Abstract

Objective: As multiple genes with small effect size are assumed to play a role in attention-deficit/hyperactivity disorder (ADHD) disease etiology, considering multiple variants within the same analysis likely increases the total explained phenotypic variance, thereby boosting the power of genetic studies. We investigated whether pathway-based analysis could bring us closer to unraveling the biology of ADHD.

Method: We describe pathway as a pre-defined gene selection based on a well-established database or literature data. Common genetic variants in pathways involved in dopamine/noradrenaline and serotonin neurotransmission and genes involved in neurite outgrowth were investigated in cases from the International Multicentre ADHD Genetics (IMAGE) study. We performed multivariable analysis to combine the effects of single genetic variants within the pathway genes. Phenotypes were DSM-IV symptom counts for inattention and hyperactivity/impulsivity (n=871) and symptom severity measured with the Conners Parent (n=930) and Teacher Rating Scales (n=916).

Results: Summing genetic effects of common genetic variants within the pathways showed significant association with hyperactive/impulsive ($p_{empirical}$=0.007), but not inattentive symptoms ($p_{empirical}$=0.73). Analysis of parent-rated Conners hyperactive/impulsive symptom scores validated this result ($p_{empirical}$=0.0018). Teacher-rated Conners scores were not associated. Post-hoc analyses showed significant contribution of all pathways to the hyperactive/impulsive symptom domain (dopamine/noradrenaline $p_{empirical}$=0.0004, serotonin $p_{empirical}$=0.0149, neurite outgrowth $p_{empirical}$=0.0452).

Conclusion: The current analysis shows association between common variants in three genetic pathways with the hyperactive/impulsive component of ADHD. This study demonstrates that pathway-based association analyses, using quantitative measures of ADHD symptom domains may increase the power of genetic analyses to identify biological risk factors involved in this disease.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a common neuropsychiatric disorder characterized by developmentally inappropriate inattentiveness and/or increased impulsivity and hyperactivity[1]. Although ADHD is highly heritable, with heritability estimates around 76%[2], discovering genetic risk variants has been challenging. A number of candidate genes have been associated, but altogether explain only a small part of the heritability[3], and so far, genome-wide association studies (GWASs) have not yielded genome-wide significant findings[4].

Difficulty in discovering genetic risk variants has been attributed to the fact that ADHD is clinically heterogeneous[5]. Factor analyses of ADHD symptoms demonstrate that ADHD is indeed multidimensional, with studies of teacher and parent ratings supporting a two-factor structure in children[6] separating inattention and hyperactivity/impulsivity. The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) diagnostic criteria make sub-classifications, distinguishing an inattentive clinical subtype (predominantly inattentive symptoms), a hyperactive/impulsive subtype (predominantly hyperactive/impulsive symptoms) and a combined type ADHD (both inattentive symptoms as well as hyperactive/impulsive symptoms)[1]. The two symptom domains of ADHD can be attributed, in part, to different brain networks[7] and twin studies show partial genetic overlap between inattentive and hyperactive/impulsive symptoms, but also clear genetic specificity[8]. For these reasons, studying the genetics of symptom domains separately might reduce phenotypic heterogeneity, increase the power of genetic studies, and enable us to identify dimension-specific genetic risk variants.

Apart from the multidimensionality, additional challenges in discovering genetic risk variants in ADHD are the small effect sizes of single common genetic variants and different genetic variants leading to similar phenotypes[9]. As genome-wide genetic analyses aimed at identifying common risk variants, mainly focused on investigating Single Nucleotide Polymorphisims (SNPs)[10-12] association, extremely large samples are needed to achieve genome-wide significance[4,13] and sample sizes in ADHD research are still small compared to other disorders[13]. A recent study performing cross-disorder GWAS using data from the Psychiatric Genomics Consortium of cases and controls for schizophrenia, bipolar disorder, major depressive disorder, autism spectrum disorders and ADHD, showed a significant polygenic component for ADHD, suggesting that searching for a combination of genetic variants might be fruitful[14]. Considering the combined effect of multiple variants in the same analysis

might increase the explained phenotypic variance[15], thereby boosting the power of genetic studies. Therefore, we investigated whether pathway-based analyses considering multiple SNPs within the same biological process, could bring us closer to unraveling the underlying genetic component of ADHD.

Alterations in dopaminergic, noradrenergic and serotonergic neurotransmission have been hypothesized to play a role in ADHD[16,17]. Medications used to treat ADHD affect these systems[18,19] and reduce behavioral symptoms[20,21]. Although serotonergic medications are not efficacious for ADHD[22], serotonin interacts with dopamine[23], therefore medication working on the dopamine system might also alter serotonin signaling. Secondly, projection sites of these neurotransmitter systems regulate cognitive processes, attention and motor behavior in ADHD, supported by structural and functional imaging data[24-26]. Thirdly, although not achieving genome-wide significance, genetic associations have been found for several candidate genes within these systems[3]. Finally, animal studies show gene knock-out of catecholaminergic genes to cause ADHD-like behavior, altered catecholamine release and symptom reduction in response to ADHD medication[27]. In addition, another biological process implicated in ADHD, mainly through genetic studies, is neurite outgrowth[9]. Genes involved in this process were found to be enriched in the top results of the five available GWASs of ADHD[28].

Prior studies investigated whether SNPs in the top results of individual analysis were overrepresented in pre-determined gene/pathways lists , or if those genes formed a network of functionally interacting proteins[29]. Others selected variants based on candidate genes/pathways from literature[30]. However, so far no studies have conducted a combined analysis of candidate genetic pathways allowing to investigate if certain genetic pathways together are associated with disorder-specific phenotypes.

In the current study we used a case-only design to investigate whether pathway-based analyses of dopamine, noradrenaline and serotonin neurotransmission and genes involved in neurite outgrowth moderate the underlying behavioral components and severity of ADHD. Common genetic variants within these pathways were included into the same analysis.

Method

Sample

The present study is part of the International Multicentre ADHD Genetics (IMAGE) study[31-33], an international collaborative study in seven European countries (Belgium, Germany, Ireland, Spain, Switzerland, the Netherlands and the United Kingdom) and Israel aiming at identifying genes that increase ADHD susceptibility. Participants were aged 5–17 years and of European Caucasian descent, based on information on ethnicity and genetic data[34](see Supplementary Figure S1). Exclusion criteria included IQ < 70, presence of autism, epilepsy, known neurological disorders and any genetic or medical disorder associated with externalizing behaviors that might mimic ADHD. Details of the sample have been described elsewhere[31,35].

ADHD phenotyping

In short, a semistructured, standardized, investigator-based interview (Parental Account of Children's symptoms [PACS][36]) and questionnaires (parent and teacher Conners long version rating scales[37], parent and teacher Strengths and Difficulties Questionnaires (SDQ)[38]) were used to establish an ADHD diagnosis in children previously clinically diagnosed with ADHD, see[39] for the standardized algorithm that was applied to derive each of the 18 DSM-IV[1] symptoms. Symptom count was defined by the number of symptoms per behavioral domain. Both symptom domains ranged from 0-9 symptoms. To investigate symptom severity a 4-point scale was used from the inattention and hyperactivity/impulsivity subscales of the Conners Parent Rating Scale (CPRS-R)[40] and the Conners Teacher Rating Scale (CTRS-R)[41].

Genotyping

Genome-wide genotyping of the IMAGE probands was performed as part of the GAIN study using the Perlegen genotyping platform, described before[42]. To increase coverage, an imputation approach was used with the Hapmap II release 22 data set[43]. The imputed data underwent quality control in which SNPs with an imputation score < 0.3 and minor allele frequency < 0.05 were excluded. After this step we had 2,182,904 SNPs across the genome. To avoid overestimation of our statistics, linkage disequilibrium-pruned genotypes were used, using the "indep" command with an r^2 threshold of 0.8 (PLINK software[44]). After this step we had 299,296 SNPs.

In this work we describe pathway as a pre-defined gene selection based on a well-established genetic database or literature data. Selection of dopamine(74 genes) and serotonin(32 genes) genetic pathways was based on Ingenuity Pathway Analysis software (www.ingenuity.com). This is a well established, frequently updated genetic database for pathway analysis. The information used in Ingenuity to produce these pathways is extracted from the scientific literature, and includes genes, drugs, biomarkers, chemicals, cellular and disease processes, and signaling and metabolic pathways. For noradrenaline, only the receptors are defined in Ingenuity, no pathway has been defined yet. Noradrenaline and dopamine share most of their synthesis-pathway as noradrenaline arises from the hydroxylation of dopamine[45]. Promiscuity has been found for transporters[46] and receptors[47,48] of both noradrenalin and dopamine, probably due to the similarities in their chemical structure. By including the noradrenaline receptors(8 genes) and transporter with the dopamine pathway, we aimed at capturing the noradrenaline pathway as well. The dopamine/noradrenaline pathway contained 82 genes. Dopamine/noradrenaline and serotonin pathways overlap in 13 genes. The selection of the neurite outgrowth genes was based on literature[28], including 45 genes from the top results of the five GWAS studies available on ADHD.

SNPs within all genes as well as 25 kilo basepairs (kb) flanking regions (capturing regulatory sequences) were selected.

Data Analysis

Association analysis to symptom counts was performed separately for hyperactive/impulsive symptoms and inattentive symptoms. Symptom count distribution were normalized and standardized using the Blom transformation (SPSS version 18).

SNP-by-SNP linear regression was performed using the “linear” command in PLINK with sex and age as covariates. To decrease genetic heterogeneity, a combined analysis approach was applied using a multivariable approach described earlier[15]. By summing single SNP association statistics, the observed summed statistic was created. To get a distribution on permuted summed statistics we ran 10,000 max(T) permutation tests using the “mperm” command, implemented in PLINK, for each SNP. The association statistics of the observed and permuted data were saved using the “mperm-save-all” PLINK command and added to create a summed statistic per run for all SNPs at the same time. The empirical p-value was determined as the number of times the observed summed statistic was smaller

than the permuted summed statistic divided by the total number of permutations. Significance threshold for the empirical p-values was set as 0.05 divided by the number of tests.

We carried out our analysis in two steps. In step 1, we analyzed the combined effect of the SNPs in the three pathways on both inattentive and hyperactive/impulsive symptom counts . We then tested the association of the combined pathways with symptom severity using parental and teacher Conners scores. Post-hoc, we investigated potential effects of single pathways, genes and SNPs that might drive the association(Figure 1). Overlapping genes were only considered once in the combined analysis, but present in both separate analysis. For gene-wide association we used the analysis program VEGAS[49]. VEGAS uses single SNP p-values to perform gene-based association tests. Significance threshold was set to 0.05 divided by the number of genes tested.

Results

Table 1 shows the general characteristics of the studied sample.

--Table 1--

Selection of dopamine/noradrenaline, serotonin and neurite outgrowth genes yielded a total of 146 unique genes (Table 2). The dopamine/noradrenaline and the serotonin pathways overlapped in 13 genes (251 SNPs). Four genes positioned on the X-chromosome (*HTR2C, MAOA, MAOB* and *PPP2R3B*) were not included in the analysis, one gene was not captured by the array used (*PRKAR1B*). The final data set contained 141 genes and 5,179 SNPs.

--Table 2--

Step 1: The combined pathway analysis for DSM-IV symptom counts showed a significant association with hyperactive/impulsive symptoms ($p_{empirical}$ =0.007), but not with inattentive symptoms ($p_{empirical}$=0.73) (Table 3). Single gene and single SNP analyses did not reveal significant associations

Step 2: Given the results of step 1, we tested symptom severity of hyperactivity/impulsivity derived from the parental Conners scores with the three genetic pathways combined and observed a significant association ($p_{empirical}$=0.0018). Post-hoc analyses showed that all pathways were independently associated with the hyperactivity/impulsivity score (dopamine/noradrenaline $p_{empirical}$=0.0004, serotonin $p_{empirical}$=0.0149, neurite outgrowth $p_{empirical}$=0.0452) (Table 3). Single gene and single SNP analysis did not reveal significant associations. For further information on single SNP

results please contact the corresponding author directly. The combined pathways were not associated with hyperactive/impulsive scores on the teacher-rated Conners scale ($p_{empirical}$=0.75).

--Table 3—

Table 4 shows a small to moderate correlation between hyperactive/impulsive and inattentive symptom counts. Hyperactive/impulsive symptom counts and Conners scores correlate moderately.

--Table 4—

Discussion

The current study is the first to show an association between dopamine/noradrenaline, serotonin pathways and neurite outgrowth genes to the hyperactive/impulsive component of ADHD using hypothesis-based pathway association analysis using a case-only design. No association to the inattentive component was observed. Post-hoc analyses showed individual contribution of all three pathways. Single genes or SNPs did not show significant association, suggesting that the observed associations are the result of combined small effects of multiple genetic variants.

The concept of biological pathways has been investigated before in ADHD. Top results from GWAS studies and rare variants were investigated for overrepresentation in certain genetic biological pathways[28,29]. Findings show overlap suggesting convergence of both rare and common variants in the risk of ADHD. When Elia et al.[50] investigated rare variants only, they showed multiple genes carrying these variants belonging to the metabotropic glutamate receptor gene family. Another approach has been to use predefined genetic pathways as a starting point for gene/variant selection and testing[31,51-53]. Oades et al.[30] selected genes that were related to serotonin function and applied a family-based multivariate approach clustering the phenotypes, to increase their statistical power. The current study extends previous approaches by including all variants in the studied pathways, by investigating both ADHD symptom domains separately, and by increasing our power by joining single SNP effects. Although IMAGE was part of these prior analysis (13 of the 45 neurite outgrowth genes were based on the single SNP effects in the IMAGE study), these 13 genes do not drive our results (see Supplementary Table S5).

Our results suggest a link between candidate genetic pathways and hyperactivity/impulsivity but not with inattentiveness. Symptom domain-specific genetic associations have previously been reported. Markunas et al.[54] identified association between the *SLC9A9* gene and hyperactive/impulsive Conners

scores. Lasky-Su et al.[55] found association between two variants within the dopamine receptor 4 gene (*DRD4*) and ADHD symptoms which was driven by the inattentive symptoms only. In a previous paper, using the IMAGE sample, Lasky-Su et al.[10] investigated domain-specific genetic associations using GWAS and reported nominal associations for variants within candidate genes included here. Also, in healthy twins, hyperactive/impulsive symptoms have been associated to variants within the dopamine pathway[56]. Further, although the neurite outgrowth network has not yet been linked to hyperactivity or impulsivity symptoms, it showed overrepresentation in the top results of a GWAS study studying motor coordination problems in ADHD[57] and some neurite outgrowth genes studied here, in particular *NOS1*[58,59] and *CTNNA2*[60], have been associated to impulsivity.

One possible explanation of lack of association with inattention may be related to a higher degree of phenotypic heterogeneity compared to hyperactivity/impulsivity. Therefore, the current sample might not have enough power to detect genetic effects. However, as both symptom domains are highly heritable (hyperactive/impulsive 88%, inattentive 79%)[61] and standard deviations are similar (Table 1), different phenotypic heterogeneity is not expected. Alternatively genetic mechanisms other than those studied here might be involved in the inattention domain.

The current study analyzed symptom count and symptom severity. Both are part of the hyperactive/impulsive domain, however, there is only moderate to small correlation between them. Symptom counts have been created through a semi-structured diagnostic interview in combination with few items from ADHD rating scales, whereas the symptom severity measures were rated by parents or teachers. Therefore we expect them to capture different aspects of the disorder.

It should be noted that the selected pathways were associated with parental but not with teacher-rated hyperactivity/impulsivity. Discrepancies between raters have been observed previously[62-64], with correlations between mother and teacher ratings ranging from .23 to .49[65,66] and significant differences in mean scores[62]. Low correlation values suggest them to capture different aspects of the disorder. Linkage analysis for parent-rated and teacher-rated Conners scores also showed rater-specific quantitative trait loci[67]. Informant differences can be attributed to several factors, like different standards and biases in reporting and scoring the symptoms[63], or setting-specific behavior observed only by one rater[68]. Teacher ratings might be more prone to measurement error, as teachers need to divide their attention over multiple children and observe each individual for a limited amount of time while performing specific school-related activities. Parents might have more opportunities to observe

their child in multiple daily life settings, but can be biased depending on having another child with similar behavior or not.

Our findings should be viewed in light of certain strengths and limitations. An important strength is the combination of multiple genetic variants in a well-characterized ADHD sample accounting for small effect sizes and genetic heterogeneity in ADHD. A limitation is that our sample shows reduced power to find associations explaining 1% or less of the variance (http://pngu.mgh.harvard.edu/~purcell/gpc/qtlassoc.html) therefore replication in an independent sample is necessary. We were unable to define the direction of the effects, or whether directions were different for the different genes/pathways studied, which we acknowledge to be a limitation.

For pathway and gene selection we took a conservative approach, only including genes selected using a well-established database or literature. Pathway selection remains difficult as the currently available databases are far from complete, therefore we feel they should be used as not more than a starting point for pathway analyses. Therefore interesting genes might have been missed. As we only included the most promising candidate pathways for ADHD, we might have missed others as new candidates are still emerging[50].

Given our case-only design, our results should be seen as moderating individual symptoms within the disorder, but not necessarily contributing to ADHD susceptibility. To investigate if these pathways increase the susceptibility to ADHD a case-control study should be performed. Also, it would be interesting to validate the current analysis in samples with an equal gender distribution, adult ADHD samples and population-based samples.

In conclusion, our results support the hypothesis that genes of the dopamine/noradrenaline and serotonin neurotransmitter pathways as well as neurite outgrowth genes are involved in ADHD through the hyperactive/impulsive component but not the inattentive one. The current study shows that pathway-based association analyses in combination with more homogeneous phenotyping may overcome power problems in association testing by taking into account allelic heterogeneity.

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Tables

Table 1. Demographic characteristics of the studied individuals.

	Value	N
Mean years of age (SD)	10.83 (2.78)	930
% male	87	930
Median symptom count hyperactivity (SD)	8 (1.27)	871
Median symptom count inattentiveness (SD)	8 (1.04)	871
Median Conners' parent hyperactive/impulsive (SD)	80 (10.15)	930
Median Conners' teacher hyperactive/impulsive (SD)	69.5 (12.16)	916

Table 2. Selection of genes.

Genes dopamine/noradrenaline pathway							Genes serotonin pathway			Genes neurite outgrowth			
ADCY1	*ADRA1D*	*DRD5*	*PPP1R10*	*PPP2CA*	*PPP2R5C*	*PTS*[a]	*DDC*[a]	*HTR3D*	*SLC18A1*[a]	*ADAMTS17*	*EMP2*	*MAP1B*	*PPM1H*
ADCY10	*ADRA2A*	*GCH1*[a]	*PPP1R11*	*PPP2CB*	*PPP2R5D*	*QDPR*[a]	*GCH1*[a]	*HTR3E*	*SLC18A2*[a]	*ASTN2*	*FAM190A*	*MBOAT1*	*RORA*
ADCY2	*ADRA2B*	*IL4I1*	*PPP1R12A*	*PPP2R1A*	*PPP2R5E*	*SLC18A1*[a]	*HTR1A*	*HTR4*	*SLC18A3*[a]	*ATP2C2*	*FHIT*	*MEIS2*	*SLCO3A1*
ADCY3	*ADRA2C*	*MAOA*[ab]	*PPP1R14A*	*PPP2R1B*	*PRKACA*	*SLC18A2*[a]	*HTR1B*	*HTR5A*	*SLC6A4*	*BMPR1B*	*FLNC*	*MMP24*	*SPOCK3*
ADCY4	*ADRB1*	*MAOB*[ab]	*PPP1R14B*	*PPP2R2A*	*PRKACB*	*SLC18A3*[a]	*HTR1D*	*HTR6*	*SMOX*[a]	*CDH13*	*GPC6*	*MOBP*	*SUPT3H*
ADCY5	*CALY*	*NCS1*	*PPP1R14C*	*PPP2R2B*	*PRKACG*	*SLC6A2*	*HTR1E*	*HTR7*	*SPR*[a]	*CDH23*	*HK1*	*MYT1L*	*TLL2*
ADCY6	*COMT*	*PCBD1*[ab]	*PPP1R14D*	*PPP2R2C*	*PRKAG1*	*SLC6A3*	*HTR2A*	*IL4I1*[a]	*TPH1*	*CREB5*	*HKDC1*	*NCKAP5*	*UGT1A9*
ADCY7	*DDC*[a]	*PPM1J*	*PPP1R1B*	*PPP2R3A*	*PRKAG2*	*SMOX*[a]	*HTR2B*	*MAOA*[ab]	*TPH2*	*CSMD2*	*ITGA11*	*NEDD4L*	*UNC5B*
ADCY8	*DRD1*	*PPM1L*	*PPP1R3A*	*PPP2R3B*[b]	*PRKAR1A*	*SPR*[a]	*HTR2C*[b]	*MAOB*[ab]		*CTNNA2*	*KCNIP4*	*NOS1*	*ZNF423*
ADCY9	*DRD2*	*PPP1CA*	*PPP1R3C*	*PPP2R4*	*PRKAR1B*[b]	*TH*	*HTR3A*	*PCBD1*[a]		*DNM1*	*KCP*	*NRXN1*	
ADRA1A	*DRD3*	*PPP1CB*	*PPP1R3D*	*PPP2R5A*	*PRKAR2A*		*HTR3B*	*PTS*[a]		*DUSP1*	*LRP1B*	*NUCB1*	
ADRA1B	*DRD4*	*PPP1CC*	*PPP1R7*	*PPP2R5B*	*PRKAR2B*		*HTR3C*	*QDPR*[a]		*DYNC2H1*	*MAN2A2*	*NXPH1*	

[a] present in dopamine/noradrenaline and in serotonin pathways

[b] no SNPs for analysis

Table 3. Association results from the combined analysis of symptom counts and combined and separate analysis of three genetic pathways with hyperactive/impulsive (HI) symptom severity measured with the parent-rated Conners Scale (n=930).

	N SNPs	IA symptom counts	HI symptom counts	HI symptom severity p-value
Combined analysis	5,179	0.73	0.007	0.0018
Dopamine/noradrenaline pathway	1,163[a]	-	-	0.0004
Serotonin pathway	407[a]	-	-	0.0149
Neurite outgrowth genes	3,860	-	-	0.0452

[a] 251 SNPs were present in the serotonin pathway as well as in the dopamine/noradrenaline pathway.

Table 4. Correlation analyses between the studied phenotypes. Correlation coefficients are shown with corresponding p-values in brackets.

	Symptom count hyperactivity/ impulsivity	Symptom counts inattentiveness	Conners parent hyperactivity/ impulsivity
Symptom counts inattentiveness	.182 (<.001)		
Conners parent hyperactivity/impulsivity	.254 (<.001)	.087 (.008)	
Conners teacher hyperactivity/impulsivity	.386 (<.001)	.111 (.001)	.238 (<.001)

Figure legends

Figure 1 shows the different steps in our analysis. Analysis started by separating the inattentive and hyperactive/impulsive symptom counts. As hyperactive/impulsive symptoms showed association, we extended the analysis by investigating hyperactive/impulsive symptom severity using Conners' scores. At step 2 post-hoc analysis were performed to investigate the pathways separately.