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Pathway Analysis in Attention Deficit Hyperactivity Disorder: An Ensemble Approach

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Abstract

Despite a wealth of evidence for the role of genetics in attention deficit hyperactivity disorder (ADHD), specific and definitive genetic mechanisms have not been identified. Pathway analyses, a subset of gene-set analyses, extend the knowledge gained from genome-wide association studies (GWAS) by providing functional context for genetic associations. However, there are numerous methods for association testing of gene sets and no real consensus regarding the best approach. The present study applied six pathway analysis methods to identify pathways associated with ADHD in two GWAS datasets from the Psychiatric Genomics Consortium. Methods that utilize genotypes to model pathway-level effects identified more replicable pathway associations than methods using summary statistics. In addition, pathways implicated by more than one method were significantly more likely to replicate. A number of brain-relevant pathways, such as RhoA signaling, glycosaminoglycan biosynthesis, fibroblast growth factor receptor activity, and

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SUPPORTING INFORMATION

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pathways containing potassium channel genes, were nominally significant by multiple methods in both datasets. These results support previous hypotheses about the role of regulation of neurotransmitter release, neurite outgrowth and axon guidance in contributing to the ADHD phenotype and suggest the value of cross-method convergence in evaluating pathway analysis results.

Keywords

ADHD; pathway analyses; GWAS

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a common and heritable neurodevelopmental disorder that affects approximately 5% of children worldwide. The disorder is characterized by symptoms of inattention, hyperactivity, and impulsivity, and frequently persists in impairing form into adulthood [Faraone et al., 2006].

While the heritability of ADHD has been estimated to be 60–80% [Faraone et al., 2005], definitive genetic mechanisms have not yet been identified. Meta-analyses of candidate gene studies have identified genes consistently associated with ADHD (DAT1, DRD4, DRD5, 5-HTT, HTR1B, SNAP25), although collectively these account for less than 5% of genetic variance in ADHD and none are diagnostic. Unsurprisingly, such studies have also highlighted the genetic heterogeneity among ADHD patients [Faraone et al., 2005; Faraone and Khan, 2006; Kebir et al., 2009; Gizer et al., 2009; Stergiakouli and Thapar, 2010].

Genome-wide association studies (GWAS) [Neale et al., 2008; Lasky-Su et al., 2008a, b; Lesch et al., 2008; Mick et al., 2010; Neale et al., 2010a; Hinney et al., 2011; Stergiakouli et al., 2012; Ebejer et al., 2013; Yang et al., 2013; Weber et al., 2014; Sánchez-Mora et al., 2015; Zayats et al., 2015] have revealed additional candidate genes (e.g., CDH13, SPOCK3, KCNC1, KCNIP1, KCNIP4), although these variants have not achieved genome-wide significance [Franke et al., 2009; Neale et al., 2010b; Hinney et al., 2011; Sánchez-Mora et al., 2015]. The most consistent finding is the CDH13 gene, which has been implicated in two family-based GWAS [Lasky-Su et al., 2008a; Neale et al., 2008] and two case-control GWAS [Lesch et al., 2008; Neale et al., 2010a]. Results from studies of other neuropsychiatric disorders [Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014] suggest that studies with tens of thousands of subjects will likely be needed to reveal more definitive single variant associations. While these are forthcoming, additional biologically relevant analytic approaches provide opportunities for insight.

Gene set methods, which test for association between groups of genes and a trait, offer one such means of extending and contextualizing the knowledge gained from GWAS for several reasons. First, ADHD, like other complex diseases, is polygenic in nature, so testing for association with sets of related variants (e.g., those influencing a biochemical pathway) can provide a functional context for multiple genetic risk factors and potentially yield new mechanisms and treatment targets.

Second, because the number of gene sets is far fewer than the number of SNPs in a GWAS, examining gene sets improves power to detect genetic correlates by reducing the multiple testing correction. A third advantage is that effects due to genetic heterogeneity can be detected. This is related to the issue of small effect sizes, since the result of genetic heterogeneity in a study population will be a mixture of small-effect variants. If multiple small effects are present within a pathway it may be possible to detect their cumulative effect using pathway analysis methods.

ADHD is an ideal candidate for pathway analysis given the evidence supporting a polygenic model of disease susceptibility [Hamshere et al., 2013; Yang et al., 2013; Groen-Blokhuis et al., 2014; Martin et al., 2014]. A few pathway analyses, using a variety of pathway definitions and statistical methods, have been conducted on ADHD datasets. Poelmans et al. [2011] identified the top 85 genes reported in five ADHD GWAS and performed a literature search for gene functions. They reported that 45 of the 85 GWAS hits could be assigned to a neurodevelopment network involved in directed neurite outgrowth. Similarly, Cristino et al. [2014] found that ADHD-associated genes are significantly more interconnected in a protein-protein interaction network than expected by chance.

Stergiakouli et al. [2012] performed a pathway analysis on an ADHD GWAS dataset comprising 727 children with ADHD and 5,081 controls. Using the ALIGATOR method [Holmans et al., 2009] they found 13 significant pathways, and these pathways were also found to contain an excess of CNV-affected genes. Pathways related to cholesterol metabolism, cation channel activity, and CNS development were implicated.

Yang et al. [2013] applied three analysis methods, INRICH [Lee et al., 2012], DAPPLE [Rossin et al., 2011], and GREAT [McLean et al., 2010], to a GWAS dataset consisting of 1,400 cases and 963 controls of Chinese descent. Although results from the three methods differed somewhat, common processes, such as cell adhesion, glutamate synaptic development, and axon development, were implicated.

A few other studies used a candidate pathway approach, rather than testing all available pathways. Bralten et al. performed a candidate pathway analysis using data from the International Multi-site ADHD Genetics (IMAGE) study [Neale et al., 2008], consisting of 909 trios. Three candidate gene sets (dopamine/norepinephrine pathway, serotonin pathway, and neurite outgrowth pathway) were defined using the Ingenuity software (www.ingenuity.com) and a literature review. The three pathways combined were associated with hyperactive/impulsive symptomatology but not inattention symptomatology [Bralten et al., 2013].

Hammerschlag et al. tested 17 expert-curated gene sets of pre-and post-synaptic genes in the IMAGE2 case-control dataset, which consists of 896 cases and 2,455 controls [Neale et al., 2010a]. However, none were more strongly associated with ADHD than random gene sets of equal size [Hammerschlag et al., 2014].

These previous studies collectively provide further evidence of the polygenic nature of ADHD. However, they also underscore the challenge of interpreting pathway analyses, due in part to the variation among methods. This challenge is substantial because of the large

number of ways to define gene sets and to test for association between a gene set and a phenotype [Mooney et al., 2014]. This issue was highlighted in the recent study [Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015] that examined gene set (a mixture of Gene Ontology and pathway models) enrichment across five different methods to rank pathways associated with schizophrenia, major depression and bipolar disorder [Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015]. They argued that combining results across analysis methods and across disorders provided the most informative and robust results.

While we follow O'Dushlaine et al. [Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015] in comparing the results of multiple methods, the present study is unlike prior pathway analyses in ADHD, in that it focuses on methods that use genotypes (rather than summary statistics) to model gene- or pathway-level association measures.

The main hypothesis was that methods utilizing genotypes would better represent the underlying genetic architecture (i.e., they do not rely solely on SNP main effects) and therefore would identify more replicable pathway associations. We applied four such methods, and compared them with two commonly used methods that utilize summary statistics.

Because we expected different results from different pathway analysis algorithms, we aimed to discover robust pathway-level effects by identifying a consensus of pathway significance across the methods and in two independent data sets. Our second hypothesis was that this ensemble approach for identifying robust pathway effects would confirm prior findings that neuro-developmental processes are important genetic mechanisms in ADHD.

DATA AND METHODS

Participants and Genotype Data

Two independent, ADHD case-control, GWAS datasets from the Psychiatric Genomics Consortium, which will be referred to as the (a) *IMAGE2* (N = 3351; mean age = 10.5, SD = 2.9) and (b) *German ADHD GWAS* (N = 1793; mean age = 11, SD = 2.7) datasets, were used for our analysis [Neale et al., 2010a; Hinney et al., 2011]. Details about these datasets and the genotype QA/QC procedures are available in the Supplementary Methods.

Gene Sets

The pathways tested were obtained from the Pathway Commons database (www.pathwaycommons.org; version 4) [Cerami et al., 2011], which included a total of 3,074 human pathways from the following sources: Reactome (www.reactome.org; v46) [Croft et al., 2014], NCI Pathway Interaction Database (pid.nci.nih.gov; 16-AUG-2012) [Schaefer et al., 2009], HumanCyc (humancyc.org; 17.1) [Romero et al., 2005], and PANTHER (www.pantherdb.org/pathway/; 3.2.1) [Mi et al., 2013]. This initial collection of pathways was filtered by removing those with only a single gene, those with more than 300 genes, and duplicates (same name and same genes). If two pathways shared the same name, but contained different members, the gene members were merged to create a single pathway.

Uniprot IDs were converted to Ensemble gene IDs using the mapping contained in the Ensembl database (version 74). The final set of 2,233 pathways ranged in size from two to 284 genes (mean = 31, SD = 39). Because of the different requirements of each analysis method, very small pathways were not tested by all methods. Of the final set of 2,233 pathways, 1980 and 2057 were tested by all methods in the IMAGE 2 and German ADHD GWAS datasets, respectively. Figure 1 provides an overview of our pathway analysis workflow.

Mapping SNPs to Genes

SNPs were mapped to pathway genes if located within 1Kb of the gene boundaries. Gene and SNP locations were obtained from the Ensembl database (www.ensembl.org; v74). For the IMAGE 2 dataset, 52921 SNPs were mapped to 5093 pathway genes. For the German ADHD GWAS dataset, 103128 SNPs were mapped to 6136 pathway genes.

Pathway Analysis Methods

Six pathway analysis methods were applied to both datasets. Four were previously published methods that use the original genotype data rather than SNP *P* values: GRASS, PCgamma, PoDA, and NBF [Chen et al., 2010; Braun and Buetow, 2011; Biernacka et al., 2012; Evangelou et al., 2014]. Two were previously published methods that utilize SNP *P* values: GSEA [Wang et al., 2007] and Fisher's method for combining *P* values [Fisher, 1932; Evangelou et al., 2014]. See the supplementary methods for more details on these algorithms. To examine the individual SNP effects contributing to pathway associations, SNP-level *P* values were calculated using the logistic regression procedure in Plink v1.07 [Purcell et al., 2007].

Adjustment for Pathway Size

Although often overlooked, an obvious confound in interpreting pathway analysis results is that pathways with more SNPs ("larger" pathways) are more likely to be associated with the phenotype [Wang et al., 2007, 2010; Ramanan et al., 2012; Mooney et al., 2014]. The degree of correlation between pathway size and pathway significance was therefore examined for all methods. When a significant correlation was seen, pathway *P* values were adjusted as follows.

For each pathway, a collection of random pathways was constructed in order to calculate a null distribution of *P* values. These random pathways were created to approximately match the number of genes and SNPs in the target pathway. This was accomplished by binning all genes according to the number of SNPs assigned to each gene. Because genes with a large number of SNPs are rare, bins were merged so that each contained approximately 25 genes. Random pathways were then created by sampling the appropriate number of genes from each bin. The adjusted *P*-value is simply the proportion of random pathways with a *P*-value smaller than the *P*-value of the target pathway.

RESULTS

Accounting for Pathway Size

We first considered the effect of pathway size in the IMAGE 2 data set. Both the PoDA and GSEA methods have built-in permutation procedures that successfully corrected for size bias (correlation *P* values >0.2). The four other methods, which do not inherently correct for pathway size, all had significant correlations between pathway size and significance of association to ADHD. These effects were small for PCgamma and GRASS (Pearson's correlation coefficients, r, of 0.169 and 0.068, respectively; *P* values <0.002). However, the results from Fisher's method were highly correlated with pathway size (r = 0.95, *P*-value $<2 \times 10^{-16}$). In addition, for the NBF method there was a significant negative correlation between pathway size and pathway significance (the inverse of the Bayes Factor) (r = −0.40, *P*-value $<2 \times 10^{-16}$).

Therefore, *P* values from the PCgamma, GRASS, and Fisher's methods were adjusted for pathway size as described in Methods. This procedure successfully corrected the size bias for PCgamma and Fisher's methods (correlation *P* values >0.13), but "overcorrected" and resulted in a slight negative correlation between size and significance for GRASS (r = −0.045, *P* value = 0.036) (Supplementary Fig. S1). However, the adjusted *P*-value was retained. The results from the NBF method could not be corrected because the hierarchical model used in that method does not allow for the application of permutation-based correction.

Similar results regarding the relationship between pathway size and significance were seen in the German ADHD GWAS (data not shown), and therefore corrections were applied in the same way. All pathway P values reported below are adjusted for pathway size either inherently or by our permutation procedure. All pathway-level association statistics (both adjusted and unadjusted) and the number of genes and SNPs in each pathway are reported in Supplementary Tables S1–S4.

Comparing Pathway Analysis Algorithms

A total of 1980 pathways were tested by all methods in the IMAGE2 dataset; the number of pathways reported as nominally significant ranged from 88 for GSEA to 61 for the NBF method. Pathways reported as nominally significant by PCgamma, Fisher's, and GRASS were most likely to also be significant by at least one other method (74.4%, 72.2%, and 63.9%, respectively), while those reported as nominally significant by NBF were least likely to be confirmed by a second method (23.0%) (Table I).

This initial finding replicated well in the German ADHD GWAS dataset, with the Fisher's, PCgamma, and GRASS methods overlapping most with other methods (73.3%, 70.6%, and 70.4%, respectively) and the NBF method overlapping the least (25.0%).

Fleiss' Kappa measure demonstrates that overall agreement among methods is low (k = 0.183 in IMAGE 2 and 0.208 in the German dataset), but that agreement increases among similar methods (for GRASS and PCgamma, k = 0.465 and 0.524 in the IMAGE 2 and German data sets, respectively; for GSEA and Fisher's method, k = 0.372 and 0.227).

With regard to cross-sample replication of particular pathways associated with ADHD, PCgamma had the highest proportion of nominally significant pathways that were also reported as nominally significant in the German ADHD GWAS dataset (17.1%), followed by GRASS (13.1%) and PoDA (10.6%). For the PCgamma method, the number of pathways found to be nominally significant in both datasets is significantly greater than would be expected by chance (Fisher's exact test *P*-value = 4.09×10^{-5}). GSEA, Fisher's Method, and the NBF method all had replication rates below 10% (Table I). This finding is consistent with our hypothesis that methods utilizing genotypes would identify more replicable associations, the NBF method being an exception.

Next, for each pathway, *P* values from both cohorts were combined, into a single *P*-value for each algorithm, using Fisher's method [Fisher, 1932]. This resulted in five *P* values for each pathway, one for each of the analysis methods (excluding NBF, which reports a Bayes factor, not a *P*-value).

Then for each pathway, we counted the number of methods reporting a *P*-value ≤0.05 and calculated the median *P*-value. Table II shows the top 25 most significant pathways ranked by median pooled *P*-value.

Examination of Discordant Pathways

Given the limited amount of overlap seen among the different methods, discordant pathways were examined in order to gain a better understanding of the differences between methods. We use the term "discordant pathway" to mean one that is reported as significant by only a single method (9.3% of pathways tested by all methods).

We hypothesize that differences in the distribution of SNP-level *P* values among pathways may explain some of the discordance across methods. In other words, different methods may detect different type of genetic effects. For instance, some methods may be more sensitive to pathways containing a few strong to moderate SNP effects, while others are more sensitive to pathways with many small SNP effects.

To examine differences in genetic effects for discordant pathways, SNP-level *P* values were calculated using the logistic regression procedure in Plink v1.07 [Purcell et al., 2007]. Next, each gene was assigned the minimum *P*-value among all SNPs in that gene. The distribution of the minimum gene-level *P*-value and the median gene-level *P*-value for each method's discordant pathways are plotted in Supplementary Figures S2 and S3. These plots show that gene-level effects within pathways implicated by one method are, in some cases, significantly different from the gene-level effects within pathways implicated by another method. For example, pathways reported as significant by only PCgamma tend to have a smaller minimum gene-level *P*-value compared to pathways reported as significant by only GSEA (*t*-test *P*-value <0.0005 for both IMAGE 2 and German ADHD GWAS datasets). This suggests that PCgamma is sensitive to pathways with only a few moderate SNP effects, while GSEA is sensitive to pathways with many small effects. This difference can also been seen when comparing the proportion of pathways reported as nominally significant between two distinct subsets of pathways: 1) pathways containing no SNPs with moderate main effects ($P \leq 0.001$), but at least 10% SNPs with weak effects ($P \leq 0.05$); and 2) pathways

with at least 1% SNPs with a moderate main effect ($P \leq 0.001$). In the IMAGE 2 dataset, the proportion of pathways reported nominally significant by PCgamma and GSEA are 9.0% and 12.7%, respectively for subset 1, and 24.1% and 8.33%, respectively for subset 2. A similar pattern is seen for the German ADHD GWAS data set (Supplemental Figures S4 and S5).

While these results are suggestive of different sensitivities across analysis methods, further study is needed. In particular, methods should be compared in GWAS datasets with different overall *P*-value distributions.

However, these observations clearly support previous assertions [Gui et al., 2011; Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015] that it may be beneficial to apply multiple analysis methods to a dataset, since the results from different methods can be complementary. Furthermore, as others have suggested [Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015], it is likely that pathways reported as significant by multiple methods are more stable and replicable (not due to spurious genetic effects).

For example, in the IMAGE2 data set 40 pathways were reported as nominally significant by three or more methods, while 179 pathways were nominally significant by only a single method. A significantly higher proportion of the pathways identified by three or more methods replicated in the German ADHD GWAS dataset (13 of 40; 32.5%), compared to the pathways identified by only a single method (27 of 179; 15.1%) (Fisher's exact test *P*-value = 0.021).

In addition to the different sensitivities among analysis methods, the small sample size of each data set is likely a contributing factor for the discordance between results in the two datasets. Because of this we place higher confidence in pathways that are implicated by multiple methods in both datasets.

Seven pathways were reported to be nominally significant by more than one method in both cohorts (pathways bold in Table II). Q–Q plots of SNP-level *P* values for all SNPs in each of these pathways show an excess of weak effects (Fig. 2). While these seven pathways do not capture all SNP associations in our data sets, the concentration of association signal within these pathways provides support for their role in the etiology of ADHD, and is consistent with a polygenic model of disease risk for ADHD, as has been demonstrated previously [Hamshere et al., 2013; Yang et al., 2013; Groen-Blokhuis et al., 2014; Martin et al., 2014; Wray et al., 2014].

Supplemental analyses were done to evaluate the use of imputed genotypes for pathway analysis (Supplementary Tables S5 and S6).

Given the small effect sizes seen for many genetic associations identified in complex diseases, increased sample sizes are going to be necessary to produce high-confidence findings. Combining data sets for a mega-analysis is not always possible, however, since array differences and population structure make it difficult to integrate genotype data from different studies. Because we focused on genotype-based analysis methods, we chose to

apply methods to each cohort individually and then compare results. Pathway analysis methods that utilize summary statistics have the advantage of more easily allowing the integration of data from multiple studies. However, even with a large sample size, it will be important to replicate findings in an independent data set.

Specific Pathway Findings for ADHD

Pathways reported as nominally significant by at least two methods in both data sets are: Ca activated K+ channels, FGFR1b ligand binding and activation, FGFR2b ligand binding and activation, Potassium Channels, Validated targets of C-MYC transcriptional repression, RhoA signaling pathway, and Chondroitin sulfate biosynthesis. All of these are expressed in the brain and are relevant to neuro-development. Given the limited sample sizes of our data sets, these results should be viewed as preliminary. However, there is ample evidence in the literature that these pathways may play a role in ADHD. Here we summarize this evidence and present biological context for these pathways.

The most significant pathway by any method in our analysis was the Potassium Channels pathway, with a pooled size-adjusted *P*-value of 4.11×10^{-5} for the GRASS algorithm. Potassium channel genes have been implicated in a number of previous GWAS and pathway analyses of ADHD using different data sets from those analyzed here [Lasky-Su et al., 2008a; Lesch et al., 2008; Neale et al., 2008; Franke et al., 2009; Yang et al., 2013]. These findings from genetic studies are supported by research on the role of potassium channels in the regulation of dopaminergic transmission [Dragicevic et al., 2015]. For instance, Fulton et al. found that a Kv1 channel blocker significantly increased dopamine release in mouse midbrain dopamine neurons, and provided evidence that the D2 dopamine autoreceptor attenuates dopamine release through regulation of Kv1 voltage-gated potassium channels [Fulton et al., 2011].

Pharmacological studies provide additional support for the role of potassium channels in ADHD. Kobayashi et al. found that atomoxetine, a norepinephrine reuptake inhibitor approved for the treatment of ADHD, significantly reduced inward currents through G-protein-activated inwardly rectifying K+ (GIRK) channels expressed in Xenopus oocytes [Kobayashi et al., 2010]. And Sasaki et al. conducted a preliminary study on the efficacy of tipepidine, reported to inhibit GIRK channel currents [Hamasaki et al., 2013], to treat childhood ADHD. They found that ADHD Rating Scale IV scores improved significantly for 10 ADHD patients after taking 30 mg of tipepidine daily for 4 weeks [Sasaki et al., 2014].

Figure 3 shows gene-level association measures (minimum SNP *P*-value) for all potassium channel genes, along with interactions from the STRING protein-protein interaction database (low-confidence interactions excluded) [Franceschini et al., 2013]. Also plotted are the distributions of distance scores, S, (as calculated by the PoDA algorithm) showing a significant difference between cases and controls (odds ratios of 1.41 and 1.81 for the IMAGE 2 and German ADHD GWAS study groups, respectively; FDR adjusted *P* values $<3 \times 10^{-13}$).

One hypothesis regarding the etiology of ADHD involves a dysregulation of developmental processes, particularly axon guidance and neurite outgrowth [Poelmans et al., 2011; Rivero et al., 2013]. A number of the pathways implicated in this study contribute to these neurodevelopmental processes, namely the RhoA signaling pathway, pathways involved in proteoglycan metabolism, and pathways involved in fibroblast growth factor receptor activation. Although the role of c-Myc in neurodevelopment has not been studied extensively [Mainwaring et al., 2010], c-Myc knockout models show significant effects on brain growth [Wey and Knoepfler, 2010], and the interaction between c-Myc and RhoA in cancer is well known [Sauzeau et al., 2010].

A recent review by Stankiewicz and others summarizes the abundance of literature describing the role of Rho family GTPases in neurodevelopment [Stankiewicz and Linseman, 2014]. RhoA in particular has been shown to regulate neuronal survival and migration during development [Sanno et al., 2010; Katayama et al., 2011; Cappello et al., 2012]. Note that 14 of 45 genes (31%) in the RhoA signaling pathway are also members of the much larger axon guidance pathway (280 genes).

Chondroitin sulfate proteoglycans (CSPGs) are thought to act as inhibitory signals to guide neuronal growth [Maeda et al., 2011; Siebert and Osterhout, 2011]. It has been proposed that the inhibitory effect of the Rho/ROCK pathway on neurite growth is mediated by CSPGs [Monnier et al., 2003; Siebert et al., 2014]. Monnier et al. demonstrated that both an inhibitor of Rho and an inhibitor of the ROCK kinase were able to block CSPG inhibition of axon growth [Monnier et al., 2003]. Siebert et al. confirmed this finding and further showed that chondroitinase ABC, which removes the glycosaminoglycan chains from CSPGs, counteracts the inhibition of axon growth [Siebert and Osterhout, 2011].

Interestingly, the SPOCK3 gene, which encodes a calcium-binding proteoglycan expressed in the brain, has previously been implicated in GWAS of ADHD and personality disorders [Neale et al., 2008; Weber et al., 2014].

Like CSPGs, heparan sulfate proteoglycans (HSPGs) have been shown to play a role in axon guidance and neuronal growth [de Wit and Verhaagen, 2007; Nishimura et al., 2010]. HSPGs may exert their effect through the activation of fibroblast growth factor receptor (FGFR) signaling pathways [Jastrebova et al., 2006], which are important in neurite outgrowth [Anderson et al., 2005; Beesley et al., 2014] and other neuronal development processes [Woodbury and Ikezu, 2014]. It has also been suggested that FGRFs may interact with the ADHD-susceptibility gene CDH13 [Rivero et al., 2013].

DISCUSSION

An abundance of data on the genetics of ADHD has been produced in recent years. Although results have been inconsistent, patterns are beginning to emerge. First, multiple studies have demonstrated the polygenic nature of the disorder [Yang et al., 2013; Hamshere et al., 2013; Groen-Blokhuis et al., 2014; Martin et al., 2014]. The observation that ADHD is likely due to the cumulative effect of many genes, each contributing only a small effect on

their own, may explain much of the discordance among previous genetic association studies, which have largely been underpowered to detect small effects.

The predictive value of polygenic risk scores provides hope that larger studies will be able to produce more definitive genetic associations [Wray et al., 2014]. Furthermore, when taking a higher-level view of the reported genetic associations, a number of cellular processes have consistently been implicated. For instance, genes involved in cell-cell signaling, adhesion, and neural development have been top hits in multiple studies.

Gaining insights by taking this process-level view is precisely the goal of pathway analyses. Given the variety of algorithms for aggregating SNP-level effects, we aimed to combine the results from multiple analysis methods to identify pathways most likely associated with ADHD—as well as to evaluate different types of pathway methods and the merits of an ensemble approach.

We identified seven pathways reported as nominally significant by multiple analysis methods in two independent data sets (Table II). Each of these pathways contains an excess of small SNP effects consistent with a polygenic model of disease risk. While confirmation in larger studies is needed, these pathway associations provide additional support for previous hypotheses about the etiology of ADHD, particularly related to the regulation of neurotransmitter release, and neuro-developmental processes.

Methods that test for the cumulative effect of multiple genes increase the strength of secondary analyses, and allow researchers to extract additional information from currently available datasets. Our results provide much needed guidance regarding the methodology of investigating gene-set (or pathway) associations in complex diseases. In particular our results suggest that identifying a consensus across multiple methods provides more replicable associations.

In addition, our results suggest that not all pathway methods are created equal. First, they do not all effectively handle gene set size bias, which must be taken into account in future analyses. Second, methods utilizing genotypes may be more robust than those that rely on summary statistics. Further investigation of this second point should be a priority.

In conclusion, these findings will help improve our ability to place individual genetic associations within a meaningful biological context, and in turn will help focus future research and guide the development of hypotheses about the mechanisms of susceptibility to ADHD and other complex diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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IMAGE2 Consortium

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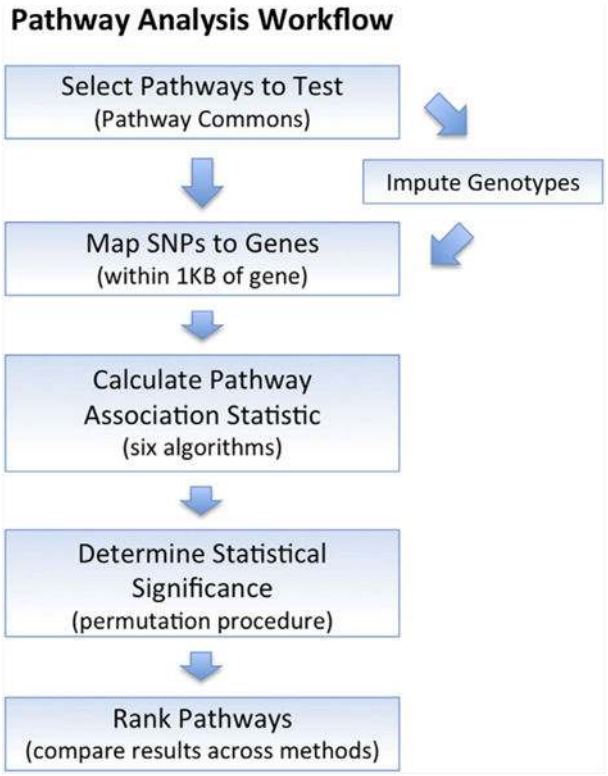

FIG. 1.

Pathway analysis workflow. Pathways tested were retrieved from the Pathway Commons database. Genotyped (and imputed) SNPs were mapped to genes in the pathways, and six pathway analysis algorithms were used to test for association with ADHD. A random pathway permutation procedure was used to adjust pathway significance for pathway size. Finally, pathways were ranked based on the number of methods reporting significance and the median *P*-value across methods. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb]

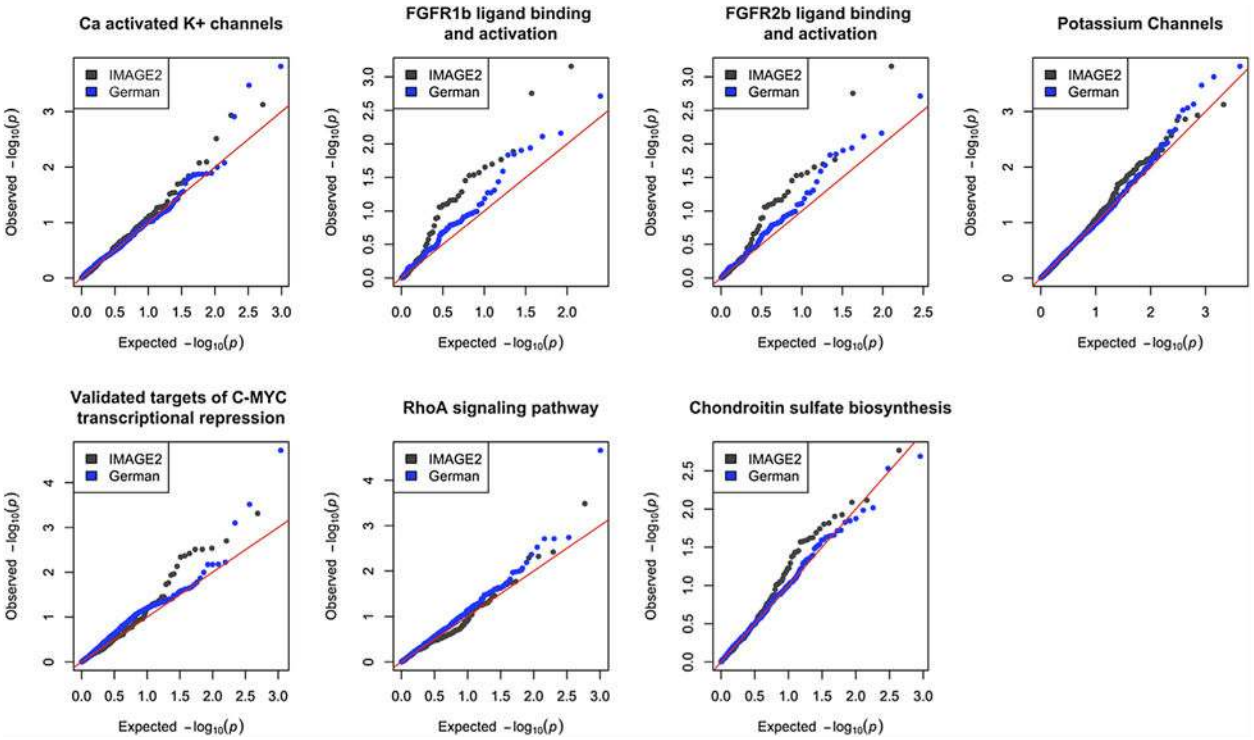

FIG. 2.

Q–Q plots for seven pathways found nominally significant in both cohorts. Each pathway shows an excess of small SNP effects consistent with a polygenic model of disease risk. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb]

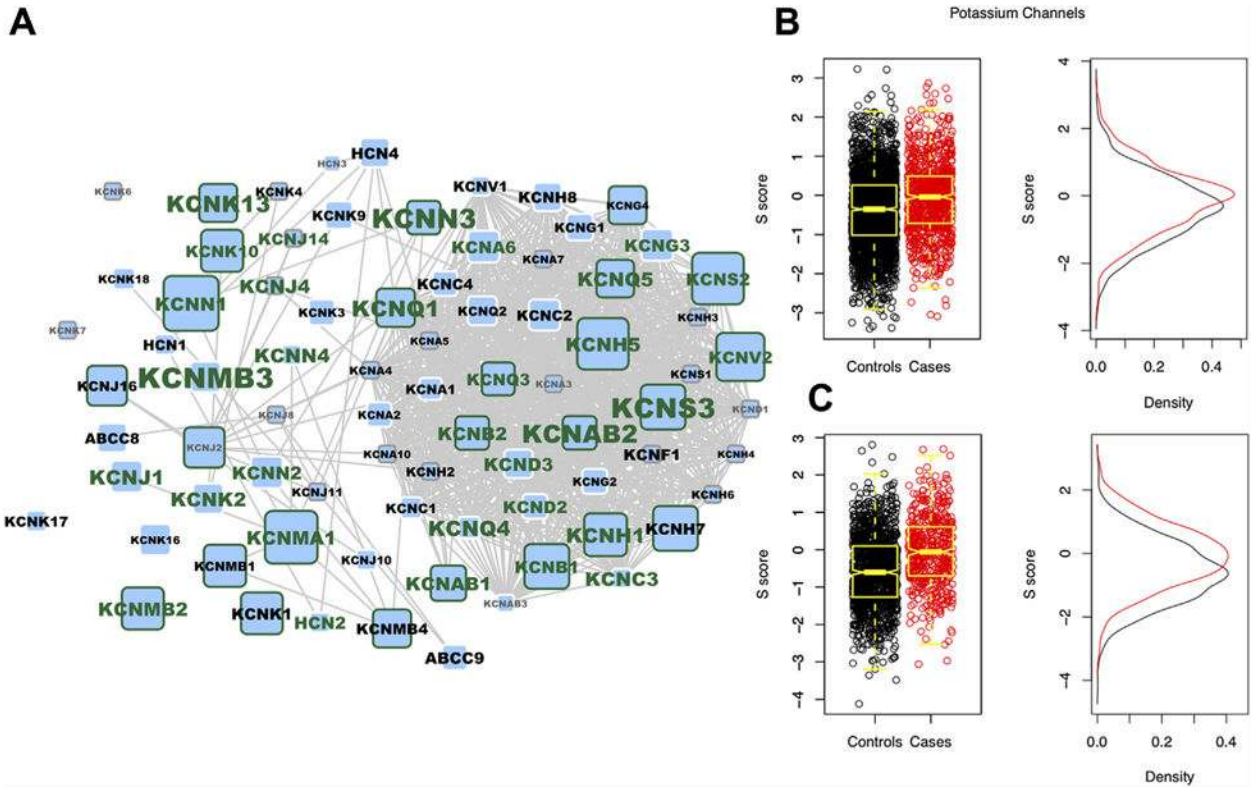

FIG. 3.

(A) The Potassium Channels pathway genes overlaid onto the STRING protein–protein interaction network (low confidence interactions, STRING score <0.5, were removed). Node size is proportion to the IMAGE2 gene *P*-value, while label size is proportional to the German ADHD GWAS gene *P*-value. Green node border indicates a gene *P*-value ≤0.05 in the IMAGE2 dataset, and a green label indicates the same in the German ADHD GWAS dataset. Gray border or label indicates no SNPs present in a particular gene. (B and C) Pathway of Distinction Analysis (PoDA) S scores showing a difference in the distribution between cases and controls in both the IMAGE2 and German ADHD GWAS datasets, respectively. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb]

TABLE I

Number of Nominally Significant Pathways in the IMAGE2 Dataset

Method	Proportion of nominally significant pathways ($P \leq 0.05$) confirmed in at least one other method	Proportion of nominally significant pathways ($P \leq 0.05$) confirmed in the German ADHD GWAS dataset
PCgamma	61/82 (74.4%)	14/82 (17.1%)
GRASS	39/61 (63.9%)	8/61 (13.1%)
PoDA	42/66 (63.6%)	7/66 (10.6%)
GSEA	45/88 (51.1%)	8/88 (9.1%)
FM	57/79 (72.2%)	3/79 (6.3%)
NBF	14/61 (23.0%)	1/61 (1.6%)

Here we refer to a pathway as confirmed in two different ways: (1) when it is nominally significant ($P \leq 0.05$) by a second analysis method in the IMAGE2 dataset (center column); or (2) when it is nominally significant using the same analysis method in an independent dataset (the German ADHD GWAS dataset; right column).

TABLE II

Top 25 Most Significant Pathways

Pathway	Pathway size (SNP count) IMAGE2/ German ADHD GWAS	Methods with nominal significance	Median pooled *P*-value
Ca activated K+ channels*	262/487	5	0.0010
FGFR1b ligand binding and activation	56/126	5	0.0011
FGFR2b ligand binding and activation	64/145	5	0.0023
Potassium channels	1065/2117	4	0.0026
Signaling mediated by p38-gamma and p38-delta	58/108	4	0.0043
Validated targets of C-MYC transcriptional repression*	243/548	5	0.0060
RhoA signaling pathway	295/507	4	0.0075
tnf/stress related signaling	111/217	5	0.0089
Histidine degradation III*	41/79	3	0.0113
Dermatan sulfate biosynthesis	85/206	4	0.0116
Chondroitin sulfate biosynthesis	219/451	4	0.0157
Metabolism of angiotensinogen to angiotensins*	70/143	3	0.0160
Clearance of nuclear envelope membranes from chromatin*	39/83	4	0.0165
Histidine catabolism*	21/52	4	0.0184
FGFR1 ligand binding and activation	69/166	5	0.0197
RAC1 signaling pathway	282/446	4	0.0197
Regulation of signaling by CBL	180/322	3	0.0224
Caspase-mediated cleavage of cytoskeletal proteins	128/208	4	0.0238
FGFR2 ligand binding and activation	81/201	4	0.0240
Human cytomegalovirus and map kinase pathways	69/143	3	0.0295
Thromboxane A2 receptor signaling*	821/1586	3	0.0299
LKB1 signaling events	305/482	3	0.0304
FGFR ligand binding and activation	103/243	3	0.0312
Nitric oxide stimulates guanylate cyclase	843/1710	4	0.0324
Role of mal in rho-mediated activation of srf*	66/107	3	0.0337

Pathways in bold were reported nominally significant by multiple methods in both the IMAGE2 and German ADHD GWAS datasets. Pathways marked with an* were also nominally significant by at least one method in the post-imputation analysis (Supplementary Tables S3 and S4). For each pathway the following information is provided: the number of SNPs assigned to the pathway in each data set, the number of analysis methods reporting the pathway nominally significant, and the median pooled *P*-value across all the analysis methods.