Protein Interaction Networks Link Schizophrenia Risk Loci to Synaptic Function

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Schizophrenia is a severe and highly heritable psychiatric disorder affecting approximately 1% of the population. Genome-wide association studies have identified 108 independent genetic loci with genome-wide significance but their functional importance has yet to be elucidated. Here, we develop a novel strategy based on network analysis of protein–protein interactions (PPI) to infer biological function associated with variants most strongly linked to illness risk. We show that the schizophrenia loci are strongly linked to synaptic transmission ($P_{\text{FWE}} < .001$) and ion transmembrane transport ($P_{\text{FWE}} = .03$), but not to ontological categories previously found to be shared across psychiatric illnesses. We demonstrate that brain expression of risk-linked genes within the identified processes is strongly modulated during birth and identify a set of synaptic genes consistently changed across multiple brain regions of adult schizophrenia patients. These results suggest synaptic function as a developmentally determined schizophrenia process supported by the illness’s most associated genetic variants and their PPI networks. The implicated genes may be valuable targets for mechanistic experiments and future drug development approaches.

Key words: genetics/pathway analysis/functional analysis/GWAS

Introduction

Schizophrenia is a severe and heritable psychiatric disorder with a lifetime risk of approximately 1%. Substantial genetic risk is conferred by a large number of common alleles with small effect sizes.1–5 The most recent large scale association study of about 150 000 individuals has identified 108 independent genetic loci linked to the disorder with genome-wide significance.5 However, the inference of affected biological pathways is a bottleneck for understanding their etiological relevance. Commonly used functional analysis approaches explore pathways for enrichment of illness-associated variants at a genome-wide level.6,7 Such analysis has recently been performed across psychiatric illnesses and has identified links to histone methylation, immune pathways and largely schizophrenia specific associations in synaptic pathways.6 However, the interpretation of etiological mechanisms and the relationship to illness risk is hampered by the large number and weak illness association of individual variants.

Therefore, functional analyses of variants with strong statistical support, such as genome-wide significance, would significantly aid interpretation but are challenging due to the small numbers of variants passing the significance threshold. For example, 2 commonly used enrichment methods did not find significantly enriched ontological categories among the 108 schizophrenia loci.3 In fact, functional analyses in the cancer field suggest that sets of genes derived statistically do not independently give insight into biological function.8 Rather, protein–protein interactions (PPI) were identified as a key element mapping genetic variation to biological processes. PPI networks have previously been associated with schizophrenia risk genes but functional analysis of such networks has thus far been limited to conventional enrichment analysis.9 For this application, inference of illness associated ontological categories depends on molecules indirectly linked to illness risk, ie, protein interaction partners of risk genes. Therefore, such analysis depends on the connectivity between the PPI network and the risk genes, which is not addressed by conventional enrichment methods. Risk genes with a high number of PPI partners within the same ontological category may lead to an overestimation of this category’s importance in relation to disease risk. Vice versa, such effects may reduce the power of identifying illness associations for other categories.

Building on substantial work by the Psychiatries Genomics Consortium (PGC) and the consortium

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behind the BrainSpan atlas, we have developed a novel approach to infer risk associated ontological categories from a network of PPI and GeneOntology information. We aimed to (1) identify ontological categories linked to the 108 schizophrenia risk loci and to show that associations are not confounded by the connectivity between risk loci and the PPI network, (2) explore whether ontological categories previously linked to schizophrenia by genome-wide enrichment analyses mapped to the 108 loci, and (3) explore gene expression within identified risk categories across developmental stages and in adult schizophrenia patients. For the latter analyses, we investigated BrainSpan data comprising expression data across multiple brain areas from healthy donors from before birth until old age. Brain expression analyses in schizophrenia patients were based on post-mortem samples from BA9, BA10, BA46, the superior temporal cortex, the parietal cortex, the hippocampus and the associative striatum in 6 independent datasets. Such analysis may increase our understanding of the genetic underpinning of schizophrenia specific and shared biological risk mechanisms.

Methods

Network Construction

Analysis was based on a network incorporating PPI (BioGRID database vs 3.2.116) and ontological category information from the Gene Ontology (mappings from 08.03.2014, derived from R library org.Hs.eg.db, vs 3.0.0, including the categories “biological process,” “molecular function,” and “cellular component; figure 1a). Our approach builds on work performed by PGC, as we are specifically focusing on the 108 genome-wide significant schizophrenia risk loci and the gene mapping identified by this consortium. As performed by the Network and Pathway Analysis Subgroup of the PGC,6 we focused analysis on ontological categories linked to at least 10 genes. Within the PPI network, only genes mapping to at least 1 ontological category were retained. The network was an unweighted, undirected graph with links representing either PPIs or ontological annotations. Further details on the construction of the network investigated here are described in the supplementary methods and a schematic overview of the procedure is shown in supplementary figure 1. This study received ethical approval from the local ethics committee (2013-842R-MA).

Analysis of Risk-Locus Associated Biological Processes

To quantify the strength of association between ontological categories and risk loci, we determined the over-representation of links between proteins linked to a given ontological category and the schizophrenia risk loci. For a given category, we determined across all i risk loci the sum \( \sum b \times d_{\text{RISK}} / (2 \times m) \), where \( b \) is the observed number of links between the category’s associated proteins and a given risk locus and \( d_{\text{RISK}} \) is the risk locus’s degree. The locus’s degree is the number of links between the gene it maps to and all interaction partners in the PPI network. A given locus’s degree is proportional to the expected number of links between the locus and the category’s proteins, which is \( \sum d_{\text{PPI}} \times d_{\text{RISK}} / (2 \times m) \), where \( \sum d_{\text{PPI}} \) is the sum of the degrees of all proteins linked to a given ontological category and \( m \) is the total number of links in the PPI network. This expected number is equal to \( c \times d_{\text{RISK}} \), where \( c \) is a constant since network configuration of the proteins linked to a given category, as well as the total number of links in the network do not change throughout the procedures applied here.

Fig. 1. Overview of network based functional analysis. a) Schematic illustration of the network linking the PPI to ontological category information. b) Selection of the 44 loci mapping to single, unique genes with at least 1 link to the PPI network. c) Network illustration of the 2 significantly schizophrenia associated ontological categories “synaptic transmission” (GO:0007268) and “ion transmembrane transport” (GO:0034765). Nodes are colored depending on their predominant link to a given ontological category or its respectively associated proteins (red-colored proteins are shared between ontological categories and red-colored risk loci predominantly associated with such nodes).
To estimate significance the overrepresentation estimate was compared against estimates obtained from (10⁶) random selections of the same number of genes from the PPI network. The frequency of estimates at least as high as that from the real schizophrenia loci was used as empirical \( P \) value and adjusted for the Family-Wise-Error-Rate using Bonferroni’s method. Statistical analyses were performed considering as risk loci those 44 that mapped to single genes (out of a total of 108 loci with genome wide significance, figure 1b, supplementary tables S1 and S2) and whose gene product had at least 1 interaction partner in the PPI network. We explored whether schizophrenia associated results were confounded by properties of the investigated network, by confounders reported in a previous enrichment analysis of GWAS data or by the selection of loci mapping to single genes. Also, we tested whether risk associated ontological categories were specific for schizophrenia compared to rheumatoid arthritis (RA) and prostate cancer (PC) (supplementary methods). Analyses were performed using R (http://cran.r-project.org/).

**Analysis of Gene Expression Across Developmental Stages**

For analysis of brain expression across developmental stages, we build on work performed by the consortium behind the BrainSpan atlas. Data preprocessing was performed as previously described, data was quantile normalized using the R library `limma`, and transformed. After filtering, the dataset comprised expression information on 16 brain regions from 47 donors. Data was aggregated into age-groups and brain regions as described previously and expression was represented by the respective mean levels within groupings. We evaluated the change of expression throughout developmental stages using Spearman’s correlation analysis and due to the exploratory nature of this analysis, determined False Discovery Rate (FDR) adjusted \( P \) values according to the method of Benjamini and Hochberg.

**Preprocessing and Analysis of Gene Expression Data**

For investigation of schizophrenia post-mortem brain expression, we utilized data from the GEO database and identified datasets by extensive keyword searches. Here, we focus on no-cerebellar datasets with at least 10 patient and control subjects per dataset. An overview of the datasets is shown in table 1. Expression was quantile normalized and log₂ transformed. Univariate case-control differences were determined using Wilcoxon tests and \( P \) values adjusted according to the method of Benjamini and Hochberg.

**Results**

The network investigated here comprised 13 934 proteins, 131 191 interactions between these, 2805 ontological categories and 161 900 links connecting ontological categories to proteins within the PPI network (figure 1a). Adjusting for the Family Wise Error Rate across all tested ontological categories (\( n = 2417 \)) linked to the schizophrenia risk loci through the PPI network, we found that “synaptic transmission” (GO:0007268, \( z = 8.98, \hat{P}_{\text{FWE}} < 10^{-6}, \hat{P}_{\text{FDR}} < .001 \)) and “ion transmembrane transport” (GO:0034765, \( z = 9.77, \hat{P}_{\text{FWE}} = 1.1 \times 10^{-4}, \hat{P}_{\text{FDR}} = .03 \)) were significantly linked to the schizophrenia risk loci. The networks relevant for these associations (figure 1c) were linked to a total of 15 out of 44 risk loci via 38 different interaction partners. Exploration of annotation quality of risk linked PPI partners showed that for GO:0007268, 87% of annotations were based on cited publications while 5% of annotations were based on computational methods. In contrast, for GO:0034765, 55% of genes were annotated computationally.

We aimed to exclude confounding due to the network’s configuration. For this, we compared schizophrenia loci to gene sets selected at random and found no significant associations (minimum \( P = .34 \)) with several parameters detailed in the supplementary methods section. Similarly, illness associations were not affected by confounders known from functional analysis of GWAS data (minimum \( P = .22 \), see supplementary methods). Analysis focused on loci that mapped to single genes, which may introduce bias if such mapping is differentially associated with biological function. Therefore, we repeatedly selected 44 genes containing at least 1 single nucleotide polymorphism (SNP) from genetic regions non-overlapping with

| Dataset | Dataset Identifier | Reference | Brain Region | No. of Subjects (SZ / HC) |
|---------|-------------------|-----------|--------------|--------------------------|
| I       | GSE17612          | Maycox et al⁸ | Anterior prefrontal cortex (Brodmann Area (BA) 10) | 28 / 23 |
| II      | GSE12679          | Harris et al⁹ | Prefrontal cortex (BA 9) | 16 / 11 |
| III     | GSE21935          | Barnes et al¹⁰ | Superior temporal cortex (BA22) | 23 / 18 |
| IV      | GSE53987          | TA Lanz (presented at ACNP 2012) | Hippocampus | 15 / 18 |
| V       | GSE35977          | Chen et al¹¹ | Prefrontal cortex (BA46) | 15 / 19 |
| VI      | GSE21138          | Narayan et al¹² | Associative striatum | 18 / 18 |
|         |                   |           | Parietal cortex | 49 / 49 |
|         |                   |           | Prefrontal cortex (BA46) | 30 / 29 |

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any other genes using gene boundaries extended by 20kb as applied for annotation of the 108 risk loci. This also identified “synaptic transmission” (GO:007268, \( P_{\text{raw}} < 10^{-6}, P_{\text{FWE}} < .001 \)) and “ion transmembrane transport” (GO:0034765, \( P_{\text{raw}} = 8.0\cdot10^{-6}, P_{\text{FWE}} = .02 \)), as well as “regulation of long-term synaptic plasticity” (GO:0048169; \( P_{\text{raw}} = 1.2\cdot10^{-5}, P_{\text{FWE}} = .03 \)) and “positive regulation of synaptic transmission” (GO:0050806; \( P_{\text{raw}} = 10^{-4}, P_{\text{FWE}} = .002 \)) as significantly schizophrenia associated.

**Associations of Ontological Categories Previously Linked to Schizophrenia**

We explored potential associations between schizophrenia risk loci and ontological categories previously implicated by the PGC cross-disorder working group. Of the 5 categories most strongly associated with schizophrenia in a GWAS enrichment meta-analysis, 4 had at least 10 links to the PPI network. The 2 ontological categories with the highest enrichment also showed significant associations to schizophrenia risk loci through the PPI network. These were “postsynaptic density” (GO:0014069; nominal \( P = .02 \)) and “postsynaptic membrane” (GO:0045211; nominal \( P < .001 \)). In contrast, the other 2 ontological categories (“dendritic spine,” GO:0043197, \( P = .66 \)) and “histone H3-K4 methylation” (GO:0051568, \( P = .62 \)), which was the most significantly enriched category across 5 psychiatric disorders, showed no significant associations in the present study. Similarly, calcium channel activity (GO:0005262), the most significant ontological category implicated by SNP-based meta-analysis GWAS across 5 psychiatric illnesses showed only a trend towards a significant association (\( P = .08 \)).

**Analysis of Disease Specificity and Positive Control**

We aimed to assess the specificity of the identified schizophrenia associations, in particular to explore potential methodological bias that could lead to identification of the same risk associated ontological categories across different illnesses without strong genetic overlap. Therefore, we performed the same analysis on 25 and 41 risk SNPs for different illnesses without strong genetic overlap. Therefore, we found significant associations with “LDL particle receptor binding” (GO:0050750, \( P_{\text{raw}} = 7.0\cdot10^{-6}, P_{\text{FWE}} = .01 \)) and “LDL particle” (GO:0034362, \( P_{\text{raw}} = 1.5\cdot10^{-5}, P_{\text{FWE}} = .03 \)). Correcting for the FDR identified associations with 25 ontological categories with FDR < 0.05 that were predominantly associated with lipid and lipoprotein pathways (supplementary table S4). For added confirmation, we used only loci where a single gene was linked to the locus or the index SNP modulated expression of only 1 gene within 500kb. Using the resulting 12 genes and the 1333 linked ontological categories yielded very similar results (supplementary table S4).

**Developmental Trajectory of Synaptic Gene Expression**

To investigate the potential neurodevelopmental role of the identified ontological categories, we examined in different developmental time-points and brain regions the expression of 36 of the 38 interaction partners linked to schizophrenia risk loci through the PPI network using the BrainSpan dataset (no data was available for GRIP1 and GNG10). This dataset contains expression data from 16 brain regions in over 50 brains acquired across the human lifespan. We found that 22 of the investigated genes had a significant change in expression patterns across time (\( P_{\text{FDR}} < .05 \), figure 2). Since expression appeared to be modulated most by birth, we split regional analyses considering separately samples taken before and after birth. This showed that after birth expression significantly increased with some regional specificity for neocortical regions.

**Expression Analysis of Synaptic Genes**

To explore potential expression differences of genes part of the 2 ontological categories, we used 6 gene expression datasets from post mortem brains of schizophrenia patients and controls (table 1). Using all genes part of the 2 ontological categories, (total \( n = 333-450 \), depending on the dataset) we found significant differences between schizophrenia patients and controls (FDR < .05) for 55 and 4 genes in the hippocampus and prefrontal cortex of dataset IV, respectively, for 22 genes in the parietal cortex (dataset V) and 5 genes in the prefrontal cortex of dataset VI. Figure 3 shows the overlap between the identified gene sets and highlights the shared schizophrenia association.
Fig. 2. Gene expression patterns throughout development stages and across brain regions. Genes were ordered using complete linkage hierarchical clustering on the Euclidean distance of their expression levels. Genes shown in bold show significant change throughout developmental stages (Spearman correlation, False Discovery Rate [FDR] < 0.05). Graphical layout adapted from ref.6

Fig. 3. Overlap of significant expression differences across datasets. Displayed genes showed expression changes with $P_{FDR} < .05$. Arrows indicate whether expression was consistently increased (up arrow) or decreased (down arrow) across the datasets where a significant change in a given gene was observed.
Discussion

Here we showed that through consideration of the biological vicinity of risk genes, synaptic function emerges as a schizophrenia risk mechanism from genetic variants with the strongest risk association. This finding is consistent with genome wide enrichment analysis, exome sequencing results, the effect of schizophrenia associated copy number variants (CNVs) on postsynaptic signaling, as well as of psychiatric risk linked mutations on postsynaptic protein scaffolding. Consistent with this, postsynaptic function, a mostly schizophrenia specific pathway identified by enrichment analysis, was associated with schizophrenia risk loci. In contrast, histone methylation, the ontological category most strongly implicated across psychiatric illnesses, was not. This discrepancy may be due to the fact that our approach focuses on the most significant risk variants and differences compared to genome-wide analysis may suggest a divergence of biological risk mechanisms depending on the underlying strength of risk association. Quantitative assessment of such divergence may provide interesting insights into biological risk mechanisms shared across and specific for individual disorders. Despite this, our results do not allow conclusions about illness specificity as synaptic processes may also be implicated by risk variants of other illnesses and their respective PPI networks. The identification of synaptic function as a schizophrenia risk process is further consistent with numerous nongenetic studies. For example, histopathological investigations have found reduced spine densities in multiple brain regions of schizophrenia patients that are thought to contribute to altered neural circuitry, as well as to reduced cortical and hippocampal volumes found in schizophrenia patients. Also, impaired synaptic neurotransmitter release and neurotransmission associated protein expression have been found in schizophrenia. A synaptic pathology in schizophrenia is further consistent with findings in patients with encephalitis, where schizophrenia-like symptoms can be induced by IgG serum and CSF antibodies against N-methyl-D-aspartate glutamate receptors (NMDA-R). These antibodies selectively decrease NMDA-R clusters in postsynaptic dendrites and have been found in serum of up to 10% of schizophrenia patients. Interestingly, the induced symptom profile appears to depend on the intensity of the antibody effect on NMDA-R density, an effect similar to that observed for different doses of NMDA-R antagonists. While low doses cause psychosis, anxiety, agitation, memory disturbance, decreased responsiveness to pain, and speech reduction, higher doses have been reported to induce unresponsiveness with catatonic features, dyskinesias, autonomic instability, and seizures.

In the present study, expression analysis indicated a substantial brain expression change of risk-protein linked interaction partners during birth and some regional specificity of this effect for neocortical brain regions. We also found significant brain expression differences within the identified synaptic categories across multiple datasets and brain regions in adult schizophrenia patients. The strongest differences were found in the hippocampus, the subcortical region showing the most pronounced structural change in MRI investigations of schizophrenia patients. Interestingly, the identified expression changes strongly overlapped across the hippocampus, parietal and prefrontal cortex, supporting synaptic alterations spanning across brain regions. Genes for the potassium channel proteins KCNK1 and KCNS3 showed consistent expression change across all of the aforementioned regions. Reduced expression of KCNS3 in prefrontal cortical parvalbumin neurons in schizophrenia and has been linked to impaired γ-oscillations that are thought to underlie the cognitive impairments in schizophrenia. Reduced KCNK1 expression in schizophrenia patients has also been detected in previous prefrontal cortex expression studies.

The strong change of synaptic gene expression we observed during the perinatal period may suggest that this period is particularly susceptible for the manifestation of genetically determined alterations in synaptic processes that may then persist into adulthood. However, it should be taken into account that the present study provides only indirect evidence since it does not directly link genetic risk to protein expression during the perinatal period, and does not take into account other potentially relevant factors, such as post-translational modification, enzyme activity, molecular trafficking or schizophrenia specific differences in PPIs. Post mortem and neuroimaging findings have highlighted the etiological relevance of synaptic function and support the hypothesis of schizophrenia being a disorder induced by developmental synaptic disturbances that lead to reduced synaptic plasticity and connectivity. Future experiments will show how genetic variation in schizophrenia risk loci impacts on perinatal gene expression. Of particular interest are interactions with environmental factors previously linked to schizophrenia risk, such as perinatal and obstetric complications. Season of birth or prenatal malnutrition. Obstetric complications have already been hypothesized...
to increase schizophrenia risk via premature synaptic pruning caused by fetal hypoxia. Similarly, even moderate malnutrition that does not impact on the offspring’s birth weight, has been shown to impair synaptic plasticity in rats. 

Limitations

Our study has several limitations. Since the network method is novel, our results should be seen as preliminary and need to be validated in independent samples and using alternative computational approaches. A limitation is our focus on risk loci that mapped to single genes, which omits a substantial amount of disorder-associated genetic variability. Such selection may be a biased representation of the schizophrenia risk architecture and although our results suggest that selection of loci mapping to single genes did not bias the identified ontological categories, we cannot exclude the possibility of residual bias. Future studies incorporating loci mapping to multiple genes may face the challenge that a higher gene number can impede inference of risk associated ontological categories, as such loci lead to stronger chance associations. Another limitation is that the data used in this study were not acquired for the objectives pursued here. Exome or whole genome sequencing data would have been more suitable in particular for mapping risk variants to genes. Another limitation is that inference was based on the content of a PPI network, whose underlying data mainly originated from nonpsychiatric research, which may have biased our results. A similar issue may affect ontological categories, since human annotations are known to be non-equally distributed across genes and this may reflect differences in research interest regarding the respective genes. Variants were selected based on a genome-wide significance threshold, which reduces the risk of false-positives but is not without limitations due its inability to account for factors such as study power or the number of likely true positives. Also, the current definition of “risk locus” relates to physical regions containing SNPs correlated with each of the 128 schizophrenia associated index SNPs with \( r^2 > .6 \) (or .5 in the case of the dyslipidemia GWAS analyses), which may miss etiologically relevant genes mapping to SNPs in lower linkage disequilibrium. We found no significant links between ontological categories and risk genes of PC or RA, which may suggest that due to the approximately 7-fold higher patient numbers used for determination of schizophrenia risk loci compared those of RA and the combination of PC risk loci across multiple independent GWAS studies, schizophrenia risk loci may currently be supported by higher statistical power and may therefore cover a higher portion of true positive associations. Also, analysis of RA and PC was based on published gene annotations, which may not sufficiently capture etiologically relevant genes. Annotation quality analysis highlighted that a large portion of risk linked PPI partners within the ontological category GO:0034765 was computationally generated, which has been associated with lower reliability compared to annotation based on experimental evidence. Finally, we did not observe significant differences in 2 prefrontal cortex datasets (BA 9 and 10), the superior temporal cortex (BA22) and the associative striatum. This may be due to regional specificity of the expression changes we identified across BA46, the hippocampus and the parietal cortex or due to the small sample numbers that make it difficult to detect more subtle changes.

Conclusions

Using a novel functional analysis method we have identified strong links between schizophrenia risk loci and synaptic function. In contrast to genome-wide analysis, this method allows identification of specific loci that mediate associations with a given risk mechanism and pinpoints sets of risk associated genes for further mechanistic investigations. Our results support the importance of PPI networks to map genetic risk to biological function. The assessment of such biological vicinity of risk genes may be a fruitful avenue for future investigations of schizophrenia associations in other data modalities, such as neuroimaging or polygenic analyses. Here, we found that brain expression of genes within the identified processes changes substantially during early development, consistent with the etiological hypothesis of developmentally determined synaptic alterations in schizophrenia. Our results link such synaptic processes with a small set of genes with strong risk association that may provide valuable targets for future drug development approaches.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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