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Semaphorin and plexin gene expression is altered in the prefrontal cortex of schizophrenia patients with and without auditory hallucinations

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ABSTRACT

Auditory hallucinations (AH) are clinical hallmarks of schizophrenia, however little is known about molecular genetics of these symptoms. In this study, gene expression profiling of postmortem brain samples from prefrontal cortex of schizophrenic patients without AH (SNA), patients with AH (SA) and control subjects were compared. Genome-wide expression analysis was conducted using samples of three individuals of each group and the Affymetrix GeneChip Human-Gene 1.0 ST-Array. This analysis identified the Axon Guidance pathway as one of the most differentially expressed network among SNA, SA and CNT. To confirm the transcriptome results, mRNA level quantification of seventeen genes involved in this pathway was performed in a larger sample. *PLXNB1, SEMA3A, SEMA4D* and *SEM6C* were upregulated in SNA or SA patients compared to controls. *PLXNA1* and *SEMA3D* showed down-regulation in their expression in the patient's samples, but differences remained statistically significant between the SNA patients and controls. Differences between SNA and SA were found in *PLXNB1* expression which is decreased in SA patients. This study strengthens the contribution of brain plasticity in pathophysiology of schizophrenia and shows that non-hallucinatory patients present more alterations in frontal regions than patients with hallucinations concerning neural plasticity.

1. Introduction

Schizophrenia (SZ) is a severe psychiatric disease with a prevalence of about 1% in the general population, but men are more frequently affected than women in a 1.4:1 ratio (McGrath et al., 2008). SZ shows complex etiology with a genetic component as a central factor in the development of the disease. Both common and rare genetic variants, including rare copy-number variants have been associated with the disease (Girard et al., 2012). Gene expression studies are also used to deal with the genetic heterogeneity in SZ and even more to identify the underlying biological pathways. Thus, different processes such as glutamatergic and GABAergic neurotransmission, neural plasticity, immune system, apoptosis and stress response have been involved in disease onset (Sequeira et al., 2012). These data reflect the pathophysiological complexity of what is defined as SZ in the Diagnostic and Statistical Manual of Mental Disorders.

A clinical hallmark of SZ are auditory hallucinations (AH), specially hearing voices with negative content that can severely disrupt normal behavior and reduce quality of life. These symptoms affect from 60% to 80% of schizophrenic patients (Chibbaro

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et al., 2005) and represent a clear endophenotype among the total schizophrenic spectrum (Sanjuan et al., 2006 a). In fact, AH are probably the symptoms with the most reliable marker in neuroimaging (Jardri et al., 2011). Over the last few decades, there has been a significant increase in the study of AH but the biological mechanisms underlying the appearance of these symptoms are still not well understood. Although genetic analysis of AH in SZ might be more feasible to address than that of SZ as a whole syndrome, little is known about the molecular genetics of these symptoms. Since the first study reporting association between AH of schizophrenic patients with the 5-HTTLPR functional polymorphism of serotonin transporter gene (Malhotra et al., 1998), several molecular genetic analyses have been carried out. The cholecystokinin type A receptor gene (*CCK-AR*) has been suggested to confer vulnerability to AH in SZ (Wei and Hemmings, 1999; Tachikawa et al., 2001; Sanjuan et al., 2004; Toirac et al., 2007). Moreover, several polymorphisms of the forkhead-box transcription factor 2 gene (*FOXP2*) have been associated with the frequency and intensity of AH in SZ (Sanjuán et al., 2006 b). Recently, by using convergent functional genomics, new blood biomarkers for hallucinations in SZ, schizoaffective disorder and substance-induced psychosis have been identified (Kurian et al., 2011). To our knowledge, gene expression profiling for AH has not yet been performed in postmortem relevant tissues from schizophrenic patients. Recent neuroimaging studies in the prefrontal cortex (PFC) of schizophrenic patients with the hallucinatory event have reported impairments in structure and connectivity of this region with the limbic system (Martí-Bonmatí et al., 2007; Amad et al., 2013).

Taking into account all these data, we hypothesized that gene expression in brain tissues may differ between SZ patients with and without AH and controls. To identify potential differences in gene expression associated to AH we analyzed postmortem brain samples from the PFC of schizophrenic patients with and without these symptoms and control subjects. First, we studied the complete transcriptome of PFC samples from three individuals of each group, finding the Axon Guidance pathway differentially expressed in the three groups. Next, we confirmed this result by quantification of the mRNA levels of several genes involved in this pathway using a larger sample.

2. Material and methods

2.1. Post-mortem human brain tissues

Thirteen postmortem brain samples from PFC of schizophrenic patients without AH (SNA), schizophrenic patients with AH (SA), and control subjects with no history of psychosis (CNT), were used in this study. Frozen tissue samples of the dorsolateral PFC were obtained from the collections of the *Sant Joan de Déu Brain Bank*, the *University of the Basque Country Brain Collection* and the *Hospital Universitari de Bellvitge Brain Bank*, all three resources included in the Spanish National Network for Research in Mental Health CIBERSAM. Demographic and tissue variables of the samples are listed in Supplementary Table S1. All the samples belong to male donor subjects of European origin from Spain in order to remove potential effects related to gender and ethnics on gene expression, which would have diminished the statistical power of this study. SZ diagnosis was established according to the DSM-IV. The presence of AH were evaluated antemortem with the Positive and Negative Symptom Scale (PANSS) by experienced psychiatrists. Because nearly 50% of schizophrenic patients (7 SNA and 6 SA subjects) showed a clinical history of Alzheimer's disease (clinical stages I–V), several samples from non-psychotic patients with a history of initial stages of Alzheimer's disease were also included in the control group. All schizophrenic patients were under treatment with antipsychotics up to death. Most of them died of natural causes with the exception of 4 SNA and 3 SA subjects who were victims of suicide. Administration of other drugs to subjects of this study is indicated in the Supplementary Table S1. In the control group, all subjects died of natural causes. Human brain samples were obtained at autopsies in compliance with policies of research and ethical boards for postmortem brain studies at the moment of sample obtaining. This study was approved by the Ethics Committee of Valencia University, Spain.

2.2. Tissue processing and RNA extraction

Cortical gray matter, from layer I to VI, was dissected with a microscalpel in sterile conditions under a stereo microscope (SZX7, Olympus), frozen in liquid nitrogen and stored at −80 °C until use. The whole procedure was performed at cold temperature and under RNase-free conditions to prevent RNA degradation.

Total RNA was extracted from the dissected brain tissues using the miRNeasy Mini Kit (Qiagen, Izasa, Spain) according to the manufacturer's procedure. Purified RNA was eluted in RNase-free water and stored at −80 °C. RNA concentration and purity was measured by spectrophotometry (Eppendorf BioPhotometer plus; Eppendorf AG, Hamburg, Germany) at 260 nm and 260/280 nm, respectively. The integrity of RNA was further assessed using an RNA 6000 Nano Chip Kit in an Agilent 2100 Bioanalyzer. The samples showed RNA Integrity Number (RIN) values between 4.0 and 8.0.

2.3. RNA microarray

Gene expression analysis was performed with the GeneChip Human Gene 1.0 ST Array (Affymetrix®, Santa Clara, CA, USA), covering more than 750,000 unique 25-mer oligonucleotide features constituting over 28,000 well-annotated genes. Hybridization experiments and microarray data generation were conducted according to the manufacturer's instructions in the Central Research Unit-INCLIVA, Faculty of Medicine, University of Valencia (Spain). RNA samples for array analysis were selected from three subjects of each group (SNA, SA and CNT) with RIN values exceeding 5.0. These samples were matched by age and pH.

2.4. Real-time reverse transcription polymerase chain reaction (RT-qPCR)

Thirty-nine samples were analyzed by RT-qPCR. For cDNA synthesis 2 µl oligo-dT plus (10 pM) were hybridized to 1 µl of total RNA (100 ng/µl) in 10.5 µl volume by heating up to 65 °C for 10 min. First strand cDNA was then synthesized by incubating the hybridized RNA at 43 °C for 60 min with dGTP, dTTP, dCTP, dATP (1 mM each), 1 µl expand reverse transcriptase (50 U/µl), 0.5 µl Protector RNase inhibitor (40 U/µl), 2 µl 1.4-dithio-DL-threitol (DTT, 100 mM) in 20 µl Buffer for Expand reverse transcriptase. All products were purchased from Roche Applied Science (Indianapolis, USA). cDNA reactions were then diluted fivefold in nuclease-free water.

Amplification was carried out with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems), gene specific primers (Table 1) at a concentration of 250 nM, and 4 µl cDNA (50 ng) of each sample. Each sample was run in triplicates. Following a 95 °C denaturation for 10 min, the reactions were cycled 40 times with a 95 °C denaturation for 15 s and a 60 °C annealing step for 1 min. After that, a melt curve was performed to assess the specificity of primers. Primers were designed by PrimerBlast free software, between exons when possible to avoid genomic DNA amplification,