

Original Research Article

Molecular Profiles of Pyramidal Neurons in the Superior Temporal Cortex in Schizophrenia

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Abstract: Disrupted synchronized oscillatory firing of pyramidal neuronal networks in the cerebral cortex in the gamma frequency band (i.e., 30–100 Hz) mediates many of the cognitive deficits and symptoms of schizophrenia. In fact, the density of dendritic spines and the average somal area of pyramidal neurons in layer 3 of the cerebral cortex, which mediate both long-range (associational) and local (intrinsic) corticocortical connections, are decreased in subjects with this illness. To explore the molecular pathophysiology of pyramidal neuronal dysfunction, we extracted ribonucleic acid (RNA) from laser-captured pyramidal neurons from layer 3 of Brodmann's area 42 of the superior temporal gyrus (STG) from postmortem brains from schizophrenia and normal control subjects. We then profiled the messenger RNA (mRNA) expression of these neurons, using microarray technology. We identified 1331 mRNAs that were differentially expressed in schizophrenia, including genes that belong to the transforming growth factor beta (TGF- β) and the bone morphogenetic proteins (BMPs) signaling pathways. Disturbances of these signaling mechanisms may in part contribute to the altered expression of other genes found to be differentially expressed in this study, such as those that regulate extracellular matrix (ECM), apoptosis, and cytoskeletal and synaptic plasticity. In addition, we identified 10 microRNAs (miRNAs) that were differentially expressed in schizophrenia; enrichment analysis of their predicted gene targets revealed signaling pathways and gene networks that were found by microarray to be dysregulated, raising an interesting possibility that dysfunction of pyramidal neurons in schizophrenia may in part be mediated by a concerted dysregulation of gene network functions as a result of the altered expression of a relatively small number of miRNAs. Taken together, findings of this study provide a neurobiological framework within which specific hypotheses about the molecular mechanisms of pyramidal cell dysfunction in schizophrenia can be formulated.

Keywords: cerebral cortex, gene expression profiling, laser-capture microdissection, microRNA, schizophrenia

INTRODUCTION

Gray matter volume in the cerebral cortex has been consistently found to be decreased in patients with schizophrenia (Ananth et al., 2002; Wilke et al., 2001). The superior temporal gyrus (STG) is one of the cortical regions that exhibit the most pronounced volumetric reduction (McCarley et al., 1999; Yamasue et al., 2004). This reduction is thought to reflect a decrease in the number of synapses (Selemon & Goldman-Rakic, 1999), the

majority of which appear to be located on the dendritic spines of pyramidal neurons (Glantz & Lewis, 2000; Sweet et al., 2009).

Pyramidal neurons in the cerebral cortex exhibit layer-specific connective properties, providing neural circuit architectures that support distinct aspects of higher cortical functions. For instance, dendritic spines on pyramidal neurons in layer 3 are targeted by both local and long-range glutamatergic projections in a highly reciprocal fashion. Synchronized activities of pyramidal

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neuronal networks through these connections, especially in the gamma frequency band (i.e., 30–100 Hz), are critical for the integrity of higher cortical functions (Buzsaki & Draguhn, 2004). Disturbances of these networks may contribute to the pathophysiology of schizophrenia by compromising gamma oscillation (Arnsten et al., 2010; Lewis & Gonzalez-Burgos, 2008; Uhlhaas & Singer, 2010). This concept is supported by the following postmortem and clinical observations. First, the density of dendritic spines on pyramidal neurons in layer 3 of the cerebral cortex, including the STG, have been shown to be decreased by 23–66% in subjects with schizophrenia (Costa et al., 2001; Garey et al., 1998; Glantz & Lewis, 2000; Sweet et al., 2009). Second, consistent with these findings, the average somal area of these pyramidal cells is significantly smaller (Sweet et al., 2003, 2004). Third, in the prefrontal cortex, the density of glutamatergic axonal boutons, of which dendritic spines are their major targets, was significantly decreased by as much as 79% in layer 3 (but not in layer 5) in subjects with schizophrenia (Bitanirwe et al., 2009). Finally, an increasing number of clinical studies have consistently demonstrated that gamma oscillatory synchrony is impaired in patients with schizophrenia (Cho et al., 2006; Kwon et al., 1999; Spencer et al., 2004; Uhlhaas & Singer, 2010). Furthermore, gamma impairment has been linked to the symptoms and cognitive deficits of the illness, and their severity has in turn been associated with the magnitude of cortical gray matter reduction (Cascella et al., 2010; Gur et al., 1999, 2000; Mitelman et al., 2003; Suga et al., 2010). Taken together, understanding the molecular underpinnings of pyramidal cell functional impairment will shed important light onto the pathophysiology and thereby the conceptualization of novel and rational treatment of cortical dysfunction in schizophrenia.

In order to identify the molecular correlates of pyramidal cell dysfunction in schizophrenia, we combined laser-capture microdissection (LCM) with Affymetrix microarray and high-throughput TaqMan-based MegaPlex quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR), respectively, to elucidate the alterations in messenger RNA (mRNA) and microRNA (miRNA) expression profiles of these neurons in layer 3 of the STG. We found that transforming growth factor beta (TGF- β) and bone morphogenetic protein (BMP) signaling pathways and many genes that regulate extracellular matrix (ECM), apoptosis and cytoskeleton were dysregulated in schizophrenia. In addition, we identified 10 differentially expressed miRNAs; the predicted targets of which included the dysregulated pathways and gene networks identified by microarray analysis. Together these findings provide a conceptual framework within which we can begin to formulate and test specific hypotheses of the neurobiological mechanisms that underlie pyramidal cell dysfunction in schizophrenia.

MATERIALS AND METHODS

Postmortem Human Brain Tissue

Liquid nitrogen vapor fresh-frozen blocks, approximately 3 mm thick and containing the STG (Brodmann's area 42), matched for age, sex, pH, and postmortem interval (PMI) (Table 1), were obtained from the Harvard Brain Tissue Resource Center (HBTRC) at McLean Hospital, Belmont, Massachusetts. Postmortem human brain collection procedures of the HBTRC, including the informed consent process, have been approved by the Partners Human Research Committee. Written informed consent for use of each of the brains for research has been obtained by the legal next of kin. All of the brains included in this study were examined by a Board-certified neuropathologist to rule out any neurological disorders. In addition, the fact that none of the subjects had any active history of substance abuse or dependence was confirmed by toxicological analysis. A detailed methodology for tissue preparation, LCM and RNA processing has been described in detail elsewhere (Pietersen et al., 2009, 2011).

LCM

Sections of 8 μ m were cut on a cryostat, mounted on slides and stored at -80°C until use. Pyramidal neurons were stained with the Histogene quick staining kit (Applied Biosystems, Foster City, CA) and identified based on the pyramidal morphology and the clearly identifiable proximal apical and basal dendrites (Figure 1). In addition, pyramidal neurons that were in close proximity to any nonpyramidal cells were excluded in order to avoid cellular contamination. Pyramidal neurons were removed using the Arcturus XT system (Applied Biosystems). Approximately 500 neurons per subject were captured onto a CapSure HS LCM cap (Applied Biosystems) for the microarray experiment whereas approximately 100 cells were captured for miRNA profiling. To avoid systematic biases, samples from schizophrenia and normal control subjects were processed for LCM in a random order.

Affymetrix Platform–Based Microarray Gene Expression Profiling

RNA Processing

RNA isolation was performed using the Picopure RNA isolation kit (Life Technologies, Grand Island, NY), with a DNase step (Qiagen, Valencia, CA). This typically resulted in approximately 1–25 ng of total RNA (Table 2) (Pietersen et al., 2009, 2011). Total RNA integrity was evaluated by RNA Quality Indicator (RQI) generated

Table 1. Subjects included in this study.

Group	Sex ^a	Age ^b	PMI ^c	pH	Smoker	Cause of death	Antipsychotic ^d
Control	F	79	15.00	6.59	Yes	Cardiac arrest	N/A
Control	M	22	21.47	6.75	Yes	Myocardial infarction	N/A
Control	M	75	20.25	6.35	Yes	Unknown	N/A
Control	M	80	15.50	6.26	Yes	Myocardial infarction	N/A
Control	F	58	21.08	6.79	No	Myocardial infarction	N/A
Control	M	61	17.00	6.64	Yes	Unknown	N/A
Control	F	71	20.50	6.89	Unknown	Unknown	N/A
Control	F	90	12.66	6.10	Unknown	Lung cancer	N/A
Control	F	86	6.92	5.74	Unknown	Myocardial infarction	N/A
Mean ± SEM	4M/5F	69.11 ± 6.85	16.71 ± 1.60	6.46 ± 0.12			
Schizophrenia	F	93	6.92	6.13	Unknown	Renal failure	Perphenazine (50)
Schizophrenia	M	55	21.40	6.51	Yes	Myocardial infarction	Perphenazine
Schizophrenia	F	67	21.80	5.80	Unknown	Pulmonary disease	Thioridazine, prochlorperazine (200)
Schizophrenia	F	55	22.00	5.90	Yes	Cancer	Clozapine, olanzapine
Schizophrenia	M	36	17.97	6.45	Unknown	Cardiac arrest	Clozapine (350)
Schizophrenia	M	62	10.75	6.50	Yes	Chronic obstructive pulmonary disease, lung cancer	Clozapine
Schizophrenia	F	92	17.80	6.34	Unknown	Cardiomyopathy	Unknown
Schizophrenia	M	56	21.83	6.75	No	Car accident	Olanzapine (150)
Schizophrenia	F	88	13.33	6.65	No	Unknown	Thiothixene (50)
Mean ± SEM	4M/5F	68.11 ± 6.60	16.90 ± 1.85	6.34 ± 0.11			

^aF = female; M = male.

^bAge is given in years.

^cPMI = postmortem interval in hours.

^dN/A = not applicable; numbers in parentheses indicate CED.

by the Experion HighSens LabChip (Bio-Rad, Hercules, CA), although in a minority of cases the amount of total RNA was too small for this analysis (Table 2).

RNA extracted from laser-captured neurons underwent two rounds of linear amplification using the RiboAmp kit (Life Technologies) to obtain amplified (a)RNA. A dilution of the resulting products (approximately 250 ng/μL) was used to determine the distribution of transcript lengths with the Experion StdSens Labchip (Bio-Rad; Figure 1). The concentration and purity of these samples were determined by absorbance measurements at the optical density of A260 and A280, using a NanoDrop spectrophotometer (Thermo Scientific, Tewksbury, MA) (Table 2).

Microarray and qRT-PCR Validation

The TURBO Biotin labeling kit (end-labeling; Life Technologies) was used to label the aRNA obtained from amplified samples (~15 μg). Gene expression profiling was performed using the Affymetrix Human X3P GeneChip®, which possesses an extreme 3' bias in its probe design and hence is particularly suitable for samples that are prone to RNA degradation, such as postmortem human brain tissue. This chip has also been shown to be superior to the more commonly used Affymetrix human U133 plus 2.0 chip in terms of data reproducibility (Caretto et al., 2008). The hybridization and scanning procedures were performed at

the Partners HealthCare Center for Personalized Genetic Medicine, Cambridge, Massachusetts. Briefly, target labeling was performed using the Message-AMP Biotin Enhanced Kit (Life Technologies). Fifteen micrograms of biotinylated RNA was fragmented, hybridized to GeneChips, stained with two rounds of streptavidinphycoerythrin (Molecular Probes, Eugene, OR) and one round of biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA), scanned twice, and inspected for hybridization artifacts before proceeding to data normalization and analysis.

For validation of microarray data, cDNA was reverse transcribed from aRNA (200 ng input) using the High Capacity RNA-to-cDNA kit (Applied Biosystems). TaqMan®-based qRT-PCR (Applied Biosystems) was subsequently performed on selected differentially expressed genes within signaling pathways identified by pathway analysis as dysregulated in schizophrenia and several randomly chosen genes (Supplementary Table 1; available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>). Samples were normalized with respect to the housekeeping gene, hypoxanthine guanine phosphoribosyltransferase (HPRT), which has been shown to produce reliable results in human brain tissue as its expression does not appear to differ in disease states (Radonic et al., 2004). Normalization was performed against only one gene because of the limited quantity of RNA available from laser-captured samples. Negative controls (negative reverse transcription

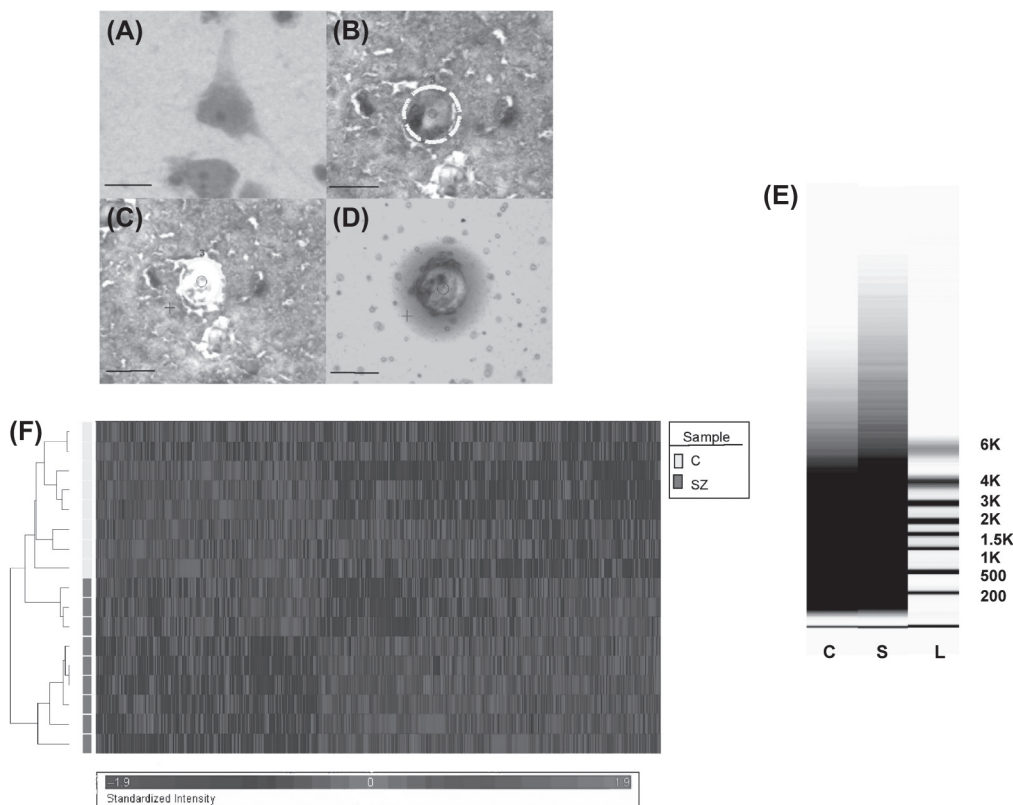


Figure 1. Identification of pyramidal neurons and LCM procedure. Photomicrograph of a pyramidal neuron in the STG visualized with Histogene stain (A). After identification (B), pyramidal neurons are captured (C) onto the LCM cap (D). (E) Representative virtual gels showing the distribution of sizes of amplified products after two rounds of linear amplification of RNA extracted from ~500 pyramidal neurons from a normal control (C) and a schizophrenia (S) subject. L = ladder. (F) Heatmap of 1331 differentially expressed genes identified based on the stringency criteria of fold-change > 1.1 and FDR-corrected $p < 0.05$. Scale bars = 25 μm .

and no template controls) were performed to detect any contamination of the samples, such as genomic (g)DNA. Where necessary, samples were treated with Turbo DNA-free kit (Applied Biosystems) and the negative controls repeated to confirm gDNA removal before qRT-PCR was performed. Samples were run in duplicate. If the difference between the duplicates exceeded $0.5 C_t$ (threshold cycle), the samples were repeated. The average of the duplicates was taken as input for quantification using the $2^{-\Delta C_t}$ method (Livak & Schmittgen, 2001) or the relative expression software tool (REST) for group-wise comparisons of expression ratios (Pfaffl et al., 2002). A Spearman's rho correlation analysis was then performed on the fold-changes determined by microarray and qRT-PCR. In accordance with Morey et al. (2006), a correlation of > 0.8 with a significance of $p < 0.05$ between qRT-PCR and microarray expression changes was considered validation of the microarray result.

miRNA Profiling Using Megaplex miRNA TaqMan Arrays

Total RNA was extracted from ~100 laser-captured cells with the *mirVana* miRNA Detection Kit (Life

Technologies). This typically resulted in approximately 4–7 ng of total RNA (Table 2). The concentration and purity of the total RNA were determined by a NanoDrop spectrophotometer, as described above. miRNA profiling was conducted according to manufacturer's specifications (Supplementary Methods 1 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>).

Data Analysis

Microarray Data Analysis

Each array was scanned twice and the Affymetrix Microarray Suite 5.1 software averaged the two images to compute an intensity value for each probe cell within each probe set. For the quality control step, we employed the dChip and Partek software's built-in function (Partek, St. Louis, MO). We then normalized the data with Partek's standard normalization method (i.e., data have a mean of 0 and a variance of 1, and each column for each sample was divided by the average of all control samples). Principal component analysis revealed the contribution

Table 2. RNA quantity and quality.

Group	RIN of total RNA extracted from homogenized cortex ^a	Microarray samples			
		Number of cells captured	Total RNA RQI ^b	Amount of RNA before/after amplification (μg)	A260/A280
Control	8.2	510	2.6	0.025/39.50	2.67
Control	7.9	530	N/A	0.004/69.30	2.37
Control	8.9	520	3.1	0.014/54.30	2.44
Control	8.8	500	2.8	0.004/39.49	2.51
Control	7.8	680	3.4	0.007/50.10	2.39
Control	9	600	3.7	0.009/59.92	2.54
Control	8.4	530	2.8	0.012/39.60	2.47
Control	8	520	3.6	0.007/29.83	2.75
Control	8.8	520	N/A	0.002/24.52	2.70
Mean ± SD	8.42 ± 0.15	545.56 ± 19.30	3.14 ± 0.16	0.009 ± 0.002/40.73 ± 5.15	2.54 ± 0.05
Schizophrenia	8.9	525	3.8	0.004/76.83	2.47
Schizophrenia	8	525	4.2	0.003/65.10	2.43
Schizophrenia	7.9	540	N/A	0.002/62.10	2.52
Schizophrenia	8.5	540	2.8	0.004/46.92	2.75
Schizophrenia	9.2	530	N/A	0.001/62.10	2.44
Schizophrenia	8.5	630	N/A	0.002/75.90	2.34
Schizophrenia	8.2	540	3.5	0.003/65.68	2.50
Schizophrenia	8.8	700	3.4	0.003/50.10	2.47
Schizophrenia	8.4	550	3.4	0.003/52.20	2.47
Mean ± SD	8.48 ± 0.16	564.56 ± 20.06	3.52 ± 0.19	0.002 ± 0.0003/61.88 ± 3.54	2.49 ± 0.04

^aRNA integrity number (RIN) of total RNA extracted from homogenized gray matter determined by an Agilent bioanalyzer.

^bRNA Quality Indication (RQI) of total RNA extracted from laser-captured neurons determined by a BioRad Experion Automated Electrophoresis System. N/A = not available: total RNA concentration too low to determine RQI.

of batch effect (i.e., scan date), but not sex, age, PMI, or antipsychotic treatment, to the observed expression variance. As such, an analysis of covariance (ANCOVA) was performed with batch effect as covariate (Simunovic et al., 2009). Differentially expressed genes were visualized by performing unsupervised hierarchical clustering as stringency of the filtering criteria (i.e., fold-change and false discovery rate [FDR]-adjusted p-value) was varied to determine a representative gene list for pathway analyses.

We employed three approaches to explore the biological significance of our expression data. First, differentially expressed genes were manually grouped to elucidate gene families that were dysregulated in schizophrenia. Second, pathway analyses were performed with two Web-based algorithms, *Ingenuity Pathway Analysis* (Ingenuity Systems, Redwood City, CA) and *MetaCore* (GeneGo, Carlsbad, CA), to map the differentially expressed genes onto biological functions and canonical pathways. With *Ingenuity*, the significance for each of the identified pathways was determined via a Fisher's exact test, whereas GeneGo *Metacore* makes use of their algorithm for hypergeometric distribution, identifying pathways overrepresented with significant genes. Third, literature mining was performed to elucidate which of these pathways or gene families might be particularly

pertinent for pyramidal neuronal functions and dendritic/synaptic architecture and plasticity. We also made note of differentially expressed genes that have been implicated as schizophrenia risk genes (Allen et al., 2008). Microarray data have been deposited into the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/projects/geo/>; accession number GSE37981).

miRNA Data Analysis

Primary analysis of the acquired signal data was performed in SDS and RQ Manager (Applied Biosystems). Quality control and differential expression analyses were performed using the qRT-PCR package for miRNA arrays (Applied Biosystems) in Bioconductor (www.bioconductor.org). The databases used to identify potential miRNA targets for specific miRNAs are summarized in Supplementary Methods 2 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>. Significant enrichment of specific Gene Ontology categories or KEGG (Kyoto Encyclopedia of Genes) pathways was estimated by hypergeometric tests or chi-square tests. Enriched pathways overrepresented by potential miRNA target genes were then determined with the p-value obtained by hypergeometric tests and adjusted for multiple comparisons (Kanehisa & Goto, 2000; Kanehisa et al., 2006, 2010).

RESULTS

Affymetrix-Based Microarray Gene Expression Profiling

We evaluated the reliability of our findings by assessing mRNA quality based on a combination of metrics, as there is not a single parameter that can serve as the sole determinant or predictor of mRNA quality. Although the quality of the total RNA extracted from homogenized cortex from all of the subjects used in this study appears to be superb (Table 2), the RQI of most samples on average tended to be relatively low, suggesting some RNA degradation. This was expected given the significantly lengthier (i.e., compared with extracting RNA from homogenized tissue) aqueous steps required for RNA extraction from single neurons (Pietersen et al., 2009, 2011). However, although RQI is a measure of total RNA integrity, it does not necessarily predict the quality of mRNA, which constitutes only 1–3% of total RNA (Vermeulen et al., 2011). In fact, virtual gels of the amplified products generated after two rounds of linear amplification of RNA extracted from pyramidal neurons revealed very healthy spreads of transcript lengths, extending well into the 5–6 kb range (Figure 1). Furthermore, the efficiency of microarray hybridization appeared to be adequate in terms of probe intensity and percentage of present calls, and these parameters were highly comparable between the schizophrenia and control groups, with average (\pm SD) probe intensity being 81.8 ± 10.5 and 70.7 ± 6.2 , respectively, and percent present calls 26.9 ± 2.6 and 27.5 ± 1.0 , respectively. Overall, these percentages of present calls are lower than what have been reported in previously published schizophrenia microarray studies performed on RNA extracted from homogenized cortical gray matter (typically in the range of 40–45%), which contains a much greater number of RNA species in much larger quantities. Our data, however, are similar in magnitude to what was reported in a recent microarray study of laser-dissected hippocampal subfields in humans (mean \pm SD = 31.8 ± 4.9) (Benes et al., 2008), a region that is cellularly much less heterogeneous than cortical gray matter but more so than our neuronal samples, and to what was described in previous microarray studies based on laser-captured cells from clinical samples or other specimens of single cells (Luzzi et al., 2001; Mahadevappa & Warrington, 1999).

Varying the stringency criteria of fold-change and FDR-adjusted p-value resulted in several lists of differentially expressed genes (Supplementary Table 2 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>). For instance, 1331 genes were identified based on the criteria of a minimal fold-change of 1.1 and $p < 0.05$, whereas only 264 genes met the slightly more stringent criteria of fold-change of 1.2 and $p < 0.05$. As a result, the 1331 genes were used for downstream

pathway analysis. Hierarchical clustering revealed that these 1331 genes appeared to be well segregated according to diagnosis (Figure 1). A complete list of these genes is shown in Supplementary Table 3 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>. The canonical pathways and functional gene networks that were found to be altered in schizophrenia included the TGF- β /BMP signaling cascades, genes that regulate cytoskeleton and dendritic morphology and ECM composition, in addition to changes in apoptosis and DNA damage canonical pathways (Tables 3a–d). A number of key genes within the pathways that were found to be significantly differentially regulated in schizophrenia ($N = 6$) and several randomly selected genes ($N = 4$) were quantified by qRT-PCR; the expression levels of these genes were significantly correlated with the microarray data ($p = 0.003$; Figure 2). HPRT expression level also did not differ between schizophrenia and normal control groups (Supplementary Figure 1 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>). Finally, correlation analyses between the expression of the validated genes and age, PMI, and chlorpromazine equivalent dosage (CED) revealed no association of gene expression changes with any of these parameters, consistent with PCA results showing that none of these parameters appear to significantly contribute to differential gene expression variance of the entire data set. A representative correlation with *SMAD4* (signal via the mothers against decapentaplegic homologs 4), the key transcription factor upon which TGF- β and BMP signaling cascades converge, is depicted in Supplementary Figure 2 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>.

TGF- β Superfamily Signaling Is Altered in Schizophrenia

The TGF- β superfamily of proteins consists of over thirty members (Derynck & Miyazono, 2008). Structurally they can be subdivided in three subfamilies: TGF- β , inhibins, and other proteins including BMPs and growth and differentiation factor (GDF). Together these proteins are involved in very diverse and complex cellular functions in both developing and adult systems, including the brain (Benes, 2011; Derynck & Miyazono, 2008; Massague & Gomis, 2006; Vivien & Ali, 2006). We found that the TGF- β and BMP signaling canonical pathways were highly dysregulated in schizophrenia (*GeneGo*: $p < 0.0001$), with up-regulation of several genes immediately downstream from the TGF- β 1 receptor (Supplementary Table 3a, Figure 3 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>; Figure 3). This is in line with a previous postmortem study revealing an up-regulation of the TGF- β signaling cascade in the hippocampus of schizophrenia subjects (Benes et al., 2007) and is consistent with previous reports showing the

Table 3a. Significantly affected genes within TGF- β /BMP signaling pathway.

Gene title	Gene symbol	p-Value	Fold-change (S vs. C) ^a
bone morphogenetic protein 1	BMP1	0.04	- 1.33
bone morphogenetic protein receptor, type IA	BMPR1A	0.04	1.19
bone morphogenetic protein 5	BMP5	0.04	1.16
bone morphogenetic protein 7	BMP7	0.03	1.21
caveolin 1, caveolae protein, 22 kDa	CAV1	0.03	1.54
CREB-binding protein	CBP	0.01	1.31
growth arrest and DNA-damage-inducible, beta	GADD45B	0.03	1.30
mitogen-activated protein kinase kinase 6	MEK6	0.04	- 1.14
ribosomal protein S6 kinase, 90kDa, polypeptide 5	RPS6KA5	0.01	1.11
SHC (Src homology 2 domain containing)-transforming protein 1	SHC1	0.03	1.16
SMAD family member 4	SMAD4	0.01	1.19
SMAD family member 5	SMAD5	0.02	1.48
SKI-like oncogene	SKIL	0.02	- 1.15
transcription factor specificity protein 1	SP1	0.03	1.29
X-linked inhibitor of apoptosis	XIAP	0.02	1.10
zinc finger, FYVE domain containing 9	ZFYVE9/SARA	0.04	1.14

^aS = schizophrenia; C = control.

Table 3b. Significantly affected genes associated with extracellular matrix.

Gene title	Gene symbol	p-Value	Fold-change (S vs. C) ^a
aggrecan	ACAN	0.03	- 1.26
ADAM metalloproteinase with thrombospondin type 1 motif, 1	ADAMTS1	0.03	2.56
ADAM metalloproteinase with thrombospondin type 1 motif, 6	ADAMTS6	0.05	1.15
hyaluronan and proteoglycan link protein 1	HAPLN1	0.05	- 1.14
leucine proline-enriched proteoglycan (leprecan) 1	LEPRE1	0.04	- 1.21
lumican	LUM	0.03	- 1.12
matrix metalloproteinase 16 (membrane-inserted)	MMP16	0.02	- 1.17
matrix metalloproteinase 25	MMP25	0.02	- 1.14
matrix metalloproteinase 24 (membrane-inserted)	MMP24	0.01	1.22
sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	SPAM1	0.04	1.15
spare/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 3	SPOCK3	0.01	1.11
spondin 1, extracellular matrix protein	SPON1	0.02	2.14
versican	VCAN	0.04	- 1.13

^aS = schizophrenia; C = control.

Table 3c. Significantly affected genes associated with apoptosis and DNA damage.

Gene title	Gene symbol	p-Value	Fold-change (S vs. C) ^a
Apoptosis and survival_BAD phosphorylation			
epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	EGFR	0.02	1.59
phosphoinositide-3-kinase, regulatory subunit 2 (beta)	PIK3R2	0.04	1.23
protein kinase, cAMP-dependent, catalytic, gamma	PRKACG	0.02	1.25
protein tyrosine phosphatase, non-receptor type 11	PTPN11	0.03	- 1.11
SHC (Src homology 2 domain containing)-transforming protein 1	SHC1	0.03	1.16
DNA damage_Role of SUMO in p53 regulation			
CREB-binding protein	CBP	0.01	1.31
SMT3 suppressor of mif two 3 homolog 1 (<i>S. cerevisiae</i>)	SUMO-1	0.03	1.17
ubiquitin-like modifier activating enzyme 1	UBA1	0.04	1.14
Other genes involved in DNA damage and oxidative stress			
HLA-B (major histocompatibility complex, class I, B)-associated transcript 3	BAT3/BAG6	0.01	- 1.21
calpain 9	CAPN9	0.05	1.15
calpain 10	CAPN10	0.02	1.14
clusterin	CLU	0.02	2.02

^aS = schizophrenia; C = control.

Table 3d. Significantly affected genes associated with cytoskeletal and dendritic integrity.

Gene title	Gene symbol	p-Value	Fold-change (S vs. C) ^a
actin, gamma 2, smooth muscle, enteric	ACTG2	0.04	1.35
dystonin	DST	0.03	1.25
major histocompatibility complex, class I, A	HLA-A	0.02	1.86
integrin, beta 5	ITGB5	0.02	1.51
microtubule-associated protein 1 light chain 3 gamma	MAP1LC3C	0.03	1.19
myosin IIIB	MYO3B	0.01	1.13
tropomodulin 3 (ubiquitous)	TMOD3	0.02	-1.14
tubulin polymerization promoting protein	TPPP	0.03	-1.50
tubulin tyrosine ligase-like family, member 12	TTL12	0.02	1.20
tubulin, alpha 4b (pseudogene)	TUBA4B	0.02	-1.17
spectrin, beta, non-erythrocytic 1	SPTBN1	0.05	1.20

^aS = schizophrenia; C = control.

involvement of TGF- β signaling in the pathophysiology of schizophrenia in general (Miller et al., 2011). Within the TGF- β superfamily, we found that the canonical BMP signaling pathway was significantly affected (*Ingenuity*: $p < 0.003$; *GeneGo*: $p < 0.005$) (Table 3a). Specifically, we observed an up-regulation of *BMP7* and BMP receptor 1A (*BMPR1A*), in addition to changes in genes

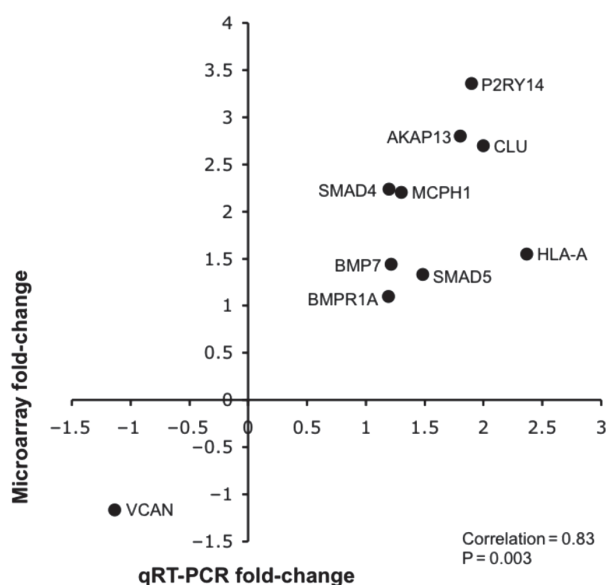


Figure 2. Correlation analysis comparing fold-changes of selected genes determined by microarray and qRT-PCR. Comparison of fold-changes of differentially expressed genes within signaling pathways identified as dysregulated in schizophrenia ($N = 6$) and several randomly selected genes ($N = 4$) determined by microarray and qRT-PCR. *BMP7* = bone morphogenetic protein 7; *BMPR1A* = bone morphogenetic protein receptor type IA; *CLU* = clusterin; *HLA-A* = major histocompatibility complex (MHC), class I, A; *HPRT* = hypoxanthine guanine phosphoribosyl transferase; *P2RY14* = purinergic receptor P2Y, G-protein coupled, 14; *MCPH1* = microcephalin 1; *SMAD4* = mothers against decapentaplegic homolog 4; *SMAD5* = mothers against decapentaplegic homolog 5; *VCAN* = versican.

further downstream in the signaling cascade (Figures 3 and Supplementary Figure 3 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>). Both the TGF- β and BMP canonical pathways propagate their signal via SMADs (Shi & Massague, 2003) (Figures 3 and Supplementary Figure 3 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>) and we found that *SMAD4* and *SMAD5* were up-regulated in schizophrenia. *SMAD4* is the central mediator of both of these signal transduction cascades and regulates the transcription of target genes (Ross & Hill, 2008). Furthermore, we observed an increase in the expression of cyclic adenosine monophosphate (cAMP) response element-binding protein

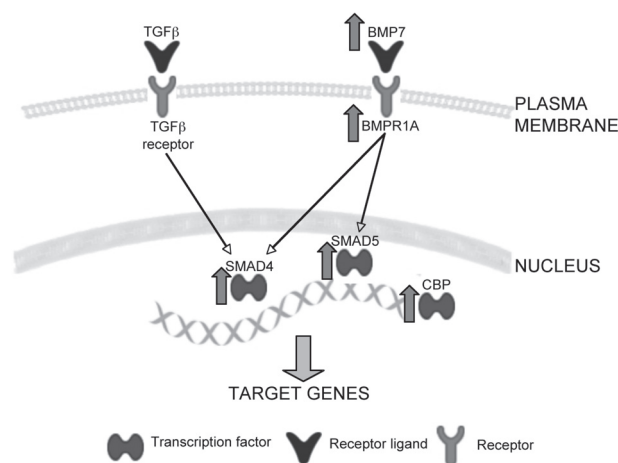


Figure 3. Representative diagram of TGF- β and BMP signaling pathways. Both TGF- β and BMP7 activate *SMAD4* and CREB-binding protein, a transcription co-activator of *SMAD*, leading to the transcription of target genes that influence extracellular matrix composition, apoptosis, and synaptic/cytoskeletal plasticity. *BMPR1A* = bone morphogenetic protein receptor type IA; *CBP* = CREB-binding protein; *SARA* (*ZFYVE9*) = zinc finger, FYVE domain containing 9; *SMAD4* = mothers against decapentaplegic homolog 4. Genes up-regulated in schizophrenia are depicted by red arrows.

(CREB)-binding protein (CBP), a transcription co-activator of SMAD (Ohta et al., 2008). Although not part of this particular canonical pathway, we noted a down-regulation of *BMP5*, which also acts via the *BMPRI1A* (Beck et al., 2001). Of interest, *BMP5*, *BMP7*, *SMAD5*, and *CBP* were all found to be significantly associated with schizophrenia in a recent genome-wide association study (Jia et al., 2010), with both *TGFB1* and *SMAD5* implicated as potential candidate genes in a linkage study (Zaharieva et al., 2008). Finally, these pathways are known to regulate the integrity of ECM (Hyytiainen et al., 2004; Smith & Strunz, 2005; Zhou et al., 2003), apoptosis (Dhandapani & Brann, 2003), and cytoskeleton (Guo et al., 1998; Moustakas & Heldin, 2008), raising the possibility that disturbances of genes that regulate these functions (see below) may in part represent downstream consequences of dysregulated TGF- β /BMP signaling.

ECM Regulation Is Altered in Schizophrenia

Gene networks related to ECM functionality, including altered cellular movement ($p < 0.03$) and changes in adhesion to the fibronectin matrix ($p < 0.05$), were altered in schizophrenia (Table 3b). Specifically, a number of genes coding for chondroitin sulfate proteoglycans (CSPGs) were down-regulated. These include aggrecan, versican, leprecan, lumican, testican, and spondin1. In addition, we found changes in genes that encode matrix metalloproteinases (MMPs), which are enzymes that are involved in the breakdown and remodeling of ECM by the proteolysis of several ECM components, including the CSPGs (Rauch, 2004). Specifically, we found an up-regulation of ADAM (a disintegrin and metalloproteinase) metalloproteinase with thrombospondin type 1 motif gene family (*ADAMTS1* and *ADAMTS6*), in addition to alterations in several other MMPs, in the schizophrenia samples (Table 3b). Together these data suggest that the decrease in ECM structural components, such as CSPGs, in addition to the alterations in the enzymes that regulate their proteolysis, contribute to the altered integrity of ECM (Ikeda et al., 2009; Mauney et al., 2013; Pantazopoulos et al., 2010).

Apoptosis Regulation Is Altered in Schizophrenia

Two apoptosis canonical pathways were found to be dysregulated in schizophrenia (Table 3c). We noted predominantly an up-regulation of genes within the p53-regulated apoptosis pathway (*GeneGo*: $p < 0.03$), suggesting pro-apoptotic events (Pietsch et al., 2008), although the exact function of sumoylation of p53 remains controversial (Melchior & Hengst, 2002). We also found changes in the expression of several genes associated with oxidative stress and DNA damage (Table 3c). Conversely, we saw increased expression of

anti-apoptotic genes within the BCL2 (B-cell chronic lymphocytic leukemia [CLL]/lymphoma 2)-associated agonist of cell death (BAD) apoptosis survival pathway (*GeneGo*: $p < 0.03$), which inhibits apoptosis through BAD phosphorylation (Bergmann, 2002). Interestingly, TGF- β signaling regulates BAD phosphorylation via the induction of anti-apoptotic proteins of the BCL-2 family (E. S. Kim et al., 1998). Together these results appear to reflect the complex interplay between the orchestrated molecular responses regulating apoptosis that may in part be mediated by TGF- β signaling.

Cytoskeletal Regulation Is Altered in Schizophrenia

We found significant alterations in key biological functions (*Ingenuity*) related to the integrity of cytoskeleton. This included the organization of actin cytoskeleton ($p < 0.05$) and actin filaments ($p < 0.05$), coalignment of neurofilaments and microfilaments ($p < 0.05$) and redistribution of F-actin ($p < 0.05$). We also noted changes in genes encoding cytoskeletal components, such as tubulin and myosin (Table 3d). Finally, we observed that the integrin $\beta 5$ receptor was up-regulated in schizophrenia. Integrin signaling is the major signaling pathway that mediates the interaction between ECM and the assembly of actin filaments that is necessary for cytoskeletal modification (Giancotti & Ruoslahti, 1999; Shi & Ethell, 2006; Wozniak et al., 2004).

miRNA Profiling by Megaplex miRNA TaqMan Arrays

miRNA expression levels were normalized to the endogenous sno-RNA MammU6, whose levels were unchanged between control and schizophrenia samples for both the Human miRNA A and B Cards (mean \pm SEM: 30.63 ± 0.35 and 31.72 ± 0.72 , respectively). Out of the 754 miRNAs investigated, analysis of variance (ANOVA) revealed 10 differentially expressed miRNAs ($p < 0.05$; Table 4). Many of these miRNAs are known to be expressed in the brain (Baskerville & Bartel, 2005; Landgraf et al., 2007), with some identified as being specifically localized in mammalian cortical neurons (e.g., miR-30b and miR-328) (J. Kim et al., 2004). A number of the differentially expressed miRNAs also correspond with previous studies on postmortem schizophrenia samples, including miR-150 (J. Kim et al., 2004), miR-328 (Santarelli et al., 2011), and miR-30b (Mellios et al., 2012; Perkins et al., 2007), with the caveat that all of the previously published miRNA profiling results were based on RNAs extracted from homogenized gray matter. Interestingly, enrichment analysis of the predicted target genes of the differentially expressed miRNAs revealed that the overrepresented signaling pathways included

Table 4. Differentially expressed miRNAs in schizophrenia.

miRNA assay name	p-Value	Log ₂ fold-change
hsa-miR-1243-002854	0.03	-0.41
hsa-miR-126-4395339	0.03	1.84
hsa-miR-150-4373127	0.05	-0.96
hsa-miR-30b-4373290	0.02	4.64
hsa-miR-328-4373049	< 0.001	2.82
hsa-miR-378-002243	0.04	-0.31
hsa-miR-520d-3p-002743	0.01	0.12
hsa-miR-628-5p-4395544	0.03	-1.05
hsa-miR-875-5p-002203	0.04	-0.41
hsa-miR-99b-4373007	0.04	2.77

many of the pathways identified by microarray as differentially regulated in schizophrenia, such as TGF- β signaling, ECM composition, apoptosis, and cytoskeleton (Table 5, Supplementary Table 3 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>). The specific genes that were found by microarray to be differentially expressed in schizophrenia and are also the predicted targets of the differentially expressed miRNAs are shown in Supplementary Table 4 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>.

DISCUSSION

In order to understand the pathophysiology of schizophrenia, which is a manifestation of functional disturbances of cortical circuits characterized by neuronal type-dependent connective complexity, it is necessary to define the molecular pathology of the illness in a neuronal type-specific manner. In this context, previous gene expression profiling studies of the cerebral cortex in schizophrenia have largely relied on the extraction of RNA from homogenized gray matter. Interpretation of these results can be difficult due to the profound cellular heterogeneity of the cerebral cortex, as gene expression changes that may be confined to a subset of neurons can be obscured, especially if transcript abundance is low or if specific transcripts are preferentially localized to cell types that are relatively uncommon. In this study, by combining LCM with mRNA profiling, we

circumvented these potential problems and identified molecular cascades that appear to be dysregulated in layer 3 pyramidal neurons in the STG in schizophrenia. Specifically, our data revealed that a number of genes within the TGF- β superfamily, especially those that regulate BMP signaling, were differentially expressed in subjects with schizophrenia. In addition, we identified abnormalities in genes that regulate apoptosis, cytoskeletal plasticity, and ECM, that may, at least in part, represent downstream consequences of TGF- β signaling dysregulation. We should also note that the majority (61%) of these genes, with some exceptions, such as those that regulate ECM, were found to be up-regulated. In contrast, previous studies interrogating the expression of mRNA extracted from homogenized gray matter have revealed an overall decrease in gene expression (Iwamoto & Kato, 2006), and, for the most part, these genes do not overlap with genes identified in the present study. These differential findings, in our opinion, highlight the argument that neuronal type-specific gene expression alterations and thus the molecular pathophysiology of the illness in terms of cortical neuronal circuit dysfunction cannot necessarily be inferred from the interrogation of expression of transcripts obtained from homogenized cortical tissue. In more specific quantitative terms, approximately 84% of the volume of the cortex, excluding layer 1, contains glia, neuropil, which comprises axonal elements, dendrites and dendritic spines, and vascular elements such as blood vessels and related cellular components, including endothelial cells and various blood cells (Braitenberg & Schuz, 1998), and the abundance of many transcripts can vary in a compartment-specific manner (Cajigas et al., 2012). Of the remaining 16% of the volume that contains neuronal bodies in layers 2–6, approximately 80% of them are pyramidal neurons and roughly 16% (i.e., 80% divided by 5 layers) are from layer 3. In other words, layer 3 pyramidal neurons represent only about 3% (i.e., 16% times 16%) of the entire volume of cortical gray matter. As such, it is difficult to envision that gene expression changes that may be restricted to this neuronal population can be readily discerned by studying RNA obtained from homogenization of the entire gray matter. This would arguably be especially true for the less abundant cell types, such as the various subsets of inhibitory neurons, including those that were examined in the accompanying study, the parvalbumin (PV)-containing neurons (Pietersen et al. 2014).

Table 5. Overrepresented KEGG pathways based on enrichment analysis of the predicted target genes of the differentially expressed miRNAs.

Pathway	Enrichment statistics
TGF- β signaling pathway	C = 155; O = 63; E = 8.75; R = 7.20; rawP = 1.27e-37; adjP = 1.45e-35
Focal adhesion	C = 201; O = 48; E = 11.35; R = 4.23; rawP = 9.92e-18; adjP = 1.46e-16
Regulation of actin cytoskeleton	C = 143; O = 37; E = 8.07; R = 4.58; rawP = 3.41e-15; adjP = 4.32e-14
Apoptosis	C = 82; O = 20; E = 4.63; R = 4.32; rawP = 2.12e-08; adjP = 8.06e-08
ECM-receptor interaction	C = 84; O = 16; E = 4.74; R = 3.37; rawP = 1.67e-05; adjP = 4.63e-05

Methodological Considerations and Limitations

The goal of this study was to delineate the gene expression profile of layer 3 pyramidal neurons in schizophrenia in order to gain insight into the molecular basis of cortical circuit dysfunction. In this context, it should be noted that obtaining RNA from a truly homogeneous source from postmortem human brains, given the technologies that are available at the present time, is probably not achievable. For instance, when capturing pyramidal neurons, it would have been inevitable to include additional elements, such as the perineuronal oligodendrocytes, GABA(γ -aminobutyric acid)ergic axon terminals that innervate the perisomatic region of pyramidal neurons, etc. In fact, we found that the mRNA for the gene that encodes the 65-kDa isoform of the GABA-synthesizing enzyme glutamic acid decarboxylase (*GAD2*) was significantly down-regulated by 1.9-fold in the schizophrenia subjects (Supplementary Table 3 available online at <http://informahealthcare.com/doi/abs/10.3109/01677063.2014.882918>). This is not a type I error, as qRT-PCR confirmed the presence of this transcript in our samples and its down-regulation by 3.1-fold. Because *GAD2* is clearly not expressed in pyramidal neurons and because our sampling approach excludes the possibility of systematic inclusion of GABAergic neurons in our samples, the *GAD2* mRNA in the samples most likely came from the GABAergic axon terminals innervating the pyramidal cell bodies. However, definitive confirmation of the presence of mRNA in axonal terminals in the human brain would be technically challenging.

Another consideration is that due to the labor-intensive nature of this methodological approach and the associated costs, the sample size of this study is small. This may have increased the likelihood of type II error. Although to a certain extent type I error can be addressed by means of validation (e.g., by qRT-PCR), type II error can only be minimized by increasing sample size. Finally, because the amount of RNA available for each sample was limited, the number of genes we were able to validate by qRT-PCR is small. For the same reason, we only used one housekeeping gene for normalization and samples were run in duplicate instead of triplicate. Because of these limitations and caveats, we consider this study proof-of-principle and our findings preliminary; it would therefore be critical to confirm these findings in a different and ideally larger cohort of subjects in future studies. Ultimately, true validation of microarray observations will have to be achieved by experimentally demonstrating mechanistic links between the disturbances of genes and pathways identified as altered in schizophrenia and known cellular and clinical phenotypes of the illness, such as decreased spine density on pyramidal neurons (Glantz & Lewis, 2000; Sweet et al., 2009) or disturbances of ECM integrity (Pantazopoulos et al., 2010), and neurocognitive

deficits (Barch et al., 2003; Bowie & Harvey, 2005; Cho et al., 2006) or gamma oscillation disturbances (Cho et al., 2006; Kwon et al., 1999; Spencer et al., 2004; Uhlhaas & Singer, 2010), respectively. In fact, this is exactly how postmortem microarray studies can uniquely contribute to the understanding of the pathophysiology of human psychiatric disorders; that is, they can inspire the conceptualization of novel hypotheses of disease mechanisms that can then be experimentally interrogated either in vitro or in animal systems.

Functional Considerations of Up-regulation of TGF- β and BMP Signaling in Schizophrenia

Apoptosis and Dendritic Atrophy

Up-regulation of TGF- β signaling typically represents a homeostatic response under a wide variety of pathological conditions, such as excitotoxicity (Chou et al., 2006; Dhandapani & Brann, 2003; Gabriel et al., 2003; Klempt et al., 1992; Petegnief et al., 2003). In schizophrenia, there has been compelling evidence indicating that the PV-containing fast-spiking inhibitory neurons, which confer perisomatic and axo-axonic inhibition to pyramidal neurons, are functionally disturbed (Lewis et al., 2005). Specifically, inhibition furnished by these neurons may be deficient, as the mRNA for the 67-kDa isoform of the GABA-synthesizing enzyme (*GAD1*) appears to be undetectable in close to half of the populations of PV neurons in schizophrenia (Hashimoto et al., 2003). In addition, the expression of the *N*-methyl-D-aspartate (NMDA) glutamate receptor NR2A subunit mRNA in some of these neurons has also been shown to be significantly decreased (Bitanhirwe et al., 2009), suggesting that excitatory inputs to PV neurons may also be impaired. Together these findings support the idea that pyramidal neurons that are postsynaptic to PV neurons may become hyperactive as a result of disinhibition of PV neurons (Homayoun & Moghaddam, 2007; Kinney et al., 2006; Olney & Farber, 1995). In this context, the observed up-regulation of TGF- β signaling may represent a homeostatic or neuroprotective response of pyramidal neurons to such excitotoxic insult (Figure 4) (Gabriel et al., 2003; Petegnief et al., 2003). One mechanism that TGF- β signaling could exert homeostatic effects is through the regulation of BAD phosphorylation pathway via the induction of anti-apoptotic proteins of the BCL-2 family (E. S. Kim et al., 1998; Prehn et al., 1994). In line with other postmortem studies indicating increased expression of anti-apoptotic genes in schizophrenia, such as BCL-2 (Benes, 2006; Benes et al., 2006), we also noted in our study an up-regulation of several genes within this pathway. Conversely, although we found an up-regulation of several pro-apoptotic genes within the p53-regulated pro-apoptosis pathway, e.g., *SUMO-1* (small ubiquitin

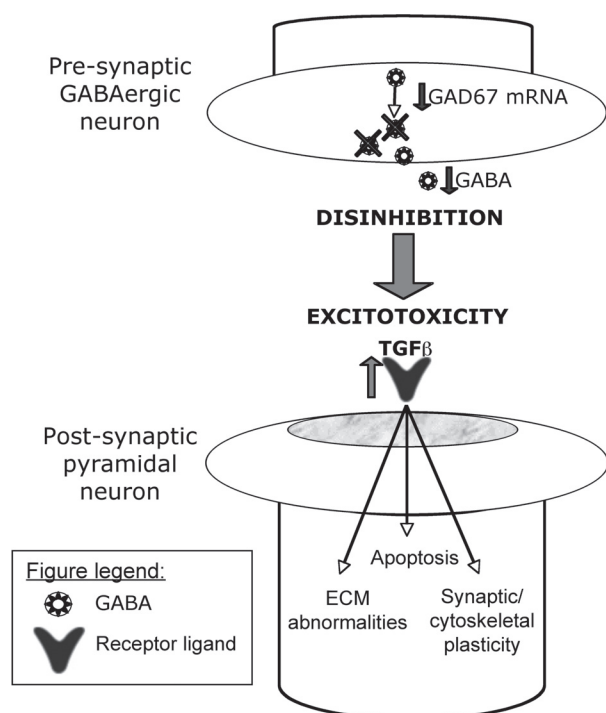


Figure 4. Hypothetical model of pyramidal cell dysfunction in schizophrenia. It is hypothesized that as a result of disinhibition-induced excitotoxic insult (see text for details), TGF β signaling within pyramidal neurons is up-regulated, which may contribute to extracellular matrix abnormalities, dysregulated apoptosis, and impaired synaptic and cytoskeletal plasticity. Shown in this highly simplified diagram are some of the hub genes that can be targeted in future experiments in the testing of this hypothesis.

related modifier precursor), *UBA1* (ubiquitin-like modifier activating enzyme 1), and *CBP*, expression of *TP53*, which encodes p53, the key initiator of apoptosis, was unaltered.

Although large-scale pyramidal cell death does not occur in schizophrenia, “nonlethal” apoptosis can lead to neuronal injury in the form of dendritic atrophy (Glantz et al., 2006). For example, chronic glutamate excess has been shown to lead to a 20% reduction in primary dendritic length without causing cell death (Esquenazi et al., 2002). The integrity of dendritic architecture depends on coordinated activities of various cytoskeletal components (Svitkina et al., 2010). Our results indicate that several cytoskeletal genes, including actin, tubulin, and microtubules, are dysregulated in schizophrenia. BMP signaling, particularly BMP7, has also been shown to affect dendrite formation in cortical neurons (Guo et al., 1998; Horbinski et al., 2002; Le Roux et al., 1999), through both the remodeling of the actin cytoskeleton (Lee-Hoeflich et al., 2004) and the stabilization of microtubules (Podkowa et al., 2010). Of interest, BMP7 has also been shown to attenuate the reduction of primary dendrites exposed to glutamate excitotoxicity, suggesting a neuroprotective response (Esquenazi et al., 2002).

Considering all of these findings together, up-regulation of TGF- β /BMP signaling may represent a molecular snapshot of the internal homeostasis of these neurons in response to disinhibition-induced excitotoxic injury.

ECM Disturbances

CSPGs are critical components of perineuronal nets (PNNs) (Figure 5A), which are ECM structures that enwrap the cell body and perisomatic region of various types of neurons, including PV and pyramidal neurons (Frischknecht & Seidenbecher, 2008). They are believed to originate predominantly from glial cells and are secreted into the extracellular space (Morgenstern et al., 2002; Pantazopoulos et al., 2008). Using biotin-labeled lectin from *Wisteria floribunda* agglutinin (WFA) to label CSPGs, it has recently been found that, in the human amygdala, CSPG-containing cells were virtually exclusively astrocytes (Pantazopoulos et al., 2008, 2010). At the same time, the density of PNNs in the amygdala has been found to be significantly decreased in schizophrenia (Pantazopoulos et al., 2010), which may be a result of impaired secretion and/or production of CSPGs by astrocytes (Pantazopoulos et al., 2010). In the present study, however, our data indicate that the transcripts of many of the genes that encode CSPGs are present in pyramidal neurons in the STG. Consistent with this, it has recently been shown that one of the CSPGs, versican, is localized to the somata and dendrites of pyramidal neurons in the mouse cerebral cortex (Horii-Hayashi et al., 2008). Cell culture studies have also shown that the production of some of the CSPGs, such as aggrecan, may be glia independent (Giamanco & Matthews, 2012). Furthermore, using WFA histochemistry, we have found that many of the cells that express CSPGs in the human cerebral cortex appear to be neurons, not astrocytes (Ikeda et al., 2009) (Figure 5B). Hence, cellular mechanisms of CSPG production and/or secretion may differ between the cortex and subcortical structures. In the present study, we found that many of the CSPG transcripts in

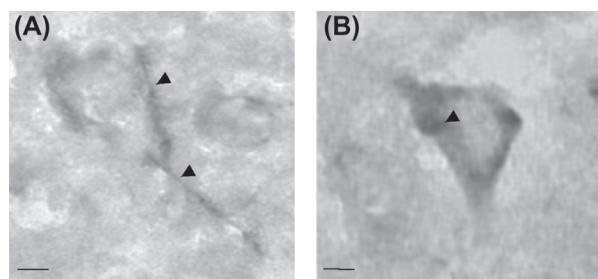


Figure 5. *Wisteria floribunda* agglutinin (WFA) histochemical labeling of chondroitin sulfate proteoglycans (CSPGs) in the human cerebral cortex. (A) CSPG-rich perineuronal nets (arrowheads). (B) WFA also labeled intracellular CSPGs (arrowhead). Scale bars = 5 μ m (A and B).

pyramidal neurons were significantly down-regulated in schizophrenia (Table 3b). Consistent with this observation and the aforementioned finding in the amygdala (Pantazopoulos et al., 2010), the density of PNNs in the prefrontal cortex has also been found to be decreased in schizophrenia (Mauney et al., 2013). A decrease in PNNs may render synaptic structures more vulnerable to alterations, contributing to reduced synaptic connectivities. It is of interest to note that TGF- β signaling is known to play a key role in regulating CSPGs (Asher et al., 2002). However, activation of TGF- β signaling typically leads to increased CSPG production (Asher et al., 2000, 2002), but we observed primarily a down-regulation of CSPG mRNAs. Further investigation is therefore needed to elucidate the precise relationship, if any, between the up-regulation of TGF- β signaling and the dysregulation of CSPGs in schizophrenia.

Altered miRNA Expression in Schizophrenia

In addition to alterations in gene expression and biological pathways, we also noted changes in the posttranscriptional regulatory environment, characterized by alterations in miRNA expression. The number of miRNAs identified in this study is relatively small, but in the same order of magnitude as found in previous studies investigating miRNA expression in the cerebral cortex in schizophrenia (Beveridge et al., 2008; Moreau et al., 2011; Santarelli et al., 2011). However, direct comparison of our data with those of the previous studies may not be appropriate, as all of these studies examined RNA extracted from homogenized cortical gray matter.

Results of pathway analysis of the predicted target genes of the differentially expressed miRNAs correlate highly with the dysregulated biological pathways identified by mRNA expression profiling, such as TGF- β signaling, regulation of actin cytoskeleton, ECM-receptor interaction, and apoptosis (Table 5), in addition to other pathways that have also been associated with schizophrenia, such as mitogen-activated protein kinase (MAPK) signaling, neurotrophin signaling, axon guidance, and WNT signaling. These data raise an intriguing possibility that the pathophysiology of pyramidal cell dysfunction in schizophrenia may in part be mediated by the concerted dysregulation of gene network functions as a result of the altered expression of a relatively small number of miRNAs.

CONCLUSION

We have identified molecular pathways that appear to be altered in layer 3 pyramidal neurons in the STG in schizophrenia. These findings provide a neurobiological

foundation upon which experimental manipulation of the expression of the differentially expressed genes may allow us to begin to characterize the molecular architectures that underlie pyramidal cell dysfunction in schizophrenia. For instance, based on our findings, one might hypothesize that neurons derived from induced pluripotent stem cells from patients with schizophrenia may be particularly vulnerable to oxidative stress, which may then lead to dysregulated TGF- β /BMP signaling and downstream consequences, such as compromised extracellular matrix, hypoplastic dendritic spines, etc. An alternative approach may involve overexpressing genes related to the TGF- β /BMP pathway using pyramidal cell-specific promoters in *in vivo* systems to see if this may result in similar molecular pathologies. Hence, this “reverse translational strategy” circumvents the traditional difficulties of studying the biology of psychiatric disorders due to the inaccessibility of the living human brain and may ultimately lead to deeper understanding of the pathogenetic mechanisms of these devastating, uniquely human disorders.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Methods 1 and 2, Tables 1–5 and Figures 1–3.