Conduct Disorder and ADHD: Evaluation of Conduct Problems as a Categorical and Quantitative Trait in the International Multicentre ADHD Genetics Study.

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Abstract

Attention-deficit/hyperactivity disorder is typically characterized by inattention, excessive motor activity, impulsivity, and distractibility. Individuals with ADHD have significant impairment in family and peer relations, academic functioning and show high co-morbidity with a wide range of psychiatric disorders including oppositional defiant disorder (ODD), conduct disorder (CD), anxiety disorder, depression, substance abuse and pervasive developmental disorder (PDD). Family studies suggest that ADHD+CD represents a specific subtype of the ADHD disorder with familial risk factors only partly overlapping with those of ADHD alone. We performed a hypothesis-free analysis of the GAIN-ADHD sample to identify markers and genes important in the development of conduct problems in a European cohort of individuals with ADHD. Using the Family-Based Association Test (FBAT) package we examined three measures of conduct problems in 1,043,963 autosomal markers. This study is part of a series of exploratory analyses to identify candidate genes that may be important in ADHD and ADHD-related traits, such as conduct problems. We did not find genome-wide statistical significance ($p<5x10-7$) for any of the tested markers and the three conduct problem-traits. Fifty-four markers reached strong GWA signals ($p<10-5$). We discuss these findings in the context of putative candidate genes and the implications of these findings in the understanding the etiology of ADHD+CD. We aimed to achieve insight into the genetic etiology of a trait using a hypothesis-free study design and were able to identify a number of biologically interesting markers and genes for follow-up studies.

KEY WORDS: ADHD; conduct disorder; genome wide association study; genetic association information network

Background

Attention-deficit/hyperactivity disorder (ADHD (MIM:143465)) affects approximately 8-12% of school-age children worldwide (Biederman and Faraone 2005). It is typically characterized by inattention, excessive motor activity, impulsivity, and distractibility. Individuals with ADHD have significant impairment in family and peer relations. Moreover, they have difficulties in academic functioning and show high co-morbidity with a wide range of psychiatric disorders including oppositional defiant disorder (ODD), conduct disorder (CD), anxiety disorder, depression, substance abuse and pervasive developmental disorder (PDD). Four types of symptoms of CD are recognized: aggression or serious threats of harm to people or animals; deliberate property damage or destruction (e.g., fire setting, vandalism); repeated violation of household or school

rules, laws, or both; and persistent lying to avoid consequences or to obtain tangible goods or privileges (American Psychiatric Association 2000).

Family studies suggest that ADHD+CD represents a specific subtype of disorder with familial risk factors only partly overlapping with those of ADHD alone (Faraone and others 1997; Faraone and others 1991; Faraone and others 2000; Frick and others 1991; Lahey and others 1988; Stewart and others 1980; Szatmari and others 1993). In a recent study by this group, we examined the sibling risk for probands with ADHD and conduct problems (Christiansen and others 2008). Families with an index case with ADHD-CT+CP (ADHD-combined type with co-morbid conduct problems) showed, when adjusted for gender and parental socioeconomic status, an increased sibling recurrence relative risk (SRRR) for both ADHD-CT (SRRR=2.9; 95%CI 1.6-5.3, $p<0.001$) and ADHD-CT+CP (SRRR=4.9; 95%CI 2.6-9.4, $p<0.001$) compared to the population risk. Additional factors, such as shared family environment, independent of socio-economic status, may also explain these findings. Nevertheless, putative shared familial environmental risk may serve to prime underlying genetic risk and facilitate expression of the disorder.

Individuals with the ADHD-CT+CP subtype manifest more severe symptoms of ADHD than those classified as having ADHD-CT alone. Moreover, ADHD-CT+CP may constitute a distinct familial disorder or a more extreme manifestation on an ADHD phenotypic continuum. Under both models one would predict ADHD-CT+CP would represent a more genetically loaded disorder compared to ADHD-CT alone. Family, twin, and adoption studies strongly support the influence of genetic factors on the etiology of ADHD (Biederman and Faraone 2005; Faraone and others 2005; Thapar and others 2005). Similarly strong genetic factors have been implicated in externalizing behaviors, such as CD (Hicks and others 2004). The influence of genetic risk in the etiology of ADHD-CT+CP has also been explored (Burt and others 2005; Thapar and others 2001; Vierikko and others 2004). Thapar and others examined categories of ADHD and CP in 2082 twin-pairs. The overlap between ADHD and ADHD+CP was explained by common genetic and common shared environment. Despite this, the environmental influence on CP would suggest that ADHD and ADHD+CP are partly distinct traits, however, ADHD-CT+CP was shown to be more genetically loaded than ADHD-CT (Thapar and others 2001). The common genetic etiology model of ADHD and CP is also supported by Vierikko and others. In the longitudinal Finnish Twin Study, FinnTwin12, they show a strong genetic correlation between aggressive and hyperactivity-

impulsivity symptoms in ADHD (Vierikko and others 2004). Contradictory evidence for a common genetic etiology has also been described in a large sample of 11 year old twins from the Minnesota Twin Study (Burt and others 2005). These data suggest that there is only marginal significant genetic contribution to a composite general externalizing behavior factor (Ext), generated from diagnosis of ADHD, CD or ODD. The variance in the Ext trait was best explained by the shared-environment. The examination of more homogenous common factors that link ADHD and the co-morbid disorders may enable greater understanding of the etiology of both the uniqueness and commonality of the disorders.

The Genetics of ADHD and Conduct Problems

Candidate gene association analysis has focused on genes within monoamine neurotransmitter systems, specifically those important in dopaminergic and serotonergic neurotransmission. Meta-analyses suggest that variation in the genes that code for the dopamine receptors D4 (DRD4) and D5 (DRD5), the 5-hydroxytryptamine (serotonin) transporter (SLC6A4), the serotonin 1B receptor (HTR1B), synaptosomal protein of 25kD (SNAP25) and the dopamine transporter (SLC6A3) influence susceptibility to ADHD (Faraone and others 2005). The genetics of antisocial externalizing behaviors has also focused on monoamine neurotransmitter systems, specifically serotonergic neurotransmission. Recent studies have identified modest association signals in the HTTLPR polymorphism of SLC6A4 (Sakai and others 2006) and a putatively functional polymorphism (RS4680) in the Catechol-O-Methyltransferase (COMT) gene (Caspi and others 2008). A linkage study in the Collaborative Study of the Genetics of Alcoholism (COGA) provides suggestive evidence that regions on chromosome 19 (D19S714; LOD score=2.82, NCBI Build36 location Chr19; 15589133 to 15589407) and chromosome 2 (D2S1331; LOD score=2.40, NCBI Build36 location Chr2; 86436445 to 86436847) may harbor susceptibility gene for conduct disorder (Dick and others 2004).

Candidate gene association studies have examined genes involved in monoaminergic neurotransmission for association with CD in an ADHD population [e.g. (Beitchman and others 2003; Comings and others 2000a; Comings and others 2000b)]. None of these studies have found strong evidence for association with the trait. To date, no linkage or genome-wide association study (GWAS) has examined the role of conduct problems in the ADHD population.

The Genetic Association Information Network

The Genetics Analysis Information Network (GAIN) is a public-private partnership between the NIH the private sector with the goal of promoting GWAS for various complex diseases (http://www.fnih.org/GAIN2/home_new.shtml). Nine hundred and fifty eight ADHD-parent trios from the International Multicenter ADHD Genetics Project (IMAGE) were genotyped as part of the GAIN initiative. We recently reported the initial findings of the IMAGE GWAS sample using a DSM-IV diagnosis of ADHD type in a family based association analysis (Neale and others 2008). These findings report no genome-wide significant associations according to the criteria suggested by Dudbridge and others (Dudbridge and Gusnanto 2008). It is possible that phenotypic and genetic heterogeneity may explain, in part, the lack of genome-wide significant findings in this dataset.

The comorbidity of CP in ADHD is readily examined in the IMAGE dataset as measurements of CP was made during the assessment process. Specifically, the ADHD diagnostic tools measure behavior including violence and cruelty to others, theft and vandalism and opposition to socially accepted behaviors and rules such as truancy and curfews. In this manuscript we use the CP symptoms to generate one categorical and two quantitative measures of CP that are used as the phenotypes of interest in GWAS analyses.

Materials and Methods

Subjects

Families were collected by the International Multicenter ADHD Genetics (IMAGE) project. IMAGE families were identified through ADHD probands aged 5 to 17 attending outpatient clinics at the data collection sites in Europe. A total of 958 affected proband-parent trios were initially selected for the GWAS scan. Family members were Caucasians of European origin from seven countries around Europe including Belgium, Germany, Ireland, the Netherlands, Spain, Switzerland, the United Kingdom and Israel. 938 probands were diagnosed as having DSM-IV combined type ADHD and 208 individuals had a DSM-IV CD diagnosis. Additional descriptive data are presented in Table 1.

Table 1.

Number of genotyped offspring	938
Diagnosed ADHD and Conduct Disorder	208
Gender Male Female	 816 122
Average Age (Standard Deviation)	10.88 (2.8)
Mean Conduct Disorder Symptom Count [PACS] (Standard Deviation)	4.55 (2.64)
Mean Conduct Disorder Symptom Count [Conners] (Standard Deviation)	20.83 (8.22)

Clinical Measures

We identified three broad phenotypic measures of conduct problems (CP) for the ADHD probands. First a categorical measure of CP was defined using DSM IV criteria of CD using a standardized algorithm applied to the Parent Account of Childhood Symptoms (PACS) (Chen and Taylor 2006; Taylor and others 1986). Two additional quantitative measures of CP were defined using the PACS and the Long Version of the Conners Parent Rating Scale (CPRS-R:L) (Conners and others 1998), where the PACS collected CP symptom information and the CPRS-R:L gathered the symptom on a less severe behavioral characteristic of an oppositional defiant individual. The CPRS-R:L and PACS were administered to the parents of the affected child by investigators at each center. There was centralized training for all who administered either the CPRS-R:L or the PACS and the responses to questions were standardized. The PACS assesses the following CP-related symptoms on a 5-point ordinal scale: 1) bully; 2) start fights; 3) used weapon; 4) cruel to animals; 5) cruel to people; 6) stay out at night; 7) tried to set fire to something; 8) run away from home; 9) broken into a building or car; 10) truanted from school; 11) threatened anyone with a gun; 12) mugging, extortion, robbery; 13) forced someone into sexual activity; 14) stealing frequency; 15) destructiveness frequency; 16) destructiveness severity; 17) aggressiveness frequency; and 18) aggressiveness severity. The 5-point scale has the following levels: 0 = never; 1= occasionally in the last 6 months; 2= frequently in the last 6 months; 3 = present in the last 7-12 months, 4 = present more than 12 months ago. Because the distribution of responses to each symptom was bimodal, indicating that the child exhibits/does not exhibit the symptom, the responses were dichotomized and then summed. The CPRS-R:L collected symptom information that was on the CP-continuum, but more likely gives a better representation of a child with ODD. The variables were also measured on a 4-point ordinal scale and the responses were spread more evenly through the four categories. Therefore the variables were kept as is and summed.

The measures included: 1) angry and resentful; 2) argues with adults; 3) loses temper; 4) irritable; 5) actively defies or refuses to comply with adults' requests; 6) temper outbursts; 7) touchy or easily annoyed by others; 8) blames other for his/her mistakes or misbehavior; 9) disturbs other children; 10) deliberately does things that annoy other people; 11) demands must be met immediately - easily frustrated; and 12) spiteful or vindictive. The 4-point scale has the following levels: 0=not true, never or seldom; 1 = just a little true, occasionally; 2 = pretty much true, often or quite a bit; 3 = very much true, very often or very frequent.

Genotyping Methodology

This study is part of the Genetic Association Information Network (GAIN), a public-private partnership of the Foundation for the National Institutes of Health, Inc. (FNIH) that currently involves the National Institutes of Health (NIH), Pfizer, Affymetrix, Perlegen Sciences, Abbott, and the Eli and Edythe Broad Institute (of MIT and Harvard University) (http://www.fnih.org/GAIN2/home_new.shtml). The IMAGE-GAIN sample was genotyped by Perlegen Sciences using a proprietary, high density oligonucleotide array-based platform. The Perlegen Array comprises approximately 600,000 tagging SNPs designed to be in high linkage disequilibrium with untyped SNPs for the three HapMap populations.

Genotype data were cleaned by The National Center for Biotechnology Information (NCBI). Quality Control analyses were processed using the GAIN QA/QC Software Package (version 0.7.4) developed by Gonçalo Abecasis and Shyam Gopalakrishnan at the University of Michigan. A copy of the software is available by e-mailing gopalakr@umich.edu or goncalo@umich.edu. The quality control procedure for cleaning this dataset is described elsewhere (Neale and others 2008). Analysis was limited to a "super-clean" set of SNPs that passed the quality control metrics for two additional GAIN Perlegen studies (for Major Depression Disorder (MDD) and Psoriasis). The use of the "super-clean" SNP selection approach is premised that the individual assay may not generally perform well and may pass quality-control metrics in one but not other studies. After excluding sex-chromosomes, additional frequency and genotyping pruning, 378,332 autosomal SNPs were examined as part of this study.

Statistical Analysis

FBAT

The Family-Based Association Test (FBAT) is a generalization of the TDT, which allows valid testing of association with any phenotype, sampling structure, and pattern of missing marker allele information (Horvath and others 2001; Horvath and others 2004; Lange and others 2004). We used Pedigree Based Association Test (PBAT) for the analyses on the three phenotypes. The dichotomous CP variable, indicating the presence or absence of CD according to DSM-IV diagnosis (American Psychiatric Association 2000) was used in FBAT analyses with an offset using the sample CD prevalence. This contrasts the genotypic information from the affected and unaffected individuals in order to identify CP disease susceptibility loci. The two quantitative phenotypes were analyzed using FBAT, while adjusting for age and gender. For all three traits we considered additive, dominant, and recessive models of inheritance. Sex chromosome markers were excluded from analysis as the FBAT/PBAT statistic is not suitable for hemizygous individuals.

Each of the nine examined phenotype and inheritance models were considered separately. All association findings are presented in the context of two evidence levels; genome-wide significance ($p \leq 5x10\text{-}7$) and strong significance ($p \leq 1x10\text{-}5$). All genotyping scatterplots for markers showing strong significance were manually examined to exclude those markers showing potential genotyping calling bias (Anney and others 2008).

SNP Labeling

All cross-referencing of SNPs was performed according to dbSNP build 128 and Human Genome build 36.2. All files are available from NCBI at ftp://ftp.ncbi.nih.gov/snp/database/organism_data/human_9606/. All SNP codes were updated to reflect dbSNP build 128 using RsMergeArch.bcp. Chromosome location was assigned using b128_SNPChrPosOnRef_36_2.bcp. Gene links were assigned using b128_SNPContigLocusId_36_2.bcp. Functional inference of linked genes was examined using Gene Ontology (GO) terms.

Linkage Disequilibrium Expansion and Functional Cross-Referencing

Linkage Disequilibrium Expansion (LDE) is a method to identify markers that are not tested directly on the Perlegen Array, but show very strong correlation with a tested marker. By expanding the dataset to include these "proxy-SNPs" we present a more inclusive list of associated markers and more importantly provide a more inclusive framework to cross-

reference association findings with previous work or genomic landmarks, such a gene loci. LDE was performed using self-authored Perl scripts. Markers that met an unadjusted P-value (P≤1x10-5) were identified and used to identify markers in near complete LD (R2≥0.98) in a 200kb window. Using the CEU HapMap data as a proxy-measure of Northern European LD structure, markers that were not on the array but met the LD criteria were identified.

Functional Cross-Referencing is a process of tagging SNPs with descriptive labels to facilitate the interpretation of the GWAS. Specifically, we tagged all associated SNPs (direct and LD expanded) with gene identifiers using the b128_SNPContigLocusId_36_2.bcp (available at http://www.ncbi.nlm.nih.gov/). Additionally, annotation of functional sites was performed by examining data from the UCSC Genome Browser (http://genome.ucsc.edu/). Specifically, we examined tracks to identify SNPs that were tagged as synonymous, non-synonymous, or located within the intron, promoter, putative transcription factor binding sites, CpG islands and regions showing conservation across species.

Analysis of whether markers within the associated regions have previously been shown to be associated with other mental health disorder was performed by cross-referencing markers and genes with the UNC Evidence Project database (https://slep.unc.edu/evidence/) (Konneker and others 2008). The search strategy used was staged according to the location of the associated marker: if the marker was found within a gene locus, we examined evidence from linkage, association and meta-analysis that specifically cross-references with the gene location; if the marker was found in an intergenic region, we examined evidence of linkage, association and meta-analysis 50kb upstream and downstream of the marker location.

Candidate Gene Enrichment

Under the candidate gene model of association, a gene is selected based upon a prior hypothesis that this gene is likely to have role in the etiology of the trait under investigation. The GWAS approach somewhat negates the need for prior hypothesis. As no selection on genes is performed prior to analysis the GWAS approach examines all genes without favor. However, it is still of interest to examine whether genes or groups of genes that have been suggested as candidate genes for a trait show an enrichment of association signals compared to genes not under selection. To test this hypothesis we examined genes selected based on a prior hypotheses, namely that they have been linked to serotonergic neurotransmission.

LDE was applied to all markers that passed QC criteria. This expanded list of markers was then annotated with gene identifiers using b128_SNPContigLocusId_36_2.bcp. Genes associated with serotonergic neurotransmission were identified through a keyword search of the GO database through GenNav (http://mor.nlm.nih.gov/perl/gennav.pl), in addition to those genes involved in the tyramine synthesis pathway. The complete list of serotonin-related genes used in this analysis are AANAT, ALDH2, AOX1, DDC, HTR1A, HTR1B, HTR1D, HTR1E, HTR1F, HTR2A, HTR2B, HTR3A, HTR3B, HTR4, HTR5A, HTR6, HTR7, INDOL1, INMT, SLC6A4, TPH1 and TPH2. ATP7A, ASMT, MAOA, MAOB and HTR2C were also considered in the original gene list but could not be examined due to their presence on the X-chromosome. All markers within the serotonergic genes and 10kb 5' and 3' of the genes were compared to all other markers. Logistic regression was performed to examine whether there was significant deviation in nominally associated SNPs (P<=0.05) from the null hypothesis for the selected genes.

Results

We performed a hypothesis-free analysis of the GAIN-ADHD sample to identify markers and genes important in the development of conduct problems in a European cohort of individuals with ADHD. Using the Family-Based Association Test (FBAT) package we examined three measures of conduct problems; a categorical measure defined using DSM IV criteria for conduct disorder, and two additional quantitative measures derived from the Parent Account of Childhood Symptoms (PACS) and the Long Version of the Conners Parent Rating Scale - Revised (CPRS-R:L). Each trait was tested under dominant, additive and recessive inheritance models.

After the quality control procedures, 378,332 markers that map to dbSNP build 128 and Human Genome build 36.2 were available for analytic use. LDE of the markers to incorporate proxy-markers altered the effective number of SNPs to 1,043,963 unique markers. A total of 938 offspring were included after the cleaning process. Of these individuals, 876 offspring had complete CPRS-R:L data and 907 offspring had complete PACS information. A summary of the total sample that contributed to at least one part of the analyses presented in this paper is listed in Table 1.

None of the markers reached genome-wide significance p≤5x10-7. Quantile-Quantile plots for each diagnosis and inheritance model indicate that there is no skew of association signals (expressed as p-value) achieved above that expected by chance alone (see Figure 1).

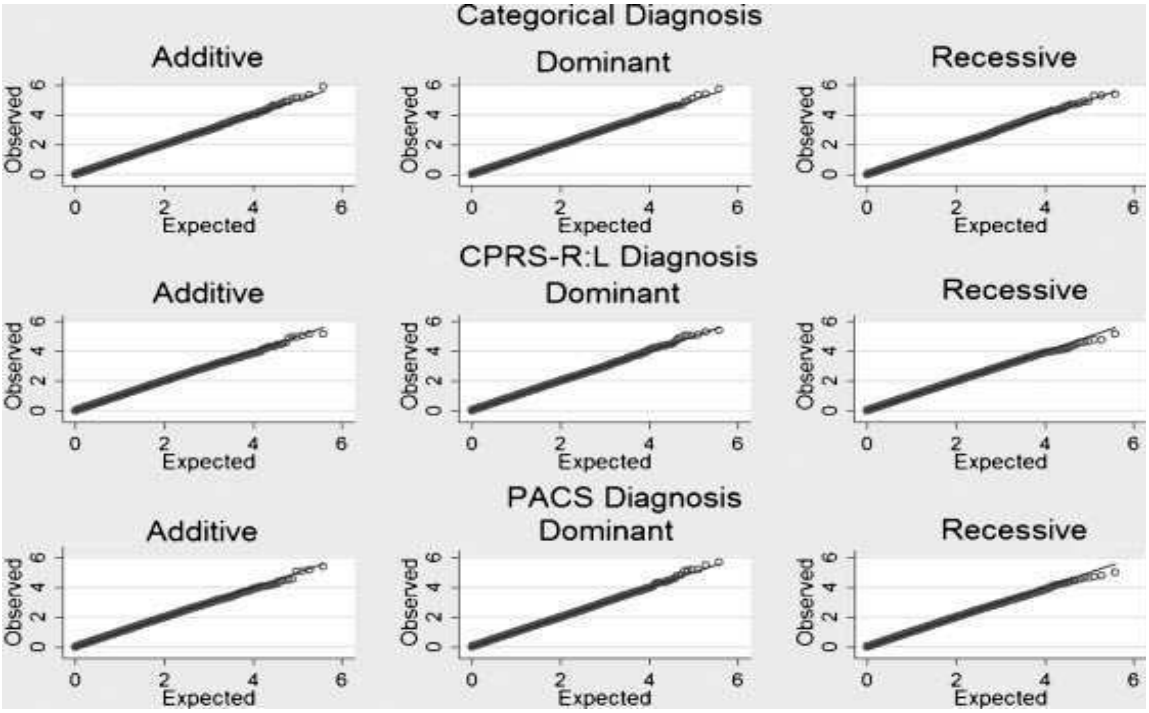

Fig. 1. Quantile–quantile plots based on expected and observed association significance (P-value) for FBAT analysis of three diagnostic measures (categorical, quantitative CPRS-R:L, and quantitative PACS) of +CP within three inheritance patterns (additive, dominant, and recessive).

Across all three phenotypes there are 28 unique modest genome-wide association signals at p≤1x10-5. Following LDE this number increased to 54 markers. A summary of the association signal for each marker is given in Figure 2 below.

Fifteen markers were located in nine genes. The associated genes include A2BP1, c12orf28, FLJ39061, KIRREL3, LOC729257, PAWR, PKD1L2, PKD1L3 and RGL1. A further twenty-three genes were identified within a 200kb window around the association signal (see Table 2). Fourteen markers reside within six "gene deserts", with no transcript present in a 200kb window. Out of the five signals that are classed as "gene deserts", three overlap directly with human expressed sequence tagged elements (EST) (sequences of expressed transcripts of the genome which may or may not code for a protein). An EST may represent a novel gene, splice variant of a gene or non-coding expressed regulatory element.

Fig. 2. Association signals for FBAT analysis of three diagnostic measures (categorical (circle), quantitative CPRS-R:L (diamond), and quantitative PACS(square)) of ADHD+CP. Two threshold lines are highlighted; blue indicates Genome-wide significance ($p \leq 5x10^{-7}$) and strong genome-wide significance ($p \leq 1x10^{-5}$)

Examination of the candidate gene enrichment using serotonin-related genes indicated that there was no signal enrichment for any of the diagnosis-inheritance groups. Conversely, there is a reduction in association signal (OR=.74; 95%CI .6-.91, p=.005) for those genes tagged as being serotonergic.

See Table 2 at the end of this report

Table 2: Summary of markers that show association signal at $p \leq 1x10^{-5}$ for the three diagnostic measures (categorical, quantitative CPRS-R:L and quantitative PACS) and three inheritance models (additive, dominant and recessive). Signal refers to the unique signal block from the GWAS, Freq refers to the risk allele frequency, and N refers to number of informative transmissions in the analysis for the SNP under the trait and inheritance model. ‡ Previous Association References were identified from the UNC Evidence Project database and confirmed by examination of the source material. Disease and analysis codes can be found at https://slep.unc.edu/evidence/. ◊ Markers highlighted as unknown are not found within the UCSC Genome Browser and could not be analyzed for functional landmarks.

Discussion

This study is part of a series of exploratory analyses to identify candidate genes that may be important in ADHD and ADHD-related traits, such as conduct problems. It is important to examine these data under the caveat that they are exploratory and like all association studies previous to this, these data will require independent replication. With this qualification considered, one must be cautious as to not over- or under-interpret these data.

In this analysis we did not find genome-wide statistical significance for any of the tested markers and the three conduct problem-traits. The top five association signals were observed on chromosome 13 (RS10492664; Categorical Diagnosis of Conduct Problems, (Additive Inheritance) p=.0000012), chromosome 21 (RS2826340; Categorical Diagnosis of Conduct Problems, (Dominant Inheritance) p=.0000018), chromosome 11 (RS10831284; Quantitative Diagnosis of Conduct Problems (PACS), (Dominant Inheritance) p=.0000019), chromosome 4 (RS6536350; Quantitative Diagnosis of Conduct Problems (PACS), (Dominant Inheritance) p=.0000033) and chromosome 12 (RS7297018; Categorical Diagnosis of Conduct Problems, (Dominant Inheritance) p=.000004).

Of these top five association signals only one, RS7297018, is located within a gene, PRKC Apoptosis WT1 Regulator (PAWR, alias PAR-4). The PAWR gene has received interest as a candidate gene for both Alzheimer's disease and schizophrenia (Kishi and others 2008; Wang and others 2008). PAWR is a leucine-zipper containing protein that has been shown to play an important role in regulating dopamine receptor D2-mediated signal transduction. The PAWR protein competes with calcium (calmodulin) for binding to the calmodulin-binding motif of the third cytoplasmic loop of the DRD2 protein (Park and others 2005). Park and colleagues found that the PAWR protein is involved in regulating the inhibitory tone of the D2-mediated cAMP

signaling in cultured cells and rat striatal neurons. Mice that lack the C-terminal domain of the PAWR protein that interacts with the D2 receptor show increased dopamine-mediated cAMP signaling activity compared to wild type. Moreover, the PAWR knockout mice show depression-like behaviors. Three recent publications have examined genetic variation in PAWR in relation to schizophrenia with varied results; both Liou et al (Liou and others 2008) and Kishi et al (Kishi and others 2008) did not find association when considering common variation within the PAWR gene. Wang et al (Wang and others 2008), performed a mutation detection approach and identified common missense mutations in exon 2 (RS8176805) and exon 3 (RS2307223) of the PAWR gene, that were in turn found to be associated with schizophrenia in the Taiwanese population. The marker RS2307223 is in complete linkage disequilibrium with the marker RS7305141 in the CEPH population, which is in turn in strong LD with RS7297018 in the CEPH sample (RS7305141:RS7297018; CEU D'=0.817, R2=0.63, LOD=12.61). In our sample RS7305141 also showed some evidence of association (p=.0012) with the Categorical Diagnosis of Conduct Problems (Dominant Inheritance). The biological role of PAWR makes it a strong candidate gene for human mental illness, specifically those related to dopaminergic dysregulation such as depression, schizophrenia and ADHD.

Of the twenty-eight regions (54 markers) that reach strong GWA signals (p<1x10-5), five have previously been linked with mental health disorder by the UNC Evidence Project (https://slep.unc.edu/evidence/). RS10797919 (Quantitative Diagnosis of Conduct Problems (PACS), (Additive Inheritance) p=.0000093) on chromosome 1 was found to be in a region that shows linkage with drive-for-thinness and obsessionality (LOD 3.46, p=.00003; (Devlin and others 2002)). Like Conduct Problems, the drive-for-thinness and obsessionality traits are considered as "dysregulation of serotonergic neurotransmission". RS10797919 is found within the RGL1 gene (Ral guanine nucleotide dissociation stimulator-like 1) and is involved in Ras and Ral GTPase signaling pathways as a downstream effector protein. To date no other markers in the RGL1 gene have been linked to CP, ADHD or other mental health disorders.

The region harboring RS10229603 (Categorical Diagnosis of Conduct Problems, (Recessive Inheritance) p=.0000049) on chromosome 7 was also shown to be linked to Autism in a meta-analysis of Strict Autism Phenotype (HEGESMA=6.24, p=.00001; (Trikalinos and others 2006)). The signal observed by Trikalinos and others (Trikalinos and others 2006) at 7p22 was discussed

in the context of potential candidate genes such as the Autism candidate gene RELN and the speech-related gene FOXP2. RS10229603 does not fall within a gene and in a 200kb window only two genes are identified; the hypothetical protein FLJ31818, and GPR85 (G-protein-coupled receptor 85). GPR85, also known as SREB2, was recently reported as the most highly conserved G-protein-coupled receptor throughout vertebrate evolution (Matsumoto and others 2008). GPR85 has also been described as a strong candidate gene for psychiatric disorders, specifically schizophrenia, following animal models showing the gene plays an important role in memory, social interaction, sensorimotor gating and brain size. *Post hoc* analysis of the region harboring the GPR85 gene in the ADHD+CP data indicate only nominal association signals ($p \sim 0.01$). The co-occurrence of linkage signals in an ADHD+CP and an Autism study is worth highlighting. As the IMAGE sample was ascertained for genetic studies of ADHD, families were excluded if the proband or sibling had a diagnosis of Autism Spectrum Disorder (ASD). Individuals who met a PACS diagnosis of ASD were excluded from the study. However, the comorbidity of Autism and ADHD traits has been examined in the IMAGE sample (Mulligan and others 2008). Mulligan and colleagues noted that a cluster of individuals with ADHD-CT, without Autism, who scored highly compared to their ADHD-CT affected peers on the Social Communication Questionnaire (mean SCQ=21.4), a screening tool for Autism, were most at risk for developing CP (Pearson's $Chi^2=22.88$, df = 4, $p< 0.001$). An SCQ-score of 15 or more on the lifetime version of the SCQ suggests the presence of a Pervasive Developmental Disorder, with a sensitivity of 0.85 and a specificity of 0.75. A cut-off score of 22 is required to differentiate autism from other PDD's, with a sensitivity of 0.75 and specificity of .60 (Berument and others 1999). Whether the region that harbors RS10229603 represents an area of common risk for ADHD, Autism and CP warrants further investigation. RS10815798 (Quantitative Diagnosis of Conduct Problems (PACS), (Dominant Inheritance) p=.0000063) on chromosome 9 correlates with a region implicated in a genome scan examining a measure of Nicotine Dependence (MP-LOD 3.19, p=.00013; (Li and others 2007). The correlation between substance use disorder and conduct disorder in ADHD is known to be high (Disney and others 1999; Elkins and others 2007).

The marker RS1951082 (Quantitative Diagnosis of Conduct Problems (CPRL), (Dominant Inheritance) p=.0000048) on chromosome 14 is part of a large 8.7Mb region that has also been shown to be linked to schizophrenia in a large study of Japanese multiplex families (RS1319956 LOD=2.87, p=.0001; (Arinami and others 2005).

The marker RS12921846 (Quantitative Diagnosis of Conduct Problems (CPRL), (Dominant Inheritance) p=.0000091) is found within the gene A2BP1 (ataxin-2 binding protein 1), which was recently found in the Wellcome Trust Case Control Consortium to be associated with Bipolar Disorder (p=.0008; (Wellcome Trust Case Control Consortium 2007). A2BP1 is thought to be an RNA-binding protein and has been linked to neuron function. Polyglutamine expansion in A2BP1have been identified as the cause of the neurodegenerative disorder, spinocerebellar ataxia 2 (Bhalla and others 2004). Moreover, Martin et al (Martin and others 2007) identified a translocation that disrupted A2BP1 in an individual with Autism. Notably, this individual showed high irritability and aggression towards herself and others. Association analysis of 27 SNP markers in 206 simplex parent-parent-autism trios from the Autism Genome Resource Exchange (AGRE) collection found two regions of the gene associated with p<.008.

As with all GWA (and linkage) studies, we aim to achieve insight into the etiology of a trait using a hypothesis-free study design. In the absence of candidate gene bias we are able to identify potentially interesting targets for follow-up studies. We have highlighted a number of chromosome regions and genes that are on the periphery of the "psychiatric candidate gene" literature, including PAWR, GPR85, A2BP1 and YHWAZ. YHWAZ (tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide) is approximately 10 kb downstream of RS931812 (Categorical Diagnosis of Conduct Problems (Dominant Inheritance) p=.0000047). The YHWAZ gene product is a binding partner for dopamine and serotonin rate-limiting enzymes, TH (tyrosine 3-monoxygenase) and TPH1/TPH2 (tryptophan 3-monooxygenase), again making it a strong candidate gene for disorders associated with dopaminergic or serotonergic dysregulation.

A number of the markers that meet strong significance (p<1x10-5) lie within regions implicated in other psychiatric traits and also harbor genes with strong candidature for these traits. It remains to be seen whether this represents true cross-trait risk or highlights the abundance of genomic regions now implicated in psychiatric disease.

To enrich the number of markers linked to genes and therefore define a more complete number of gene-tagging SNPs in this dataset we used a LDE protocol to identify proxy markers in

complete linkage disequilibrium with the tested marker. We assumed that the white European sample examined in this study shows similar LD patterns to the CEPH European HapMap sample. The test and proxy marker sets were then aligned to gene co-ordinates from build36 of the human genome project. This approach is somewhat transcript-centric and may exclude regulatory elements not captured by LD. Moreover, the FBAT/PBAT statistic is not suitable for X-linked markers and consequently X-linked genes are excluded.

None of the genes tagged as "serotonin-related" according to our inclusion criteria achieved association signals at $p \leq 1x10\text{-}5$. The caveat for any network-based analysis is the annotation used to define the network of genes, and the methodology used to assign given markers to gene terms. GO terms, are reasonably well annotated but keywords such as serotonin may exclude genes that influence monoamines in general. Moreover, the inclusion of general keywords reduces the specificity of the network. We focused on a mixture of GO terms, KEGG annotations and evidence from the literature to tag genes as being "serotonin-related". From the markers included, a marker on chromosome 13 in the Serotonin 2A Receptor (HTR2A), RS6314 showed the strongest association (RS6314, Categorical Diagnosis of Conduct Problems (Dominant Inheritance) p=.00087).

The strongest signal from those serotonin-related genes that we have previously studied in relation to ADHD-CT in a subset of the IMAGE dataset (Brookes and others 2006) was observed for RS363052, a marker on chromosome 20 in the Synaptosomal-Associated Protein, 25kDa (SNAP25) (RS363052, Quantitative Diagnosis of Conduct Problems for CPRL (Recessive Inheritance) p=.000025). Of the notable candidate genes previously studied for their putative role in conduct problems, namely SLC6A4 and DRD4, no marker within the gene or a 10kb window upstream or downstream of the gene were found to be associated at $p<1x10\text{-}2$.

This study is the first to perform a hypotheses-free genome-wide association analysis of comorbid conduct problems in ADHD. By using the FBAT approach we are able to examine categorical and quantitative measures of conduct disorder as well as specific inheritance patterns. Moreover, the FBAT approach has over 75% power to detect SNPs with heritability estimates of 0.01 or more at a nominal alpha level of 0.001 using this methodology.

A major question over the use of derived non-standard phenotype measures is their "genetic" relevance. Although Conduct Disorder *per se* has been shown to have a modest to strong heritable component (h2=0.4 to 0.8) (Hicks and others 2004; Maes and others 2007), we have not quantified the heritability of the specific traits examined in this analyses. The two quantitative measures of CP are moderately correlated ($R2^{=}0.45$). It is appropriate to consider these traits as quantitative and not discrete as both show normal distributions. Clinically, it is accepted that, among individuals with ADHD, comorbid CP predicts persistence of ADHD, and increased levels of functional impairment (Biederman and others 1996; Lahey and others 2004). However, we must again offer caution regarding whether ADHD+CP represents a more extreme presentation with regards to genetic burden. Moreover, these findings may not be relevant to CP in a general population but reflect risk to a clinical subtype of ADHD with CP.

This study is the first to perform a hypothesis-free genome-wide analysis of comorbid conduct problems in ADHD. As such, this study should be viewed as a hypothesis-generating study. Consequently, strict statistical evidence is required. No markers met genome-wide significance and the distribution of association signals did not indicate any strong enrichment of association signals greater than expected by chance alone. However, we identified a number of markers that reached strong GWA significance of p<1x10-5 and highlight, where available, putative links that may inform testable biological hypotheses regarding their candidature in the etiology of the ADHD+CP trait. It is now important to examine these markers in independent samples to investigate whether these represent true risk factors or strong examples of type I error.

Finally, this study examines the trait under the assumption that environmental risk factors are common to all individuals and all individuals have been exposed to risk at a level that is necessary for expression of the phenotype. This may be the case; however, recent approaches to examining externalizing behaviors have considered specific environmental exposures such as childhood maltreatment, parental substance use, pre- and peri-natal insult in the analytical model (Langley and others 2008). Stratification of samples by exposure to environmental predictors of disease in the analytical model may create a more homogenous phenotype and consequently, improve our power to identify true genetic risk loci from GWAS data.

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Table 2:

Signal	Chr	Pos	Marker	Gene	Nearby Genes	Allele	Freq	Phenotype (Model)	N	P-Value	Test
1	1	30392050	RS1543424		GENE DESERT	C	0.31	CPRS-R:L (Dominant)	472	0.0000086	Proxy
	1	30400298	RS2180233		GENE DESERT	C	0.31	CPRS-R:L (Dominant)	472	0.0000086	Perlegen Chip
	1	30406269	RS4949546		GENE DESERT	C	0.31	CPRS-R:L (Dominant)	472	0.0000086	Proxy
	1	30410602	RS2064648		GENE DESERT	C	0.31	CPRS-R:L (Dominant)	472	0.0000086	Proxy
2	1	155395328	RS11264625		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
	1	155397189	RS6427356		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Perlegen Chip
	1	155399011	RS6661210		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
	1	155399760	RS10796972		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
	1	155399773	RS6700498		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
	1	155400099	RS1176542		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
	1	155400218	RS1176543		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
	1	155402279	RS1176551		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
	1	155403164	RS1176555		ETV3L, ETV3	G	0.28	CPRS-R:L (Dominant)	493	0.0000082	Proxy
3	1	182113906	RS4079923	RGL1	GLT25D2	G	0.59	PACS (Additive)	635	0.0000093	Proxy
	1	182119536	RS10797919	RGL1	GLT25D2	G	0.59	PACS (Additive)	635	0.0000093	Perlegen Chip
4	1	228741448	RS701157	LOC729257	-	C	0.45	CPRS-R:L (Dominant)	467	0.0000041	Perlegen Chip
5	2	34333578	RS6733379		GENE DESERT	G	0.28	Categorical (Dominant)	493	0.0000043	Perlegen Chip
6	2	77918911	RS1487044		GENE DESERT	T	0.69	Categorical (Additive)	620	0.0000085	Perlegen Chip
	2	77918965	RS1487045		GENE DESERT	T	0.69	Categorical (Additive)	620	0.0000085	Proxy
	2	77928751	RS7595103		GENE DESERT	A	0.64	Categorical (Additive)	651	0.0000074	Perlegen Chip
7	2	202649554	RS939745	FLJ39061	FZD7	A	0.82	PACS (Additive)	444	0.0000078	Proxy
	2	202657916	RS1521883	FLJ39061	FZD7	A	0.82	PACS (Additive)	444	0.0000078	Proxy
	2	202658095	RS1521882	FLJ39061	FZD7	A	0.82	PACS (Additive)	444	0.0000078	Perlegen Chip
	2	202661471	RS1521879	FLJ39061	FZD7	A	0.82	PACS (Additive)	444	0.0000078	Proxy
8	3	22203580	RS13061352	FLJ17340	-	T	0.39	Categorical (Dominant)	499	0.0000084	Perlegen Chip
9	4	159660266	RS6536350	FLJ16077	RXFP1	G	0.2	PACS (Dominant)	422	0.0000033	Perlegen Chip
10	5	133227447	RS1644308		c5orf15	A	0.41	PACS (Dominant)	499	0.0000076	Proxy
	5	133231494	RS1644305		c5orf15	A	0.41	PACS (Dominant)	499	0.0000076	Perlegen Chip
11	7	112415608	RS10229603		FLJ31818, GPR85	C	0.31	Categorical (Recessive)	246	0.0000049	Perlegen Chip
12	8	101986896	RS4734494		YWHAZ	C	0.74	Categorical (Additive)	554	0.0000047	Proxy
	8	101986992	RS4734495		YWHAZ	C	0.74	Categorical (Additive)	554	0.0000047	Proxy
	8	101988496	RS931812		YWHAZ	C	0.74	Categorical (Additive)	554	0.0000047	Perlegen Chip

13	9	8225632	RS10815798		PTPRD	A	0.48	PACS (Dominant)	459	0.0000063	Perlegen Chip
14	10	3273384	RS2764978		PITRM1	A	0.51	CPRS-R:L (Additive)	638	0.0000089	Proxy
	10	3274006	RS2764980		PITRM1	A	0.51	CPRS-R:L (Additive)	638	0.0000089	Perlegen Chip
	10	3274060	RS2814925		PITRM1	A	0.51	CPRS-R:L (Additive)	638	0.0000089	Proxy
15	11	94307611	RS10831284		AMOTL1, CWC15, JMJD2D	G	0.13	PACS (Dominant)	357	0.0000019	Perlegen Chip
16	11	126120770	RS10736554	KIRREL3	-	T	0.18	Categorical (Recessive)	95	0.0000053	Proxy
	11	126124399	RS1557488	KIRREL3	-	T	0.18	Categorical (Recessive)	95	0.0000053	Perlegen Chip
	11	126124790	RS1557487	KIRREL3	-	T	0.18	Categorical (Recessive)	95	0.0000053	Proxy
17	12	68618093	RS789560	C12orf28	-	G	0.87	CPRS-R:L (Additive)	331	0.0000072	Perlegen Chip
18	12	78586360	RS7297018	PAWR	-	A	0.19	Categorical (Dominant)	426	0.0000040	Perlegen Chip
19	13	27327737	RS9512900		GSX1, PDX1	C	0.37	PACS (Dominant)	532	0.0000085	Perlegen Chip
20	13	107614225	RS10492664		LIG4, ABHD13	C	0.84	Categorical (Additive)	410	0.0000012	Perlegen Chip
	13	107616885	RS8002852		LIG4, ABHD13	T	0.9	Categorical (Additive)	305	0.0000072	Perlegen Chip
21	14	26329882	RS1951082		GENE DESERT	T	0.43	CPRS-R:L (Dominant)	428	0.0000048	Perlegen Chip
	14	26333356	RS8021717		GENE DESERT	T	0.43	CPRS-R:L (Dominant)	428	0.0000048	Proxy
22	15	95063430	RS4533251		SPATA8	T	0.15	Categorical (Recessive)	58	0.0000041	Perlegen Chip
23	16	6850385	RS12921846	A2BP1		T	0.19	CPRS-R:L (Dominant)	407	0.0000091	Perlegen Chip
24	16	62512947	RS1381102		GENE DESERT	A	0.4	PACS (Dominant)	491	0.0000063	Perlegen Chip
25	16	70522696	RS16973500	PKD1L3	KIAA0174, DHODH	C	0.86	CPRS-R:L (Additive)	372	0.0000074	Perlegen Chip
26	16	79714022	RS4889240	PKD1L2	c16orf46	T	0.45	CPRS-R:L (Recessive)	385	0.0000073	Perlegen Chip
27	18	53585199	RS7236632		ATP8B1	A	0.86	PACS (Additive)	384	0.0000063	Perlegen Chip
28	21	20807172	RS2826340		GENE DESERT	T	0.17	Categorical (Dominant)	390	0.0000018	Perlegen Chip