Association Analysis of Candidate Genes for ADHD on Chromosomes 5p13, 6q12, 16p and 17p

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Abstract: Attention-deficit/hyperactivity disorder (ADHD) is a common, heterogeneous psychiatric disorder of childhood displaying inattentive, hyperactive, and impulsive symptoms. Although the biological basis of ADHD remains unknown, it has been shown that genetic factors substantially contribute to the aetiology of the disorder. Our group has previously reported significant linkage to four chromosomal regions on 5p13, 6q12, 16p13 and 17p11 in genome-wide scans and subsequent fine-mapping. We selected nine positional candidate genes within the linkage intervals for study based on biological plausibility. We analyzed at least 189 ADHD trios to determine if common variants in these genes have a major affect on ADHD risk. None yielded significant association. This does however not completely exclude these genes as potential susceptibility genes for ADHD since it is plausible for common causal variants with low effect size to go undetected due to insufficient power of the study sample. We conclude that none of the tested alleles confer a major risk for developing ADHD and that investigation of other genes within the linked regions is warranted.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) [MIM 143465] is one of the most common childhood disorders with a disease prevalence ranging from 5%-10% [1, 2]. While most studies of ADHD are focused on affected children, a disease prevalence of 3% is observed among adults. ADHD diagnosis is four times as frequent in males as in females, and the worldwide prevalence across different populations and cultural settings are found to be very similar [2-8]. As in most psychiatric disorders, there is no definite test for ADHD and both diagnosis and classification rely on behavioral description. The clinical phenotype has clearly been refined through the replacement/advances of DSM-III-R to DSM-IV, however further understanding of endophenotypes and the impact of co-morbidities are necessary for proper clinical intervention. Diagnostic assessment generally defines two symptom dimensions of ADHD, inattentive and hyperactive-impulsive, that are used to discern three categorical subtypes including inattentive (I), hyperactive-impulsive (HI) or a combined form (C).

To date, five genome-wide scans have been conducted in ADHD worldwide, of which two were carried out by our group in U.S population samples [9, 10]. The most prominent linkage findings from each study are summarized in Table **1**. In summary, four regions exceeded the genome-wide threshold for significance [11] in the genome-wide linkage analyses and their subsequent fine-mapping on chromosomes 5p13 [2, 13], 6q12 [13], 16p13 [13, 14] and 17p11 [13, 15]. Nevertheless, the original linkage findings are generally considered type I errors until they have been replicated in an independent study sample (LOD $\geq$ 1.2 or P $\leq$ 0.001). The chromosome 5p region satisfies these criteria for declaring linkage. In a German population sample, Hebebrand *et al*. (2006) defined a significant linkage peak spanning 5p15-p13 (HLOD 4.75 at 17cM, multipoint LOD = 3.37 at 40 cM) [12]. Additionally, Ogdie and colleagues reported a two-point MLS of 3.24 and multipoint MLS of 2.55 on 5p13 [13], within the 1-LOD support interval reported by Hebebrand *et al*. (2006) [12]. Bakker *et al*. (2003) performed a genome-wide linkage study in a Dutch population sample that yielded additional supporting evidence with a multipoint MLS of 1.43 on 5p13.1 [16]. Due to the overlap on chromosome 5p between the studies, a meta-analysis was conducted by combining the Dutch and U.S linkage data. This resulted in increasingly significant support for linkage to 5p13 (MLS: 3.67) [17]. A study of multigenerational families in a Colombian population isolate presented nominal support for linkage to 5p13 [15]. Another promising region is found on chromosome 17p11 where Ogdie *et al*. (2004) observed significant linkage with a multipoint MLS of 3.63 [13]. This finding was replicated in a Colombian sample by Arcos-Burgos *et al.* (2004) who observed significant linkage (LOD: 3.4 – 3.9) stretching from chromosome 17p13.2 - 17p13.1 [15].

Both of the replicated chromosomal loci on 5p15-p13 and 17p13-p11 have linkage peaks with broad 1-LOD support intervals in the range of 10 – 40cM, both within the individual studies and across regions defined by meta-analyses. It could therefore be argued that these findings do not represent the same linkage signal and hence would not be true replications of a common locus. However, it has been demonstrated in several simulation studies that the position of the linkage peak for complex-trait susceptibility loci is influenced by factors such as locus heterogeneity, sample size and incomplete penetrance. The localization of the

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Table 1. Genome-Wide Scans of ADHD and Subsequent Fine-Mapping (The Replicated Regions are Bolded)

Study	Year	Sample (Population)	Linkage Findings
Fisher *et al*. [9]	2002	126 ASP (U.S)	**2q24, 5p12**, 10q26, 12q23, 16p13
Smalley *et al*. [14]	2002	203 ASP (U.S)	16p13
Ogdie *et al*. [10]	2003	270 ASP (U.S)	**5p13**, 6q14, 11q25, 16p13, **17p11**, 20q13
Bakker *et al*. [16]	2003	164 ASP (Dutch)	**5p13**, 7p13, 9q33, 13q33, 13q33, 15q15
Ogdie *et al*. [13]	2004	308 ASP (U.S)	**5p13**, 6q12, 16p13, **17p11**
Acros-Burgos *et al*. [15]	2004	16 multiplex families (Columbian)	4q13, 5q33, 11q22, **17p11**
Hebebrand *et al*. [12]	2006	155 ASP (German)	**5p15-p13**, 12q24

linked region may therefore be susceptible to variations from study to study [18-20]. In light of these observations, it is a reasonable hypothesis that these linkage peaks represent the same underlying ADHD susceptibility locus, especially considering the differences between the four studies in the ascertainment strategies, phenotypic definition methods, pedigree structures and populations studied.

In fact, no association in ADHD to date has satisfied the criteria for genome-wide significance [$P < 10^{-7}$] [21]. Motivated by the assumption that dysfunction in dopaminergic systems underlies the etiology of ADHD most candidate gene studies have focused on genes involved in dopamine metabolism and transmission. Perhaps the most consistently replicated finding is association to the DRD5 (CA) n repeat. Lowe *et al*. (2005) combined data from 14 independent samples comprising of 1980 cases and 3072 parents reported strong association to the DRD5 locus (p = 0.00005). However, the odds ratio is small (OR 1.24; 95% CI 1.12-1.38) indicating a modest effect size, and the necessity to expand sample size in order to reach significance [22]. Other dopaminergic genes reported to be associated with ADHD include dopamine receptor 4 (DRD4), the dopamine transporter (DAT1), dopamine beta-hydroxylase (DBH), and the synaptosome associated protein 25 (SNAP25). Again, independent studies have been equivocal and the failure to replicate may result from small underlying effect and inadequate sample sizes.

While association analyses present a more statistically powerful methodology to detect effect loci, findings in ADHD have been equivocal. The vast majority of the study samples to date are underpowered to detect common causal variants with low to moderate effect sizes. The current report exploits the relatively strong linkage data published to date. Focusing on genes within the four significant linkage peaks, of which two have been replicated (5p13 and 17p11) greatly increases the probability of detecting common effect alleles. The prior significant evidence that a causative variant exists within a region containing on average 170 genes enables a more efficient strategy of identifying effect alleles. Within the linked regions we prioritized genes that are central to brain processes that are involved in neurotransmitter systems that play a central role in the biology of ADHD.

We have investigated candidate genes within the linked regions on chromosomes 5p13, 6q12, 16p13 and 17p11 that have emerged from our genome-wide linkage scans and fine mapping. Performing association analysis of candidate genes in a population sample previously presenting evidence of linkage in the selected regions increases the precision of the overall genetic model; the association signal observed can be evaluated for the likelihood that the variants tested can account for the observed linkage signal. Altogether, nine candidate genes were analyzed across four chromosomal regions (5p13.2: SLC1A3, GDNF; 6q13: RIMS1; 16p: SNN, NDE1, DOC2A; 17p: VAMP2, PSD95, EPN2). We found nominal association between ADHD and the RIMS1 gene located on chromosomes 6q13, however the association did not withstand the correction for multiple testing.

MATERIAL AND METHODS

Study Sample

The study sample derives from families that were recruited to a UCLA ADHD sibpair study. Each family was recruited by advertisement from multiple sources, including previous research studies of ADHD at UCLA, clinics, hospitals, and schools in the greater Los Angeles area [23] and the major inclusion criteria was parental indication of at least two children with ADHD. Further details of the ascertainment strategy of the study sample have been described previously [10, 23]. After all participating families provided an informed consent approved by the UCLA institutional review board diagnostic evaluations were conducted by clinical psychologists or highly trained interviewers with extensive experience and reliability training in psychiatric assessment. A wide range of diagnostic assessment tools were utilized, including the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version (K-SADS-PL) [24] for ages 5 to 17 and the SADS-Lifetime version, Modified for the Study of Anxiety Disorders, Updated for *DSM-IV*) (SADS-LA-IV) [25] for ages 18 and older. The SADS-LA-IV was then supplemented with the Behavioral Disorders section of the K-SADS-PL to elucidate lifetime and current diagnoses of ADHD, CD, and ODD in subjects 18 years of age and older. Additional measures such as the parent and teacher versions of the SNAP-IV [26], the Child Behavior Checklist, and the Teacher's Report Form [27] was also collected. Teacher's Reports Forms were used to supplement information obtained in the direct interview to achieve best-estimate diagnoses using all available information. Each parent also completed 2 self-report questionnaires, the Wender Utah Rating scale [28] and the ADHD-IV scale [29]. The ADHD-IV scale was also completed by each parent about his/her partner's current behavior.

Diagnosis was determined using a best-estimate procedure, with senior psychiatrists (J.J.M and J.T.M) reviewing all positive diagnosis. All psychiatric conditions were coded

according to *DSM-IV* criteria. The mean weighted κ for diagnoses was 0.84 (SD = 0.14), including values for ADHD (1.0), oppositional defiant disorder (0.93), and conduct disorder (1.0). Included families had to have at least one proband that met full *DSM-IV* criteria for definite ADHD, the other proband could have a diagnosis of either definite or probable ADHD. Probable ADHD was defined as a subject falling one symptom short of diagnostic criteria (including behavioral symptoms, age at onset, duration, or presence in 2 settings), but with evidence of impairment. Both family members had to have a full scale IQ greater than 70 (measured by using the Wechsler Intelligence Scale for Children, 3rd edition) [30], and no known genetic conditions associated with ADHD (eg. tuberous sclerosis, fragile X, generalized resistance to thyroid hormone), or diagnosed with schizophrenia and autism. In the present study we analyzed ADHD 189 trios (320 ADHD cases) for 6 of the 9 genes (EPN2, PSD95, RIMS1, SLC1A3, SNN, VAMP2) and 220 trios (335 ADHD cases) for 3 of the 9 genes based on additional sample availability (DOC2A, GDNF, NDE1). The detailed information of the study sample is presented in Table **2**.

Table 2. Demographics and Clinical Characteristics of the Total Study Sample of 258 Trios (n=774) (Including Both 189 and 220 Trio Sets)

Characteristic	No. of Affected	% of Sample
Sex:		
Male	440	56.8
Female	334	43.2
Ethnicity:		
White	627	81.0
Hispanic	59	7.6
Black	18	2.3
Asian	15	2.0
Other	55	7.1
SES:		
I	191	24.7
II	278	35.9
III	220	28.4
IV	66	8.5
V	12	1.6
unknown	7	0.9
ADHD diagnosis:		
Definite	364	47.0
Probable	45	5.8
ADHD subtype:		
Combined	169	21.8
Inattentive	204	26.4
Hyperactive-impulsive	35	4.5
Comorbidity:		
ODD	146	18.9
CD	78	10.1
Mood	314	4.1
Anxiety	284	36.7

Selection of Positional Candidate Genes

The genes of interest were selected from within the linked regions on chromosomes 5p13 (15.5 Mb; NCBI Build36.1: 38,095,281-53,578,586), 6q12 (33.2Mb; NCBI Build36.1: 51,272,146-84,442,438), 16p13 (6.9 Mb; NCBI Build36.1: 11,193,827-18,054,753) and 17p11 (22.1 Mb; NCBI Build36.1: 6,189,992-28,314,303). Some of these four linkage regions were relatively gene-rich containing hundreds of genes. Naturally genes within these regions that previously have been associated to ADHD were prioritized in the present study. Since ADHD is considered to be al disorder of the central nervous system, all candidate genes were required to be expressed in brain. The next level of prioritizing included genes involved in the neurotransmitter systems presumed to play a role in the biology of ADHD and others that are involved in various aspects of brain function and development. Altogether nine such candidate genes were selected from the four chromosomal regions. Many of the genes are involved in synaptic neurotransmitter release, among these are regulating synaptic membrane exocytosis 1 (RIMS1), vesicle-associated membrane protein 2 (VAMP2), solute carrier family 1 member 3 (SLC1A3) and postsynaptic density protein 95 (PSD95). Others like solute carrier family 1 member 3 (SLC1A3) and glial cell derived neurotrophic factor (GDNF) that are part of the glutamatergic and dopaminergic pathway, respectively. And three of the genes; nuclear distribution gene E homolog 1 (NDE1), epsin 2 (EPN2) and stannin (SNN) are involved in various features of brain function.

Selection of the SNPs

The single nucleotide polymorphisms (SNPs) spanning genes of interest were selected from NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/), the international HapMap project (http://www.hapmap.org/), and SNPBrowser (http://www.appliedbiosystems.com). Markers were selected for minor allele frequency > 0.10 according to the frequencies reported in the Caucasian sample set in the HapMap database. Additionally, we maximized coverage of coding regions by selecting markers with the highest proximity to exons in order to capture the underlying linkage disequilibrium (LD) and haplotype structure as defined by the HapMap database.

Genotyping

The study sample was genotyped for the SNPs using the TaqMan Allelic Discrimination Assays (Applied Biosystems, Foster City, CA, USA). 5uL genotyping reactions were amplified on a MJ Research PCR system and fluorescence was detected on an ABI Prism 9700 (Applied Biosystems).

In general the genotyping success rate exceeded 90% SNPs with exception to SNPs rs2463694 (72%), rs3027172 (85%), rs11150581 (83%), rs4781679 (88%), rs3749692 (78%), rs2910797 (62%), and rs12514589 (58%). Nevertheless, we are presenting the genotyping results for all SNPs in order to provide an overview of the overall performance of the individual SNPs across the study. Also all SNPs that deviated from Hardy Weinberg equilibrium are shown (rs3027172, rs4781679, rs9922180, rs34493374). The detailed information of the SNPs tested is presented in a supplementary table.

Even though the HapMap (http://www.hapmap.org/) SNP coverage of the genome is not complete, it should be considered the current standard. Therefore, in order to estimate not only the LD structure between the genotyped SNPs in the current study but also how well the entire gene had been covered compared to the HapMap project, we pairwise tagged each of the SNPs tested here and compared the coverage against all of the existing HapMap SNP for the gene at r2 ≥ 0.8. The results are presented in Table **3**. However, since all of the nine genes were screened with SNPs that were not represented in HapMap, the presented gene coverage is an underestimation.

Statistical Analysis

A test for deviation from Hardy-Weinberg equilibrium was performed on all unaffected individuals (parents). Haploview 4.0 beta 15 [31] was used to determine the r^2 and D' measures of linkage disequilibrium (LD) for the single SNPs. Haploview was also used for construction of haplotypes and their analysis by using an expectation-maximization (EM) algorithm. According to the statistical guidelines provided for association studies by Freimer and Sabatti (2005), we considered *P*-values lower than ~ 10^{-7} to be significant (21). We applied the Bonferroni correction for all multiple tests. We used the Genetic Power Calculator to determine the power of the study sample to detect a risk allele with 20% frequency with an additive genotype relative risk of 2 (Aa) and 4 (AA). The genotype relative risk (GRR) ratios were calculated according to Risch and Merikangas (1996) from the TDT tables [32].

RESULTS

Based on our genome-wide scans and the subsequent fine-mapping four promising linkage regions emerged on chromosomes 5p13, 6q12, 16p13 and 17p11. We have genotyped nine genes located under the linkage signals at these four chromosomal regions in 189 or 220 ADHD trios (based on additional sample availability). The genotype distribution of all SNPs except for four markers (rs3027172, rs4781679, rs9922180, rs34493374) followed Hardy–Weinberg equilibrium (significance level 0.05). The detailed association results for the individual SNPs can be found in Table **4**. Overall we did not see any association for the haplotypes constructed across all the candidate genes. Our power analysis for the smaller study sample of 189 trios, under an additive model with GRR (Aa) ~2 and $\alpha = 0.05$, indicates ~85% power for a causal variant frequency of 10% and ~96% power for a causal variant frequency of 20% (see Methods).

The nine genes investigated here all represent promising candidate genes in ADHD based on their function and expression patterns. Most of them are involved in neurotransmitter related pathways such as dopamine and glutamine or in other central functions of the brain. Only one gene, RIMS1 showed nominal evidence of association in our study ($P = 0.0295$). RIMS1 is 514 Kb in size and is known to consist of 34 exons. Altogether 26 SNPs were genotyped across this entire gene. One SNP (rs2463694) located in intron 6 produced evidence of nominal association ($P = 0.0295$), however it did not withstand correction for multiple testing ($P = 0.767$). This SNP was in HW equilibrium (significance level 1.0), however the molecular assay for this SNP would not type on several samples and had the lowest genotype success rate of all the SNPs in the study (72%). In the HapMap Project data, no heterozygosity was seen for this particular polymorphism in the 29 European Caucasian families genotyped, nevertheless we found a minor allele frequency of 0.221 (A) in our study sample. The flanking SNPs downstream and upstream of the nominally associated SNP were within a ~ 28 Kb distance, but again no trend of association was seen with either of these SNPs. Five haplotype blocks spanned the gene, none of which showed any association.

DISCUSSION

Nine candidate genes selected based on biological plausibility were evaluated in this study that coincided with the four chromosomal regions highlighted by our genome-wide scans and subsequent fine-mapping in ADHD (5p13, 6q12, 16p and 17p). The nine candidate genes examined here are involved in diverse neurotransmitter systems that are thought to be altered in ADHD patients, and other genes that are involved in various aspects of brain and nervous system devel-

Table 3. Estimation of LD Coverage of the Nine Genes

Gene	Genotyped SNPs	Genotyped HapMap SNPs	Alleles Captured of Possible Genic HapMap SNPs (%)
VAMP2	7	2	5 of 7 (71%)
DOC2A	3	0	-
EPN2	9	5	11 of 86 (13%)
GDNF	7	5	13 of 53 (24%)
NDE1	8	7	31 of 65 (47%)
PSD95	6	5	10 of 23 (43%)
RIMS1	26	19	222 of 788 (28%)
SLC1A3	2	1	1 of 166 (0%)
SNN	5	4	18 of 82 (21%)

The overall number of genotyped SNPs per gene is presented as well as how many of these SNPs are genotyped in HapMap. The last column represents an estimation of how many of the possible HapMap SNPs within the distinct genes have been captured in this study by the genotyped HapMap SNPs alone. Since only a fraction of the genotyped SNPs for each of the genes are represented in HapMap the expected gene coverage is an underestimation.

Table 4. The Bolded SNPs Show Nominal Association (Uncorrected for Multiple Testing)

GENE	SNP	Genomic Position	TR	NT	χ^2	P Value
RIMS 1	rs1147530	6q13: 72634830	37	35	0.06	0.814
	rs13218069	6q13: 72654258	51	36	2.59	0.108
	rs1328710	6q13: 72659285	85	83	0.02	0.877
	rs1936022	6q13: 72723073	75	66	0.57	0.449
	rs535435	6q13: 72765270	69	60	0.63	0.428
	rs9341359	6q13: 72857598	92	87	0.14	0.709
	rs1482574	6q13: 72869603	75	67	0.45	0.502
	rs2449425	6q13: 72882532	88	81	0.29	0.590
	rs2496497	6q13: 72940842	88	86	0.02	0.880
	rs12526524	6q13: 72942330	51	43	0.68	0.409
	rs2463694	**6q13: 72970002**	**42**	**25**	**4.31**	**0.038**
	rs1971561	6q13: 72998188	76	70	0.25	0.620
	rs9293872	6q13: 73009642	78	78	0.00	1.000
	rs9351902	6q13: 73019073	71	70	0.01	0.933
	rs17783700	6q13: 73026388	36	31	0.37	0.541
	rs2746198	6q13: 73052051	76	74	0.03	0.870
	rs1569720	6q13: 73063041	85	79	0.22	0.639
	rs11751326	6q13: 73067286	80	68	0.97	0.324
	rs13212600	6q13: 73086596	94	83	0.68	0.408
	rs9442769	6q13: 73086596	93	81	0.83	0.363
	rs10943003	6q13: 73097442	80	78	0.03	0.874
	rs12202907	6q13: 73108605	77	67	0.69	0.405
	rs12202544	6q13: 73148973	51	47	0.16	0.686
	rs2815739	6q13: 73158635	68	61	0.38	0.538
	rs7738055	6q13: 73166669	86	86	0.00	1.000
	rs9446646	6q13: 73180101	69	52	2.39	0.122
SNN	rs2868980	16p13.13: 11661955	81	80	0.01	0.937
	rs1050068	16p13.13: 11677449	77	75	0.03	0.871
	rs17606098	16p13.13: 11696691	77	76	0.01	0.936
	rs933573	16p13.13: 11700896	81	73	0.42	0.519
	rs12149831	16p13.13: 11713513	86	81	0.15	0.699
EPN2	**rs959071**	**17p11.2: 19082819**	**54**	**34**	**4.55**	**0.033**
	rs1027872	17p11.2: 19084103	12	09	0.43	0.513
	rs3862148	17p11.2: 19109505	45	39	0.43	0.513
	hCG1811979	17p11.2: 19114716	20	17	0.24	0.622
	rs868698	17p11.2: 19125804	49	35	2.33	0.127
	rs2296981	17p11.2: 19153928	62	47	2.06	0.151
	rs1472932	17p11.2: 19161259	68	47	3.84	0.050
	rs6587216	17p11.2: 19164990	60	48	1.33	0.248
	rs12603815	17p11.2: 19182051	22	16	0.95	0.330
PSD95	rs314253	17p13.1: 7032374	88	74	1.21	0.271
	rs13331	17p13.1: 7034190	76	62	1.42	0.233
	rs2242449	17p13.1: 7036231	89	80	0.48	0.489
	rs390200	17p13.1: 7050719	92	78	1.15	0.283
	rs507506	17p13.1: 7059046	93	76	1.71	0.191
	rs739669	17p13.1: 7063101	89	73	1.58	0.209
VAMP 2	rs3027178	17p13.1: 7993810	90	76	1.18	0.277
	rs3027172	*17p13.1: 7996448*	*104*	*99*	*0.12*	*0.726*
	rs2518023	17p13.1: 7997331	43	38	0.31	0.579
	rs3027160	17p13.1: 7998297	61	56	0.21	0.644
	rs2278637	17p13.1: 8002827	81	78	0.06	0.812
	hcv25610873	17p13.1: 8005504	58	58	0.00	1.000
	rs8067606	17p13.1: 8007508	83	80	0.06	0.814
SLC1A3	rs12651999	5p13.2: 36728190	92	78	1.15	0.283
	rs1025052	5p13.2: 36745001	62	60	0.03	0.856
DOC 2A	rs12851	16p11.2: 29924478	25	20	0.56	0.456
	rs11150581	16p11.2: 29938200	79	75	0.10	0.747
	rs4788212	16p11.2: 29941970	92	90	0.02	0.882
NDE1	***rs4781679***	***16p13.11: 15668934***	*77*	***53***	***4.43***	***0.035***
	rs3784859	16p13.11: 15672904	99	89	0.53	0.466
	rs9922180	*16p13.11: 15692030*	*166*	*159*	*0.15*	*0.698*
	rs12922717	16p13.11: 15696196	11	08	0.47	0.491
	rs34493374	*16p13.11: 15703477*	*178*	*176*	*0.01*	*0.915*
	rs1050162	16p13.11: 15718563	98	91	0.26	0.611
	rs2384933	16p13.11: 15723647	94	78	1.49	0.223
	rs760023	16p13.11: 15726556	07	06	0.08	0.782
GDNF	rs2910710	5p13.2: 37846989	63	56	0.41	0.521
	rs11111	5p13.2: 37849859	41	29	2.06	0.152
	rs3749692	5p13.2: 37849905	65	51	1.69	0.194
	rs2910797	5p13.2: 37856122	51	38	1.90	0.168
	rs1862574	5p13.2: 37866334	53	47	0.36	0.549
	rs12518844	5p13.2: 37868516	60	58	0.03	0.854
	rs12514589	5p13.2: 37878188	43	37	0.45	0.502

(Table 4) contd…..

The SNPs shown in italic deviated from Hardy-Weinberg equilibrium.

opment. However, this does not rule out that other mechanisms such as epigenetic regulation and posttranslational modification could be involved in the disease phenotype. Our aim was to apply the common disease variant hypothesis and thus selecting for highly informative SNPs within these genes with minor allele frequencies above 10%. In order to obtain an extensive coverage of the genes using haplotypes, we further selected the SNPs based on known LD structures.

Two candidate genes on chromosome 5p were analyzed; GDNF (Glial Cell Derived Neurotrophic Factor) and SLC1A3 (Solute Carrier Family 1 Member 3). The GDNF gene is involved in the dopaminergic pathway, while the SLC1A3 gene is

part of the glutamatergic pathway. Genes involved in the dopamine pathway have long been the targets for a great deal of research mainly because of the overwhelming evidence from the treatment efficacy of the stimulant ADHD medications that seem to primarily act by regulating the dopamine levels in the brain. Additionally, deactivation of specific dopaminergic genes in knockout mice that have shown behavioral effects similar to ADHD further demonstrate the potential relevance of genes within this neurotransmitter system. In particular the dopamine transporter 1 (DAT1) knockout mice demonstrated that the mice lacking both copies of the gene exhibited behaviors analogous to ADHD, such as greater motor activity, compared to wild-type controls and mice with a single intact copy of the gene [33]. Another prominent animal model for ADHD is the Coloboma mouse as it shows high levels of spontaneous motor behavior [34]. These mice exhibit spontaneous hyperactivity caused by a semi-dominant deletion mutation that includes the *Snap25* gene. This mutation results in a 50% reduction in the expression of the protein SNAP-25 [34]. On the other hand, glutamine, the most abundant excitatory neurotransmitter in the nervous system, plays a role in synaptic plasticity and hence is also involved in cognitive functions that are defective in ADHD such as learning and memory. Even though the dopamine and glutamine neurotransmitter systems could be considered to represent two different hypotheses of biological mechanisms underlying ADHD, they are not necessarily mutually exclusive [35, 36]. For example, it has been shown that striatal dopamine release is modulated by glutamatergic corticostriatal pathways [37, 38]. In addition, dopaminergic and glutamatergic heteroreceptors have been found on glutamatergic and dopaminergic terminals, respectively, in the caudate-putamen [39]. Therefore, the dysregulation of reciprocal dopaminergic/glutamatergic modulation may be at the root of the deficits in ADHD, and may also explain the treatment response to methylphenidate [35]. Despite the fact that both genes present valid candidate genes for ADHD, no association was found for them in this study set.

On chromosome 6q12 only one gene was investigated, regulating synaptic membrane exocytosis 1 (RIMS1) gene. RIMS1 is involved in pre-synaptic neurotransmitter release and has been shown to play an important role in regulating both short- and long-term glutamatergic presynaptic plasticity at certain synapses. This was the only gene that in this study setting showed nominal association with a single SNP (rs2463694: P = 0.0295). This association is not significant after correction for multiple testing. Further, the haplotype analysis for this gene yielded no significantly associated halotypes. However, allelic variants of this gene could play a role in ADHD risk but are likely to be modest if they cause any affect at all.

On chromosome 16p three candidates genes, double C2-like domain-containing protein, alpha (DOC2A), stannin (SNN) and nuclear distribution gene E homolog 1 (NDE1) were evaluated. The DOC2 gene is also involved in the pre-synaptic neurotransmitter release and has been shown to form a protein complex with the RIMS1 gene on chromosome 6q13 that take part in the priming process of the synaptic vesicles. The SNN gene is a mitochondrial membrane protein that has been identified as the specific marker for neuronal cell apoptosis induced by trimethyltin chloride (TMT) intoxication that inflicts severe injury to specific brain regions. The NDE1 gene has been implicated in playing a role in microtubule organization as well as in neuron migration during brain development in higher organisms [40]. Moreover, Nde1 knockout mice are characterized by a phenotype of small brain mass resulting from defects in development of the cerebral cortex [40]. However, none of the genes on chromosome 16p showed any association.

Chromosome 17p locus presented three candidate genes, vesicle-associated membrane protein 2 (VAMP2), postsynaptic density protein 95 (PSD95) and epsin 2 (EPN2) that were investigated. The VAMP2 gene is as well involved in pre-synaptic neurotransmitter release and is part of the main complex in vesicle docking. The active zone or the so-called SNARE complex consists of synaptobrevin/VAMP on the synaptic vesicle and SNAP-25 and syntaxin on the presynaptic membrane. A rise in intracellular calcium ($[Ca^{2+}]_i$) triggers the fusion of vesicles with the presynaptic membrane. Also the PSD95 gene is involved in the synaptic pathway, but on the postsynaptic side of the synaptic cleft. This protein is a scaffold protein that has several protein binding domains, these include binding sites for *N*-methyl-D-aspartate (NMDA) and kainate acid (KA) receptors that are crucial for the clustering of NMDA receptors and KA receptors in the postsynaptic membrane [41-43]. The EPN2 gene is enriched in brain and present in a brain-derived clathrin-coated vesicle fraction. It is concentrated in the peri-Golgi region and at the cell periphery of transfected cells, and partially co-localizes with clathrin. Nevertheless, none of these three candidate genes showed any association in our study set.

CONCLUSION

From all nine positional candidate genes only RIMS1, which has not previously been linked to ADHD, yielded nominal association. The RIMS1 gene maps to chromosome 6q13 and plays an important role in the synaptic vesicle docking during the presynaptic neurotransmitter release. The estimated GRR for the nominally associated polymorphism (rs2463694) within this gene was 1.68 (OR = 1.63, CI = 1.13-2.45). However, since the association did not withstand multiple testing corrections, this nominal result has to be presumed as a type I error. While the results here do not highlight a strong role for any of these genes in ADHD, the possibility of a minor role exists due to limitations of our sample size. However, the risk alleles that exist within these linked regions are not yet identified and further work on common and rare allele associations with larger datasets are needed to elucidate the genetic affect under the four linkage peaks.

To conclude, we tested nine positional candidate genes underlying our linkage peaks on chromosomes 5p13.2, 6q12, 16p and 17p. However, we failed to see evidence of significant association to ADHD in our study sample with any of the genes tested. Large linkage regions such as in this study contain numerous genes of which many are of unknown function but may harbor causative variants associated with ADHD. Thus, instead of directly targeting only specific candidate genes we propose a more informative way of searching for underlying disease variations by constructing a dense SNP marker map spanning the entire linked region.

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SUPPLEMENTS

Supplementary Table 1. Description of the Positional Candidate Genes Studied (The Bolded SNPs Showed Nominal Assocation)

Gene	SNP (rs #)	Genomic Position	HWpval	% Geno	MAF
RIMS 1	s1147530	Chr 6q13: 72634830	0.152	92.1	0.133
	rs13218069	Chr 6q13: 72654258	0.712	98.7	0.147
	rs1328710	Chr 6q13: 72659285	0.290	98.4	0.400
	rs1936022	Chr 6q13: 72723073	0.239	96.2	0.400
	rs535435	Chr 6q13: 72765270	0.873	98.6	0.281
	rs9341359	Chr 6q13: 72857598	0.383	98.7	0.424
	rs1482574	Chr 6q13: 72869603	0.715	96.2	0.378
	rs2449425	Chr 6q13: 72882532	0.495	98.0	0.386
	rs2496497	Chr 6q13: 72940842	0.792	99.3	0.405
	rs12526524	Chr 6q13: 72942330	0.309	95.9	0.164
	rs2463694	Chr 6q13: **72970002**	**1.0**	**71.9**	**0.221**
	rs1971561	Chr 6q13: 72998188	0.923	98.7	0.279
	rs9293872	Chr 6q13: 73009642	0.988	96.8	0.359
	rs9351902	Chr 6q13: 73019073	1.0	94.6	0.347
	rs17783700	Chr 6q13: 73026388	0.333	95.5	0.104
	rs2746198	Chr 6q13: 73052051	0.962	94.8	0.355
	rs1569720	Chr 6q13: 73063041	1.0	99.3	0.348
	rs11751326	Chr 6q13: 73067286	0.933	99.5	0.266
	rs13212600	Chr 6q13: 73086596	0.339	97.7	0.486
	rs9442769	Chr 6q13: 73086596	0.969	98.6	0.385
	rs10943003	Chr 6q13: 73097442	1.0	96.8	0.365
	rs12202907	Chr 6q13: 73108605	1.0	94.8	0.368
	rs12202544	Chr 6q13: 73148973	1.0	94.8	0.205
	rs2815739	Chr 6q13: 73158635	0.342	95.7	0.251
	rs7738055	Chr 6q13: 73166669	0.769	98.9	0.481
	rs9446646	Chr 6q13: 73180101	0.542	98.0	0.235
SNN	rs2868980	16p13.13: 11661955	0.146	99.8	0.426
	rs1050068	16p13.13: 11677449	0.959	95.8	0.463
	rs17606098	16p13.13: 11696691	0.921	94.2	0.490
	rs933573	16p13.13: 11700896	0.846	94.6	0.446
	rs12149831	16p13.13: 11713513	0.757	99.8	0.391
EPN2	**rs959071**	**17p11.2: 19082819**	**0.092**	**95.9**	**0.176**
	rs1027872	17p11.2: 19084103	1.0	98.4	0.029
	rs3862148	17p11.2: 19109505	1.0	98.9	0.133
	hCG1811979	17p11.2: 19114716	1.0	98.0	0.058
	rs868698	17p11.2: 19125804	0.899	98.6	0.138
	rs2296981	17p11.2: 19153928	0.348	94.6	0.243
	rs1472932	17p11.2: 19161259	0.120	98.2	0.242
	rs6587216	17p11.2: 19164990	0.359	95.9	0.224
	rs12603815	17p11.2: 19182051	0.908	99.5	0.060

(Supplementary Table 1) contd…..

Gene	SNP (rs #)	Genomic Position	HWpval	% Geno	MAF
PSD95	rs314253	17p13.1: 7032374	1.0	98.7	0.365
	rs13331	17p13.1: 7034190	0.628	93.2	0.319
	rs2242449	17p13.1: 7036231	0.269	95.9	0.408
	rs390200	17p13.1: 7050719	0.306	98.9	0.383
	rs507506	17p13.1: 7059046	0.285	98.7	0.385
	rs739669	17p13.1: 7063101	0.378	99.6	0.333
VAMP 2	rs3027178	17p13.1: 7993810	0.524	99.5	0.309
	rs3027172	**17p13.1: 7996448**	**$2.25x10^{-12}$**	**84.7**	**0.406**
	rs2518023	17p13.1: 7997331	0.115	98.4	0.154
	rs3027160	17p13.1: 7998297	0.333	97.5	0.210
	rs2278637	17p13.1: 8002827	0.715	95.9	0.379
	hcv25610873	17p13.1: 8005504	0.591	97.5	0.235
	rs8067606	17p13.1: 8007508	0.687	98.6	0.380
SLC1A3	rs12651999	5p13.2: 36728190	0.622	98.4	0.394
	rs1025052	5p13.2: 36745001	0.168	96.3	0.206
DOC 2A	rs12851	16p11.2: 29924478	1.0	98.0	0.070
	rs11150581	16p11.2: 29938200	0.206	83.4	0.410
	rs4788212	16p11.2: 29941970	0.495	97.2	0.449
NDE1	**rs4781679**	**16p13.11: 15668934**	**$1.39x10^{-14}$**	**88.4**	**0.252**
	rs3784859	16p13.11: 15672904	1.0	98.5	0.473
	rs9922180	16p13.11: 15692030	$1.32x10^{-85}$	96.9	0.462
	rs12922717	16p13.11: 15696196	1.0	98.3	0.024
	rs34493374	16p13.11: 15703477	$7.85x10^{-100}$	99.4	0.477
	rs1050162	16p13.11: 15718563	0.676	97.8	0.493
	rs2384933	16p13.11: 15723647	0.387	98.9	0.316
	rs760023	16p13.11: 15726556	1.0	98.2	0.020
GDNF	rs2910710	5p13.2: 37846989	0.493	92.2	0.429
	rs11111	5p13.2: 37849859	0.621	93.0	0.181
	rs3749692	5p13.2: 37849905	0.46	77.7	0.479
	rs2910797	5p13.2: 37856122	0.022	61.8	0.284
	rs1862574	5p13.2: 37866334	0.897	95	0.240
	rs12518844	5p13.2: 37868516	1.0	89.7	0.406
	rs12514589	5p13.2: 37878188	0.873	58.3	0.396

The bolded SNPs show nominal association before correction for multiple testing.