Innate immune response is differentially dysregulated between bipolar disease and schizophrenia

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ABSTRACT

Schizophrenia (SZ) and bipolar disorder (BD) are severe psychiatric conditions with a neurodevelopmental component. Genetic findings indicate the existence of an overlap in genetic susceptibility across the disorders. Also, image studies provide evidence for a shared neurobiological basis, contributing to a dimensional diagnostic approach. This study aimed to identify the molecular mechanisms that differentiate SZ and BD patients from health controls but also that distinguish both from health individuals. Comparison of gene expression profiling in post-mortem brains of both disorders and health controls (30 cases), followed by a further comparison between 29 BD and 29 SZ revealed 28 differentially expressed genes. These genes were used in co-expression analysesthat revealed the pairs *CCR1/SERPINA1*, *CCR5/HCST*, *C1QA/CD68*, *CCR5/S100A11* and *SERPINA1/TLR1* as presenting the most significant difference in co-expression between SZ and BD. Next, a protein-protein interaction (PPI) network using the 28 differentially expressed genes as seeds revealed *CASP4*, *TYROBP*, *CCR1*, *SERPINA1*, *CCR5* and *C1QA* as having a central role in the diseases manifestation. Both co-expression and network topological analyses pointed to genes related to microglia functions. Based on this data, we suggest that differences between SZ and BP are due to genes involved with response to stimulus, defense response, immune system process and response to stress biological processes, all having a role in the communication of environmental factors to the cells and associated to microglia.

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1. Introduction

Schizophrenia (SZ) and bipolar disorder (BD) are severe psychiatric conditions, with a lifetime prevalence of about 1% (Merikangas et al., 2007; Alaerts and Del-Favero, 2009; Doherty et al., 2012). Both disorders have a neurodevelopment component, with onset of symptoms occurring most frequently during late adolescence or early adulthood (Maier et al., 2006; Doherty et al., 2012). Family studies demonstrate that the recurrence risk in families of SZ patients is 8-12% and the recurrence risk in BD families is approximately 10% (Barnett and Smoller, 2009; Ivleva et al., 2010). The estimates of heritability range between 40 - 80% for both diseases (Sullivan et al., 2003; Bienvenu et al., 2011) with genetic findings indicating an overlap in familial-genetic susceptibility across the diseases (O'Donovan et al., 2008; Lichtenstein et al., 2009; Ivleva et al., 2010). In addition, chromosomal regions, including risk variants show linkage to both BD and SZ (Barnett and Smoller, 2009; Moskvina et al., 2009; Williams et al., 2011a,b). Global gene expression analyses revealed common genes for SZ and BD, which were associated with synapse, neuronal and glial functions, metabolism, cellular and mitochondrial function, nervous system development, immune system development and response, and cell death (Iwamoto et al., 2005; Choi et al., 2008; Shao and Vawter, 2008; Lin et al., 2012).

Due to the similarities between both disorders, gene expression profiling of BD and SZ were first compared as one entity to controls to identify common alterations. Further, genes from this comparison were analyzed in co-expression and protein-protein interaction (PPI) networks contexts allowing the identification of changes in expression

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and biological processes potentially involved in the different clinical phenotypes observed in SZ and BD.

2. Material and methods

2.1. Sample

RNA samples were obtained from the frontal cortex of 104 subjects from the Stanley Neuropathology Consortium. Potential donors for the brain collection were identified by the pathologist who contacts the family of the deceased, request permission for donation, make a preliminary diagnosis and require psychiatric records; if necessary a psychiatrist contacts one or more family members and make a telephone call to clarify the symptoms. All records are reviewed for DSM-IV psychiatric diagnosis independently by two senior psychiatrists. For normal controls, a structured telephone interview with a first-degree family member was carried out in all cases. Detailed sample collection is available in (Torrey, 2000). RNA concentration was determined by spectrophotometry (Nanodrop, Thermo Scientific, US), and integrity was accessed using the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Due to low RNA integrity, 16 samples were discarded. The final samples included 30 non-psychiatric controls, 29 bipolar patients and 29 schizophrenic patients. A summary of subject characteristics is shown in Table 1.

The study protocol was approved by the ethics committee of A.C.Camargo Cancer Center and was performed in accordance with the Declaration of Helsinki.

2.2. cDNA microarray experiments

The Agilent 4x44K human oligonucleotide microarray assay was used (Agilent 4112 F; Agilent Technologies, CA) for microarray analyses. Slides were scanned with the Agilent Bundle Microarray Scanner System (Agilent Technologies) and data were processed using the Feature Extraction 10.7.3.1 software (Agilent Technologies). Among the 45,015 spots present in each array, only those with none flag for quality control (i.e. low intensity, saturation, controls, etc.) were selected for analysis. For analysis of replicate spots, the average intensity after background correction was calculated, normalization was performed using locally weighted linear regression (LOWESS) with $\alpha = 0.2$ within slides using the R software version 2.11.1 (R Development Core Team, 2010). For statistical analyses only transcripts that were presented in at least 24 cases of each group were considered. In total, 22,639 transcripts were analyzed. Gene expression data described in this study are available from GEO with accession ID GSE6210.

2.3. Statistical analysis

For analysis of genes related to pathological changes, patients with either BD or SZ (PSY) were compared to individuals without pathology (CON). From this list of differentially expressed genes, BD and SZ patients were compared to identify possible disease-specific genes.

Analyses using Multiple Significant Analysis of Microarray (SAM) identified differentially expressed genes. Five hundred permutations were performed using a False Discovery Ratio (FDR) of 1% for both the PSY vs. CON and BD vs. SZ analyses. To assess similarity patterns, Pearson correlation and complete linkage were used for non-supervised hierarchical clustering, and reliability was assessed by bootstrapping using multiExperiment Viewer (MeV) software (Saeed et al., 2003).

Differentially expressed genes were annotated using biological process categories in the Gene Ontology Database (GO) and a hyper-geometric test with multiple test adjustments was applied to find over-represented chromosome regions with WebGestalt, using the human genome as reference, p-value <0.05 and Benjamin Hochnerg adjustment (Zhang et al., 2005).

To assess differences in network organization between individuals with BD and SZ, co-expression of pairs of genes were evaluated as previously described (Silva et al., 2012). Briefly, the Pearson Correlation Coefficient (PCC) for each gene and partners in each group was calculated. PCC absolute difference between groups was used to identify genes whose co-expression was different between BD and SZ. To identify genes that were significantly different between patient groups, an empirical p-value distribution was created as follows: cases were randomly assigned to two groups and the PCC was calculated for each group and a difference ranking was calculated. These analyses were repeated 1,000 times to create a random distribution of PCC's difference rankings. Real PCC differences for genes between patient groups were compared to the random distribution to generate p-values (Supplemental table 1) defining a network of genes whose co-expression was significantly different ($p \leq 0.05$) between SZ and BD. The network was visualized using Cytoscape (Cline et al., 2007).

To identify additional properties potentially associated with the differentially expressed genes between SZ and BD, a Protein-Protein Interaction (PPI) network was used. By querying three human interactome databases: HPRD (Keshava Prasad et al., 2009), MINT (Licata et al., 2012) and IntAct (Kerrien et al., 2012), a network starting with differentially expressed genes (seeds) and their first neighbors (genes with direct interaction in the interactome databases) and genes that connected first neighbors from seed was constructed. To identify broker (i.e., a gene that connects different genes that do not connect directly with each other) and bridge (i.e., a gene that has only a few connections but connects broker genes and their associated partners (i.e., hubs)) genes, previously published algorithms (Cai et al., 2010) were used in the Interactome Graph website (http://bioinfo.lbhc.hcancer.org.br/interactomegraph/). Top 5% genes were selected. Using the entire set of genes available in the three banks (14,276), the probability of a node appearing in a random network was estimated by generating 1,000 networks using a random collection of 25 genes (an equivalent number of genes differentially expressed found in this study and present at the PPI data). For each gene in the original network, the number of times it appeared in the 1,000 networks was computed. Genes that appeared more than 20% times (5th percentile) were considered random, because the gene has a high probability of appearing in any human PPI.

3. Results

3.1. Characterization of differentially expressed genes between BD and SZ

Gene expression profile analyses of PSY (i.e., BD and SZ) versus CON identified 1,264 differentially expressed genes. These genes were involved with nervous system, vasculature and ectoderm development, regulation of metabolism and the immune system biological processes.

Of the 1,264 genes, 28 were differentially expressed when comparing individuals with BD and SZ (FDR < 1%), all having a higher expression in BD. These genes are involved with immune system response, immune system regulation, and response to stimulus (Table 2). Non-

Table 1
Summary of subject characteristics.

	Bipolar patients	Schizophrenic patients	Non-psychiatric controls
Number of Samples	29	29	30
Age	44,46	42,17	44,43
Gender	52% Male	79% Male	76% Male
Race	93% White	96% White	100% White
PMI	36,89 ± 18,26	31,40 ± 16,93	29,97 ± 12,71
Brain pH	6,49 ± 0,25	6,45 ± 0,25	6,61 ± 0,28

For each variable, mean ± standard error or percentage value is reported. PMI: post-mortem interval.

Table 2

Genes differentially expressed between BD and SZ with respective genomic location, fold change and main biological processes according Gene Ontology functional annotation.

Gene Symbol	Gene ID	Location	BD/SZ	GO Biological Processes
SST	6750	3q28	1,36	response to stimulus, locomotion, response to stress
APOC1	341	19q13.2	1,42	Apolipoprotein C1 associated to LOAD (late onset Alzheimer Disease)
A_24_P41890[1]	Not_found		1,46	None informed
PGDS	27306	4q22.3	1,48	Apoptosis-increase ROS, IL6, TNFa
AK126405	1240	12q24.1	1,50	Chemokine-like receptor 1 – CMKLR1 – multifunctional receptor with pro-anti-inflammatory effect
S100A11	6282	1q21	1,51	response to stimulus
CCDC71	64925	3p21.31	1,54	Coiled coil domain containing 71
LAIR1	3903	19q13.4	1,55	Leucocyte Associated IG like receptor1 immune inhibitory receptor
CMTM7	112616	3p22.3	1,59	response to stimulus, locomotion
U80773[1]	Not_found	chr1:83151695-83151636	1,61	None informed
AIF1	199	6p21.3	1,63	immune response, immune system process, regulation of immune system process, response to stimulus, response to stress
HLA-DMB	3109	6p21.3	1,63	immune response, immune system process, response to stimulus
PYCARD	29108	16p11.2	1,63	immune response, immune system process, regulation of immune system process, response to stimulus, response to stress
CYBB	1536	Xp21.1	1,65	immune response, defense response, immune system process, response to stimulus, response to stress
CASP4	837	11q22.2-q22.3	1,66	Inflammation and natural immunity regulation activates CASPASE1, that in turn activates IL1b and IL18 that regulat inflammation and immunity
TREM2	54209	6p21.1	1,68	immune response, immune system process, response to stimulus, locomotion
CCR5	1234	3p21.31	1,69	immune response, immune system process, defense response, response to stimulus, response to stress
HCST	10870	19q13.1	1,71	immune response, immune system process, regulation of immune system process, response to stimulus
TLR1	7096	4p14	1,73	immune response, immune system process, regulation of immune system process, response to stimulus, response to stress
SERPINA1	5265	14q32.1	1,75	response to stimulus, response to stress
CD14	929	5q31.1	1,75	immune response, immune system process, regulation of immune system process, response to stimulus, response to stress
CCR1	1230	3p21	1,77	immune response, immune system process, regulation of immune system process, response to stimulus, response to stress
TYROBP	7305	19q13.1	1,77	immune response, immune system process, regulation of immune system process, response to stimulus, response to stress
RGS1	5996	1q31	2,05	immune response, immune system process, response to stimulus
C1QA	712	1p36.12	2,08	immune response, immune system process, regulation of immune system process, response to stimulus, response to stress
CD68	968	17p13	2,09	response to stimulus
PSPH	5723	7p11.2	2,30	response to stimulus
CAMK2N2	94032	3q27.1	3,57	Calcium calmodulin dependent protein kinase II inhibitor 2 regulation of synaptic plasticity implicated in neurodegeneration

[1] Probes in the platform without identified gene.

supervised hierarchical clustering based on the expression of these 28 genes did not reveal different patterns of gene expression between SZ and BD (Supplemental Fig. 1).

To identify chromosome regions potentially involved in the differentiation between BD and SZ, the 28 genes were mapped revealing over-representation of genes located at 3p21 (p < 0.001); CCDC71, CCR1
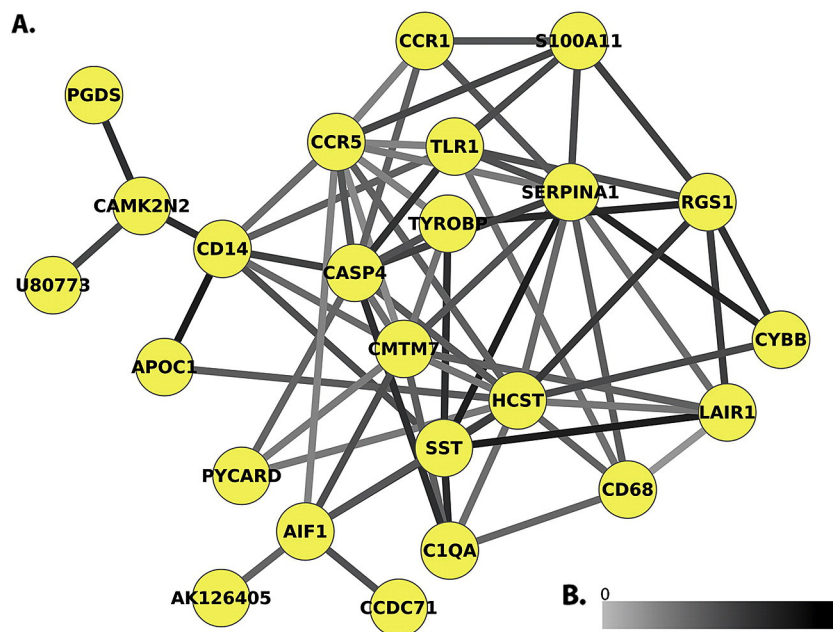

Fig. 1. A. Network showing gene pairs with significant altered correlations (p < 0.05). The color of edges is based on absolute differences of correlations. As the correlations were not inverted but increased, the color of edges represents the difference in absolute correlation. **B**. Legend of colors of edges.

and *CCR5* found in our analyses in this region were part of the chemokine receptor gene cluster.

3.2. Construction of networks based on the differentially expressed genes between BD and SZ patients

3.2.1. Co-expression gene network

To identify differences in co-expression among the 28 genes (see Material and Methods), we used Pearson Coefficient Correlation (PCC) between gene pairs in SZ and BD. This analysis revealed 65 significant gene pairs ($p < 0.05$) between SZ and BD, with the gene pairs *CCR1/SERPINA1*, *CCR5/HCST*, *C1QA*/CD68, *CCR5/S100A11* and *SERPINA1/TLR1* presenting the most significant differences of co-expression between the disorders (Supplemental Table 1). These were used to construct a co-expression network (Fig. 1). It is importantto note that the significant differences in co-expressed gene pairs between SZ and BP presented the same sign, suggesting that it represents variances in the magnitude of the co-expression rather than inversion of the PCC. Of

note, there were not gene pairs that correlated in a positive way in one disorder and in a negative way in the other.

3.2.2. PPI network

To better address the cellular mechanisms involved in the differences between SZ and BD, 25 out of 28 differentially expressed genes were found to be represented in the human PPI databases and used as seeds to construct an interaction network rendering 285 genes (Fig. 2). This set of 285 genes has over-representation of response to stimulus, defense response, immune system process and response to stress biological processes (Supplemental Table 2).

Next, we identified the genes that could affect the network topology. Fifteen bridge and 15 broker genes were initially identified. All 15 bridges and eight out of 15 brokers after bias correction (appeared in less than 20% of the random networks (Supplemental Table 3) due to the fact that commonly studied proteins are more likely to be included in PPI databases (Das and Yu, 2012) were selected. We observed that six
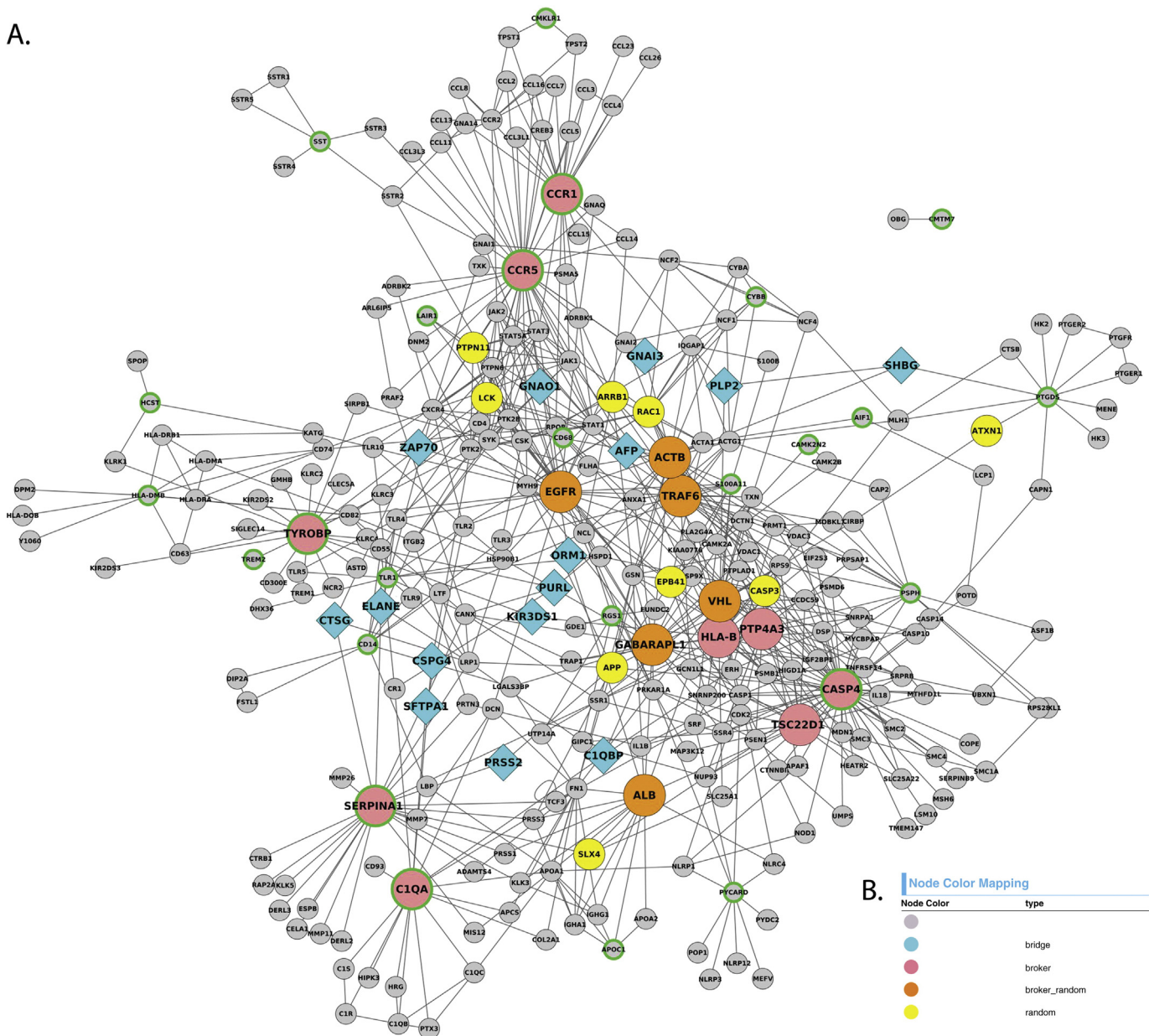

Fig. 2. A. Gene network based on the 25 differentially expressed genes which presented interactions in the human interactome. Nodes present their sizes proportional to the number of neighbors. **B.** Legend of colors. The 28 genes are indicated with green border.

of the 28 differentially expressed genes were brokers in the network: *CASP4*, *TYROBP*, *CCR1*, *SERPINA1*, *CCR5*, *C1QA*.

4. Discussion

In this study, gene expression profiling and PPI network in post-mortem brain samples of SZ, BD and controls subjects allowed us: 1) to detect common alterations in both diseases when compared to healthy controls, and 2) to characterize molecular alterations that differentiate SZ from BD. Gene network approaches allow the identification of genes that are not necessarily central but have a crucial biological role in signal transduction or in the regulation of the expression of other genes. Typically found in complex diseases, these genes tend to have an intermediate impact in the protein-protein interaction networks as is the case for brokers and bridges (Feldman et al., 2008; Cai et al., 2010).

The integration of gene expression with a PPI network to study patients with schizophrenia and bipolar disorder have been used by other studies. Lee et al analyzed BD and SZ separately in relation to controls revealing genes involved in housekeeping functions (translation, transcription, energy conversion, and metabolism), in brain specific functions (signal transduction, neuron cell differentiation, and cytoskeleton), and in stress responses (heat shocks and biotic stress) (Lee et al., 2011). A recent whole transcriptome analysis of post-mortem brain tissues identified differentially expressed genes between SZ and BD, compared to control subjects, many of the genes showing concordant expression level, also revealing the involvement of lysosomal function and regulation of actin cytoskeleton with both diseases (Zhao et al., 2014). Different from previous studies, we first identified the common alterations of SZ and BD in relation to control and subsequently focused on the differences between both disorders. The common alterations included 1,264 genes possibly involved with the clinical symptoms shared by SZ and BD. They are involved with nervous system, vasculature and ectoderm development as well as regulation of metabolism and the immune system.

A much smaller subset of 28 genes were found differentially expressed between BD and SZ. Others have noted the small difference in the molecular profiles of BD and SZ and the concordant expression level of genes in both diseases in relation to control (Zhao et al., 2014). A comparison of inflammatory monocyte gene expression profiles revealed three subsets of strongly correlating genes characterized by different sets of transcription/MAPK regulating factors, with only one subset showing a different profile: up-regulated in the monocytes of BD but down-regulated in SZ (Drexhage et al., 2010).

Functional analyses of the 28 genes revealed that they are associated with response to stimulus, defense response, immune system process and response to stress. Previous *in silico* analyses suggested that inflammatory response markers are able to differentiate patients with SZ or BD (Griffiths et al., 2010; Ricklin et al., 2010). A systematic meta-analysis of 13 studies reviewed evidence of peripheral cytokine alterations in BD and found differences between patients and controls and between phases of disease within patients (Munkholm et al., 2013), suggesting that different symptoms are related to activation of different processes within the cell. A meta-analysis based on 40 studies of SZ also indicated that some cytokines may be state markers for acute exacerbations, and others may be trait markers, suggesting that cytokine alterations in schizophrenia may also vary with clinical status (Miller et al., 2011). Accordingly, inflammatory cytokines such as IL6, IL12 and TNF-α were found at high levels in the peripheral blood of patients with SZ (Kunz et al., 2011; Pedrini et al., 2012).

The set of 28 genes had over-representation of genes located at 3p21 in the chemokine receptor gene cluster (*CCDC71*, *CCR1* and *CCR5*). The 3p21 locus were pointed as a putative altered locus in BD by GWAS (genome-wide association studies) (McMahon et al., 2010) and an increased susceptibility for late onset SZ was related to a 32-bp deletion in *CCR5* (Rasmussen et al., 2006). Down-regulation of *CCR1* was also observed in human peripheral mononuclear cells from SZ patients

under treatment with antidepressant and antipsychotic (Chertkow et al., 2007). Chemokines have fundamental roles in regulating immune and inflammatory responses. *CCR1* and *CCR5* encode members of the β-chemokine receptor family critical for the recruitment of mononuclear phagocytes to the central nervous system (CNS) in neuroinflammatory diseases (Eltayeb et al., 2007).

Prenatal inflammation is thought to be a risk factor for the development of neuropsychiatric disorders such as schizophrenia and autism spectrum disorders in the unborn child (Gertig and Hanisch, 2014), whereas dysfunctional innate immunity in bipolar disorder could make patients more susceptible to stressful events during life (Najjar et al., 2013; Stertz et al., 2013). Prenatal inflammation triggers the activation of microglia, the tissue-resident macrophages of the central nervous system (CNS) and are active participants in the development and homeostasis of the CNS (Marin-Teva et al., 2004; Takahashi et al., 2005). Using differentially gene expression, co-expression and PPI network topological analyses we found genes related to microglia functions as important genes in the differentiation of SZ and BD: *TREM2*, *TLR1*, *TYROBP*, *C1QA*, *CD68*, *SERPINA1*, *CD14*, and *AIF1*.

TREM2 promotes microglial phagocytosis of apoptotic cells (Hsieh et al., 2009). It inhibits macrophage response to ligation of toll-like receptors (TLR) (Ito and Hamerman, 2012). *TLR1* plays a fundamental role in activation of innate immunity and is found in microglia, neurons, astrocytes and endothelial cells (Lampron et al., 2013). *TREM2* also interacts with *TYROBP (DAP12)* to maintain brain homeostasis (Paradowska-Gorycka and Jurkowska, 2013). The complex DAP12-TREM2 is detected in embryonic day 14 CNS co-localized with markers of microglia/macrophages (Thrash et al., 2009; Cameron and Landreth, 2010). *CD68* can be considered an immunological marker of the density of activated microglia (Monier et al., 2007). CD68-positive microglial cells have frequently been observed in human fetal white matter and present a topographical relationship with growing axons. The protein encoded by *CD14* is a surface antigen that mediates the innate immune response. Socially-defeated mice show increased surface expression of several inflammatory proteins including *CD14* in microglia and CNS macrophages (Wohleb et al., 2011, 2012). Another protein expressed in microglial cells is *AIF1*, a marker of activated macrophages (Fukui et al., 2012). *SERPIN1* has an important role as a self-defense protein that modulates the activation of microglia (Griffiths et al., 2010).

Microglia is also related to neurodevelopment in neurogenic niches such as the hippocampal dentate gyrus, which contains neural precursor cells (NPCs). In such regions microglia shows a more activated phenotype, detected by *CD68* expression (Mosher et al, 2012). Moreover, during development more synapses tagged by complement signaling are pruned by microglia. *C1q* is an upstream member of the complement signaling cascade critical for the elimination of weak or dysfunctional synapses during postnatal neurodevelopment. C1q is found co-localized with synapses in the developing CNS and its ablation results in an excess number of synapses during adolescence (Stevens et al, 2007). Microglia play a significant role in determining the neuronal and the behavioral responses to chronic psychological stress and, as such, may contribute to the development of stress-related psychopathologies (Hinwood et al., 2012). Altogether, these results suggest that changes in expression of genes involved in microglial function would account to environmental stimulus during neurodevelopment. Also, emerging data show fundamental roles for microglia in the control of neuronal proliferation and differentiation as well as in the formation of synaptic connections, clearance of apoptotic cells and debris, production of trophic factors and in the more long term wiring of neuronal circuits (Graeber, 2010; Hughes, 2012).

In summary, the results from this study revealed genes and biological processes that may underlie differences between SZ and BD, supporting the hypothesis that SZ and BD arise from shared genetic factors, but that the resulting clinical phenotype is modulated by additional alterations mediated by microglia, possibly caused by interference of environmental factors at different times during neurodevelopment

and early life, and/or epistatic interactions among groups of genes and environment (Rovaris et al., 2013). Our study has limitations since the expression data was provided from post-mortem brain samples and because patients had been medicated. Additionally, it can be conceived that this group of differently expressed genes could represent spurious association due to limited number of cases, regardless all statistical corrections. Lager sample sizes could confirm or refute these findings. Further studies with larger patient and control groups using samples from distinct brain regions are needed to validate our findings and to elucidate how they precisely contribute to disease pathology in these psychiatric disorders.

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