ASD symptoms in children with ADHD

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Quantitative Linkage for ASD symptoms in ADHD: Significant Locus on Chromosome 7q11

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The study described in this chapter has been submitted for publication.
Abstract

Objective: In this study, the genetic basis for Autism Spectrum Disorders (ASD) symptoms in children with Attention-Deficit/Hyperactivity Disorder (ADHD) was addressed using a genome-wide linkage approach, followed by locus-wide association analysis (LWAS). Method: Dutch participants of the International Multicenter ADHD Genetics (IMAGE) study comprising 261 ADHD probands and 354 of their siblings were analyzed, using the total and subscale scores of the Children’s Social Behavior Questionnaire (CSBQ) and the Conners parent total score as quantitative traits to run multipoint regression-based linkage analyses. Significant and suggestive linkage peaks were fine-mapped using LWAS. Results: A genome-wide significant locus (LOD 4.241 after correction for Conners’ scores) for the CSBQ subscale addressing social interaction was found on chromosome 7q11, with suggestive signals supporting this locus on three other CSBQ subscales. We identified eight other suggestive loci for the CSBQ total scale and individual subscales on chromosomes 1q42, 2q37, 3p24, 4q35, 7p12, 8p21, 16q12, 18q21, and 22q11. Neither suggestive nor significant loci were identified for ADHD symptoms as a quantitative trait. Fine-mapping resulted in one locus-wide significant association for rs9935845 for the chromosome 16 locus for the CSBQ understanding scale. Conclusions: Through quantitative trait linkage analysis, followed by LWAS, we identified a significant locus on chromosome 7q11 and a significant SNP on chromosome 16 related to ASD symptoms in children with ADHD, and generated hypotheses for further testing.
Introduction

Children with Attention-Deficit/Hyperactivity Disorder (ADHD) and Autism Spectrum Disorders (ASD) often show phenotypic overlap (Nijmeijer et al., 2009; Nijmeijer et al., 2008; Reiersen, Constantino, Volk, & Todd, 2007). Substantial genetic influences have been established for both disorders, and ADHD and ASD traits in the general population have shown high heritability as well (Constantino & Todd, 2003; Rommelse, Franke, Geurts, Hartman, & Buitelaar, 2010; Ronald, Happé, Price, Baron-Cohen, & Plomin, 2006). Two general population twin studies have suggested that both unique and shared genetic influences underlie ADHD and ASD (Reiersen, Constantino, Grimmer, Martin, & Todd, 2008; Ronald, Simonoff, Kuntsi, Asherson, & Plomin, 2008). The study by Ronald et al. (2008) quantified the genetic correlation between ADHD and ASD symptoms to be >0.50, suggesting a significant role for pleiotropic risk loci. Support for genetic overlap is also found in genetic linkage studies for ADHD and ASD, in which overlapping sets of suggestive disease loci have been found (Faraone et al., 2005; Waldman & Gizer, 2006; Yang & Gill, 2007).

In the present study, we aimed to identify genetic factors that underlie ASD symptoms in children with ADHD. The quantitative trait locus (QTL) approach is a suitable method to find those loci, given that children diagnosed with ADHD according to Diagnostic and Statistical Manual of Mental Disorders, fourth edition criteria (DSM-IV; American Psychiatric Association, 1994) criteria have substantial variance in ASD symptoms below diagnostic ASD cut-offs. The QTL approach preserves all variation in ASD severity, which increases the statistical power of our analyses (Almasy & Blangero, 1998). We analyzed data from the Dutch participants of the International Multicenter ADHD Genetics (IMAGE) project. The Dutch subsample was selected, since only here the Children’s Social Behavior Questionnaire (CSBQ; Hartman, Luteijn, Serra, & Minderaa, 2006) had been used. The CSBQ is particularly suited for measuring the continuous distribution of ASD traits in the population, as well as in clinical groups other than ASD patients (Hartman et al., 2006; Luteijn et al., 2000). QTL linkage analyses for different ASD domains were carried out using 5407 single nucleotide polymorphisms (SNPs) spanning all autosomes of the human genome. Subsequently, significant and suggestive linkage peaks were fine-mapped using locus-wide association testing (LWAS).

Methods

Participants

Subjects were Dutch European-Caucasian ADHD-affected probands aged 5-17 who participated in the IMAGE project (Kuntsi, Neale, Chen, Faraone, & Asherson, 2006; Brookes et al., 2006). A detailed description of sample selection is described in...
Nijmeijer et al. (2008a). Exclusion criteria were an IQ < 70, autism, epilepsy, brain disorders, and any genetic or medical disorder associated with externalizing behaviors that might mimic ADHD. The final sample included 261 ADHD probands and 354 siblings from 261 families. Fifty-four (42 probands, 12 siblings) had Social Communication Questionnaire (SCQ; Berument, Rutter, Lord, Pickles, & Bailey, 1999) scores ≥15, but no diagnosis of autistic disorder based on the Parental Account of Childhood Symptoms (PACS; Taylor, Schachar, Thorley, & Wieselberg, 1986) interview. Ethical approval was obtained from National Institutes of Health recognized local ethical review boards, and all families gave written informed consent prior to participation.

Measures
The CSBQ was used to assess ASD symptoms. It has six empirically-derived subscales entitled ‘not optimally tuned to the social situation’ (tuned; 11 items addressing emotional overreacting and stubbornness/disobedience), ‘reduced contact and social interest’ (social; 12 items), ‘orientation problems in time, place, or activity’ (orientation; 8 items), ‘difficulties in understanding of social information’ (understanding; 7 items), ‘fear of and resistance to changes’ (change; 3 items), and ‘stereotyped behavior’ (stereotyped; 8 items). Estimates for internal, test-retest, and inter-rater reliability, as well as convergent and divergent validity of the CSBQ are good (Hartman et al., 2006). The CSBQ can differentiate between autism and pervasive developmental disorders-not otherwise specified (PDD-NOS) on the one hand, and ASD and ADHD on the other (Hartman et al., 2006). Furthermore, a strong correlation of 0.75 was found between the total scores of the Autism Behavior Checklist (Krug, Arick, & Almond, 1980) and the CSBQ in a large Dutch population sample (Hartman, Luteijn, Moorlag, de Bildt, & Minderaa, 2007). In our sample, the Pearson’s correlation between the SCQ and CSBQ total scores was 0.44 (p<0.01). To assess ADHD symptom severity in both probands and siblings, the DSM-IV inattentive, hyperactive-impulsive, and total scales of the Conners parent rating scale, long form (Conners, 1996) were used.

Linkage Genotyping and Data Cleaning
An extensive description of DNA extraction and genotyping is provided elsewhere (Brookes et al., 2006; Zhou et al., 2008a). Briefly, DNA was extracted from blood samples or immortalized cell lines at Rutgers University Cell and DNA Repository, New Jersey, USA. In a few cases mouth swabs were used to extract DNA at the Social Genetic and Developmental Psychiatry laboratories in London, UK. Illumina BeadArray™ technology on a BeadLab system was used for genotyping, which was provided by the Center for Inherited Disease Research (http://www.cidr.jhmi.edu/). A total of 5,545 autosomal SNPs from the Illumina Linkage IVb SNP panel were successfully assayed with a call rate of 99.6% and a reproduction rate of 99.994%. The markers were ordered...
and placed on the physical map according to Genome Build 35. Interpolated genetic distances from the deCODE genetic map were used to estimate map distances (Kong et al., 2002).

Pedigree errors were identified and corrected by testing pair-wise subject relationships with the program Relpair (Epstein, Duren, & Boehnke, 2000). Genotypes causing Mendelian inconsistencies were identified by PEDCHECK and removed by a custom script (O’Connell & Weeks, 1998). Unlikely genotype combinations leading to double recombinations over short genetic distances in a few cases were removed by MERLIN (Abecasis & Wigginton, 2005; Wigginton & Abecasis, 2005). Following data cleaning, 5,407 autosomal SNPs with an average resolution of 1.66 SNP/centiMorgan (cM) were entered into the linkage analyses.

**Linkage Data Analyses**

Multivariate QTL linkage analysis was performed for the CSBQ total scale and each of the CSBQ subscales. Our first model included age and gender as covariates. All analyses were repeated with scores on the ADHD/DSM-IV inattentive and hyperactive-impulsive scales of the Conners parent rating scale-long form simultaneously added as covariates. The latter analyses served to investigate whether QTLs found in the first analyses were related to ADHD (which would be the case if signals disappeared after inclusion of ADHD scores), and also whether new QTLs would appear. Signals that would disappear could be suggestive of pleiotropic effects, while remaining and new QTLs could harbor genes uniquely contributing to ASD symptoms. Subsequently, QTL linkage analysis was performed for the Conners parent DSM-IV total score, adjusting for age and gender, and repeated with the CSBQ total score as additional covariate. These analyses served to verify potential pleiotropic effects of loci identified in the CSBQ analyses, i.e., pleiotropic loci with substantial effects on both ADHD and ASD were expected to appear in both the analyses for CSBQ and Conners, and LOD scores for these loci were expected to decrease after addition of the Conners or CSBQ score, respectively, as a covariate.

The linkage analyses were carried out using Merlin-regress software, which implements a regression-based procedure using trait-squared sums and differences to predict Identity by Descent sharing between relative pairs (Abecasis, Cherny, Cookson, & Cardon, 2002; Sham, Purcell, Cherny, & Abecasis, 2002). With the population distribution parameters of mean, variance, and heritability specified, this method can be applied to selected samples with a statistical power similar to variance component linkage tests (Zhou et al., 2008a). For this study, heritability estimates were based on values previously reported for the broader ASD spectrum in twins from the general population (Constantino & Todd, 2000; Ronald et al., 2006). Because treating tightly linked markers as independent markers can inflate LOD scores, we applied the criterion of r2 <0.05 between SNPs to cluster SNPs into combined markers (Wigginton et al., 2005).
Empirical p-values were derived using Merlin software by running 1,000 simulations under the null-hypothesis of no linkage, while preserving the original phenotypes, family structures, allele frequencies, linkage disequilibrium (LD) structure, and missing data pattern (Abecasis et al., 2002; Zhou et al., 2008a). In each simulated data set, linkage was defined as peak LOD scores equal to or higher than the experimental LOD score.

**Fine-mapping Methods**

Fine-mapping of selected loci was performed using the SNP data available from the Genetic Association Information Network (GAIN) for ADHD. GAIN is a public-private partnership of the Foundation for the National Institutes of Health, Inc., which involves the National Institutes of Health, Pfizer, Affymetrix, Perlegen Sciences, Abbott, and the Eli and Edythe Broad Institute (Massachusetts Institute of Technology and Harvard University; http://www.fnih.org). Genotyping and data cleaning details for the ADHD GAIN study (Study Accession, phs00016.v1.p1) have been reported elsewhere (Neale et al., 2008). Briefly, genotyping was conducted at Perlegen Sciences using the 600K genotyping platform, which comprises approximately 600,000 tagging SNPs designed to be in high LD with untyped SNPs for the three Haplotype Map (HapMap) populations. Genotype data were cleaned by the National Center for Biotechnology Information. Quality control was performed using the GAIN Quality Assurance/Quality Control Software Package, version 0.7.4, developed by Gonçalo Abecasis and Shyan Kopalakrishnan at the University of Michigan. Briefly, we selected SNPs with minor allele frequencies ≥ 0.05 and Hardy Weinberg Equilibrium p ≥ 1x10^{-6}. Mendelian inconsistencies were identified by PLINK and removed (http://pngu.mgh.harvard.edu/purcell/plink/; Purcell et al., 2007). We additionally removed SNPs that failed quality controls in the two other GAIN Perlegen studies, i.e. for major depression (dbGAP Study Accession, phs00020.v1.p1) and psoriasis (dbGAP Study Accession, phs00019.v1.p1). The final data set included 384,401 SNPs. To increase coverage in the targeted genomic areas, we used the imputation approach implemented in PLINK (v1.07), which imputes genotypes of SNPs that are not directly genotyped in the data set, but that are present on a reference panel. The PLINK algorithm is an extension of multimarker tagging. The reference panel used consisted of 2,543,285 polymorphic autosomal SNPs genotyped on the 60 HapMap CEU (Caucasians from Utah, USA) founders which are publicly available for download from the HapMap website (http://www.hapmap.org). For each SNP with missing data for individuals in the IMAGE data set, PLINK selects a set of neighbouring observed SNPs (“proxy” SNPs) using the reference panel information, and then phases these proxy SNPs using a standard Expectation Maximization algorithm in both reference panel and IMAGE sample jointly. By subsequently grouping haplotypes based on the allele at the reference position, genotype data for the reference SNP can be inferred, as discrete “hard”
genotype calls. For the present analysis, we selected up to 5 SNPs either side of the reference SNP, from a search of 15 SNPs either side of the reference, at most 250 kb away. All potential proxies were considered one at a time in order of strongest to weakest LD with the reference. A proxy had to be r² > 0.05 with the reference to be added; additionally, a proxy should not have an r² > 0.5 with any proxy already selected. An exception is that PLINK always attempted to add at least two proxies even if the best proxy did not meet the r² > 0.05 criterion, to ensure a 2-SNP haplotype could be found that might tag the reference SNP, even if no single SNP did. In addition, proxies were selected only if the minor allele frequency ≥ 0.01 and genotyping failure rate were < 0.05. A threshold of 0.95 confidence level was set for a hard call to be included in further association testing. Most likely genotypes on imputed SNPs were then included in association analyses. In total, 710,447 SNPs were available for analyses after imputation.

LWAS Genotyping and Data Analysis
Genome-wide genotyping data were available for the 261 ADHD probands. For the LWAS, we selected all SNPs that lay within loci identified in the linkage analyses. The width of the peaks was defined by the LOD-1 approach, applied to suggestive and significant linkage regions. Linear regression in PLINK was used for the association analyses of the 261 probands. All analyses were corrected for age, gender, and Conners’ DSM-IV inattentive and hyperactive-impulsive scores. In addition to a standard regression analysis, a permutation test was conducted to give p-values corrected for multiple comparisons across all the SNPs in a locus assessed for potential association. We applied the Max(T) permutation, which estimates a value that controls for the fact that thousands of other SNPs were tested. This permutation strategy maintains the correlation structure between SNPs, providing a less stringent correction for multiple testing. This process was repeated 5,000 times for each locus.

Results

Linkage Results
In Table 1, sample characteristics are presented. Figure 1 and Tables 2 and 3 show the results of the multivariate QTL linkage analyses. We found one significant linkage peak for rs1859293 on 7q11.23 for the CSBQ social subscale, which in the primary analyses (Conners’ scores not included as covariates) had a LOD score of 3.977. Suggestive LOD scores for this same genomic region were found for the understanding and change subscales. Other regions that showed suggestive linkage in the analyses uncorrected for Conners’ scores were found on 4q35, 18q21, and 22q21 for the social subscale, and on 7p12 for the change subscale.
### Table 1 Phenotypic characteristics of the Dutch IMAGE sample

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (sd) probands (n=261)</th>
<th>Mean (sd) siblings (n=354)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>11.3 (2.6)</td>
<td>11.0 (3.5)</td>
</tr>
<tr>
<td>IQ</td>
<td>98.3 (12.2)</td>
<td>102.1 (11.5)</td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>14.2</td>
<td>51.1</td>
</tr>
<tr>
<td>CSBQ scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSBQ total</td>
<td>40.3 (16.6)</td>
<td>18.8 (14.6)</td>
</tr>
<tr>
<td>Tuned</td>
<td>13.7 (5.1)</td>
<td>7.0 (5.3)</td>
</tr>
<tr>
<td>Social</td>
<td>5.5 (4.5)</td>
<td>2.7 (3.7)</td>
</tr>
<tr>
<td>Orientation</td>
<td>7.7 (3.7)</td>
<td>3.2 (3.4)</td>
</tr>
<tr>
<td>Understanding</td>
<td>6.9 (3.6)</td>
<td>3.7 (3.1)</td>
</tr>
<tr>
<td>Stereotyped</td>
<td>4.4 (3.7)</td>
<td>1.4 (2.4)</td>
</tr>
<tr>
<td>Change</td>
<td>2.1 (2.0)</td>
<td>0.8 (1.3)</td>
</tr>
<tr>
<td>Conners’ scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSM-IV inattentive</td>
<td>18.8 (4.9)</td>
<td>7.6 (7.4)</td>
</tr>
<tr>
<td>DSM-IV hyperactive-impulsive</td>
<td>17.5 (4.7)</td>
<td>5.9 (6.1)</td>
</tr>
</tbody>
</table>

Note. ADHD Genetics project; CSBQ=Children’s Social Behavior Questionnaire; DSM-IV=Diagnostic and Statistical Manual of Mental Disorders, 4th Edition; IMAGE=International Multicenter

### Table 2 Thresholds for suggestive and significant linkage (LOD scores)

<table>
<thead>
<tr>
<th>Quantitative trait</th>
<th>Suggestive threshold</th>
<th>Significant threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSBQ total</td>
<td>1.95</td>
<td>4.01</td>
</tr>
<tr>
<td>Tuned</td>
<td>1.97</td>
<td>3.8</td>
</tr>
<tr>
<td>Social</td>
<td>1.97</td>
<td>3.9</td>
</tr>
<tr>
<td>Orientation</td>
<td>1.94</td>
<td>3.89</td>
</tr>
<tr>
<td>Understanding</td>
<td>1.98</td>
<td>4.00</td>
</tr>
<tr>
<td>Stereotyped</td>
<td>1.90</td>
<td>3.75</td>
</tr>
<tr>
<td>Change</td>
<td>1.93</td>
<td>3.79</td>
</tr>
<tr>
<td>Conners DSM-IV total</td>
<td>1.92</td>
<td>3.95</td>
</tr>
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</table>

Note. *Thresholds were derived by running 1,000 simulations in Merlin under the 0-hypothesis of no linkage, while preserving original phenotypes, family structures, allele frequencies, linkage disequilibrium structure, and missing data pattern. CSBQ=Children’s Social Behavior Questionnaire; DSM-IV=Diagnostic and Statistical Manual of Mental Disorders, 4th Edition; LOD=Logarithm of the odds*
Figure 1  Logarithm of the odds (LOD) scores for the Children's Social Behavior Questionnaire (CSBQ) social, understanding, and change subscales for chromosome 7.
The signals found on 7q11.23 remained present (and significant in the case of the social subscale) in the analyses corrected for Conners’ inattentive and hyperactive-impulsive scores, with an additional signal in this region arising for the CSBQ total score. New suggestive loci were found on chromosome 1q42 and 2q37 for the total score, on 8p21 for the tuned subscale, 16p12 for the understanding subscale, and 3p24 for the change subscale. LOD scores for the loci on 4q35, 18q21, and 22q11 decreased below the empirically derived levels for suggestive linkage upon correction for Conners’ scores.

Analyses for the Conners DSM-IV total score did not yield any loci of interest, neither significant nor suggestive. Addition of the CSBQ total score as a covariate did not change this finding.

### Table 3  Linkage results for the CSBQ total score and the CSBQ subscales

<table>
<thead>
<tr>
<th>CSBQ scale</th>
<th>Chromosome</th>
<th>Position (cM)</th>
<th>Location</th>
<th>Marker</th>
<th>LOD</th>
<th>No. of markers</th>
<th>Gene</th>
</tr>
</thead>
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<tr>
<td>Gender and age as covariates</td>
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<td></td>
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<tr>
<td>CSBQ total</td>
<td></td>
<td></td>
<td></td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Tuned</td>
<td>4</td>
<td>4q35.1</td>
<td>183.66</td>
<td>rs2063906</td>
<td>2.294</td>
<td>2</td>
<td>ODZ3</td>
</tr>
<tr>
<td>Social</td>
<td>7</td>
<td>7q11.23</td>
<td>87.77</td>
<td>rs1859293</td>
<td>3.977</td>
<td>9</td>
<td></td>
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<tr>
<td></td>
<td>18</td>
<td>18q21.1</td>
<td>71.1</td>
<td>rs521861</td>
<td>2.056</td>
<td>3</td>
<td>MYO5B</td>
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<tr>
<td></td>
<td>12</td>
<td>12q11</td>
<td>0</td>
<td>rs7288876</td>
<td>1.975</td>
<td>1</td>
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<td>Orientation</td>
<td>7</td>
<td>7q11.23</td>
<td>87.77</td>
<td>rs1859293</td>
<td>3.083</td>
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<tr>
<td>Understanding</td>
<td>7</td>
<td>7q11.23</td>
<td>87.77</td>
<td>rs1859293</td>
<td>3.500</td>
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<tr>
<td>Stereotyped</td>
<td>7</td>
<td>7q11.23</td>
<td>87.77</td>
<td>rs1859293</td>
<td>3.083</td>
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<tr>
<td>Change</td>
<td>7</td>
<td>7q11.23</td>
<td>87.77</td>
<td>rs1859293</td>
<td>3.500</td>
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<tr>
<td></td>
<td>7</td>
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<td>70.13</td>
<td>rs921630</td>
<td>2.876</td>
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</table>

Note: 1DeCODE Genetic map position 2Most likely cytogenic location 3Number of consecutive SNP markers with LODs above thresholds for suggestive linkage and (in brackets) the genetic distance they span. AGAP1=ArfGAP with GTPase domain ankyrin repeat and PH domain 1, CSBQ=Children’s Social Behavior Questionnaire, FZD3=Frizzled Homolog 3, LOD=Logarithm of the odds, MYO5B=Myosin Vb; ODZ3=Odd; Ots/tm homolog 3, TOX3=TOX high mobility group box family member 3, **Bold** are significant results given LODs derived from simulations.

The signals found on 7q11.23 remained present (and significant in the case of the social subscale) in the analyses corrected for Conners’ inattentive and hyperactive-impulsive scores, with an additional signal in this region arising for the CSBQ total score. New suggestive loci were found on chromosome 1q42 and 2q37 for the total score, on 8p21 for the tuned subscale, 16p12 for the understanding subscale, and 3p24 for the change subscale. LOD scores for the loci on 4q35, 18q21, and 22q11 decreased below the empirically derived levels for suggestive linkage upon correction for Conners’ scores.

Analyses for the Conners DSM-IV total score did not yield any loci of interest, neither significant nor suggestive. Addition of the CSBQ total score as a covariate did not change this finding.
Fine Mapping Results

We performed a total of 14 LWAS: five for the model without adjustment for Conners' scores and nine in the Conners' adjusted model. In Table 4, the ten SNPs with the lowest p-values from our LWAS are displayed. A significant permutation p-value was found for rs9935845 (empirical p = 8.8E-03) in the locus on chromosome 16 linked to the understanding subscale. No other SNPs were significant after permutation testing.

In the significant linkage peak on chromosome 7q11.23 for the social subscale, the SNP with the lowest p-value (4.5E-03) was rs12699099, with a p-value of 5.3E-01 after permutation testing.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Position (cM)a</th>
<th>Locationb</th>
<th>Marker</th>
<th>LOD</th>
<th>No. of markersc</th>
<th>Gene</th>
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Note. aDeCODE Genetic map position  
bMost likely cytogenic location  
cNumber of consecutive SNP markers with LODs above thresholds for suggestive linkage and (in brackets) the genetic distance they span.

AGAP1 = ArfGAP with GTPase domain ankyrin repeat and PH domain 1; CSBQ = Children's Social Behavior Questionnaire; FZD3 = Frizzled Homolog 3; LOD = Logarithm of the odds; MYO5B = Myosin Vb; ODZ3 = odd; Oz/ten-m homolog 3; TOX3 = TOX high mobility group box family member 3; Bold are significant results given LODs derived from simulations.

Fine Mapping Results

We performed a total of 14 LWAS: five for the model without adjustment for Conners' scores and nine in the Conners' adjusted model. In Table 4, the ten SNPs with the lowest p-values from our LWAS are displayed. A significant permutation p-value was found for rs9935845 (empirical p = 8.8E-03) in the locus on chromosome 16 linked to the understanding subscale. No other SNPs were significant after permutation testing.

In the significant linkage peak on chromosome 7q11.23 for the social subscale, the SNP with the lowest p-value (4.5E-03) was rs12699099, with a p-value of 5.3E-01 after permutation testing.
### Table 4 LWAS markers with the lowest p-values for the CSBQ total score and CSBQ subscales

<table>
<thead>
<tr>
<th>CSBQ subscale</th>
<th>Chromosome</th>
<th>Position</th>
<th>Marker</th>
<th>Gene</th>
<th>p-value</th>
<th>p-value after permutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Understanding</td>
<td>7</td>
<td>75090650</td>
<td>rs1179620</td>
<td>HIP1</td>
<td>0.0005999</td>
<td>0.1546</td>
</tr>
<tr>
<td>Understanding</td>
<td>7</td>
<td>75093427</td>
<td>rs1179625</td>
<td>HIP1</td>
<td>0.0005999</td>
<td>0.1546</td>
</tr>
<tr>
<td>CSBQ Total</td>
<td>7</td>
<td>82169463</td>
<td>rs4236670</td>
<td>intergenic</td>
<td>0.0003999</td>
<td>0.2422</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>82186075</td>
<td>rs970867</td>
<td>intergenic</td>
<td>0.0003999</td>
<td>0.1522</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>82185964</td>
<td>rs7806760</td>
<td>intergenic</td>
<td>0.0002</td>
<td>0.1562</td>
</tr>
<tr>
<td>Understanding</td>
<td>16</td>
<td>50405299</td>
<td>rs9935845</td>
<td>intergenic</td>
<td>0.0002</td>
<td>0.008798</td>
</tr>
<tr>
<td>Understanding</td>
<td>16</td>
<td>54493517</td>
<td>rs12325081</td>
<td>CES7</td>
<td>0.0005999</td>
<td>0.1338</td>
</tr>
<tr>
<td>Understanding</td>
<td>16</td>
<td>54510789</td>
<td>rs6499802</td>
<td>CES7</td>
<td>0.0003999</td>
<td>0.215</td>
</tr>
<tr>
<td>Understanding</td>
<td>16</td>
<td>54555118</td>
<td>rs7195759</td>
<td>intergenic</td>
<td>0.0003999</td>
<td>0.235</td>
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<tr>
<td>Understanding</td>
<td>16</td>
<td>54479842</td>
<td>rs6499799</td>
<td>CES7</td>
<td>0.0002</td>
<td>0.2026</td>
</tr>
<tr>
<td>Understanding</td>
<td>16</td>
<td>54479990</td>
<td>rs4784604</td>
<td>CES7</td>
<td>0.0002</td>
<td>0.2116</td>
</tr>
</tbody>
</table>

*Note:* CES7=carboxylesterase 7 isoform 1; CSBQ=Children’s Social Behavior Questionnaire; HIP1=huntingtin-interacting protein 1; LWAS=locus wide association analysis; **Bold** are results significant after permutation testing.
Discussion

In the current study, we performed genome-wide QTL linkage analyses for CSBQ-based ASD symptom domains in 261 children with ADHD and 354 of their siblings, followed by fine-mapping in identified loci using association testing. We identified a genome-wide significant locus on chromosome 7q11.23 for the CSBQ subscale addressing social interaction, as well as eight suggestive loci for the CSBQ total scale and individual subscales. The LOD scores at the loci on 4q35, 7p12, and 18q21, 22q11 decreased after including ADHD symptom scores in the analyses. Nevertheless, the pleiotropy of these loci could not be confirmed when we analysed ADHD symptom severity as a QTL, which resulted in no linkage signals at all. Suggestive loci that appeared to be primarily associated with ASD symptoms were found on 1q42, 2q37, 3p24, 8p21, and 16q12. LOD scores for these regions did not change substantially, or even appeared after inclusion of Conners' scores as covariates. Fine mapping within the peaks resulted in interesting candidate genes and one intergenic SNP (rs9935845) on 16q12 showing a significant permutation-based association p-value.

The most prominent, genome-wide significant (LOD score 4.241) linkage result was found on chromosome 7q11.23 for the CSBQ social subscale, with suggestive linkage to this region for the change, understanding, and total subscales after correction for ADHD symptom severity. Interestingly, the 7q11.23 area has been reported to be involved in Williams Beuren syndrome (in which extraordinary pro-social behavior is a hallmark feature) and possibly in autism (Allen-Brady et al., 2009b; Berg et al., 2007; Depienne et al., 2007; Sultana et al., 2002). These findings, together with the association with social impairments in children with ADHD as suggested by the present study, imply that this region may harbor genes that are important for social interaction. Although our results appear to show that the 7q11 area has ASD-specific effects, for ADHD, suggestive linkage to a region encompassing our peak SNP was reported in a meta-analysis by Zhou et al. (2008a). It cannot be precluded that previous ADHD linkage results were driven by ASD symptoms or a common phenotype, but clearly, this needs further investigation.

Fine-mapping within the 7q11.23 peak resulted in several interesting SNPs. The SNP (rs12699099) with the smallest p-value (0.004599) is located within the Calneuron-1 (CALN1) gene, which encodes a neuron-specific protein (Wu, Lin, Liu, Jamrich, & Shaffer, 2001). Moreover, of the top ten association signals, two are located in the Huntington-interacting protein 1 gene (HIP1) on chromosome 7 (for the total scale), lying at 5 kb from our linkage peak SNP. HIP1 encodes a membrane-associated protein that colocalizes and interacts with huntingtin, and possibly plays a role in the origins of Huntington's disease (Moores, Roy, Nicholson, & Staveley, 2008).

Regions identified in the present study that may be particularly interesting in explaining the overlap between ADHD and ASD are 4q35, 18q21, and 7p12. As
mentioned previously, their linkage results suggest pleiotropic effects. Although 4q35 and 18q21 have not been reported in earlier ADHD or ASD linkage publications, the 7p12.3 signal overlaps with a significant linkage peak reported by Bakker et al. (2003; marker D7S1818; 69.6 cM), who studied ADHD-affected sibling pairs, in whom autism had not been an exclusion criterion. We found that the LOD score for 7p12 for the change subscale slightly decreased when the Conners total score was added as a covariate. This confirms the potential importance of 7p12 for ADHD, but also suggests it may in addition have an effect on ASD symptoms, particularly rigidity, and could harbor pleiotropic risk genes. In accordance with this, a suggestive linkage region encompassing our peak SNP on 7p12 has been reported for autism (Allen-Brady et al., 2009a). Furthermore, a candidate gene that may warrant further investigation as a potentially pleiotropic risk gene is Myosin Vb (MYO5B), in which our linkage peak SNP on chromosome 18q was located. Previously, a relation with bipolar disorder was found for this gene (Sklar et al., 2008).

Although the present study indicates 8p21 to have ASD specific effects, this region overlaps with suggestive linkage peaks reported for ADHD (Faraone et al., 2008) and for reading ability in sibling pairs with ADHD (Loo et al., 2004). Language impairments are a hallmark characteristic of ASD, and interestingly, language impairment in itself appears to be associated with weaker social adaptation (Loucas et al., 2008). For ASD, there are no studies showing linkage for chromosome 8p21, although evidence of Copy Number Variants in this region has been reported (for a review, see Tabares-Seisdedos & Rubenstein, 2009). The 8p21 region may therefore be a compelling target for further investigation with regard to the overlap between ADHD, ASD, and dyslexia, with the frizzled homolog 3 (FZD3) gene, in which our linkage peak SNP (rs352436) was located, deserving special attention. Recently, this gene has been identified as a potential candidate gene for several neuropsychiatric disorders, including autism and schizophrenia (Tabares-Seisdedos et al., 2009). Our linkage peak on 2q37 also points to a candidate gene that may deserve further investigation as a potential ASD risk gene, i.e., ArfGAP with GTPase domain ankyrin repeat and PH domain 1 (AGAP1), which has been indicated as a putative autism risk gene in an earlier study (Wassink et al., 2005).

Our fine-mapping analyses resulted in a significant association finding for rs9935845 on chromosome 16q12 for the CSBQ understanding subscale. This result appeared to be ASD-specific, as the 16q12 peak appeared only after correction for ADHD symptom severity. The understanding subscale addresses communication problems, in particular pragmatic language problems, which are obviously associated with ASD, but also with ADHD (e.g., Geurts et al., 2004; Ketelaars, Cuperus, Jansonius, & Verhoeven, 2009). The linkage peak SNP lies within 1Mb of a region found in a previous ADHD linkage meta-analysis (Zhou et al., 2008b), which raises the question whether this meta-analysis result perhaps reflects ASD-like communication problems.
in children with ADHD, rather than DSM-IV-defined ADHD. Interesting genes in the 16q12 region are the carboxylesterase 7 isoform 1 gene (CES7), given that four of the top ten association signals were located in this gene, and the TOX high mobility group box family member 3 gene (TOX3), in which the SNP with the highest LOD score was located. Thus far, the role of these genes in neuropsychiatric disease is unexplored territory.

Notably, our results appear to underline both the existence of a general autism factor and the genetic independence of the different autism symptom domains. The QTL on chromosome 7q11 may be considered support for a general autism factor, as it was found for subscales related to different ASD domains, i.e., the social subscale, which focuses on impaired social interaction, the understanding subscale, addressing communication problems, in particular pragmatic language problems, and the change subscale, which refers to resistance to change and is related to the ASD restricted and repetitive behavior domain as described in DSM-IV. The eight other loci showed suggestive linkage to individual traits, which may support findings of the independent heritability of distinct ASD subdomains (for a review see Happé & Ronald, 2008). However, careful interpretation of these results is necessary as our analyses mostly revealed suggestive linkage findings and may contain false positives.

A limitation of our study may have been that the most severe ASD cases were excluded from participation in the IMAGE sample. The exclusion of children with autism from the IMAGE sample was one of the reasons for applying a QTL approach in this study, as opposed to studying affection status. Nevertheless, although ASD-symptoms are generally considered to represent a continuous trait (Constantino et al., 2003; Constantino & Todd, 2005; Spiker, Lotspeich, Dimicelli, Myers, & Risch, 2002), we did not actually investigate the most severe end of the ASD spectrum, and therefore cannot be sure whether our findings pertain to narrowly defined autism as well. Our modest linkage results for ADHD symptoms as a quantitative trait may be a consequence of the restriction of range in ADHD scores, inherent to the clinical ADHD sample we used, or to the relatively small sample size. This renders our interpretation of the loci we found as being specific for ASD or potentially pleiotropic interesting, but preliminary. Ideally, subsequent studies would use samples with extremes of both the ADHD and ASD spectra represented, in which parent reports as well as a detailed developmental history and observational data would be collected to assess ASD and ADHD symptomatology.

We did not adjust for testing multiple ASD traits, since a Bonferroni correction would have been overly conservative due to the high correlations between the ASD symptom scales. We did, however, account for the multiple testing that resulted from analyzing several thousands of markers through simulation, and produced conservative LOD scores for significant and suggestive linkage. Replicating the suggestive QTLs identified in the present study with genome-wide significance will likely require larger
samples, since our findings suggest that effects are not large. Similarly, follow-up studies are needed to confirm whether the observed, and obviously modest, fine mapping association findings represent true effects.

To our knowledge, our study represents the first attempt to identify QTLs underlying the occurrence of (subtle) ASD symptoms in children with ADHD. The strengths of this study are the use of a well-defined combined type ADHD sample, and the relatively large sample size. This resulted in the identification of a genome-wide significant QTL on chromosome 7q11, suggestive QTLs on 1q42, 2q37, 3p24, 4q35, 7p12, 8p21, 16q12, 18q21, and 22q11, and a significant association signal for rs9935845 on chromosome 16. Moreover, we found preliminary evidence for the association of the CES7, CALN1 and HIP1 gene with ASD symptoms. These genes may therefore be interesting new candidate genes for ASD symptoms, at least in the context of ADHD.
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