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Review Article

Schizophrenia as a neuronal synaptic disorder related to multiple rare genetic mutations

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ABSTRACT

Schizophrenia is a highly heritable disorder, but many aspects of its etiology and pathophysiology remain poorly understood. Synaptic pathology has been reported as a feature of the brain in schizophrenia. Abnormal expression of some synaptic proteins (e.g., SYP, GAP-43, and NRGN) in different brain regions has been linked to this disorder in postmortem brain studies. In our series of genetic studies, we used a resequencing strategy to search for genetic variants in these candidate genes in a sample of patients with schizophrenia and nonpsychotic controls, all of whom were Han Chinese from Taiwan, and conducted further association and functional studies. After resequencing these candidate genes, no common polymorphisms appeared to play a major role in conferring susceptibility to schizophrenia in our population. In contrast, we identified some rare patient-specific variants. The results of the reporter gene assays and software analysis demonstrated the influence of reporter genes on the function of each studied gene, suggesting that they may contribute to the pathogenesis of schizophrenia. These data lend support to the hypothesis that multiple rare mutations are involved in the pathogenesis of schizophrenia, and provide genetic clues that indicate the involvement of synaptic pathology in this disorder.

1. Introduction

Schizophrenia is a complex disorder with a high degree of genetic influence in its etiology [1]. It is now generally accepted that this illness involves variants in multiple genes that are individually insufficient to cause the illness, but which act in combination and with environmental factors to increase the risk of development [2]. Although the majority of the genetic loci that contribute to schizophrenia most likely have weak effects, their identification is essential to determining the neurobiological molecules that play a crucial role in the disorder.

Over the past two decades, structural anomalies have been identified in the brains of patients with schizophrenia [3]. This can be seen in *in vivo* neuroimaging studies that have demonstrated significant ventricular enlargement and decrease in cortical mass [4]. Postmortem brain studies have shown a reduction in the total brain volume, particularly in the cerebral cortex [5], and functional brain-imaging studies have indicated impaired connectivity between the frontal lobe and other brain regions [6,7]. At the cellular and molecular level, microscopic histopathological studies have demonstrated reduced neuronal size and decreased density of the dendritic spines [8]. Taken together, these changes in the synaptic components may reflect a decrease in the cortical volume, and it is believed that such changes may underlie the aberrant functional connectivity in schizophrenic patients [9,10].

For these reasons, some synaptic proteins have been utilized as proxy markers of synapses to determine whether synaptic alterations are a feature of schizophrenia [9,10]. Three such synaptic proteins that have been repeatedly reported to be involved in schizophrenia are synaptophysin (SYP), growth-associated protein 43 (GAP-43), and neurogranin (NRGN) [9].

2. Genetic and functional analyses of the *SYP* gene in schizophrenia

SYP is an abundant integral membrane protein in the synaptic vesicles that is expressed in 95% of cortical synaptic terminals [11,12]. Its expression occurs early in neurogenesis and is greatly upregulated during synaptogenesis [13]. This protein is known to

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regulate neurotransmitter release and synaptic plasticity [14,15]. In addition, it participates in the biogenesis and recycling of synaptic vesicles [16,17].

Several studies have examined SYP expression in the postmortem brains of patients with schizophrenia. Using *in situ* hybridization, histochemistry, immune autoradiography, and Western blot analysis, decreased SYP expression has been demonstrated in the prefrontal cortex, medial temporal cortex, visual association cortex, hippocampus, and thalamus [18–33]. Conversely, elevated SYP protein levels have been found in the anterior cingulate cortex [34,35]. Microarray studies have also highlighted lower levels of SYP in the postmortem brains of patients with schizophrenia, along with other presynaptic markers [36]. These findings lend support to the notion that SYP disturbance in specific brain regions might be part of the pathogenesis of schizophrenia [9].

The *SYP* gene (gene ID: 6855) has been mapped to chromosome Xp11.23-p11.22. This region has been linked to schizophrenia [37,38]. The function of *SYP*, to a large extent, is believed to be affected by its genetic variations [39]. To test this possibility, we searched for genetic variants in the promoter region, including all exons and both UTR ends of the *SYP* gene, using direct sequencing in a sample of patients with schizophrenia ($n = 586$) and nonpsychotic controls ($n = 576$), all of whom were Han Chinese from Taiwan, and conducted further association and functional studies [40].

After sequencing all of the amplicons in the 586 patients and 576 control patients, we identified two common polymorphisms (c.*4+271A>G and c.*4+565T>C) in the *SYP* gene. Single nucleotide polymorphism (SNP)- and haplotype-based analyses indicated no associations with schizophrenia. In addition, we identified six rare variants in seven of the 586 schizophrenic patients, including one variant (g.-511T>C) located in the promoter region, one synonymous (A104A) variant and two missense variants (G293A and A324T) located in the exonic regions, and two variants (c.*31G>A and c.*1001G>T) located at the 3′UTR. No rare variants were found in the control subjects. The results of the reporter gene assay demonstrate the influence of g.-511T>C and c.*1001G>T on the regulatory function of the *SYP* gene, while the influence of c.*31G>A may be tolerated. *In silico* analysis demonstrated the functional relevance of other rare variants. These findings lend support to the hypothesis of multiple rare mutations in schizophrenia and provide genetic clues that indicate the involvement of *SYP* in this disorder.

3. Genetic and functional analyses of the *GAP-43* gene in schizophrenia

GAP-43 is a neuron-specific phosphoprotein that is localized to the presynaptic membrane and is a substrate of protein kinase C (PKC). Its phosphorylation by PKC in response to extracellular guidance cues could regulate the behavior of F-actin in neuronal growth cones [41]. In transgenic mice, overexpression of GAP-43 results in the spontaneous formation of new synapses and enhances sprouting after injury [42,43], whereas manipulations that abolish GAP-43 expression result in the disruption of axon outgrowth and could lead to premature death [44,45]. GAP-43 is expressed primarily during brain development and declines sharply in most brain regions after synaptogenesis is completed [41]. High levels of GAP-43 persist in neocortical-association areas and the limbic system throughout life, where the protein might play important roles in mediating experience-dependent synaptic plasticity and long-term potentiation [41].

Several studies have examined GAP-43 expression in the postmortem brains of patients with schizophrenia. Alterations in GAP-43 mRNA levels have been demonstrated in the dorsolateral prefrontal cortex, primary visual cortex, anterior cingulate gyrus, and hippocampus [46–48]. Additionally, GAP-43 protein levels have been found to be altered in the frontal cortex, visual association cortex, and hippocampus [31,49,50]. These findings lend support to the notion that GAP-43 disturbances in specific brain regions might be part of the pathogenesis of schizophrenia.

The *GAP-43* gene (gene ID: 2596) has been mapped to chromosome 3q13.1-q13.2 [41]. This region has been linked to schizophrenia in a Japanese single multiplex pedigree, and in a meta-analysis of 32 genome-wide linkage studies that were performed on different populations [51,52]. As part of our series on molecular genetic studies on schizophrenia, we were interested in understanding whether the *GAP-43* gene plays a role in conferring genetic liability to schizophrenia. To test this possibility, we searched for genetic variants in the promoter region and three exons (including both UTR ends) of the *GAP-43* gene using direct sequencing of a sample of patients with schizophrenia ($n = 586$) and nonpsychotic controls ($n = 576$), all of who were Han Chinese from Taiwan, and conducted further association and functional studies [53].

After sequencing all of the amplicons of the 586 patients and 576 control patients, we identified 11 common polymorphisms in the *GAP-43* gene. SNP- and haplotype-based analyses indicated no associations with schizophrenia. Additionally, we identified four rare variants in five of the 586 patients, including one variant located in the promoter region (c.-258-4722G>T) and one synonymous (V110V) and two missense (G150R and P188L) variants located on exon 2. No rare variants were found in the control patients. The results of the reporter gene assay demonstrate that the regulatory activities of constructs containing c.-258-4722T was significantly lower when compared with the wild-type construct (c.-258-4722G). *In silico* analysis also demonstrated the functional relevance of other rare variants. These findings lend support to the hypothesis of multiple rare mutations in schizophrenia, and they provide genetic clues that indicate the involvement of *GAP-43* in this disorder.

4. Genetic and functional analyses of the *NRGN* gene in schizophrenia

NRGN is a neural-specific, calmodulin (CaM)-binding protein localized to the postsynaptic membrane and is a substrate of PKC [54]. Glutamate stimulation of N-methyl-D-aspartate (NMDA) receptors results in calcium influx to the neuron and NRGN oxidation [55]. These induce dissociation of the NRGN-CaM complex and stimulate the phosphorylation of NRGN by PKC, which prevents the rebinding of NRGN and CaM [56]. As a CaM reservoir, NRGN regulates the release of CaM and the activities of downstream CaM-Ca^{2+}-dependent enzymes that play important roles in the neuroplasticity mechanisms of learning and memory [57,58]. Therefore, altering NRGN activity could mimic the effects of NMDA-receptor hypofunction that has been suggested by several studies, thereby implicating NRGN in the pathophysiology of schizophrenia [59].

NRGN has been found in neurons in the cerebral cortex, hippocampus, striatum, and amygdala [60]. During development, NRGN expression is regulated by thyroid hormones [61], and its highest expression is coincident with the developmental period characterized by rapid dendritic growth and the formation of the majority of the cortical synapses [62]. Broadbelt et al. (2006) examined NRGN expression in the postmortem brains of patients with schizophrenia. Lowered NRGN immunoreactivity was demonstrated in areas 9 and 32 of the schizophrenic prefrontal cortex [63]. Therefore, NRGN disturbance in specific brain regions might be part of the pathogenesis in schizophrenia.

Genome-wide association (GWA) studies have identified one SNP (rs12807809) located upstream of the *NRGN* gene that is associated with schizophrenia [64,65], but causal variants that account for the association signal have not been determined. GWA data usually indicate the indirect association of a proxy of a strongly correlated causal variant that has a similar frequency or synthetic association with one or more rarer causal variants in the linkage disequilibrium [66,67]. Causal variants require extensive resequencing and association analysis. In order to find the causal variants of the *NRGN* gene associated with schizophrenia, we searched for genetic variants in the promoter region and all exons (including both UTR ends and rs12807809) using direct sequencing in a sample of patients with schizophrenia ($n = 346$) and nonpsychotic controls ($n = 345$), all of whom were Han Chinese from Taiwan, and conducted further association and functional studies [68].

After sequencing all the amplicons of the 346 patients and 345 control subjects, we identified seven common polymorphisms in the *NRGN* gene. SNP- and haplotype-based analyses indicated no associations with schizophrenia. Additionally, we identified five rare variants in six of the 346 patients, including three rare variants located in the promoter region (g.-620A>G, g.-578C>G, and g.-344G>A) and two rare variants located at 5′UTR (c.-74C>G, and c.-41G>A). No rare variants were found in the control patients. The results of the reporter gene assay demonstrate that the regulatory activities of constructs containing g.-620G, g.-578G, g.-344A, c.-74G, or c.-41A are significantly lower when compared to the wild-type construct. *In silico* analysis also demonstrated their influence on the regulatory function of the *NRGN* gene. These data also lend support to the hypothesis that multiple rare mutations are involved in schizophrenia, and provide genetic clues that indicate the involvement of *NRGN* in this disorder.

5. Summary and future research

In our series of genetic studies of schizophrenia, we used a resequencing strategy to search for genetic variants in each candidate gene in a sample of schizophrenic and control patients, and assessed their associations with schizophrenia. Three candidate genes related to synaptic pathology in schizophrenia were resequenced, but no common polymorphisms appeared to play a major role in conferring susceptibility to schizophrenia in our population. Additionally, we identified some rare patient-specific variants. The results of reporter gene assays and software analysis demonstrate the influence of reporter genes on the function of each studied gene, suggesting that they may contribute to the pathogenesis of schizophrenia.

Our findings support the “common disease, rare alleles” model for explaining some cases of schizophrenia [69]. The hypothesis is that the many mutations that predispose an individual to developing schizophrenia are highly penetrant and individually rare, sometimes even specific to a single patient or family. In this model, different patients harbored different mutations, either in the same gene or in different genes, but each one carried only one or two mutations. Given the fact that these individually rare variants may not contribute, to a significant degree, to the heritability of schizophrenia, their discovery is likely to be much more rewarding than that of common polymorphisms in terms of practical applications, including our understanding schizophrenia’s etiology.

After identifying these rare patient-specific variants that are related to synaptic pathology, family studies on the patients with these rare variants should be conducted in order to clarify their inheritance model, genotype/phenotype correlation, phenotypic variability, and penetrance rate. Furthermore, cell-based and electrophysiological experiments are warranted to verify their influence on signal transduction, membrane potential, cell proliferation, migration, and differentiation (e.g., axonal outgrowth, dendrite branching, and synaptogenesis). Furthermore, these findings will be essential to the development of model animals, further pathogenic studies, and novel drugs that could be used to treat this devastating disorder.

It is noteworthy that the targeted resequencing of genes has been used to successfully find associations between rare variants with quantitative traits. However, this approach is currently limited to selected candidate genes. Recently, massive parallel-sequencing technologies, in conjunction with new DNA-enrichment technologies (e.g., exome capture), have been developed that allow the sequencing of targeted regions in large samples of the human genome [70]. In addition, exome capture allows unbiased investigations into complete protein-coding regions of the genome. Because rare variants are usually associated with a high rate of penetrance, they may be much more likely to become the basis for some sort of personalized medicine than those usually discussed in relation to the common polymorphisms.

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