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Association of the Cannabinoid Receptor Gene (CNR1) With ADHD and Post-Traumatic Stress Disorder

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Abstract

Attention deficit hyperactivity disorder (ADHD) is a highly heritable disorder affecting some 5–10% of children and 4–5% of adults. The cannabinoid receptor gene (*CNR1*) is a positional candidate gene due to its location near an identified ADHD linkage peak on chromosome 6, its role in stress and dopamine regulation, its association with other psychiatric disorders that co-occur with ADHD, and its function in learning and memory. We tested SNP variants at the *CNR1* gene in two independent samples—an unselected adolescent sample from Northern Finland, and a family-based sample of trios (an ADHD child and their parents). In addition to using the trios for association study, the parents (with and without ADHD) were used as an additional case/control sample of adults for association tests. ADHD and its co-morbid psychiatric disorders were examined. A significant association was detected for a SNP haplotype (C-G) with ADHD ($P = 0.008$). A sex by genotype interaction was observed as well with this haplotype posing a greater risk in males than females. An association of an alternative SNP haplotype in this gene was found for post-traumatic stress disorder (PTSD) ($P = 0.04$ for C-A, and $P = 0.01$ for C-G). These observations require replication, however, they suggest that the *CNR1* gene may be a risk factor for ADHD and possibly PTSD, and that this gene warrants further investigation for a role in neuropsychiatric disorders.

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ELECTRONIC DATABASE INFORMATION

The accession number and URLs for data presented herein are as follows:

National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=retrieve&dopt=graphics&list_uids=1268; NCBI dbSNP database, <http://www.ncbi.nlm.nih.gov/snp/>; International Hap-Map Project, <http://hapmap.org/>; TaqMan SNP Genotyping Assays, <http://www.appliedbiosystems.com/>; FBAT software, <http://www.biostat.harvard.edu/~fbat/default.html>; Haploview software, <http://www.broad.mit.edu/mpg/haploview/>; PBAT, <http://www.biostat.harvard.edu/~clange/default.htm>; SAS, <http://www.sas.com/>.

Keywords

genes; attention; association; post-traumatic stress disorder

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a common neurobehavioral disorder affecting some 5-10% children and adolescents and 4-5% of adults [Brown et al., 2001; Cuffe et al., 2005; Faraone and Biederman, 2005; Kessler et al., 2006]. ADHD is defined by symptoms of inattention and/or hyperactivity-impulsivity, impairment in at least two settings, and onset in childhood [APA, 1994]. While ADHD has its onset in childhood, the chronic nature of the disorder is evident by the persistence of ADHD into adulthood in 60-70% of cases [McGough et al., 2005]. Neurobiological and genetic research support the hypothesis that ADHD likely represents an extreme on one or more continua of neural processing and that multiple risk genes are responsible for the underlying liability to ADHD, with estimates of heritability at 76% [Faraone et al., 2005]. Despite strong genetic and neurobiological underpinnings, environmental factors are also indicated in both the etiology of ADHD [Hudziak et al., 2005] and its associated impairment [Pressman et al., 2006].

ADHD is a known risk factor for other psychiatric disorders, most notably mood, anxiety, disruptive disorders (oppositional defiant disorder, conduct disorder), and substance abuse or dependence [Biederman et al., 1995; McGough et al., 2005]. However, the mechanism(s) underlying this heightened risk for co-morbid illness is poorly understood. Identification of etiological factors in ADHD and psychiatric co-morbidity are topics of extant research. Recent molecular genetic studies suggest several chromosomal regions may harbor risk genes of moderate effect in ADHD, while candidate gene studies, thus far, support a likely role of dopamine-related systems in ADHD [Maher et al., 2002; Bakker et al., 2003; Arcos-Burgos et al., 2004; Kustanovich et al., 2004; Ogdie et al., 2004; Hebebrand et al., 2006].

The purpose of the present study is to investigate the role of variants of the cannabinoid receptor gene (*CNR1*) in relation to ADHD and its co-morbid psychiatric disorders. We selected this gene for investigation for several reasons. First, the *CNR1* locus (~88.9 Mb or 96-98 cM) falls near one of the regions of interest on chromosome 6 supported by our genome-wide studies (MLS = 3.3 at 89 cM) [Ogdie et al., 2004]. Second, studies of endogenous cannabinoid (EC) regulation in animal models suggest that it plays a role in some aspects of memory, emotional recognition or processing, and reward, each an area of putative dysregulation in ADHD [King et al., 1998; Nigg and Casey, 2005]. Third, individuals with ADHD have increased rates of addiction [Kessler et al., 2005; McGough et al., 2005; Smalley et al., 2007b] which has been suggested to be associated with *CNR1* variants.

There are numerous investigations of *CNR1* gene variants in psychiatry, including one study of ADHD among alcoholic individuals where no association was detected [Ponce et al., 2003]. Most studies have utilized a polymorphism (AAT)_n in the promoter region of the *CNR1* gene, although a few recent studies also include SNP variants within the gene as well [Schmidt et al., 2002; Roeder et al., 2005; Hopfer et al., 2006]. There are positive association studies of *CNR1* variants with substance abuse/dependence and schizophrenia [Ujike et al., 2002; Ballon et al., 2006; Martinez-Gras et al., 2006] although results are not consistent, with failure to replicate also reported for each condition analyzed [Tsai et al., 2000; Heller et al., 2001; Herman et al., 2006]. There are also positive and negative reports of *CNR1* gene variants with other psychiatric disorders as well, including a positive finding with eating disorder [Siegfried et al., 2004], a negative finding with Tourette syndrome

[Gadzicki et al., 2004], a negative finding with bipolar illness [Tsai et al., 2001], and a positive finding with psychiatric sequelae in Parkinson's disease [Barrero et al., 2005]. Taken together, the findings support a plausible role of the *CNR1* gene in a variety of psychiatric disorders but the specific gene variants, strengths of associations, and specificity to particular psychiatric disorders (or underlying liabilities) are unclear.

MATERIALS AND METHODS

Samples

The *CNR1* (NM_016083 and NM_033181) gene variants were investigated in two samples: First, a sample of 187 trios (ADHD child and 2 parents) randomly drawn from the affected sibling pair (ASP) sample of the UCLA ADHD Genetic study (the eldest child was selected within an ASP for trio formation) were available to apply family-based association tests (FBATs). There were 130 (69.5%) males and 57 (30.5%) females among 187 children with a mean age of 12.7 ± 3.3 years (range, 5-25). Among the children, 81.8% are Caucasians, 3.2% Hispanic, 4.8% are other ethnic groupings, and 10.2% are mixed-ethnic group, in which parents were from different ethnicity. Of the 187 trios, 374 parents (187 mothers, 187 fathers) were available as a case-control sample using their phenotype status on ADHD and psychiatric diagnoses. However, only Caucasian parents ($n = 320$) are used for the purpose of the present study to reduced effects due to population stratification. The parental mean age was 43.2 ± 5.2 years (range 31-66 years). The second independent sample consists of adolescent ADHD cases ($n = 159$) and controls ($n = 151$) drawn from a Northern Finnish Birth Cohort (NFBC) [Smalley et al., 2007a]. Among that sample, there are 201 (64.8%) males and 109 (35.2%) females with a mean age of 16.1 ± 0.3 years. All subjects signed informed consent prior to data collection as approved by the University of California Institutional Review Board and/or the University of Oulu Institutional Review Board (for the NFBC) as appropriate for the specific sample.

Measures

A similar diagnostic system is in place for samples used in the current analyses. ADHD and other psychiatric diagnoses were determined using a semi-structured psychiatric interview, KSADS-PL for children 5-18 [Kaufman et al., 1997], and SADS-LAR for adults [Fyer et al., 1995] with the Behavioral Disorders section of the KSADS-PL used to determine lifetime and current ADHD, oppositional defiant disorder and conduct disorder. A best estimate procedure [Leckman et al., 1982] is utilized to determine final diagnosis [see Smalley et al., 2000, and Smalley et al., 2007 for a review of assessment procedures]. Lifetime diagnoses are used in the current study.

Genotyping

The *CNR1* gene spans 5,469 bp on chromosome 6 [88906306bp-88911775bp; UCSC Human Mar. 2006 (hg18) assembly] and contains a single exon that can be alternatively spliced within the coding sequence. There are two distinct RefSeq transcripts (NM_033181.2 and NM_016083.3) that span the same genomic sequence, and differ solely by alternative splicing. NM_016083.3 contains a single contiguous coding region representing the complete exon1 sequence and encoding a 471 amino acid protein. NM_033181.2 contains a form of exon1 spliced internally that encodes a 411 amino acid protein. SNPs spanning the *CNR1* gene were selected from the NCBI dbSNP database and the International HapMap Project (HapMap Rel 19 Oct 05 Build), and physical positions were determined from the NCBI Build 35 human genome assembly. According to the most recent HapMap build (HapMap Rel 19 Jan 07 Build), the SNPs included in this study capture common variation at $R^2 > 0.8$ and $MAF > 0.05$ for all genotyped SNPs in NM_033181 and NM_016083 with three exceptions (*rs12720071*, *rs16880248*, *rs4707436*)

which were not genotyped due to technical difficulties. Genotyping was performed using TaqMan SNP Genotyping Assays and the 7900HT Fast Real-Time PCR System for fluorescent read detection and allelic clustering. All assays were performed in 5 μ l volume in 384-well plates according to the manufacturers' specifications. All SNPs included in the current study were successfully genotyped in >95% of the samples, were in Hardy-Weinberg equilibrium (HWE, P -value cut off = 0.001) in parental samples, and had Mendelian error rates of less than 1%.

Haplotypes Construction

Haplotypes were used to test for association in addition to individual SNPs because they are known to often provide more information [Hugot et al., 2001; Rioux et al., 2001]. The Haploview 3.2 software [Barrett et al., 2005] was used to define blocks using the default setting based on Gabriel et al. [2002]. Specifically, criteria included that the upper bound of $D' > 0.98$ and the lower bound > 0.7 , a 0.9 as the upper confidence interval maximum for strong recombination, and a fraction of strong linkage disequilibrium (LD) in information comparison of 0.95. The haplotypes were constructed using an EM algorithm, available in Haploview and FBAT. For analyses using logistic regression, the EM algorithm under SAS 9.1 PROC HAPLOTYPE was used to construct haplotypes.

Analyses

Associations of SNPs and haplotypes with the psychiatric diagnostic phenotypes under analysis were assessed using FBAT for LA parent-child trios or a case-control test for the Finnish sample and Caucasian parents in the LA trios. For analysis of the trios, the FBAT [Horvath et al., 2001] was conducted using the FBAT software, which tests for transmission distortion from the null hypothesis of no association: no distortion from the expected 0.5 transmission [Laird et al., 2000]. An offset option (FBAT-O/HBAT-O) was used for adding an offset in the FBAT parametric model in analyzing both affected and unaffected psychiatric diagnostic phenotypes. This option makes the score statistic more efficient [Lunetta et al., 2000]. For all analyses, we reported both a global P -value and the individual P values. The global P -value is found from a χ^2_{H-1} distribution of a test of association between a disease status and a gene with "H" haplotypes. The individual P values are computed for a test of one allele versus all others. In addition, permutation P values, based on 10,000 replicates, were also computed to confirm any significant findings as they may achieve a higher precision level.

For any significant associations detected at $P < 0.05$, logistic regression was used to test for gender by gene interactions and to estimate an odds ratio. In this test, the gene variant (haplotype) is treated as binary (Present or Absent) so that individuals homozygous for the marker allele are assigned twice the weight of heterozygotes in the logistic regression.

The study is considered to be hypothesis generating with the level of significance set at 0.05 in each case. In order to test *CNR1* associations with psychiatric disorders co-morbid with ADHD, 5 out of 18 psychiatric disorders were selected because there were ~50 individuals with the diagnosis in at least two of the samples (Finnish, Trios, or Parents). We selected a sample size of 50 to minimize our test comparisons while providing adequate power to detect relatively small odds ratios. Based on power analyses for a case control design in a sample size of 50, we would have 80% power to detect an odds ratio (OR) > 1.8 for haplotype frequencies > 0.15 if the level of significance was set at 0.05. For family based analyses and comparable parameters, power is adequate for OR of 2.3 or higher. Although somewhat arbitrary, this criterion kept the number of test comparisons relatively small per group while affording sufficient power to detect a relatively minor gene effect. The power analyses were carried out using the SAS PROC POWER procedure for the case control

design and PBAT [Lange and Laird, 2002a,b] for the family based design. We made two exceptions to this sample size selection criterion by including an analysis of substance abuse/dependence (only one sample had the $n > 50$) and PTSD (maximum sample size of 25) because of previous reports of *CNR1* with the former [Ballon et al., 2006] and the specific biological function of ECs (to modify memory of traumatic events) as a compelling argument for a *CNR1* role in the latter.

The disorders available for analysis are shown in Table I and include ADHD, “any” anxiety disorder, mood disorder which includes either major depression or dysthymia, disruptive disorder which includes either oppositional defiant disorder or conduct disorder, post-traumatic stress disorder (PTSD) and substance abuse/dependence. “Any anxiety” was defined if any of the following were present: simple phobia, social phobia, PTSD, obsessive compulsive disorder (OCD), generalized anxiety disorder (GAD), separation anxiety, agoraphobia or panic, and adjustment disorder.

RESULTS

SNP and Haplotype Description

The *CNR1* gene in the current study was investigated using four SNPs. Table II displays their positions and allele information. We note that the minor allele frequencies in our samples were close to those found for Caucasian people in the HapMap project. Using Haploview, two haplotype blocks were detected in both the LA and Finnish samples with the same block boundaries. The first block consists of SNPs 1 and 2, and the second one consists of SNPs 3 and 4. D' and R^2 [Gabriel et al., 2002] are summarized in Table III. We note that the D' was the same in both samples and the R^2 was higher in the second block (0.38). The blocks in the LA sample met the default setting [Gabriel et al., 2002] under the Haploview blocking algorithm. The blocks in the Finnish sample did not and were generated based on the solid spine option of LD [Barrett et al., 2005], a method appropriate for cases where $D' > 0.8$. We let superscript 1 denote the haplotype that consists of SNPs 1 and 2, and superscript 2 to denote the haplotype consisting of SNPs 3 and 4. Table III also summarizes the haplotype frequencies across samples.

Results From SNP and Haplotype Associations

We performed an association test of individual SNPs for ADHD and the disorders listed in Table I. At the single SNP level, only PTSD in the LA Caucasian parents showed a significant association, with SNP *rs1049353* (allele A, $P = 0.011$).

As shown in Table IV(A), haplotype 2 (C-G²) was associated with ADHD in the Finnish sample ($\chi^2 = 7.06$, $P = 0.008$ and global $P = 0.05$). The proportion of C-G² was higher in cases (19%) than in controls (11%). No significant association in either the LA Caucasian parent sample ($\chi^2 = 1.93$, $P = 0.16$ and global $P = 0.29$) or the LA trios ($Z = -0.57$, $P = 0.57$, global $P = 0.91$) was observed.

Two variants (C-A¹, C-G¹) within haplotype 1 were associated with PTSD in the LA Caucasian parents (global $P = 0.03$) as shown in Table IV(B). No difference was observed in LA trios (global $P = 0.37$), although very few children and comorbid PTSD ($n = 6$). Although no difference was observed in the Finnish sample (global $P = 0.20$) either, a similar trend in C-A¹ and C-G¹ distributions was observed. C-A¹ was more common in PTSD cases (71%) compared to non-PTSD controls (55%), while C-G¹ was less common among the PTSD (20%) compared to non-PTSD (28%) in that sample as well. Using the permutation method, the permutation P values for C-G² and ADHD was 0.02 while the

permutation P values for C-A¹, C-G¹, and PTSD were 0.11 and 0.04, respectively. A depiction of the SNPs, haplotypes and significant associations is shown in Figure 1.

Testing for haplotype by gender interactions, we found a significant interaction between the C-G² haplotype and gender for ADHD ($P = 0.03$) in the Finnish sample. The interaction reflects the approximate threefold increased risk of ADHD in males carrying the haplotype compared to females (carrying it or not) or males without the haplotype.

DISCUSSION

In the current study we find support that variants at *CNR1* are associated with ADHD and PTSD. The findings are similar to those of other investigations of this gene, in that they are of relatively small effect size, and do not replicate necessarily across samples. In the current study, we see support for an association of *CNR1* and PTSD but findings require replication in larger samples. The failure to replicate across samples may reflect shared underlying constructs embedded in diagnostic classifications (e.g., emotional regulation difficulties) and/or inadequate power to detect small effect sizes. There may be an important role of gender in mediating the association of *CNR1* and psychiatric illness, however, and future research needs to include a possible role of genotype by gender interactions.

ECs play an important role in neural plasticity, stress response, and learning and memory. *CNR1* receptors are found in particularly high density in the hippocampus and amygdala, regions known to play a role in emotional regulation and memory, and regions suggested to play a role in ADHD [Plessen et al., 2006], emotional regulation [Urry et al., 2006], and other psychiatric disorders (e.g., bipolar, mood, anxiety disorders). They are found on axonal terminals of GABAergic inhibitory neurons containing neuropeptide cholecystokinin (CCK), a neuropeptide known to bind to CCK_B receptors and play a role in anxiety. ECs are known to increase CCK release and also have interactions with other neurotransmitter systems including dopamine [Price et al., 2007] and serotonergic systems [Braidia et al., 2007]. Genes involved in dopamine and serotonin regulation have already been implicated in both ADHD [Kim et al., 2005], anxiety and mood disorders [Hariri et al., 2006] and are also associated with variation in hypothalamic-pituitary-axis (HPA) stress response [King et al., 1998].

In rodents, endogenous cannabinoids have numerous influences on cognition and behavior as evident by the use of antagonists and agonists of cannabinoids. Agonists impair memory in rats, increase slow wave sleep and rapid eye movement sleep at the expense of wakefulness, impair prepulse inhibition, and recognition memory [Castellano et al., 2003]. The specific mechanism of ECs and *CNR1* activity in the brain are currently being delineated but it is well known that many psychiatric disorders, including ADHD, schizophrenia, PTSD, anxiety, and mood disorders have various memory and inhibition impairments. The potential involvement of reward systems in multiple psychiatric disorders, including ADHD, again suggests the plausible association of *CNR1* and a range of conditions.

These data provide support for a putative role of endogenous cannabinoids in ADHD, and PTSD. The *CNR1* gene may contribute to shared underlying risk continua, such as emotional dysregulation in response to stress, across these diverse diagnostic groups. Increased amygdala activity, poor stress reactivity as reflected by HPA response, and poor prefrontal cortical modulation is a plausible underlying mechanism of liability that may be shared across disorders. Recent imaging studies support amygdala and hippocampal differences in ADHD [Plessen et al., 2006], studies of siblings of ADHD individuals support prefrontal-limbic circuitry differences as a putative endophenotypes [Durston et al., 2006],

and studies of stress response in ADHD support abnormalities in HPA axis responsivity [King et al., 1998]. Taken together with the current findings, we suggest that this gene may be an important risk variant in the emotional regulation difficulties underlying ADHD, PTSD, and possibly other co-morbid conditions (such as mood disorder); however, the role of *CNR1* is likely small, particularly at the level of psychiatric diagnosis, so future work using more refined phenotypes or endophenotypes of affect regulation are necessary.

The current findings require additional work and replication. Further investigation of affect related traits associated with ADHD may improve our understanding of the role the *CNR1* gene may have in this condition and co-morbid disorders. Further refinement of putative DNA variants in *CNR1* with functional outcomes is needed to delineate the causal variants contributing to psychiatric illness.

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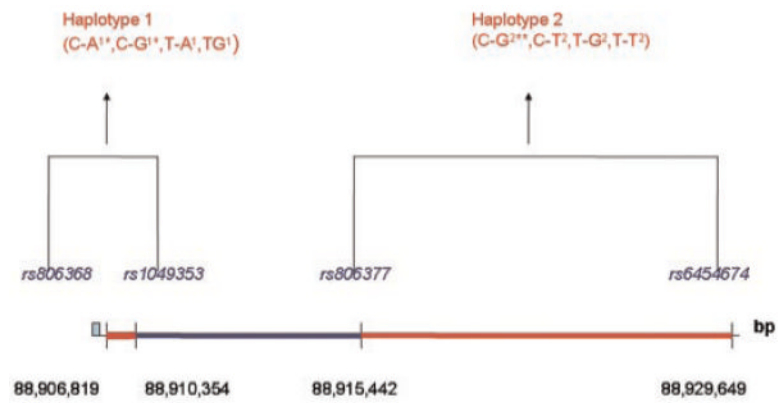


Fig. 1. Haplotypes and associations with attention deficit hyperactivity disorder (ADHD) and PTSD at *CNRI*. *Haplotypes associated with post-traumatic stress disorders (PTSD), **haplotype associated with ADHD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.].

TABLE I
 Numbers of ADHD Affected and Non-Affected Having Associated Psychiatric Disorders

Affection status ^d	LA child (n=187) ^b		LA Caucasian parent (n=320) ^b		Finland (n=310) ^b	
	Yes	No	Yes	No	Yes	No
ADHD	187 (70%) ^c	0 (0%)	115 (46%)	193 (50%)	159 (71%)	151 (58%)
Any anxiety	82 (67%)	104 (72%)	141 (41%)	171 (57%)	71 (54%)	238 (68%)
Mood	49 (67%)	137 (70%)	158 (39%)	158 (60%)	57 (47%)	253 (69%)
Disruptive disorder	104 (74%)	83 (64%)	53 (57%)	262 (48%)	78 (60%)	232 (66%)
PTSD	6 (67%)	181 (70%)	25 (24%)	291 (52%)	17 (29%)	292 (67%)
Substance abuse/dependence	11 (55%)	176 (70%)	110 (65%)	206 (42%)	32 (66%)	278 (65%)

^aLifetime diagnoses.

^bNumbers vary due to missing data on genotypes or phenotypes for subjects across conditions.

^cNumber in parentheses reflects the percent that are male.

TABLE II

SNPs Information on *CNR1*

Ref SNP ID	Function	Alleles major/minor	Position (bp)	LA children MAF	LA parents ^d MAF	LA HWE	Finland MAF	Finland HWE
<i>rs806368</i> ^b	3' UTR	C/T	88906819	0.23	0.22	0.14	0.17	0.73
<i>rs1049353</i>	Synonymous	A/G	88910354	0.23	0.26	0.77	0.27	1.00
<i>rs806377</i> ^c	Non-coding	C/T	88915442	0.47	0.48	1.00	0.47	0.87
<i>rs6454674</i> ^d	Non-coding	G/T	88929649	0.33	0.32	0.85	0.39	0.18

MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium *P*-value

^aFrequencies are reported for all parents but the values found in the subset of only Caucasian parents are comparable (data not shown).

^bhttp://www.hapmap.org/cgi-perl/snp_details_b35?name=rs806368.

^chttp://www.hapmap.org/cgi-perl/snp_details_b35?name=rs806377.

^dhttp://www.hapmap.org/cgi-perl/snp_details_b35?name=rs6454674.

TABLE III
Linkage Disequilibrium Information and Estimated Frequencies of Haplotypes in Two Samples

	LA children	LA parents	Finnish
Haplotype 1			
D' (CI)	NA	1.0 (0.84, 1.0)	1.0 (0.80, 1.0)
R ²	NA	0.10	0.07
C-A ¹ ^a	0.56	0.54	0.57
C-G ¹	0.21	0.24	0.27
T-A ¹	0.21	0.20	0.16
T-G ¹	0.02	0.02	0.0
Haplotype 2			
D' (CI)	NA	0.94 (0.87, 0.98)	0.94 (0.87, 0.97)
R ²	NA	0.38	0.49
C-G ² ^b	0.22	0.23	0.15
C-T ²	0.31	0.29	0.38
T-G ²	0.45	0.45	0.46
T-T ²	0.02	0.03	0.01

CI denotes 95% confidence interval; NA denotes not available because LD construction was based on founders.

^aDenotes haplotypes for SNPs 1 and 2.

¹Denotes haplotypes for SNPs 1 and 2.

^bDenotes haplotypes for SNPs 3 and 4.

²Denotes haplotypes for SNPs 3 and 4.

TABLE IV

(A) ADHD Association with Haplotype 2 (SNPs 3 and 4) in the Finnish Sample (n=310), (B) PTSD Association With Haplotype 1 (SNPs 1 and 2) in LA Caucasian Parent Sample (n=320)*

(A) Phenotype	Estimate haplotype frequencies		
	C-G ²	C-T ²	T-T ²
ADHD (n=159)	0.19	0.37	0.43
Not ADHD (n=151)	0.11	0.39	0.49
<i>P</i> -value	7.10	0.15	1.95
	0.008	0.50	0.16
	0.56		

(B) Phenotype	Estimate haplotype frequencies		
	C-A ¹	C-G ¹	T-A ¹
PTSD (n=22)	0.65	0.12	0.23
Not PTSD (n=280)	0.50	0.29	0.21
<i>P</i> -value	4.21	6.53	0.08
	0.04	0.01	0.78

(A) $\chi^2_3=7.75$, *P*-value=0.05 global analysis of association

* Variation in sample sizes reflects cases that were missing either genotype data or specific diagnoses were unknown

(B) $\chi^2_2=6.86$, *P*-value=0.03 global analysis of association.