mean of 48 years. Two patients had been ill for 73 years. Thirteen patients were never discharged after their first admission, while only five had more than four periods of discharge in between their first admission and their death in hospital. Eleven patients had ECT in the past. Most patients had been treated with neuroleptic drugs when they became available, the mean duration of treatment was 33 years. One patient was neuroleptic naïve at death. Doses were relatively low, and only four patients took doses as expressed in chlorpromazine equivalents of more than 750mg per day. The ethnicity of patients was Caucasian. All patients with the agreement of their nearest relative or authorised representative, have given written informed consent for use of tissue obtained post mortem for research.

Control brain samples were obtained from mentally normal individuals, The control group were Caucasian tissue donors for research from the community, Charing Cross Hospital and local nursing homes. The causes of death were similar in the control and schizophrenia cohorts, bronchopneumonia being the most common cause followed by carcinoma (lung, bowel, prostate, bladder, oesophagus) and to a lesser extent, ischaemic heart disease and coronary artery occlusion. At autopsy, gross examination of brains revealed no major atrophy. As for the patient group, the right hemisphere was sampled. Cases of widespread damage due to stroke were excluded. Histological screening was carried out and cases with evidence of Alzheimer's disease, Parkinson's disease or multiple sclerosis were excluded. The control and schizophrenia cohorts were collected over the same period and the storage conditions are identical. This study has been approved by the West London Mental Health Ethical Research Committee and complies with the conditions of the Research Governance Office of the Imperial College of Science, Technology and Medicine Clinical Research Office.

Tissue Collection Harvard Brain Bank (HBB). The collection from the Harvard Brain Bank (HBB) comprises dorsolateral prefrontal cortex (BA9) brain regions from 16 schizophrenic (13 male, 3 female), 18 bipolar (12 male, 6 female), 27 controls (19 male, 8 female) and 3 schizo-affective (3 female) donors. Schizo-affective disorder and Bipolar samples were excluded from our analysis. Samples were collected as described by Glatt *et al*, 2005 (18). Demographics include: age, gender, PMI, handedness, morbidity (see Methods in Ref. 22). The ethnicity of the HBB cohort is not absolutely clear as many samples are referred to as "white" and some are annotated with unknown ethnicity..

Array Processing of CCHPC Samples. Total RNA was extracted from frozen BA10 obtained from 62 donors using a Polytron type homogeniser (YellowLine DI 25 Basic) and TriZol reagent (Invitrogen, Paisley, UK) in a ratio of 1 ml of TriZol to 20 mg of tissue. RNA was further purified using RNeasy mini-columns (Qiagen, Valencia, CA) including on-column DNAse-1 step and elution in water. Although pH was analysed in brain lysates using a pH meter, this was not considered to be rigorous enough to exclude or include samples and instead the RNA Integrity Number (RIN) was used to assess the quality of the RNA as the primary inclusion criterion. The quantity of extracted RNA was determined by spectrophotometry and quality was assessed using an Agilent 2100 Bioanalyzer (South Plainfield, NJ, USA to determine the RIN. Based on the RIN, samples were classified into three quality groups – pass (RIN > 7.0; borderline (RIN 6.0 – 7.0); fail (RIN< 6.0). Following classification there were 41 pass (RIN range of samples 7.0 – 9.0: average = 7.7, 16 borderline (RIN range of samples 6.0 – 6.9; average = 6.4) and 5 fail samples. Samples in the fail category were excluded from the study, and the remaining samples were randomized into 4 batches, containing an equal number of schizophrenic/control and male/female samples, for target generation and hybridization. For each batch, 10 µg total RNA was processed to Biotin-labeled cRNA and hybridized to HG-U133_Plus_2.0 GeneChips[®] in accordance with the Affymetrix protocol (Affymetrix, Santa Clara, CA, USA,). Arrays were scanned on a GeneChip Scanner 3000 and fluorescence intensity for each feature of the array was obtained by using GeneChip[®] Operating Software (Affymetrix, Santa Clara, CA, USA). A total of 57 samples were successfully hybridized. Sample progression for CCHPC is shown in Supplementary Fig.1.

Array Processing of Harvard Brain Bank (HBB) Samples. Samples were processed and hybridized to Affymetrix HG-U133A GeneChips[®] as described by Glatt *et al*, 2005 (18). Agilent profiles were available for all HBB samples but not the RINs. Supplementary data in ref 22 indicate that the RNA samples were quality controlled by several indices, including the mean 28S:18S RNA ratio (1.11 vs. 1.07) and the mean 3':5' ratio of the RNA transcripts of the

housekeeping genes *G3PDH* (1.57 vs. 1.44) and ACTB (2.42 vs. 2.33). These data suggest that the samples are of high RNA quality. In addition, we analysed the agilent profiles qualitatitively and as no HBB samples showed complete degradation of both 18S and 28S peaks by Agilent Bioanalyser, 43 samples were included for further analysis from the deposited RAW data. Sample progression for HBB is shown in Supplementary Fig.1.

Microarray Quality Control of CCHPC Samples. Standard MAS5.0 Affymetrix quality control criteria were examined to determine the quality of the CCHPC microarray data. All samples had background levels (46.6 – 74.7) and scale factors (0.64 – 1.49) within the acceptable range with an average percent present of 44.4%. In order to ensure only the highest quality microarray data were used in the analysis, β-actin 3'/5' ratios were assessed as a surrogate for quality. Six samples with a β-actin 3'/5' ratio >3.8 failed this measure of quality and were excluded from further analysis. A gender check was performed as an additional quality control to ensure that all samples had been annotated correctly by assessing the gene expression levels of DDX3Y (205000_at) which is male specific and XIST (224589_at) which is female specific. From the final 51 samples selected for analysis, all males showed high expression of DDX3Y and all females showed high expression of XIST indicating that the samples had the correct gender annotation. The final number of samples used for statistical analysis comprised 23 control and 28 schizophrenia samples.

Microarray Quality Control of HBB samples. MAS5.0 Affymetrix quality control was also used to analyse the HBB microarray data. All samples had background levels (47.1 – 99.9) and scale factors (0.63 – 4.22) within the acceptable range, with an average percent present of 45.7 %. β-actin 3'/5' ratios were also assessed as a surrogate for quality, and showed an evenly distributed range from 1.17 – 4.98. No outliers were detected from the microarray QC metrics and all samples were progressed to analysis. A gender check was performed to ensure that all samples were annotated correctly by assessing the gene expression levels of DDX3Y (205000_at) and XIST (224589_at). One sample (control, assay 1029) was labelled as female, but showed high expression of DDX3Y and low expression of XIST, a male-specific pattern indicating a mis-labelled sample. Due to the inconsistency between gene expression and gender allocation, this sample was excluded from further analysis. The final set of samples for analysis included: 26 controls (19 male, 7 female), 16 schizophrenia samples (13 male, 3 female).

All measures used to compare samples showed that the two collections had comparable metrics, despite hybridization of the HBB samples to HG_U133A Affymetrix

(half genome) microarrays and the CCHPC samples to HG_U133_plus_2.0 Affymetrix (whole genome) microarrays.

CCHPC Data Analysis. The raw signal intensities (.cel files) for each scan were imported into the gene expression analysis software, Resolver[TM] version 4.0 (Rosetta Biosoftware, Seattle, USA). Signal extraction was performed within Resolver and the normalised data were then exported for further analysis (21).

An initial Principal Components Analysis (PCA) was performed on detected probe sets (28,065 probe sets, defined as detected in Rosetta Resolver v4.0 as probe sets with P<0.01 for detection in ≥ 20 samples) and indicated that the major source of variability (first principal component, PC1) was due to sample quality, measured using β-actin 3'/5' ratios. There was no obvious structure due to disease (control vs schizophrenic) or gender. In order to account for the variability seen by the expression PCA, a PCA was performed on the QC metrics (average signal, background, standard deviation of the background, number present, raw q, scale factor, GAPDH 3'/5' ratio, β-actin 3'/5' ratio) and the scores from the first principal component (PC1) were taken to be used as a covariate in a subsequent analysis. This approach allows us to combine all of the information from the QC metrics into a single value that can be used in an analysis model to account for variability in data quality, due to sample degradation and other factors. The following model was used to analyse the final 51 (23 control vs 28 schizophrenia) CCHPC BA10 samples: Scores from PCI of QC metrics PCA (covariate), age (covariate), gender (factor), disease (factor), disease*gender (interaction) and gender*age (interaction). All probe sets (54,613) were analysed, negative intensity values were floored to 1 and data were analysed on a $\log_{10}$ scale using SAS[TM] v9.1.

HBB Data Analysis. In order to allow comparison between the CCHPC and HBB collections, the HBB data were processed in a comparable manner to the CCHPC. The raw signal intensities (.cel files) for each scan were imported into the gene expression analysis software, Resolver[TM] version 4.0 (Rosetta Biosoftware, Seattle, USA). Signal extraction was performed within Resolver and the normalised data exported for further analysis (18).

The HBB samples had been binned into groups representing 10 year intervals rather than classified according to discrete age. For this reason, age was fitted as a categorical factor as opposed to a continuous covariate as used in the CCHPC analysis. The final factors used in the analysis were age, gender and disease. No interactions between factors were fitted. All negative intensity data were floored to 1 before $\log_{10}$ was taken. All data were analysed on a $\log_{10}$ scale using SAS Enterprise Guide v3.0 in concordance with the CCHPC data.

Real Time PCR for CCHPC. Total RNA, as described previously for microarray analysis was further DNased through incubation (37°C, 10 min) with 1U DNase/10 µg total RNA (Ambion) to ensure removal of any contaminating genomic DNA. β-actin gene expression was analysed in the absence of reverse transcriptase to ensure the RNA samples were free of genomic DNA before converting to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems − according to protocol). Samples were diluted to a final concentration of 10 ng/µl of cDNA in 384 well format or 5 ng/µl in 96well format. PCR results were generated using the 5' nuclease assay (TaqMan) (22) and the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Each reaction included cDNA from 20 ng of RNA, 900 nM of each primer and 100 nM of probe and Universal PCR Master Mix (Applied Biosystems). Assay sequence information is indicated in Supplementary Table 2. PCR parameters were: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. A linear regression line calculated from the standard curves of serially diluted genomic DNA allowed relative transcript levels in RNA-derived cDNA samples to be calculated from the fluorescent signal in each run. The geometric mean of calculated abundance levels were calculated for technical replicates thus giving one abundance measure per sample. To identify which genes show a statistically significant difference in expression between the disease groups a linear mixed model analysis of variance was fitted to the data using SAS version 9.1. The same terms were used as were fitted to the Affymetrix data i.e., disease, gender, age. In addition a covariate was also included in the model to account for any change in expression due to the RNA loading of the samples (often referred to as normalising the data). This covariate was represented by the scores from the first principal component obtained from a PCA analysis of the three housekeeper genes (cyclophilinA[PPIA], glyceraldehyde phosphate dehydrogenase [G3PDH] and beta-actin [ACTB]). It can be shown that this technique is far more efficient at normalising Taqman data than more traditional techniques e.g. using ratios between the test genes and a housekeeper (23).

Bioinformatic Data Analysis. Evaluating the entire microarray dataset, we assessed whether the functions of all genes showing differential regulation between cases or controls in both the CCHPC and HBB studies (p<0.05, intensity>30; n=1662) might be related to pathways and biological processes with relevance to schizophrenia. We compared differentially regulated genes using parametric analysis of gene expression (PAGE), a modified gene set enrichment analysis (GSEA) method (24). Our method was previously described in detail (25) and is able to identify genes that are over-represented in a pathway or defined functional grouping. Our analysis was undirected (i.e. no *a priori* selection of pathways was made) and only detected enrichment against a background of the genes tested in the experiment (i.e. the genes assayed

on the HG_U133A chip; n=8444) rather than all genes. In order to enable this analysis, we have compiled a comprehensive database of interactions assembled from a diverse combination of public and commercial databases, supplemented by our own curated pathway data. Data sources included a number of GSK curated pathways, public domain curated data from the Gene Ontology (GO) consortium and the KEGG pathways database, and also commercially curated pathway data derived from the Ingenuity Pathways Knowledge Base (IPA) (Ingenuity Systems, Redwood City, CA), GeneGo (Encinitas, CA), NetPro Molecular Connections (Singapore) and Jubilant (Berkley Heights, NJ).

RESULTS

Charing Cross Hospital Prospective Collection (CCHPC). Following analysis of all probe sets (54,613) on the HG_U133_Plus_2.0 Affymetrix GeneChip® using the linear model described in the methods, the differentially expressed gene list was filtered to remove all low intensity probe sets (defined as median intensity <30 in either schizophrenic or control group) and probe sets that map to intronic regions or to the antisense strand. This left a total of 29464 probe sets, of which 1846 (880 up-regulated, 966 down-regulated) showed a significant (P≤0.05) disease effect, with 1792 showing a significant (P≤0.05) gender effect, 8702 showing a significant (P≤0.05) age effect and 13886 showing a significant (P≤0.05) effect due to quality correction (covariate from PC1 of PCA on QC metrics). A summary of probe sets for the 1846 significant changes in provided in Supplementary Table 1.

Harvard Brain Bank Cohort (HBB). Following analysis of all probe sets (22,215) on the HG_U133A Affymetrix GeneChip® using the linear model described in the methods section above, the differentially expressed gene list was filtered to remove all low intensity probe sets (defined as median intensity <30 in either schizophrenic or control group) and probe sets that map to intronic regions or to the antisense strand. This left a total of 14608 probe sets, of which 870 (300 up-regulated, 570 down-regulated) showed a significant (P≤0.05) disease effect, with 3036 showing a significant (P≤0.05) gender effect, 3895 showed a significant (P≤0.05) age effect and 9982 showed a significant (P≤0.05) effect due to quality correction (covariate from PC1 of PCA on QC metrics). A summary of probe sets for the 870 significant changes is also provided in Supplementary Table 1

Comparison of gene expression changes between the CCHPC and HBB cohorts. In order to compare the differentially expressed genes between the CCHPC and HBB cohorts, we identified probe sets that were assayed in common since the HG_U133_Plus_2.0 comprises probe sets from the HG_U133A and HG_U133B GeneChips®. The 22,215 probe sets represented on the HG_U133A chip (and assayed for HBB samples) were extracted for the CCHPC samples and integrated with the HBB data. The commonly detected probe sets (median intensity >30 in either schizophrenic or control groups) from both studies were identified and those designed to intronic or antisense strands were removed, leaving 11,912 reliable probe sets detected in both cohorts. Of these, 797 showed a significant (P≤0.05) change in the CCHPC cohort, and 725 showed a significant (P≤0.05) change in the HBB cohort, with 51 probe sets showing a significant (P≤0.05) change in both. Of these 51 probe sets, 49 probe sets (representing 49 genes) showed the same direction of disease-associated regulation (96 % similarity: 33 down-regulated versus 16 up-regulated). The gene symbol,

HUGO symbol, Entrez geneID, AffyID, functional group and difference in expression between schizophrenia and control patients and corresponding p-value are shown in Table 2

The 49 commonly regulated genes were categorised according to functional properties, using recent literature reports and Uniprot definitions for function. The genes clustered into a relatively small number of functional groups that comprised synaptic vesicle recycling/secretion (e.g., ZnT3, RABGGTB, VAMP2, SYT5, WNK1, Doc2A), cytoskeletal regulation (e.g., ELMO1, OLFM1, WASF1, SEPT8), signal transduction (e.g., CACNB3, CACNG3, CAPNS1, CMKK2, PIK4CB) and transcription (e.g., ZBTB1, ZNF395). Additional genes were identified that represented other functions such as mitochondrial function (e.g., MRPS28, SLC25A11 and SNN), neurodevelopment (e.g., PHYHIP), and there was a small set of genes with unknown function (e.g., FLJ10925, KIAA0082 and TMEM24). The housekeeping genes (including β-actin) did not show any significant difference between control and schizophrenic groups.

The predicted mean expression and 95% confidence intervals were generated for the 51 common genes in each cohort (Table 2). The 95% confidence intervals of the ratio (schizophrenic/control) between the means of the two disease groups were also calculated for both cohorts and the results for six example genes are shown in the interval plots in Fig. 1. The interval plots show the size of the up- or down-regulation for each gene in each cohort as well as the associated precision (variability). By viewing the data in this way we can easily compare the level of similarity in results between the two cohorts which, in this case, shows a high level of consistency between two independent cohorts. In addition, we also confirmed some of the overlapping (SLC30A3, VEGF, SYT5, TMEM24) and non-overlapping (FZDB, S100A12) genes within the CCHPC cohort by RT-PCR (Supplementary Fig. 2). Many of the genes altered in the arrays have not been confirmed by RT-PCR and although they are common to both disease cohorts should be considered to some extent as provisional observations.

In order to compare the broader pathway characteristics of each cohort, we performed an enrichment analysis of differentially regulated genes against all transcripts measured. We also combined the pathway enrichment results for the transcriptomics experiments to generate a combined p value and a combined false discovery rate (FDR) corrected p-value. In the combined analysis, genes showing differential regulation in our sample were significantly over-represented in a number of pathways which have been implicated in the pathogenesis of schizophrenia (see Table 3), including dysbindin signalling and glutamate signalling, cytoskeletal protein binding, neurogenesis, synaptogenesis, synaptic transmission, protein kinase signalling and previously curated network of schizophrenia responsive genes (Table 3). In addition, when the gene sets were examined as a whole, we identified a significant

enrichment of genes localised in the postsynaptic synaptome (based on data from Emes *et al*, 2008 (26). Eighteen of the fifty one genes are localised in the synaptome; the expected nEumber is 3.2 (p=7e-10) and this analysis survived FDR correction (see supplementary data in ref.26).

DISCUSSION

Our objective was to conduct a transcriptional analysis of post-mortem tissue samples from schizophrenic donors compared to matched controls. We compared data from the CCHPC and HBB cohorts as the latter represented an independent study of comparable size and quality.

This enabled the first direct comparison between two well-archived and demographically distinct cohorts (European and North American). If it is assumed that both cohorts are independent and there is no correlation between genes then the proportion of genes that would be expected to be detected at the 5% level in both cohorts purely by chance, ignoring direction is 29.7 (0.05*0.05 = 0.0025 i.e., 0.25% of 11,912). However, if direction is taken into account then the proportion of genes that would be expected to be detected at the 5% level that are up/down regulated in both cohorts purely by chance is 14.9 (0.05*0.025 =0.00125 i.e., 0.125% of 11,912) and therefore, in the CC and HBB overlap we observe 3.3 times the number of significant changes in the same direction than would be expected by chance. In addition, by using confidence intervals to view the data, we can easily compare the level of similarity in results between the two cohorts which, in this case, shows a high level of consistency between two independent cohorts.

In agreement with previous studies, we did not detect any genes that are linked to the dopamine hypothesis in either cohort. Neither were genes associated with myelin function observed to change in either cohort. This contrasts with some previous array studies which report modulation of several oligodendroglial genes (11, 12, 13). There is, however, little consistency between the specific genes in these studies and the significance of these observations is unclear. Furthermore, several of these genes (CNP, MAG, OLIG2) were analysed by Mitkus *et al.*, (14) who did not observe any difference between levels in schizophrenic patients and controls. We did not detect astrocyte or microglial markers or significant numbers of metabolic genes It is possible that the latter reflects the condition of the samples as mitochondrial gene modulation has been linked to agonal/pH state (27, 28, 29). We also did not detect any genes which have been proposed as disease candidates based on genetic studies, although our data support modulation within the reported networks for DISC1 and dysbindin (30, 31) A major confounding factor for microarray analysis is patient exposure to drugs of abuse, to smoking and to treatment medication. The patients in the CCHPC were institutionalised and had no access to drugs of abuse including alcohol. We analysed for a correlation with drug exposure (chlorpromazine equivalents at death, neuroleptic treatment years, high/low dose) in the CCHPC but none was detected for the forty nine common genes. In addition, genes thought to be regulated by chronic smoking (32 and

Supplementary Table 2 therein) were also assessed but none were present in the overlapping gene set described here.

Fifty one differentially expressed genes were identified that were common between the cohorts and forty nine of these showed the same direction of regulation. Many have not been identified previously in post-mortem studies of schizophrenia or in genetic association studies and cluster broadly into three functional groups; synaptic vesicle function, cytoskeletal regulation and signal transduction.

Synaptic Vesicle Function: A large number of genes were present that regulate synaptic vesicle (SV) release and recycling (33, 34). These include the SV integral membrane proteins, VAMP2 (synaptobrevin 2), synaptotagmin 5 and zinc transporter 3 (down-regulated) and the vesicle associated protein Rab geranyl geranyl transferase (up-regulated). VAMP2 (synaptobrevin 2) forms part of the tripartite vesicle fusion complex together with the presynaptic plasma membrane proteins, syntaxin and SNAP-25. This release complex is thought to mediate the fusion event between the vesicle and plasma membranes and is tightly regulated by a hierarchy of modulating proteins that include the Munc family members, Munc 13 and Munc 18 (see below) (33). Synaptotagmin 5 belongs to the calcium-sensitive synaptotagmins that regulate vesicle release (33) and it appears to be preferentially localised to large dense core vesicles that are associated with peptide hormone release (35). The zinc transporter type 3 (ZnT3) is particularly intriguing as it is the only modulated gene that regulates vesicle content. ZnT3 is highly brain-specific (36) and is localised to synaptic vesicles in various neuronal populations (37). ZnT3 is responsible for vesicular zinc accumulation although the role of vesicular zinc is unclear. Studies in ZnT3 targeted–null mice have not revealed a phenotype associated with schizophrenia symptoms (38, 39) and there are no robust hypotheses supporting Zn disruption in the illness. However, ZnT3 is up-regulated following a series of pathological stimuli (40) and may warrant further investigation as expression appears to correlate with the expression of the vesicular glutamate transporter type 1 (41) which in turn shows an inverse relationship with dysbindin expression (42). Reduced levels of vesicular zinc release may have pleiotropic effects, for example, released zinc regulates NMDA receptor activity, which is linked to schizophrenia (43). In addition, a series of genes that are dependent on zinc binding for their function are present in the overlapping gene set e.g., RABGGTB, ZBTB1, ZNF395, ZBBX. Rab3a undergoes a cycle of association and detachment from the synaptic vesicle membrane during vesicle release. The geranylation step, performed by Rab geranyl geranyl transferase (RABGGTB), is essential for the re-attachment of Rab3a to the SV membrane during the regeneration of the releasable vesicle pool in the nerve terminal (33, 34). In addition, ARF3, ARL4L, ARPP-19, genes associated with vesicle trafficking, are down regulated. Several regulators of vesicle secretion

are also modulated. DOC2α, a double cortin containing protein specifically expressed in neurons and localised to SVs (44) is involved with transmitter release via Munc13 regulation. WNK1, a kinase associated with MUNC-18 regulation (45) and therefore calcium-dependent fast transmitter release is up-regulated. Septin 8 belongs to an emerging family of presynaptic proteins that appear to regulate vesicle release (46).

Additional genes that are likely to influence vesicle release were also detected. The beta3 and gamma3 subunits of voltage-dependent calcium channels were decreased and increased, respectively, suggesting a possible modulation of voltage dependent calcium activation in the nerve terminal (47). Furthermore, calpain small subunit was also down-regulated. This is the common heterodimeric partner for the calcium dependent calpains 1 and 2 which target, amongst other proteins, components of the cytoskeleton and are involved in long-term potentiation and nerve terminal remodelling.

Taken together, these data strongly suggest a primary deficit of vesicle release (fast transmitter vesicles and large dense core granules) and possibly cellular trafficking. The molecules discussed above function at different points in the hierarchy of vesicle regulation ranging from vesicle trafficking (ARF3, ARL4L, ARPP-19) to priming (DOC2a) and release (VAMP2, SYT5, RABGGTB) (Fig. 2).

Signal Transduction: Expression changes in genes associated with signal transduction were observed representing the inositol pathway (PI4Kinase), GPCR signalling (GNG3, VIPR1) and trophins (VEGF, SH2B adaptor protein). PI4 kinase regulates the first committed step in the synthesis of IP_3 which is the primary mediator of rapid intracellular calcium release. VEGF, a potent endothelial mitogen involved in vascularisation and angiogenesis has, more recently, been shown to exert a number of trophic effects in the nervous system, promoting neurite outgrowth, promoting nerve regeneration and causing proliferation of astrocytes and Schwann cells. VEGF has not previously been implicated in schizophrenia but it has been shown to be neuroprotective for dopaminergic neurons in models of Parkinson's disease. Furthermore, tumor–derived VEGF promotes angiogenesis and tumour growth but this mechanism is attenuated by dopamine (48, 49).

Cytoskeletal Regulation: In addition to calpain small subunit, expression changes in other genes associated with modulation of the actin cytoskeleton and microtubule structure were identified including ELMO1, WASF1, SEPT8 and OLFM1 which is a confirmed interactor with the schizophrenia-associated gene, DISC-1 (which in turn is a modulator of nerve terminal function). Actin is a major cytoskeletal protein found both pre- and post-synaptically and the modulation of actin dynamics by the genes described here may be linked to cyto-architectural changes associated with synaptic modulation (50) The WASF1 downregulation

we observed is of particular interest as Kim *et al*. 2006 (51) showed it to be a key regulator of actin-dependent morphological processes in mouse neurons. Loss of WASF1 function *in vivo* or in cultured neurons resulted in a decrease in mature dendritic spines. This suggests that WASF1 may play an important role in the regulation of dendritic spine morphology in schizophrenia.

Smaller groups of genes represented in the overlap set included those associated with brain development such as phytanoyl-CoA hydroxylase interacting protein and a series of transcription factors and genes associated with transcriptional regulation/RNA processing, for example, GATA3 and ZNF395. In order to try to place these observations into a wider pathway context, we carried out an enrichment analysis on the total detected gene set in each cohort for dominant gene interactions and pathways. This identified an over-representation of genes involved in dysbindin signalling (31) and synaptic transmission (33, 34), further supporting the hypothesis from the common gene set for synaptic dysfunction in schizophrenia. Several important caveats nonetheless remain and we cannot at this point exclude the possibility that these gene changes are in some way influenced, for example, by medication (although our data suggest otherwise) or are secondary to an underlying pathology or its consequences.

In summary, we identified 49 genes that are differentially regulated and common to two independent schizophrenia cohorts. Furthermore, the genes cluster into a relatively small number of functional groups that are associated with synaptic function via synaptic vesicle regulation, signal transduction or cytoskeletal dynamics. The broader bioinformatics comparison between the cohorts supports this and the data strongly suggest more than any previous microarray study that one of the primary sites of schizophrenia disease pathology is localised at the nerve terminal and in particular at the presynaptic site. This is in agreement with previous suggestions for a synaptic pathology in schizophrenia based on genetic observations (52) and genomic studies (7 and reviewed in 53). Although the transcriptional effects are small, as reported in previous studies, the cumulative effect of disturbance of many genes acting at multiple points in the vesicle life cycle and at hierarchies of regulation within the presynaptic terminal, is likely to have significant impact on synaptic transmission and remodelling that may underlie the pathology of the illness.

Acknowledgements

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Legends to Figures

Figure 1 Confidence interval Comparisons

Six forest plots showing the 95% confidence intervals of the disease ratio (schizophrenic / control) between the means of the two disease groups for the CCHPC and HBB cohorts for selected genes. The width of the blue boxes in each plot represents the size of the difference between the ratios of the two cohorts i.e. there is no box in the plot of results from VIPR as the disease ratios from the two cohorts are identical.

Figure 2. Schematic representation of a nerve terminal showing the functional localisation of a subset of genes directly associated with presynaptic function.

Genes associated with cytoskeletal regulation and signal transduction are also shown as they are likely to influence nerve terminal function. Pale blue circles = small synaptic vesicles; purple circle = large dense cored vesicle; red hatching = postsynaptic receptors; small green circles = actin polymers.

Supplementary Figure 1. Flow schematic for CCHPC and HBB samples

CCHPC samples were processed for microarray analysis and compared to microarray data reported in Glatt *et al.* (18). This schematic illustrates the steps of QC progression and reasons for sample omission in each cohort.

Supplementary Figure 2. Confirmation of microarray findings using QPCR

(A) Confirmation of a sub-set of the overlapping CCHPC and HBB genes (SLC30A3, VEGF, SYT5, TMEM24) using QPCR. Genes are also shown from the CCHPC set that were not in the overlapping group (FZDB, S100A12). (B) Fold changes and p values are shown for both microarray and QPCR analysis.

Table1: Summary of patient demographics for samples that were included in the statistical analysis.

		Schizophrenic	Control
BA10 region			
Number of samples		28	23
Gender (Males / Females)		19 / 9	13 / 10
Age	Mean	73.3	69.0
	SD	15.2	21.6
	Median	78.0	71.0
	Range	28-97	25-94
PMD (Hours)	Mean	8.1	9.4
	SD	6.5	4.4
	Median	4.8	9.5
	Range	3-30	4-17
Brain pH	Mean	6.2	6.5
	SD	0.2	0.3
	Median	6.2	6.5
	Range	5.7-6.6	5.7-6.9

Table 2. Gene identifications of transcripts showing common changes in expression between CCHPC and HBB cohorts

Gene Symbol	HUGO symbol	Entrez Gene ID	Affy ID	Functional Category	CC				HBB			
					BA10 Fold Change (Model)	Lower 95% CI Limit	Upper 95% CI Limit	BA10 Disease P value	BA9 Fold Change (Model)	Lower 95% CI Limit	Upper 95% CI Limit	BA9 Disease P value
ARF3	ARF3	377	200011_s_at	Vesicle Function	-1.11	-1.20	-1.02	0.0224	-1.10	-1.20	-1.01	0.0245
ARF4L	ARL4D	379	203586_s_at	Vesicle Function	-1.18	-1.31	-1.06	0.0028	-1.20	-1.44	-1.00	0.0469
ARPP-19	ARPP-19	10776	221482_s_at	Vesicle Function	-1.12	-1.21	-1.04	0.0041	-1.20	-1.35	-1.07	0.0030
CACNB3	CACNB3	784	209530_at	Vesicle Function	-1.18	-1.31	-1.05	0.0055	-1.26	-1.44	-1.10	0.0011
CACNG3	CACNG3	10368	206384_at	Vesicle Function	-1.25	-1.53	-1.01	0.0375	-1.47	-1.99	-1.08	0.0144
DOC2A	DOC2A	8448	205744_at	Vesicle Function	-1.12	-1.27	-1.00	0.0497	-1.30	-1.63	-1.04	0.0225
RABGGTB	RABGGTB	5876	209181_s_at	Vesicle Function	1.08	1.02	1.15	0.0061	1.24	1.05	1.48	0.0152
SEPT8	SEPT8	23176	208999_at	Vesicle Function	1.17	1.03	1.32	0.0139	1.31	1.00	1.71	0.0482
SLC30A3	SLC30A3	7781	207035_at	Vesicle Function	-2.00	-3.11	-1.28	0.0033	-2.42	-5.21	-1.13	0.0247
SYT5	SYT5	6861	206161_s_at	Vesicle Function	-1.22	-1.40	-1.08	0.0030	-1.40	-1.95	-1.01	0.0452
VAMP2	VAMP2	6844	201557_at	Vesicle Function	-1.11	-1.23	-1.00	0.0403	-1.28	-1.60	-1.01	0.0379
WNK1	WNK1	65125	211994_at	Vesicle Function	1.10	1.01	1.19	0.0245	1.16	1.00	1.34	0.0457
DKFZP564K0822	ECOP	81552	208091_s_at	Transcription /RNA processing	-1.11	-1.18	-1.04	0.0018	-1.06	-1.12	-1.00	0.0484
GATA3	GATA3	2625	209604_s_at	Transcription	-1.15	-1.33	-1.00	0.0472	-1.11	-1.22	-1.02	0.0228
HNRPM	HNRNPM	4670	200072_s_at	Transcription/	1.08	1.01	1.15	0.0223	1.15	1.01	1.31	0.0368
HTATIP	KAT5	10524	214258_x_at	Transcription /	-1.05	-1.11	-1.00	0.0415	-1.12	-1.24	-1.01	0.0352
PNN	PNN	5411	212037_at	Transcription/	1.09	1.04	1.14	0.0011	1.19	1.04	1.37	0.0151
SAP30	SAP30	8819	204900_x_at	Transcription	1.10	1.01	1.21	0.0370	1.21	1.02	1.43	0.0274
FLJ23049	ZBBX	79740	220269_at	Transcription/	-1.19	-1.42	-1.00	0.0475	-1.29	-1.66	-1.01	0.0401
ZBTB1	ZBTB1	22890	213376_at	Transcription	1.11	1.02	1.20	0.0137	1.21	1.03	1.43	0.0238
ZNF395	ZNF395	55893	218149_s_at	Transcription	1.17	1.00	1.37	0.0462	1.33	1.01	1.76	0.0459
CAMKK2	CAMKK2	10645	207359_at	Signalling	-1.16	-1.31	-1.03	0.0165	-1.49	-2.09	-1.06	0.0230
CAPNS1	CAPNS1	826	200001_at	Signalling	-1.09	-1.17	-1.01	0.0323	-1.19	-1.40	-1.01	0.0420
GNG3	GNG3	2785	222005_s_at	Signalling	-1.12	-1.27	-1.00	0.0475	-1.30	-1.66	-1.02	0.0372

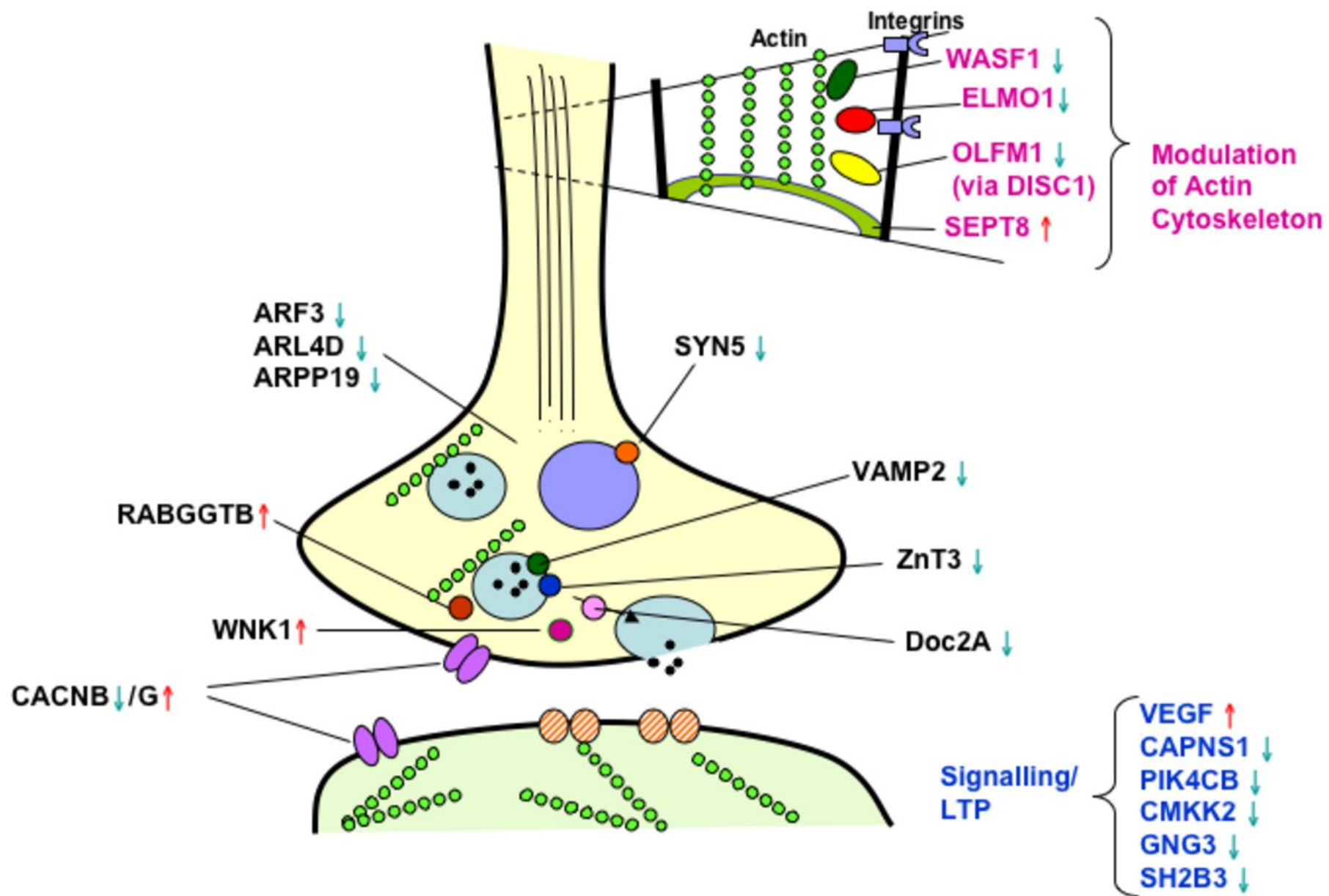|												
LRCH4	LRCH4	4034	204692_at	Signalling	1.20	1.04	1.38	0.0151	1.15	1.03	1.27	0.0117
PIK4CB	PI4KB	5298	206139_at	Signalling	-1.16	-1.31	-1.04	0.0109	-1.11	-1.21	-1.01	0.0236
LNK	SH2B3	10019	203320_at	Signalling	1.11	1.03	1.20	0.0108	-1.09	-1.18	-1.01	0.0325
VEGF	VEGFA	7422	210513_s_at	Signalling	1.33	1.03	1.71	0.0309	1.23	1.01	1.50	0.0369
VIPR1	VIPR1	7433	205019_s_at	Signalling	-1.32	-1.70	-1.02	0.0371	-1.32	-1.62	-1.07	0.0105
RCBTB1	RCBTB1	55213	218352_at	Nuclear protein	1.10	1.00	1.21	0.0455	1.15	1.01	1.30	0.0301
JMJD1A	JMJD1A	55818	212689_s_at	Nuclear protein	1.14	1.06	1.22	0.0008	1.20	1.06	1.37	0.0062
NAP1L1	NAP1L1	4673	208754_s_at	Nuclear protein	1.07	1.02	1.14	0.0114	1.19	1.03	1.37	0.0187
RPL39L	RPL39L	116832	210115_at	Nuclear Protein	-1.35	-1.65	-1.11	0.0037	-1.27	-1.54	-1.05	0.0161
CLIPR-59	CLIP3	25999	212358_at	Cytoskeleton	-1.14	-1.26	-1.04	0.0090	-1.17	-1.33	-1.02	0.0228
MCF2L		23263		Cytoskeleton	1.23	1.01	1.50	0.0416	1.22	1.02	1.47	0.0309
NRBP	NRBP1	29959	217765_at	Cytoskeleton	-1.22	-1.40	-1.07	0.0038	-1.25	-1.53	-1.02	0.0313
OLFM1	OLFM1	10439	213131_at	Cytoskel/ DISC1	-1.16	-1.32	-1.02	0.0212	-1.18	-1.36	-1.03	0.0222
NEK7	NEK7	140609	212530_at	Centrosome	1.11	1.03	1.19	0.0088	1.22	1.01	1.48	0.0412
ACTR1A	ACTR1A	10121	200720_s_at	Cytoskeleton	-1.12	-1.25	-1.01	0.0405	-1.25	-1.54	-1.01	0.0418
ELMO1	ELMO1	9844	204513_s_at	Cytoskeleton	-1.12	-1.24	-1.02	0.0190	-1.26	-1.55	-1.02	0.0340
HRIHFB2122	TRIOBP	11078	202795_x_at	Cytoskeleton	1.13	1.04	1.23	0.0075	1.19	1.02	1.40	0.0292
WASF1	WASF1	8936	204165_at	Cytoskeleton	-1.11	-1.22	-1.01	0.0291	-1.15	-1.27	-1.04	0.0091
ABCA1	ABCA1	19	203504_s_at	Cholesterol	1.14	1.01	1.29	0.0337	1.28	1.04	1.57	0.0220
PHYHIP	PHYHIP	9796	205325_at	Development	-1.11	-1.21	-1.02	0.0217	-1.24	-1.40	-1.10	0.0007
UQCRC1	UQCRC1	7384	201903_at	Mitochondrial	-1.11	-1.23	-1.01	0.0344	-1.18	-1.36	-1.02	0.0272
MRPS28	MRPS28	28957	219819_s_at	Mitochondrial	-1.09	-1.16	-1.01	0.0215	1.20	1.03	1.39	0.0191
SLC25A11	SLC25A11	8402	209003_at	Mitochondrial	-1.10	-1.17	-1.02	0.0101	-1.16	-1.30	-1.03	0.0121
SNN	SNN	8303	218032_at	Mitochondrial	-1.11	-1.21	-1.01	0.0292	-1.11	-1.21	-1.01	0.0232
PSMD13	PSMD13	5719	201233_at	Ubiquitin	-1.18	-1.32	-1.05	0.0054	-1.21	-1.41	-1.05	0.0116
FLJ10925	C7ORF43	55262	220659_s_at	Unknown	-1.18	-1.35	-1.03	0.0182	-1.52	-1.96	-1.18	0.0020
TMEM24	C2CD2L	9854	204757_s_at	Unknown	-1.22	-1.40	-1.08	0.0027	-1.44	-1.72	-1.20	0.0002
KIAA0082	FTSJD2	23070	212380_at	Unknown	-1.11	-1.23	-1.01	0.0398	-1.15	-1.26	-1.04	0.0071

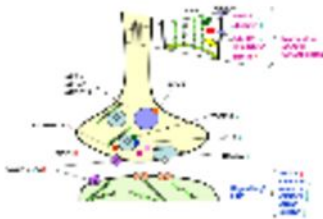**Table 3. Summary of top 20 pathways following PAGE analysis (FDR < 0.0002)**

Pathway name	Source	no. genes*	FDR P combined	Psychiatric Rationale†
Kinase Protein families	Jubilant	(23/228)	1.68E-11	General
Receptor protein tyrosine kinase docking protein:SH3/SH2 adaptor protein activity	NetPro	(37/300)	1.20E-06	General
NEF Mediated Pathway--Acquired Immuno Deficiency Syndrome	Jubilant	(10/64)	1.20E-06	
Intracellular Signaling Cascade:Phosphatidylinositol 3-Kinase Mediated Signaling	NetPro	(44/428)	2.80E-06	General
Dysbindin signalling	GSK	(14/63)	3.49E-06	Schizophrenia
Glutamate signalling network	GSK	(34/273)	3.94E-06	General
Insulin_Signaling	GenMAPP	(19/160)	6.89E-06	
Regulation_of_Actin_Cytoskeleton_KEGG	GenMAPP	(15/142)	7.61E-06	General
Focal_adhesion_KEGG	GenMAPP	(20/185)	2.20E-05	
Amyloid beta-peptide Signaling Pathway--Alzheimers	Jubilant	(11/113)	3.97E-05	Cognition
Alzheimers Responsive Genes	Jubilant	(15/157)	4.11E-05	Cognition
MAPKinase Signaling Pathway	Biocarta	(7/87)	4.11E-05	General
TGFbeta Signaling Pathway--Growth and Differentiation	Jubilant	(10/66)	4.69E-05	General
Ligand-dependent nuclear receptor activity:Vitamin D3 receptor activity	NetPro	(21/158)	5.44E-05	Schizophrenia
Transcription_Ligand-dependent activation of the ESR1/SP pathway	GeneGo	(5/39)	6.46E-05	Schizophrenia
Calcium_regulation_in_cardiac_cells	GenMAPP	(16/137)	6.67E-05	
Jubilant:Thrombopoietin Signaling Pathway--Thrombopoiesis	Jubilant	(16/137)	0.000212	
Jubilant:AR Mediated Pathway--Prostate Cancer	Jubilant	(12/99)	0.000212	
GOA:Neurotransmitter Secretion: BP	GOA	(7/65)	0.000343	General
NetPro:Regulation of transcription	NetPro	(20/152)	0.000345	

*Column indicates number of genes showing significantly different regulation between cases and controls versus the total number of genes in the pathway tested in the experiment. † Pathway rationale in psychiatric diseases is based on co-occurrence of disease and pathway terms in literature.

Figure 1: Confidence interval Comparisons

Actin
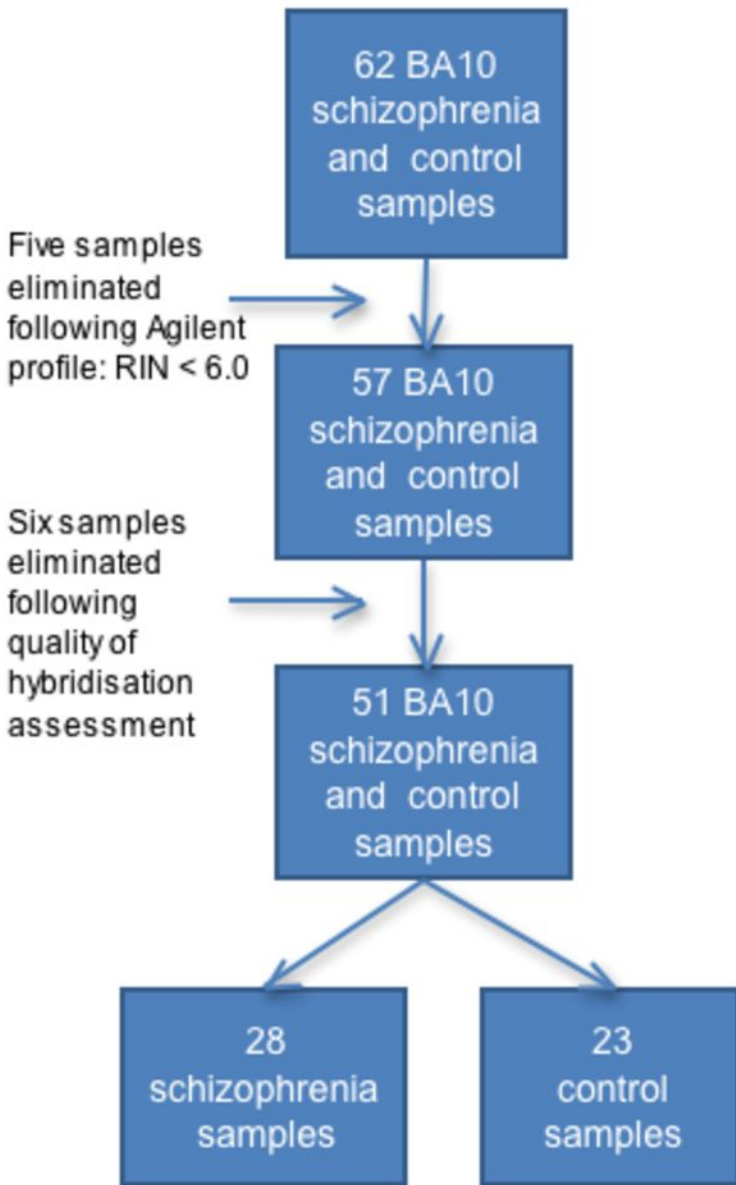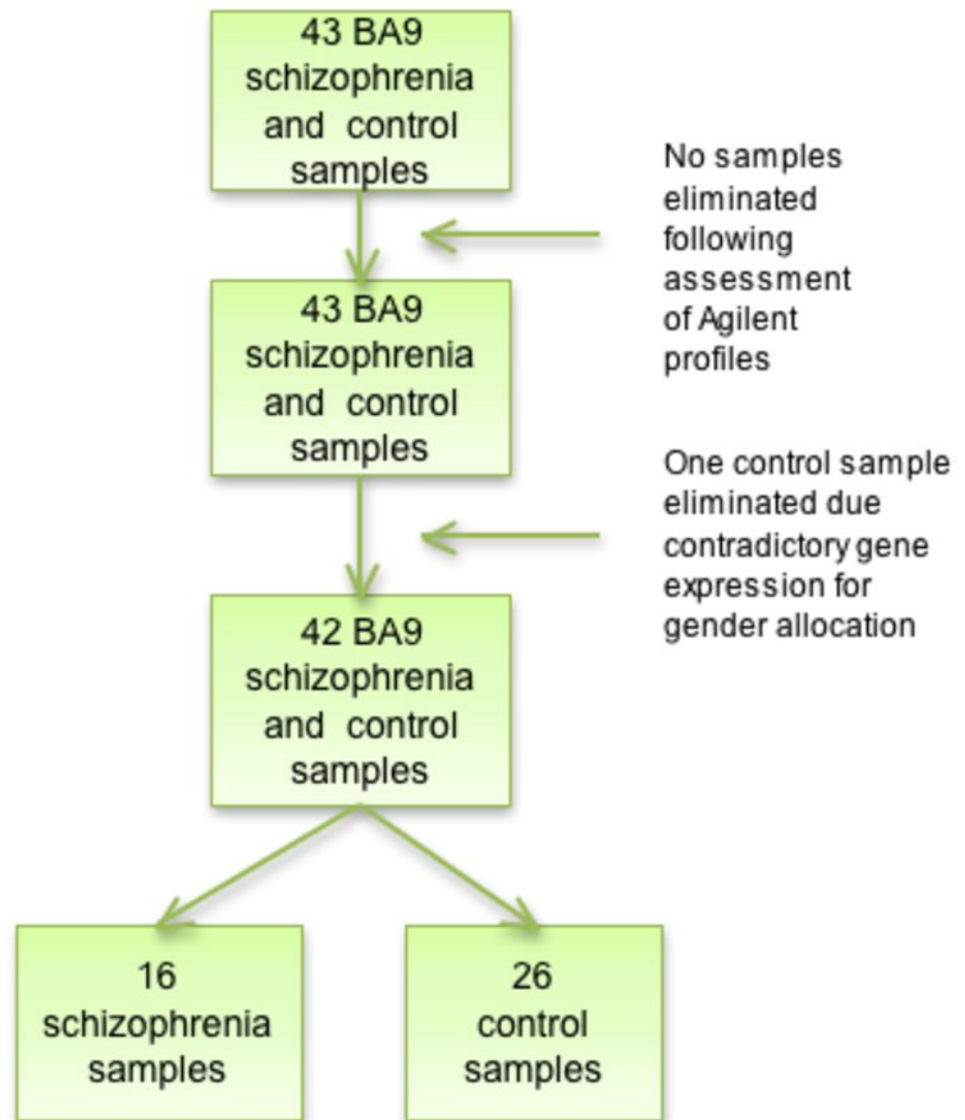Integrins
WASF1 ↓
ELMO1 ↓
OLFM1 ↓
(via DISC1)
SEPT8 ↑

Modulation
of Actin
Cytoskeleton

ARF3 ↓
ARL4D ↓
ARPP19 ↓

SYN5 ↓

VAMP2 ↓

RABGGTB ↑

ZnT3 ↓

WNK1 ↑

Doc2A ↓

CACNB ↓ /G ↑

Signalling/
LTP

VEGF ↑
CAPNS1 ↓
PIK4CB ↓
CMKK2 ↓
GNG3 ↓
SH2B3 ↓

CCHPC

HBB

62 BA10 schizophrenia and control samples

43 BA9 schizophrenia and control samples

Five samples eliminated following Agilent profile: RIN < 6.0

No samples eliminated following assessment of Agilent profiles

57 BA10 schizophrenia and control samples

43 BA9 schizophrenia and control samples

Six samples eliminated following quality of hybridisation assessment

One control sample eliminated due contradictory gene expression for gender allocation

51 BA10 schizophrenia and control samples

42 BA9 schizophrenia and control samples

28 schizophrenia samples

23 control samples

16 schizophrenia samples

26 control samples
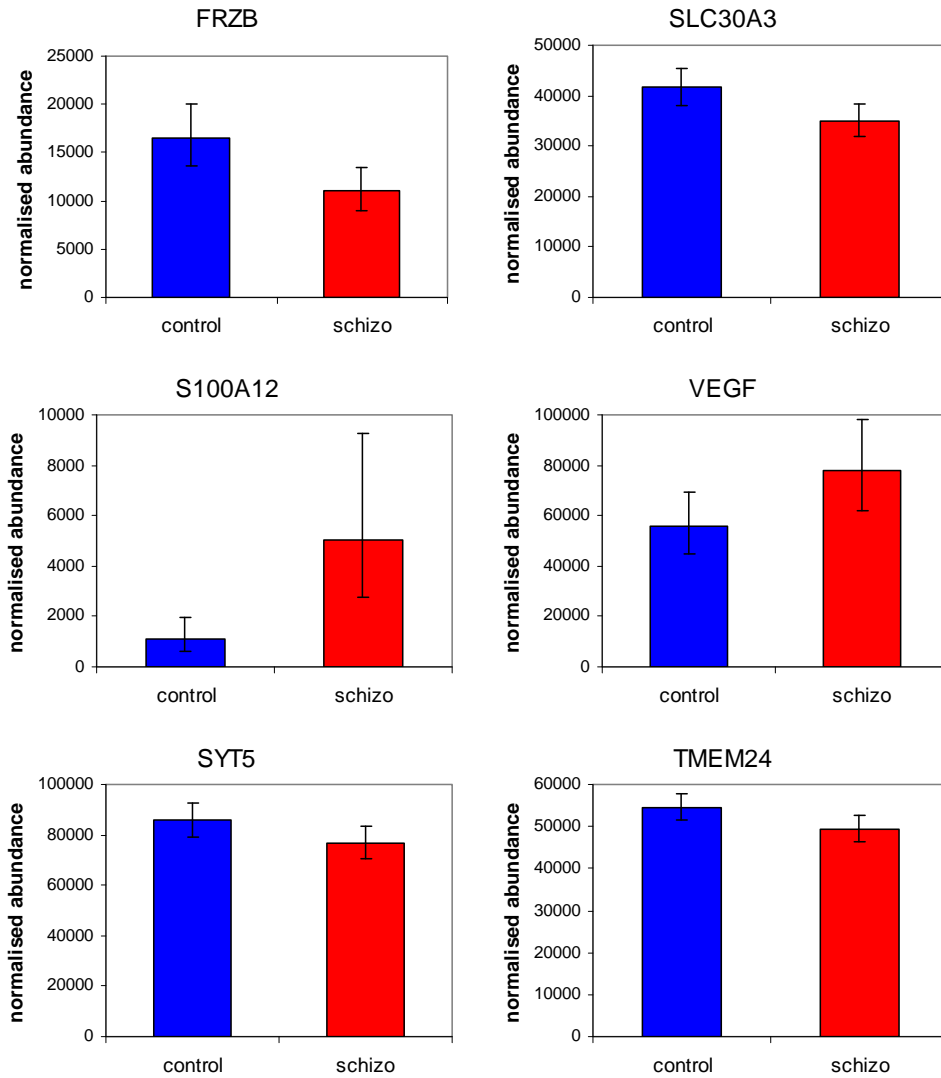
Supplementary Figure 2.
A) mRNA expression levels measured by Real Time PCR in post-mortem brain
samples of schizophrenics and controls (Geometric Mean with 95% confidence
intervals of normalised abundance, copies of total RNA detected per 20ng of total
RNA). Details of primers used are given in Supplementary Table 2.

B) Comparison of fold changes obtained for Microarray and QPCR and associated levels of significance.

	Microarray		QPCR	
	Fold Change	Corrected P-Value	Fold Change	Corrected P-Value
FRZB	-1.61	0.003	-1.45	0.008
SLC30A3	-2.00	0.003	-1.19	0.009
SYT5	-1.22	0.003	-1.12	0.050
TMEM24	-1.22	0.003	-1.11	0.020
VEGF	1.33	0.031	1.40	0.041
S100A12	1.75	0.0134	4.04	0.0007

Supplementary Table 2. Primers used for QPCR

Gene	Forward Primer Sequence	Reverse Primer Sequence	Probe sequence (5' FAM, 3' TAMRA)
FRZB	GAAGCTTCGTCATCTTGGACTCAG	ATTTAGTTGCGTGCTTGCCG	TCAGAAGTCTGGCAGGAACTCGAACCC
SLC30A3	TGTGTCTCTGTGCCTATGTGGC	CAGCACCAACAGAGGGATGG	TCTGCTCCATCCATGTGTCTGTTTGGG
SYT5	GAGACCGGCCAAAGCCAAG	AGAAGACAGGAATGGTTCAGATGG	CCCACCCTTAAGCTTCCTCTGACGGTT
TMEM24	GACCTCTCCAACGCAACGG	CCTTGGGTTTGGATTTGAAGG	TTTCTCGCCGCCTTATCAAGCGCTTT
VEGF	ATCCCTGTGGGCCTTGCTC	CCTCGGCTTGTCACATCTGC	TCCTGCAAAAACACAGACTCGCGTTGC
S100A12	GCTCCACATTCCTGTGCATTG	TGGTGTTTGCAAGCTCCTTTG	CATCTGGAGGGAATTGTCAATATCTTCCACC
ACTB	GAGCTACGAGCTGCCTGACG	GTAGTTTCGTGGATGCCACAGGACT	CATCACCATTGGCAATGAGCGGTTCC
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG	ACCACAGTCCATGCCATCACTGCCAT
PPIA	CATCTGCACTGCCAAGACTGA	CCACAATATTCATGCCTTCTTTCA	CCAAACACCACATGCTTGCCATCCA