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Association of Synapsin 2 with schizophrenia in families of Northern European ancestry

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Abstract

The synapsin 2 (*Syn2*) gene (3p25) is implicated in synaptogenesis, neurotransmitter release, and the localization of nitric oxide synthase to the proximity of its targets. In this study we investigated linkage and association between the *Syn2* locus and schizophrenia. 37 pedigrees of Northern European ancestry from the NIMH Human Genetics Initiative collection were used. Four microsatellites and twenty SNPs were genotyped. Linkage (FASTLINK) and association (TRANSMIT, PDTPHASE) between markers and schizophrenia were evaluated. A maximum heterogeneity LOD of 1.93 was observed at marker D3S3434 with a recessive mode of inheritance. Significant results were obtained for association with schizophrenia using TRANSMIT (minimum nominal $p=0.0000005$) and PDTPHASE (minimum nominal $p=0.014$) using single marker analyses. Haplotype analysis using markers in introns 5 and 6 of *Syn2* provided a single haplotype that is significantly associated with schizophrenia using TRANSMIT (nominal $p<0.00000001$) and PDTPHASE (nominal $p=0.02$). Simulation studies confirm the global significance of these results, but demonstrate that the small p -values generated by the bootstrap routine of TRANSMIT can be consistently anticonservative. Review of the literature suggests that *Syn2* is likely to be involved in the etiology or pathogenesis of schizophrenia.

Keywords

schizophrenia; linkage; linkage disequilibrium; family based association tests; TRANSMIT; PDTPHASE; Synapsins

1. Introduction

Schizophrenia (SCZD [MIM 181500]) is a debilitating psychiatric disorder that affects approximately 1% of the human population worldwide. Twin, family and adoption studies are the principal methods that have been used to elucidate the genetic contribution to schizophrenia (McGuffin et al. 1994). It is considered a complex disorder since it cannot be explained by a single gene effect or environmental influence alone (Bassett et al. 2001, Lewis et al. 2003). Numerous regions in the human genome may influence susceptibility to the disease, as shown by several published linkage analysis studies, implying a complex mode of inheritance, incomplete penetrance and polygenic involvement (Bassett et al. 2002, Lewis et al. 2003).

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Synapsins are a family of three genes in higher vertebrates whose products are associated with the cytoplasmic surface of the synaptic vesicles and implicated in synaptogenesis and neuronal development, neurotransmitter release, and the localization of nitric oxide (NO) synthase to the proximity of NO targets in presynaptic neurons. All synapsins have a similar domain structure at the amino terminal region, which is composed of short A and B domains and a longer C domain. Domain A contains a phosphorylation site for protein kinase A and Ca^{++} /calmodulin-dependent protein kinase I and regulates neurotransmitter release in a phosphorylation dependent manner. Domain C accounts for more than half of the synapsin sequences, and is the most highly conserved domain with over 50% homology between vertebrate and invertebrate synapsins. Together with domain E, domain C is responsible for binding to the actin scaffold and maintaining the vesicle pool in the periphery. Domains D-I make up the carboxyl-terminal region, which varies among the different isoforms. As a result of alternative splicing, synapsin genes are known to be transcribed into three isoforms named “a”, “b”, and “b-like” with a structurally different C-terminal part of the molecule. Synapsins “a” are grouped together since they share a conservative domain E. Domains F and I are found in the “b” type of synapsins. Domains B, D, G, H, and J demonstrate an abundance of proline, glutamine, alanine, and serine and contain a site for phosphorylation by mitogens-activated protein kinase and Ca^{++} /calmodulin-dependent protein kinase II (Hilfiker et al. 2005, Hilfiker et al. 1999, Hosaka and Sudhof 1998).

A family of tissue inhibitors of metalloproteinase genes (*TIMP*) is associated physically with the synapsin family. TIMPs inhibit the matrix metalloproteinases, a group of zinc-binding endopeptidases, and are expressed in many tissues, with highest expression in the placenta. The *TIMP4* gene (OMIM 601915) is located within the sixth intron of *Syn2* and transcribed in the opposite direction. *TIMP1* (OMIM 305370) is located within the sequence of *Syn1* on chromosome X, and *TIMP3* (OMIM 188826) resides within *Syn3* on chromosome 22. While the evolutionary development of both synapsins and *TIMP* genes through duplications explains the physical link between the two families, there is currently no strongly established functional interaction between them (Olson et al. 1998, Pohar et al. 1999).

In humans, the *Syn2* gene (MIM 600755) is located on chromosome 3p25 and is translated into two proteins of 547 (*Syn2a*) and 478 (*Syn2b*) aminoacids. It exhibits high homology across a broad range of major phyla within the animal kingdom with conserved functionality (Kao et al. 1999), though the whole synapsin family does not appear to be required for survival, as shown by knockout experiments (Gitler et al. 2004). Brain and spinal cord are the major sites of expression.

Many theories regarding the etiology of schizophrenia emphasize the misregulation of neurotransmitters at the synapse. When an action potential reaches a nerve terminal, calcium ions enter via voltage-gated ion channels, triggering the fusion of vesicles to the plasma membrane and the subsequent release of neurotransmitters into the synaptic cleft (Greengard et al. 1993). There are two pools of synaptic vesicles present in the nerve terminal: a releasable pool of vesicles floating freely in the cytoplasm that can fuse to the plasma membrane following an action potential, and a reserve pool of vesicles that are reversibly tethered to the actin-based cytoskeleton. These reserved vesicles effectively replenish those that have undergone fusion, lessening the synaptic depression. Synaptic vesicles, the associated synapsin protein, and actin filaments form a complex that is anchored to the cytoskeleton. The phosphorylation of synapsin is believed to cause the dissociation of this complex, thereby increasing the density of free synaptic vesicles in the cytoplasm (Augustine et al. 1999, Greengard et al. 1993, Hosaka and Sudhof 1998).

Another important function of synapsins that relates to schizophrenia is their ability to localize NO in proximity of its target. Neurally produced NO is a highly reactive molecule with a broad

spectrum of functions that involve neurotransmitter release and extension of neuronal processes. As NO cannot be stored in vesicles, it must be synthesized by neuronal NO synthase (nNOS) on demand. To minimize the undesired reactivity of NO molecules in neurons, adapter proteins are used for the localization of nNOS. One such adapter protein is the nitric oxide synthase 1 (neuronal) adaptor protein encoded by the *NOS1AP* gene (MIM605551), previously known as the C-terminal PDZ domain ligand of neuronal nitric oxide synthase or *CAPON*, (Jaffrey et al. 2002), which was recently identified as a major candidate susceptibility gene for schizophrenia in a Canadian population of Northern European descent (Brzustowicz et al. 2002, Brzustowicz et al. 2000, Brzustowicz et al. 2004, Xu et al. 2005) and in Han Chinese (Zheng et al. 2005). Synapsins are known to colocalize with CAPON and nNOS in neurons and the absence of the *Syn1* and *Syn2* genes in knockout mice markedly alters the subcellular localizations of these molecules (Jaffrey et al. 2002).

Several published studies have investigated the mRNA expression profile of *Syn2* in the brains of subjects affected with schizophrenia. A microarray expression profile of the prefrontal cortex revealed that *Syn2* transcript levels were significantly decreased in nine out of ten subjects with schizophrenia as compared to ten matched controls (Mirnics et al. 2000). Although this finding was not confirmed by real-time PCR and immunoblotting experiments in another sample (Imai et al. 2001), another Western immunoblotting study found a decrease in synapsin (IIa and IIIa) expression in the hippocampus of individuals with schizophrenia (Vawter et al. 2002). *Syn2* transcript levels were significantly up-regulated in immunoblotting experiments in rats following chronic haloperidol treatment (Chong et al. 2002), implying a possible role in reaction to perturbations of the dopamine system. More recently it has been shown in the rat that *Syn2* expression may be up or down regulated by manipulations of the dopamine D1 and D2 receptors, further supporting the theory of neurotransmitter misregulation in the etiology of psychotic phenotypes (Chong et al. 2006).

Though no significant linkage results have been reported in the area of the *Syn2* gene, a suggestive LOD score of 2.34 was reported in this region from an affecteds only genome scan analysis of 57 multiplex schizophrenia families (Pulver et al. 1995). Recently, two association studies of *Syn2* polymorphisms and schizophrenia reported highly significant results in Han Chinese using case-control (Chen et al. 2004) and transmission-disequilibrium (Chen et al. 2004) approaches. In addition, significant haplotype association was observed in Korean sample (Lee et al. 2005).

In this study we used a data set of 37 multiplex schizophrenia families of Northern European ancestry to investigate linkage and association to the *Syn2* locus. Four microsatellite markers were used for the linkage analysis and 20 single nucleotide polymorphism markers (SNPs) that spanned almost the entire *Syn2* gene were used for two distinct tests of association.

2. Materials and Methods

2.1 Subjects

The sample of families used in this study was obtained from the National Institute of Mental Health (NIMH) Schizophrenia Genetic Initiative Collection. Schizophrenia pedigrees were ascertained by three independent medical sites (Columbia University, Harvard University, Washington University). The subjects were diagnosed according to DSM-III-R and DSM-IV classifications with the following systematic and comprehensive examination of affected individuals and their relatives using the Diagnostic Interview for Genetic Studies (Cloninger et al. 1998, Faraone et al. 1996, Nurnberger et al. 1994). Nuclear and extended pedigrees have been ascertained which have at least two individuals affected with schizophrenia who are first-degree biological relatives. Data on family structure and individual family members, including psychiatric diagnosis and other clinically relevant information, as well as lymphoblastoid cell

lines and DNA, are stored, maintained and distributed by the NIMH Center for Genetic Studies. All data and biological material is stored and distributed without any links to personal identifying information. To reduce the impact of genetic heterogeneity on association with the schizophrenia phenotype, families of exclusively Northern European ancestry were selected for this study. This set includes 292 individuals (98 founders) in 37 pedigrees with an average family size of 7.9 subjects (ranging from 4 to 28) and an average number of generations of 2.3 (ranging from 2 to 4). 229 DNA samples were available for genotyping from the cell repository at Rutgers University. For this study, a single schizophrenia phenotype definition was used with subjects diagnosed with schizophrenia or schizoaffective disorder designated as affected. Subjects unavailable for assessment were designated phenotype unknown. The remaining individuals, including those with schizophrenia spectrum diagnoses, were designated as unaffected. Overall, 89 subjects were assigned affected, 153 unaffected, and 50 unknown phenotypes. Written informed consent was obtained from all participants after explanation of possible consequences according to the protocols of NIMH Human Genetic Initiative.

2.2 Genotyping

The dbSNP database was used to select SNPs rs308969, rs308964, rs308965, rs931676, rs3817004, rs17035945, rs308952, rs99365, rs308950, rs308953, rs795009, rs795010, rs795011, rs3755724, and rs794999 for PCR amplification and genotyping assays. Three additional SNPs were identified by sequencing intron 6 of the *Syn2* gene in a set of 16 random founders from the sample described above (ss35528974, ss35528972, ss35528973). SNPs rs308969 (intron 2), rs308964 (intron 3), rs308965 (intron 4), rs931676 (intron 5), rs3817004, rs17035945, rs308952, rs99365, rs308950, rs308953, ss35528974 (intron 6), rs795009 (intron 8), rs795010 (intron 9), rs795011 (intron 10) were genotyped using simplex Pyrosequencing™ assays on the automated PSQ HS96A platform (Ahmadian et al. 2000, Ronaghi et al. 1998). PCR primers were designed using Primer3 (Whitehead Institute) and the sequencing primer used for the Pyrosequencing assay was designed using the Pyrosequencing SNP Primer Design Software v1.0 (Pyrosequencing). PCR reactions contained 40 ng of template DNA, 0.5 U AmpliTaq Gold polymerase (Applied Biosystems), 0.01 μM of each primer, dNTPs (200 μM each, Invitrogen), 1.5 mM of MgCl₂, 1 μl of GeneAmp 10x buffer II (Applied Biosystems), in a 10 μl volume. After 3 min at 95°C, 30 cycles were performed at 94°C for 30 s, at 50-60°C for 30 s, and at 72°C for 15 s, followed by a final extension step at 72°C for 3 min.

DNA fragments for SNPs rs3755724, ss35528972, ss35528973 (intron 6), rs794999 (exon 13) were amplified using a recently described multiplex PCR approach that minimizes primer complementarity, especially between their 3'-bases (Wang et al. 2005). Multiplex PCR was performed in 30-μl of PCR mix containing 1x PCR buffer (50 mM KCl, 100 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, and 100 μg/ml gelatin), dNTPs (200 μM each, Invitrogen), primers (20 nM each), 0.5 U AmpliTaq Gold polymerase (Applied Biosystems) and 20ng of template DNA. The samples were heated to 94°C for 15 min, followed by 40 PCR cycles of 40 sec at 94°C, 2 min at 55°C, and 5 min of ramping from 55°C to 70°C with 0.01°C/s increase. A final extension step was carried out at 72°C for 3 min. PCR amplifications were performed with the PTC-200 Programmable Thermal Controller (MJ Research). A DNA fragment containing SNPs rs598704 and rs598747 (intron 2) was amplified as a single PCR product. PCR reactions contained 40 ng of template DNA, 1U AmpliTaq Gold polymerase (Applied Biosystems), 0.01 μM of each primer, 2mM of MgCl₂, 1 μl of GeneAmp 10x buffer II (Applied Biosystems), in a 10 μl volume. After 10 min at 94°C, 40 cycles were performed at 94°C for 4 s, at 50°C for 60s, ramping from 50°C to 72°C with 0.2°C/s increase, at 72°C for 45 s, followed by a final extension step at 72°C for 3 min.

These 6 SNPs were genotyped using the Ligase Detection Reaction (LDR) combined with Luminex flow cytometry (Bortolin et al. 2004, Iannone et al. 2000). Three primers were

designed for each LDR assay: two allele-specific primers incorporating different 5'-FlexMAP™ Tags (Luminex® Corporation) and ending with the variant base, and a single SNP-specific common primer complementary to the sequence 3' to the SNP, 5'-phosphorylated, and ending with a 3'universal tag (CTATCTTTAACTACAAATCTAAC). LDRs were performed in a 20 µl volume containing 2 µl of multiplex PCR product, 1 µl of rs598704-rs598747 PCR product, 6 U Taq DNA Ligase (New England Biolabs), 0.15 pmol of allele specific and common primers for each SNP, 2 µl of 10x Taq DNA Ligase buffer (New England Biolabs), and distilled water. LDR was carried out at 95°C for 60 s followed by 22 cycles of 95°C for 15 s and 58°C for 2 min. The bead hybridization step was performed with 0.5 µl of each Luminex® FlexMap™ bead conjugated to anti-tag probes complementary to the FlexMAP™ Tags on the allele-specific primers, 0.48 pmol of 3'-biotinalated universal oligonucleotide (GTTAGATTTGTAGTTTAAAGATAG-biotin) complementary to the universal tag at the 3' end of the common SNP-specific primer, and 46.02 µl of hybridization buffer (3 M tetramethylammonium chloride, 50 mM Tris-HCl, pH 8.0, 3mM EDTA, pH 8.0, 0.1% SDS). After heating to 95 °C for 60 s, the hybridization reaction was carried out at 37°C for 20 min. Fluorescent labeling was performed by adding 0.12 µl of 1mg/ml streptavidin-R-phycoerythrin (Molecular Probes) to the hybridization buffer and incubating at 37°C for 40 min. Detection of allele-specific LDR-bead complexes was performed using a Luminex® 100™ Total System.

To facilitate the detection of SNP genotyping errors and to assess linkage in the *Syn2* area, four microsatellite markers spanning 4 Mb and located at the 5'-end (D3S4545, D3S3680), within intron 2 (D3S1259), and at the 3'-end (D3S3701) of the *Syn2* gene were genotyped using WellRED labeled oligonucleotides (Proligo®) in conjunction with automated fluorescent fragment analysis on the CEQ8000 (Beckman Coulter).

PCR, pyrosequencing and LDR primers are listed in Supplemental Materials.

2.3 Error-checking and statistical analysis

As genotyping errors are known to affect family-based tests of association (Mitchell et al. 2003), we undertook several error-checking steps. Genotype data from all *Syn2* SNPs and microsatellite markers were first checked for Mendelian inconsistencies using the program PEDCHECK (O'Connell and Weeks 1998). Second, the genotyping error probabilities were estimated with five runs of SIMWALK v2.82 (Sobel and Lange 1996, Sobel et al. 2002, Sobel et al. 2001) under different starting conditions using all genotyped SNP and microsatellite markers. Three genotypes with a probability of mistyping ≥ 0.25 were re-genotyped and since the apparent errors were not resolved, they were removed from further analysis. Overall, all of the genotypes for a single subject with multiple Mendelian errors and 5 other genotypes in the remainder of the sample were removed from the final analysis due to Mendelian inconsistencies and high probability of mistyping.

The PEDSTATS program was used to characterize the family structures and to assess deviations from Hardy-Weinberg equilibrium (Abecasis et al. 2002).

Parametric two- and multi-point linkage analyses of the microsatellite markers and the phenotype locus were performed using FASTLINK v.4.1P (Cottingham et al. 1993, Schaffer et al. 1994). Heterogeneity testing was conducted using the HOMOG program (Ott 1986). Parametric linkage analysis was conducted, as it is more powerful than nonparametric methods and is a robust method for detecting linkage despite errors or simplifications in the analyzing model, as long as both a dominant and a recessive model are used (Durner et al. 1999). Dominant and recessive genetic models and the narrow phenotype definition were the same as used previously (Brzustowicz et al. 2000, Saviouk et al. 2004).

Association between the genotyped SNPs and the phenotype was assessed using two different methods. Allelic transmission of markers from parents to affected offsprings was evaluated by the Transmission Disequilibrium Test (TDT) using the TRANSMIT software (version 2.5.4) (Clayton 1999). TRANSMIT can analyze single marker data as well as multi-locus phase-unknown haplotypes and allows for unknown parental genotypes. Rare haplotypes with frequency less than 0.03 were aggregated by using the “-c3” option. Multiple nuclear families from a single pedigree were allowed (“-mf”) with robust estimation of the variance of the vector score (“-r”). Reported p-values for association were based on up to 10,000,000 internal bootstrap procedures.

To estimate the linkage disequilibrium (LD) between SNP markers, and the significance of association between SNP markers and the phenotype locus in pedigrees of various structures, we used an extension of the pedigree disequilibrium test (PDT) (Martin et al. 2000) implemented in the program PDTPHASE, which is a part of the UNPHASED package (Dudbridge 2003) of association analysis programs. We used the “missing genotypes” and “equal weight to all families” options for our analyses. The PDTPHASE output was used to report global p-values for association of individual SNPs with the disease phenotype based on 1,000 permutations and their corresponding transmission statistics.

To assess the effect of linkage in the area on the results of TRANSMIT and PDTPHASE a simulation study was conducted. Two SNPs with minor allele frequencies (MAF) of 0.1 and 0.5, respectively, were simulated by SLINK (Ott 1989) with 0%, 25%, 50%, 75%, and 100% of families linked (α) at $\theta = 0.1$ to the affection locus using the original affection status and pedigree structures but without any association between the markers and the affection locus. For each SNP, MAF, and value of α , 10,000 replicates were generated and analyzed using TRANSMIT and PDTPHASE under the same conditions as in the real data. For MAF=0.1 and $\alpha=0$, an additional 75,000 replicates were generated to estimate small empirical p-values for TRANSMIT. Output of the simulation was arranged into a table to allow empiric evaluation of the results.

3. Results

All of the tested microsatellite and SNP markers were in Hardy-Weinberg equilibrium among the founders ($p \geq 0.70$) and in the whole sample ($p \geq 0.08$). Four groups of SNPs appeared to be in four different, partially interdigitated, haplotype blocks in complete LD ($D'=1$, $r^2=1$) and therefore were considered as four single markers for subsequent analyses. Block 1 consisted of rs308969, rs308964, rs308965, and ss35528974 which spread through introns 2, 3, 4, and 6. Block 2 included rs931676 in intron 5, and ss35528973 and rs308953 in intron 6. Three SNPs in intron 6, rs99365, rs308952, and rs308950, were grouped into Block 3. Block 4 was formed by rs795009, rs795010, and rs795011, covering introns 8, 9, and 10. Seven SNPs fell outside of these defined LD blocks and so were included individually in further analysis.

LD between the eleven markers defined above was analyzed using PDTPHASE. Table 1 demonstrates that all SNPs exhibit high level of LD between each other. For most of the markers $D'=1$, implying that transmission of markers can be analyzed jointly, using haplotypes.

No significant ($LOD \geq 3$) or suggestive ($LOD \geq 2$) linkage results were obtained in linkage analysis with the microsatellite markers in the *Syn2* area. A maximum two-point heterogeneity LOD (HLOD) score of 1.93 was obtained at marker D3S3434 ($\theta = 0$) under a recessive model of inheritance and with 35% of families linked. Multi-point analysis using all of the genotyped microsatellites in a five-point parametric linkage analysis did not produce an increase in LOD or HLOD values, with a maximum HLOD of 1.21 at D3S3434 under a recessive model and 25% of families linked. The addition of SNPs data did not significantly affect the outcome of

the multipoint linkage analysis. Results of the two-point linkage analysis under homogeneity and heterogeneity for microsatellite markers in the *Syn2* region are presented in Table 2.

The PDTPHASE and TRANSMIT statistics for each marker are provided in Table 3. Single marker transmission disequilibrium to affected and unaffected siblings (PDTPHASE) demonstrated the most interesting results for Block 3 and rs17035945, both in intron 6, where significant transmission disequilibrium was observed at both markers (nominal $p=0.014$ and $p=0.021$, respectively). Additionally, slight overtransmission of the common allele to affecteds was observed for SNP rs598747 in intron 2 ($p=0.034$) and slight overtransmission of the rare allele to affecteds was detected for markers in Block 2 ($p=0.037$). Analyses of allelic transmissions from parents to affected subjects (TRANSMIT) demonstrated significant disequilibrium for Block 2 (nominal global $p=0.0000005$), Block 3 ($p=0.00024$), rs598747 ($p=0.0039$), ss35528972 ($p=0.0042$), and rs3817004 ($p=0.0097$).

Although we did not demonstrate suggestive linkage ($LOD \geq 2.0$) in the area, a review of modern literature on the genetics of complex traits provides numerous examples where any LOD score greater than 1.0 is presented as “worthy of attention”. Therefore, we conducted a simulation study to assess how various strength linkage signals could affect the results of the TRANSMIT and PDTPHASE association tests. Two SNPs with MAF of 0.1 and 0.5 were analyzed for evidence of association in simulated datasets of the same family structure as our sample, with varying proportions of families linked to either simulated SNP. Overall, our data shows that for pedigrees of this structure TRANSMIT and PDTPHASE p -values are not distorted by the presence of linkage in the area (Table 4). However, for all tests the TRANSMIT bootstrap-based p -values seem to be anticonservative with a p -value of 0.05 or less being seen in approximately 7% of replicates for MAF=0.5 and 8% for MAF=0.1 when unassociated data was analyzed, regardless of the degree of linkage in the sample. Interestingly, the anticonservative nature of the TRANSMIT p -values increased as the probability of obtaining smaller and smaller p -values by chance was examined. P -values of 0.001 were seen approximately 50 times more often than expected by chance when MAF=0.5 and 92 times more often when MAF=0.1. Overall, inflation of the TRANSMIT p -values was greatest when the MAF=0.1 and all families were unlinked. For this case, a nominal bootstrap p -value of 0.0202 would be equivalent to an empiric p -value of 0.05, while a nominal p -value of 0.00038 would be equivalent to an empiric p -value of 0.01, and a nominal p -value of 0.00001 would be equivalent to an empiric p -value of 0.005.

PDTPHASE performance was closer to expected from its reported statistic with a p -value of 0.05 or less observed in approximately 3.6% of the replicates regardless of the degree of linkage or MAF. The PDTPHASE p -values were almost always overly conservative, with the least conservative p -values occurring with the MAF=0.5 and 25 to 50% of the sample linked. Based on the least conservative condition observed, a nominal p -value of 0.062 would be equivalent to an empiric p -value of 0.05. Table 4 summarizes the results of the simulation study.

A limited number of haplotype analyses were performed on the data. First, we performed a PDTPHASE haplotype analysis with markers rs5987747, Block2 (rs931676), Block 3 (rs99365) and rs170035945, each of which demonstrated some evidence of association with schizophrenia (nominal $p < 0.05$) in the single marker test. One out of the four observed haplotypes, haplotype C-C-T-C, showed a significant transmission disequilibrium with 19 transmissions to affected and 12 transmissions to non-affected siblings ($p=0.024$), although the global permutation-based p -value for all haplotypes was not significant ($p=0.056$). TRANSMIT haplotype analysis with markers rs598747, Block 2 (rs931676), Block 3 (rs99365), rs3817004, and ss35528972 (nominal $p < 0.05$ in single marker TRANSMIT tests) did not yield any significant results for any of 5 observed haplotypes. Since the majority of markers with evidence for association under the single marker tests fall within intron 5 and

intron 6, we performed haplotype analysis with markers only from this area of the gene. Seven haplotypes were observed for markers Block 2 (rs931676), Block 3 (rs99365), rs17035945, rs3817004, ss35528972, and rs3755724 in both the TRANSMIT and PDTPHASE tests. Three of the observed haplotypes produced evidence of association with schizophrenia in both tests. The most interesting results were obtained for haplotype C-C-T-A-A-C that demonstrated the strongest association with both the TRANSMIT ($p < 0.00000001$) and PDTPHASE ($p = 0.02$) tests, with overtransmission to affected individuals. The global p-value for all haplotypes tested was significant only for the TRANSMIT test ($p = 0.0066$). Table 5 summarizes results of both haplotype tests using markers in intron 5 and 6 of the *Syn2* gene.

4. Discussion

In this study we investigated linkage and association of *Syn2* gene polymorphisms with schizophrenia using four microsatellite markers and twenty SNPs. We failed to identify significant ($\text{LOD} \geq 3.0$) or suggestive ($\text{LOD} \geq 2.0$) linkage to the area in this sample. However, family-based association analysis of the transmission of *Syn2* SNPs individually and as haplotypes from parents to affected offspring and transmission disequilibrium between affected and unaffected siblings provided evidence that *Syn2* is associated with the development and/or pathogenesis of this debilitating disorder.

Analysis of twenty SNPs spanning 117 kb in the *Syn2* gene revealed a complex LD/haplotype structure of the gene. We observed four groups of SNPs in tight LD blocks with $D' = r^2 = 1$. Physically, these blocks ranged in size with the largest spreading over 20.6 kb and covering almost 10% of the gene (Block 1), and the smallest encompassing only 2.4 kb (Block 4). While in our study Block 4 appeared as a discreet haplotype unit, the other blocks interdigitated significantly. The largest block (Block 1) encompassed the proximal 96.5% of the 9.6 kb Block 2 and the proximal 40.7% of the 8.8 kb Block 3, while the distal 40.9% of Block 2 overlapped with the proximal 44.6% of Block 3. We demonstrated that strong LD encompassed almost the entire gene, with the majority (39 of 55) of non-redundant marker pairs being in complete LD ($D' = 1$), a large number (14 pairs) demonstrating very strong LD (D' from 0.76 to 0.92) and only two marker pairs with $D' < 0.6$ ($D' = 0.45$ for Block 2 vs Block 4 and $D' = 0.53$ for rs17035945 vs Block 4). Public LD databases, such as that provided by Perlegen Sciences and Hapmap, reveal a similarly complex pattern of LD in this region, although with differences in the details of the LD/haplotype structures observed, presumably due to differences in the markers and populations used for the different analyses.

Out of eleven SNPs and SNP block markers analyzed for association with schizophrenia, four showed disequilibrium of transmission to affected subjects and their unaffected siblings and five demonstrated deviation of transmission from parents to affected offspring. Although these results of association were obtained in an area without significant evidence of linkage to schizophrenia in this sample, in light of the mildly positive lod scores (maximum HLOD of 1.9) we wished to explore the possible effect of linkage on the tests of association. A simulation study to assess the effect of linkage on the results of the tests of association revealed that the PDTPHASE and TRANSMIT statistics appear to be independent of the effect of linkage for this sample. While TRANSMIT appeared to be anticonservative when calculating p-values by its internal bootstrap procedure, our simulation analysis demonstrated that the observed associations with schizophrenia are still highly significant. According to the most conservative scenario observed in our simulation results, an empiric p-value of 0.05 is equivalent to a nominal p-value of 0.02 in TRANSMIT and of 0.06 in PDTPHASE. Five out of eleven SNP markers reached this level of empiric significance for each test; markers rs598747, Block 2, Block 3, and ss35528972 were empirically significant in both tests, while markers rs17035945 and rs3817004 were empirically significant in only PDTPHASE or TRANSMIT, respectively.

While three of the SNPs tested produced nominal p-values <0.05 with both PDTPHASE and TRANSMIT, more SNPs were detected as associated using TRANSMIT than PDTPHASE, and the significant p-values obtained with TRANSMIT were one to five orders of magnitude smaller than the PDTPHASE p-values for the same markers. Part of this difference appears to be due to the increasingly large distortion of the TRANSMIT nominal p-values for smaller values. So while, under the most conservative scenario, a nominal p-value of 0.05 occurred in 8.5% of the replicates by chance, a nominal p-value of 0.001 occurred 1.2% of the time, and a nominal p-value of 0.0001 occurred 0.7% of the time. However, even accounting for this inflation does not appear to fully explain the differences in the results. Further differences can be explained by the different statistical approaches of the two methods. TRANSMIT, a TDT based method, evaluates the transmission of markers from parents to affected offspring. It calculates a score vector averaged over all possible configurations of parental haplotypes and transmissions. Data from unaffected siblings and siblings with unknown phenotype is used only to narrow the possible parental genotypes and haplotype configurations. PDTPHASE is based on the PDT test (Martin et al. 2000) that uses a broader range of information from an extended pedigree by including unaffected siblings into the statistical analysis of association. For the PDT, a measure of linkage disequilibrium is defined for each triad and each discordant sib pair within a pedigree, and an average is determined for each pedigree. The difference in what is considered the fundamental unit for each test (heterozygous parents for TDT, triads and discordant sib pairs for the PDT) can also lead to differences in the test statistics. For example, in the case of triads with two heterozygous parents, the test statistic for the TDT can be double that of the PDT in the situation where the allelic transmission of the two heterozygous parents is concordant (Martin et al. 2000). We assume that *Syn2* is a susceptibility gene that is neither necessary nor sufficient for the development of schizophrenia. Individuals inheriting certain alleles of this gene are at increased risk for developing the schizophrenia phenotype. Environmental factors or susceptibility alleles at other genes may also be required before a sufficient threshold is reached for the phenotype to manifest, resulting in apparent reduced penetrance of the *Syn2* disease-associated allele. The use of risk allele carriers who do not manifest the illness by the PDT-based tests, but not the TDT-based test, could also contribute to a weaker association result for the PDT in situations with greatly reduced penetrance. While TRANSMIT appears to have greater power to detect the association present between *Syn2* and schizophrenia in this sample, small p-values generated by this program must be evaluated with great caution due to the anticonservative nature of the internal bootstrap procedure.

Overall, 11 markers were tested for association. Given the strong marker to marker association in this region, considering these as 11 independent tests would be overly conservative. While it unclear if the PDTPHASED results would reach significance if corrected for multiple testing, our simulations do indicate that the TRANSMIT results are significant. Applying the overly conservative Bonferroni correction would require a p-value of 0.0045 to reach study-wide significance. Our simulation results suggest that to obtain an empiric TRANSMIT p-value of 0.005 would require a nominal p-value of 0.00001. Thus even with a Bonferroni correction, the Block 2 nominal p-value of 0.0000005 would reach study-wide significance.

Haplotype analysis demonstrated that simply combining all markers with evidence of association under the single marker tests was not helpful in identifying a haplotype that is likely to harbor schizophrenia associated polymorphism within the *Syn2* gene. One explanation could be that the associated SNPs are in LD with more than one susceptibility allele, so combining them all into a single analysis with markers spanning over 90 kb was not beneficial. Most of the associated SNP makers were located in introns 5 and 6 which are known for their evolutionary conservation due to the presence of the *TIMP4* gene in intron 6 of *Syn2*. A positional haplotype analysis approach that densely covered a 14.4 kb interval from this area identified seven haplotypes consisting of markers from introns 5 and 6 of *Syn2*, with one of these haplotypes significantly overtransmitted to affected individuals.

Although the majority of the schizophrenia associated SNPs and haplotypes within the *Syn2* gene fall into the area of the *TIMP4* gene, *TIMP4* seems to be an unlikely functional candidate for schizophrenia. The expression profile of the TIMP family is diverse and includes multiple tissues, including the central nervous system. The literature on this family is limited, and fails to report a significant association of TIMP genes with schizophrenia on genetic or functional levels (Hung et al. 2001). Moreover, the review of the clinical manifestations caused by alterations within the TIMP family (TIMP1 [MIM305370], TIMP2 [MIM188825], TIMP3 [MIM188826], TIMP4 [MIM601915]) reveals a single established human phenotype, Sorsby fundus dystrophy, that does not resemble schizophrenia or any other psychotic illnesses. However, *TIMP4* cannot be ruled out as a susceptibility gene on the basis of our association data. The nested relationship of the TIMP and synapsin family members will likely necessitate a functional approach to differentiate the possible role of *Syn2* and *TIMP4* in the etiology of schizophrenia.

This sample represents the first population of Northern European descent to exhibit an association between *Syn2* and schizophrenia. Recently, evidence of schizophrenia associated polymorphisms in case-control and family based studies were found in the Han Chinese (Chen et al. 2004, Chen et al. 2004) and Korean (Lee et al. 2005) populations. Further association studies may provide a more precise location of the schizophrenia associated polymorphism(s) within the *Syn2* gene. Functional studies of polymorphisms identified through association studies should aid in the identification of the schizophrenia risk-allele within this region. Interestingly, samples from both Northern European descent (Brzustowicz et al. 2004) and Han Chinese (Zheng et al. 2005) populations have also demonstrated association between schizophrenia and *NOS1AP*, a gene that is functionally linked with *Syn2* (Jaffrey et al. 2002). A combined influence of *NOS1AP* and *Syn2* on schizophrenia susceptibility is plausible and warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

LD between SNP markers

SNP markers	rs598747	rs598704	Block 1	Block 2	Block 3	rs17035945	rs3817004	ss35528972	rs3755724	Block 4	rs794999
rs598747		1	1	1	1	1	1	0.92	0.86	0.87	0.89
rs598704	0.36		1	1	1	1	1	0.91	0.76	1	1
Block 1	0.58	0.21		1	1	1	1	0.90	1	1	1
Block 2	0.02	0.20	0.01		1	1	1	1	1	0.45	1
Block 3	0.92	0.33	0.65	0.02		1	1	0.86	1	1	1
rs17035945	0.04	0.41	0.02	0.49	0.04		1	1	0.84	0.53	0.82
rs3817004	0.25	0.09	0.01	0.005	0.01	0.28		1	1	1	1
ss35528972	0.26	0.09	0.42	0.01	0.01	0.25	0.003		1	0.86	0.92
rs3755724	0.07	0.15	0.05	0.05	0.08	0.08	0.01	0.03		0.88	0.90
Block 4	0.38	0.74	0.30	0.06	0.17	0.45	0.13	0.11	0.14		1
rs794999	0.35	0.85	0.25	0.24	0.33	0.38	0.11	0.11	0.17	0.84	

D' measures are in the top-right part and R² measures are in the bottom-left part of the table.

Table 2
Maximum two-point LOD scores under homogeneity and heterogeneity for microsatellite markers in *Syn2* region.

Model	Loci	cM	Max LOD	θ	Max HLOD	θ	α
Dominant	D3S4545	0	0.22	0.2	0.69	0	0.3
	D3S3680	7.4	0.48	0.2	0.49	0.2	0.9
	D3S1259	8.9	0	0.5	0	0.5	1
Recessive	D3S3701	11.8	0.56	0.2	1.06	0	0.25
	D3S4545	0	0.97	0.2	1.93	0	0.35
	D3S3680	7.4	0.08	0.3	0.10	0.3	0.7
	D3S1259	8.9	0	0.5	0.05	0.1	0.1
	D3S3701	11.8	0.09	0.3	0.43	0.05	0.2

cM - Map position in centimorgans from the first marker (D3S4545)

θ – Recombination fraction;

Max LOD - maximum LOD score under homogeneity;

Max HLOD - maximum LOD score under heterogeneity;

α – Proportion of linked families.

LOD values of > 1.0 are in bold.

Table 3 Transmission statistics to affected and nonaffected siblings (PDTPHASE) and from parents to affected subjects (TRANSMIT)

SNP markers	Location	Alleles ^d	Frequency ^b	Transmission ^c		χ^2	p-value	
				A	NA		PDTPHASE	TRANSMIT
rs598747	Intron 2	C/T	0.8438	261	243	4.50	0.034	0.0039
rs598704	Intron 2	C/T	0.6574	214	212	0.29	0.62	0.70
Block 1	Intron 2	T/C	0.9011	278	270	2.38	0.12	0.34
rs308969	Intron 3	G/A						
rs308964	Intron 4	G/C						
rs308965	Intron 6	T/C						
ss35528974								
Block 2	Intron 5	T/C	0.9041	289	296	3.74	0.037	0.0000005
rs931676	Intron 6	G/A						
ss35528973	Intron 6	A/G						
rs308953								
Block 3	Intron 6	C/T	0.8563	273	254	7.80	0.014	0.00024
rs99365	Intron 6	G/A						
rs308952	Intron 6	G/C						
rs308950								
rs17035945	Intron 6	C/T	0.8224	264	284	4.4	0.021	0.09
rs3817004	Intron 6	A/G	0.9552	311	300	3.65	0.13	0.0097
ss35528972	Intron 6	A/T	0.9456	279	271	3.33	0.058	0.0042
rs3755724	Intron 6	C/T	0.6695	181	183	0.03	0.86	0.42
Block 4	Intron 8	A/C	0.7296	221	222	1.06	0.59	0.30
rs795009	Intron 9	A/T						
rs795010	Intron 10	A/C						
rs795011								
rs794999	Exon 13	G/A	0.6962	213	208	0.08	0.78	0.42

^a Common alleles are shown first

^b Frequency of the common allele

^c Transmission as calculated by PDTPHASE to affected (A) and nonaffected (NA) siblings (shown for major alleles only)

^d χ^2 values as reported by TRANSMIT

Nominal p-values < 0.05 are in bold

Frequency of observing target p-values using TRANSMIT and PDTPHASE in an area of linkage with various proportions of linked families, based on 10,000 simulations for each condition tested.

Observed p-value ^a	Method	Proportion of linked families (<i>a</i>)									
		0		0.25		0.5		0.75		1	
		0.5 ^b	0.1 ^b	0.5 ^b	0.1 ^b	0.5 ^b	0.1 ^b	0.5 ^b	0.1 ^b	0.5 ^b	0.1 ^b
0.05	TRANSMIT	0.067	0.085	0.072	0.084	0.071	0.081	0.071	0.078	0.068	0.070
0.05	PDTPHASE	0.038	0.033	0.039	0.035	0.038	0.033	0.035	0.036	0.035	0.034
0.01	TRANSMIT	0.020	0.033	0.022	0.032	0.025	0.03	0.023	0.028	0.019	0.023
0.01	PDTPHASE	0.006	0.004	0.007	0.004	0.010	0.010	0.006	0.0039	0.006	0.0039
0.005	TRANSMIT	0.013	0.023	0.015	0.022	0.015	0.021	0.014	0.018	0.013	0.016
0.005	PDTPHASE	0.0021	0.0018	0.0028	0.0018	0.0017	0.0016	0.0019	0.0016	0.0023	0.0012
0.001	TRANSMIT	0.0049	0.0119	0.005	0.0119	0.0058	0.0105	0.0045	0.0089	0.0047	0.0029
0.001	PDTPHASE	0.0003	0.0002	0.0004	0.0003	0.0004	0.0001	0.0004	0.0004	0.0003	0.0002

^aThe frequency was calculated for observing this or a smaller p-value in PDTPHASE and TRANSMIT analysis of simulated replicates with no association and varying degrees of linkage

^bFrequency of minor allele

Haplotype analysis with markers covering exclusively introns 5 and 6 of *Syn2*: Block 2 (rs931676), Block 3 (rs99365), rs17035945, rs3817004, ss35528972, rs3755724.

Haplotype	PDT phase statistics		Haplotype Probability ^c	Transmit statistics			p-value
	Transmission A ^a	NA ^b		Transmission to affecteds O ^d	E ^d	χ ²	
T-T-C-G-A-C	5	16	0.04	4	8	4.20	0.0000043
T-C-C-A-A-C	95	89	0.59	59	60	0.03	0.86
C-C-T-A-A-C	19	12	0.02	18	13	4.83	<0.00000001
T-C-T-A-A-C	28	17	0.20	15	14	0.07	0.68
T-T-C-A-A-C	9	14	0.04	6	5	0.16	0.27
T-T-C-A-T-C	29	32	0.04	6	10	4.50	0.0015
T-C-C-A-A-T	126	125	0.76	55	53	0.25	0.63
Global p- values			0.087				0.0066

^aTransmission to affected and non-affected siblings
^bTransmission to affected and non-affected siblings
^cHaplotype probabilities as defined by transmit
^dObserved and expected transmissions
^eObserved and expected transmissions

p-values ≤ 0.05 are in bold; global p-values are based on 1,000,000 bootstraps for TRANSMIT and 1,000 permutations for PDTPHASE.