Integrated Genome-Wide Association Study Findings: Identification of a Neurodevelopmental Network for Attention Deficit Hyperactivity Disorder

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Objective: Attention deficit hyperactivity disorder (ADHD) is a highly heritable neuropsychiatric disorder. In the present study, the authors investigated the presence of genomic convergence in the top findings of the five published genome-wide association studies (GWASs) of ADHD.

Method: The authors carried out bioinformatics pathway analyses, using the Ingenuity and BiNGO tools, as well as a systematic literature analysis of 85 genes from the five published GWASs containing single nucleotide polymorphisms associated with ADHD at a p value <0.0001.

Results: Findings revealed that 45 of the 85 top-ranked ADHD candidate genes encode proteins that fit into a neurodevelopmental network involved in directed neurite outgrowth. Data on copy number variations in patients with ADHD and data from animal studies provide further support for the involvement of this network in ADHD etiology. Several network proteins are also directly modulated by stimulants, the most commonly used psychopharmacological treatment for ADHD.

Conclusions: The authors have identified a protein network for ADHD that contributes to our understanding of the molecular basis of the disorder. In addition, the data suggest new candidate genes for ADHD and provide clues to future research into psychopharmacological ADHD treatments.

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Attention deficit hyperactivity disorder (ADHD) is a common neuropsychiatric disorder that is observed in children and adults. ADHD is characterized by age-inappropriate levels of hyperactive, impulsive, and inattentive behaviors and leads to significant clinical and psychosocial impairments (DSM-IV-TR). The disorder has a worldwide prevalence of 5%–6% in children (1) and 1%–4% in adults (2, 3). The most effective and commonly used medications for ADHD are the stimulants methylphenidate and amphetamine and the nonstimulant drug atomoxetine, which (predominantly) modulate dopaminergic and noradrenergic neurotransmission (4, 5).

Converging neurobiological evidence suggests that ADHD involves alterations of catecholaminergic brain circuits (6). Neuroimaging studies in children with ADHD also show neurodevelopmental brain anomalies, such as reduced cortical volume and folding and gray matter heterotopia (7–10), which indicate that, neuroanatomically, ADHD is a disorder of early brain development. In addition, specific neuroimaging modalities, such as diffusion tensor imaging (11–13), (resting-state) functional magnetic resonance imaging (fMRI) (14–16), and EEG (17), have shown that both structural and functional brain connectivity are impaired in ADHD patients.

Evidence from family and twin studies shows that ADHD is a highly heritable disorder, and approximately 76% of the phenotypic variance can be explained by genetic factors (18). ADHD behaves as a multifactorial (or complex) disorder, in which different combinations of genetic and environmental factors contribute to the overall risk of developing the disorder. The genetic model underlying most cases of ADHD is likely one in which multiple genetic factors of small-to-moderate effect size contribute to the disease etiology (19). Recent data indicate that rare copy number variations of genes may also be relevant to the etiology of ADHD (20–22), but pending further studies, it is unclear which proportion of ADHD cases could be explained by these variants.

To date, eight independent genome-wide linkage scans have been conducted for ADHD (23, 24). A recent meta-analysis of seven of these studies identified genome-wide significant linkage for a chromosomal region on 16q (23). In addition, a large number of candidate gene association studies have been published, and these have primarily focused on genes involved in catecholaminergic neurotransmission. Recent meta-analyses of these studies have yielded significant meta-association for six ADHD candidate genes (i.e., the dopamine and serotonin transporter genes *DAT1*/*SLC6A3* and *5-HTT*/*SLC6A4*, the *DRD4* and *DRD5* dopamine receptor genes, the *HTR1B* serotonin receptor gene, and the *SNAP25* gene involved in neurotransmission) (25, 26). In 2008, the results of three genome-wide association studies (GWASs) for ADHD were published (27–29) in two independent data sets. Lasky-Su et al. (28) reported the following two findings that reached (trait-specific) genome-wide significance: 1)

a single nucleotide polymorphism (SNP) in *CDH13* and 2) a SNP in *GFOD1*.

Very recently, the results of two additional GWASs for ADHD in two independent samples were reported (30, 31). Apart from *CDH13*, which maps to the only chromosomal region that yielded genome-wide significant linkage for ADHD in a recent meta-analysis (23), overlap between GWAS findings and any of the reported linkage or candidate gene-based association findings has been limited (32), although this is probably due in part to the fact that the published GWASs for ADHD are strongly underpowered.

In the present study, we attempted to integrate the most significant findings from the five reported GWASs for ADHD into protein signaling networks that would not only increase our understanding of the genetic etiology of ADHD but also provide clues for future research into more effective (psychopharmacological) treatments for the disorder. Based on bioinformatics analyses and a systematic literature analysis of the (putative) function of the proteins encoded by the 85 top-ranked genes emerging from the five GWASs for ADHD, we describe a signaling network involved in neurite outgrowth that includes 45 of these proteins. The other 40 proteins could not be convincingly linked to the network. Corroborating evidence for the involvement of this gene network from chromosomal aberrations in humans, from animal models, and from psychopharmacological studies is also presented.

Method

Identification of ADHD Candidate Genes From GWASs

To date, the results of five GWASs of ADHD have been reported. Neale et al. (27) reported GWAS results for a categorical ADHD phenotype in 909 Caucasian case-parent trios that were collected as part of the International Multicenter ADHD Genetics study in children. Lasky-Su et al. (28) performed a GWAS on the same sample using quantitative phenotypes of ADHD.

Lesch et al. (29) conducted a GWAS for a categorically defined ADHD phenotype using pooled DNA from 343 ADHD-affected adults and 304 comparison subjects. Recently, Mick et al. (30) also reported the results of a GWAS of a categorical ADHD phenotype in a sample of 735 trios from three different U.S. clinical sites (30). In addition, the International Multicenter ADHD Genetics II consortium reported the results of a GWAS using a case-control design in 896 unrelated participants with ADHD and 2,455 comparison subjects (31). From these five GWASs, we compiled a list of top SNPs, taking into account SNPs located within exonic, intronic, or untranslated regions of genes and in association with the ADHD phenotype at p<1.00E-04 (Table 1).

Ingenuity and BiNGO Pathway Analyses

In order to detect significantly enriched gene categories in the selected ADHD candidate genes from the five GWASs, we performed analyses using the Ingenuity Pathway Analysis software package (http://www.ingenuity.com) and the BiNGO (Biological Network Gene Ontology) bioinformatics tool (33). We performed similar analyses on a set of top genes from four GWASs for diabetes type I and Crohn's disease and compared the results with those for ADHD in order to identify possible overrepresentation bias of large (brain-expressed) genes in the top findings of the ADHD GWASs.

The Ingenuity software package (http://www.ingenuity.com) uses the Ingenuity Knowledge Base, which is based on information from the published literature as well as many other sources, including gene expression and gene annotation databases, to assign genes to different groups and categories of functionally related genes. Each of these categories can be further divided into many subcategories. Ingenuity calculates single p values for the enrichment of each gene category using the right-tailed Fisher's exact test and taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules that is linked to the same gene category according to the Ingenuity Knowledge Base. Furthermore, for each gene category, a multiple testing corrected p value of enrichment, calculated using the Benjamini-Hochberg correction, is provided.

The BiNGO tool also assigns genes to different functional categories, but these are specifically linked to gene ontology terms that group functionally related genes (33) and can be found in the Gene Ontology database (http://www.geneontology.org/). The gene ontology terms can be further assigned to three main gene ontology subgroups or domains (i.e., cellular component, biological process, and molecular function). As with Ingenuity, the BiNGO tool provides single p values, calculated with the hypergeometric test and taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules that is linked to the same gene ontology term, as well as multiple testing corrected p values, calculated using the Benjamini-Hochberg correction, for the enrichment of each gene ontology term (33).

In the presentation of the results of the Ingenuity and BiNGO analyses for ADHD, only categories/processes with significant enrichment (i.e., multiple testing corrected p<0.05) and containing more than one gene were taken into account. Only in the comparison between ADHD, diabetes type I, and Crohn's disease were the functional categories that only contain one gene also considered.

Literature Analysis

Subsequent to the bioinformatics analysis, we systematically searched the literature for the (proposed) function of all 85 proteins derived from the ADHD candidate genes using PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) and the UniProt Protein Knowledge Base (UniProtKB), a comprehensive catalog of protein sequences and functional annotations that is updated every 3 weeks and can be accessed online (http://www.uniprot.org/uniprot [34]). For each ADHD candidate, we first looked at the available information in UniProtKB, which in several cases already provided a general indication of the (putative) function of the gene/protein in question. Subsequently, we searched PubMed using the search terms "brain," "neuron," and "neurite" in combination with the name of each of the candidate genes/proteins from the GWASs. Lastly, guided by the literature we found, we searched PubMed for functional interactions between several of the candidate genes/proteins.

Results

Table 1 shows the 85 genes from the five GWASs for ADHD fulfilling the inclusion criteria of the present study. When more than one SNP in a single gene was found among the top findings, only the SNP yielding the lowest p value for association with (a quantitative phenotype of) ADHD is presented. As can be derived from Table 1, the only overlap between the top findings of the five published

TABLE 1. The Top Single Nucleotide Polymorphisms (SNPs) From the Five Reported Genome-Wide Association Studies of Attention Deficit Hyperactivity Disorder (ADHD)[a]

GWAS	SNP	p	Locus	Gene	Position Within Gene	Gene Size
Neale et al. (27)[b]						
	rs9676447	1.00E-05	19q13.33	***NUCB1***	intronic	23 kb
	rs876477	2.70E-05	4p15.31	***KCNIP4***	intronic	1,220 kb
	rs7657608	3.00E-05	4q32.3	***SPOCK3***	intronic	501 kb
	rs3782309	5.50E-05	12p11.23	*ITPR2*	intronic	498 kb
	rs922781	5.50E-05	15q22.2	***RORA***	intronic	741 kb
	rs12505502	5.60E-05	4q22.1	***FAM190A***	intronic	1,360 kb
	rs1062793	7.00E-05	6q14.1	*SH3BGRL2*	3′ untranslated region	72 kb
	rs703965	9.20E-05	10q22.3	*ZMIZ1*	intronic	248 kb
	rs4149601	9.30E-05	18q21.31	***NEDD4L***	intronic	357 kb
Lasky-Su et al. (28)[c]						
	rs6565113	[d]	16q23.3	***CDH13***	intronic	948 kb
	rs552655	[d]	6p24.1	*GFOD1*	intronic	130 kb
	rs4128767	1.28E-06	15q25.1	*IL16*	intronic	117 kb
	rs11719664	2.48E-06	3p24.3	*ZNF385D*	intronic	955 kb
	rs7577925	2.55E-06	2q21.2	***NAP5***	intronic	897 kb
	rs7495052	2.83E-06	15q26.1	***SLCO3A1***	intronic	319 kb
	rs17281813	3.46E-06	16q12.1	***ZNF423***	intronic	336 kb
	rs363512	3.89E-06	21q21.3	*GRIK1*	intronic	403 kb
	rs8041675	3.98E-06	15q14	***MEIS2***	intronic	210 kb
	rs17641078	4.73E-06	9p24.3	*DMRT2*	nonsynonymous, coding	7 kb
	rs930421	5.64E-06	2p21	*MTA3*	3′ untranslated region	262 kb
	rs17651978	6.05E-06	3p13	*FOXP1*	intronic	629 kb
	rs478597	8.08E-06	12q24.22	***NOS1***	intronic	163 kb
	rs6791644	8.32E-06	3p14.2	***FHIT***	intronic	1,502 kb
	rs260461	8.38E-06	19q13.43	*ZNF544*	intronic	35 kb
	rs2290416	8.51E-06	8q24.3	*NAPRT1*	synonymous, coding	4 kb
	rs1202199	8.52E-06	6p22.3	***MBOAT1***	intronic	110 kb
Lesch et al. (29)[e]						
	rs864643	1.30E-08	3p22.2	***MOBP***	3′ untranslated region	59 kb
	rs7995215	1.35E-08	13q31.3	***GPC6***	intronic	1,180 kb
	rs11243897	5.63E-08	9q34.13	*C9orf98*	intronic	153 kb
	rs7164335	1.30E-07	15q23	***ITGA11***	intronic	130 kb
	rs220470	1.34E-07	17p13.2	*ITGAE*	intronic	87 kb
	rs10983238	1.37E-07	9q33.1	***ASTN2***	intronic	990 kb
	rs2587695	2.73E-07	2q14.2	*PCDP1*	intronic	118 kb
	rs2281597	5.41E-07	1p35.1	***CSMD2***	intronic	652 kb
	rs10514604	8.06E-07	16q24.1	***ATP2C2***	intronic	96 kb
	rs2677744	9.69E-07	15q26.1	***MAN2A2***	intronic	18 kb
	rs2502731	1.58E-06	9q34.11	***DNM1***	intronic	52 kb
	rs2199161	1.62E-06	5q13.2	***MAP1B***	intronic	100 kb
	rs10786284	1.96E-06	10q24.1	***TLL2***	intronic	149 kb
	rs1555322	3.60E-06	20q11.22	***MMP24***	intronic	50 kb
	rs16928529	3.90E-06	10q22.1	***UNC5B***	intronic	90 kb
	rs515910	4.36E-06	10q25.1	*C10orf79*	intronic	102 kb
	rs2237349	4.63E-06	7p15.1	***CREB5***	intronic	527 kb
	rs4964805	4.74E-06	12q23.3	*NT5DC3*	intronic	71 kb
	rs3799977	4.90E-06	6p21.1	***SUPT3H***	intronic	569 kb
	rs11646411	7.40E-06	16q23.3	***CDH13***	intronic	948 kb
	rs469727	7.54E-06	5q22.2	*REEP5*	intronic	46 kb
	rs2241685	8.11E-06	2p25.3	***MYT1L***	intronic	542 kb
	rs2242073	8.27E-06	2q33.3	*CRYGC*	intronic	2 kb
	rs13395022	9.68E-06	2p12	***CTNNA2***	intronic	1,460 kb
	rs11594082	1.00E-05	10q22.1	***CDH23***	intronic	419 kb
	rs3893215	2.56E-05	11p15.1	*KCNC1*	intronic	47 kb

(Continued)

TABLE 1. The Top Single Nucleotide Polymorphisms (SNPs) From the Five Reported Genome-Wide Association Studies on Attention Deficit Hyperactivity Disorder (ADHD)[a] *(Continued)*

GWAS	SNP	p	Locus	Gene	Position Within Gene	Gene Size
Mick et al. (30)[f]						
	rs2823819	6.70E-07	21q21.1	*C21orf34*	intronic	539 kb
	rs11074889	6.71E-07	16p13.13	***EMP2***	intronic	52 kb
	rs8074751	1.18E-06	17q24.1	*CCDC46*	intronic	556 kb
	rs1859156	2.05E-06	4q22.3	***BMPR1B***	intronic	400 kb
	rs438259	3.71E-06	6p24.3	*AL024506.1*	intronic	122 kb
	rs2602381	3.85E-06	2q37.1	***UGT1A9***	intronic	101 kb
	rs10011926	8.07E-06	4q25	*ELOVL6*	intronic	153 kb
	rs11953346	1.59E-05	5q15	*MCTP1*	intronic	578 kb
	rs7702178	1.84E-05	5q35.1	***DUSP1***	intronic	3 kb
	rs17023218	1.88E-05	3p24.1	*RBMS3*	intronic	1,166 kb
	rs6561686	2.08E-05	13q14.3	*LECT1*	intronic	37 kb
International Multicenter ADHD Genetics II consortium (31)[g]						
	rs10823973	6.30E-07	10q21.1	*PRKG1*	intronic	1,307 kb
	rs2291567	2.00E-06	7q32.1	***FLNC***	intronic	29 kb
	rs13238831	5.60E-06	7q32.1	***KCP***	intronic	48 kb
	rs11593241	5.70E-06	10q26.3	*TCERG1L*	intronic	219 kb
	rs12317552	9.10E-06	12q14.1	***PPM1H***	3′ untranslated region	291 kb
	rs17151821	1.10E-05	7p21.3	***NXPH1***	intronic	319 kb
	rs17095690	1.60E-05	1p31.1	*C1orf173*	intronic	106 kb
	rs8045006	2.30E-05	16q23.3	***CDH13***	intronic	948 kb
	rs1023556	2.60E-05	7p14.3	*AC005493.1*	intronic	14 kb
	rs2394538	2.80E-05	10q22.1	***HK1***	intronic	132 kb
	rs1278776	2.90E-05	13q34	*ATP11A*	intronic	197 kb
	rs17495366	3.10E-05	2p16.3	***NRXN1***	intronic	1,114 kb
	rs906219	3.70E-05	10q22.1	***HKDC1***	nonsynonymous, coding	47 kb
	rs1472147	4.10E-05	7q32.1	*ATP6V1F*	intronic	3 kb
	rs589943	4.90E-05	11q22.3	***DYNC2H1***	intronic	370 kb
	rs2962393	5.60E-05	5q34	*GABRB2*	intronic	260 kb
	rs846971	5.60E-05	6q21	*SOBP*	intronic	170 kb
	rs10445861	6.50E-05	2q14.3	*CNTNAP5*	intronic	890 kb
	rs4261672	7.20E-05	2q22.2	***LRP1B***	intronic	1,900 kb
	rs6986578	7.30E-05	8q13.2	*PREX2*	intronic	280 kb
	rs12090000	7.40E-05	1q25.2	*FAM5B*	intronic	111 kb
	rs1565630	8.00E-05	7q32.1	*CCDC136*	intronic	31 kb
	rs4533267	8.30E-05	15q26.3	***ADAMTS17***	intronic	370 kb
	rs2979307	9.00E-05	3q21.2	*OSBPL11*	intronic	66 kb

[a] Data show SNPs from the GWASs (27–31) located within genes and with a p value <1.00E-04 for association with ADHD after correction for linkage disequilibrium. The 45 (different) genes encoding proteins that could be directly placed in the putative ADHD network are indicated in bold.
[b] GWAS of a categorical DSM-IV ADHD phenotype in 909 Caucasian case-parent trios collected as part of the International Multicentre ADHD Genetics study in children.
[c] GWAS of quantitative phenotypes of ADHD carried out in the same International Multicentre ADHD Genetics sample used in the GWAS conducted by Neale et al. (27).
[d] Findings revealed genome-wide significance for a single quantitative trait using the family-based association testing principal component algorithm.
[e] GWAS of a categorical DSM-IV ADHD phenotype using pooled DNA from 343 ADHD-affected adults and 304 comparison subjects. (Reported p values in this study were ranked according to the mean rank calculated across three statistics.)
[f] GWAS of DSM-IV-TR ADHD in a combined sample of 735 trios from three U.S. clinical sites.
[g] GWAS conducted in 896 unrelated case patients with DSM-IV ADHD and 2,455 comparison subjects using a case-control design.

GWASs was for *CDH13*, in which (three different) SNPs were associated with ADHD at p<1.00E-04 in three GWASs (28, 29, 31).

Analysis of these 85 top ADHD candidate genes using the Ingenuity pathway software revealed a significant enrichment (p=4.11E-08 after correction for multiple testing) of the functional gene category neurological disease, with 44 of the 85 genes falling into this category (Table 2). Furthermore, analysis with the BiNGO bioinformatics tool revealed that the gene ontology processes (calcium)

TABLE 2. Enrichment Analyses of the Top 85 Attention Deficit Hyperactivity Disorder (ADHD) Candidate Genes in the Five Reported Genome-Wide Association Studies of ADHD[a]

Category	Genes	Significance	Adjusted Significance
Ingenuity Pathway Tool[b]			
Neurological disease (44/85 genes)	***ADAMTS17***, ***ASTN2***, ***ATP2C2***, ***BMPR1B***, *C10ORF79, C21ORF34, C9ORF98, CCDC46,* ***CDH13***, ***CDH23***, *CNTNAP5,* ***CREB5***, ***CSMD2***, ***CTNNA2***, ***DYNC2H1***, ***FAM190A***, ***FHIT,*** *FOXP1, GABRB2, GFOD1, GRIK1,* ***ITGA11***, *ITPR2,* ***KCNIP4***, ***LRP1B***, ***MAP1B***, *MCTP1,* ***MEIS2***, ***MOBP, MYT1L,*** *NAPRT1,* ***NOS1, NRXN1, NXPH1, PPM1H,*** *PRKG1, RBMS3,* ***RORA***, ***SLCO3A1***, ***SPOCK3***, ***TLL2***, ***UNC5B,*** *ZNF385D,* ***ZNF423***	4.67E-10	4.11E-08
Biological Network Gene Ontology tool[c]			
Gene ontology term[d]			
Ion binding	***ADAMTS17****, ATP11A,* ***ATP2C2****,* ***BMPR1B****,* ***CDH13, CDH23****,* ***CREB5,*** *DMRT2,* ***DUSP1, FHIT****, FOXP1, GABRB2,* ***HK1****,* ***ITGA11,*** *ITGAE, ITPR2, KCNC1,* ***KCNIP4****,* ***LRP1B, MAN2A2****, MCTP1,* ***MMP24****, MTA3,* ***MYT1L****,* ***NOS1, NRXN1,*** *NT5DC3,* ***NUCB1****,* ***RORA,*** *SOBP,* ***SPOCK3, TLL2,*** *ZMIZ1, ZNF385D,* ***ZNF423,*** *ZNF544*	4.10E-06	2.94E-03
Metal ion binding	***ADAMTS17****, ATP11A,* ***ATP2C2****,* ***BMPR1B****,* ***CDH13, CDH23****,* ***CREB5,*** *DMRT2,* ***DUSP1, FHIT****, FOXP1,* ***HK1****,* ***ITGA11,*** *ITGAE, ITPR2, KCNC1,* ***KCNIP4, LRP1B, MAN2A2****, MCTP1,* ***MMP24****, MTA3,* ***MYT1L****,* ***NOS1, NRXN1,*** *NT5DC3,* ***NUCB1, RORA****, SOBP,* ***SPOCK3, TLL2,*** *ZMIZ1, ZNF385D,* ***ZNF423,*** *ZNF544*	7.45E-06	2.94E-03
Cation binding	***ADAMTS17****,* ***ATP2C2****,* ***BMPR1B****,* ***CDH13, CDH23****,* ***CREB5,*** *DMRT2,* ***DUSP1, FHIT****, FOXP1,* ***HK1****,* ***ITGA11,*** *ITGAE, ITPR2, KCNC1,* ***KCNIP4****,* ***LRP1B, MAN2A2****, MCTP1,* ***MMP24****, MTA3,* ***MYT1L****,* ***NOS1, NRXN1, NUCB1, RORA,*** *SOBP,* ***SPOCK3, TLL2,*** *ZMIZ1, ZNF385D,* ***ZNF423,*** *ZNF544*	1.19E-05	3.14E-03
Calcium ion binding	***ATP2C2****,* ***CDH13, CDH23****,* ***ITGA11,*** *ITGAE, ITPR2,* ***KCNIP4****,* ***LRP1B,*** *MCTP1,* ***MMP24****,* ***NRXN1, NUCB1, SPOCK3, TLL2***	2.76E-05	5.43E-03
Hexokinase activity	***HK1, HKDC1***	1.71E-04	2.69E-02

[a] The genes (in the reported GWASs [27–31]) encoding proteins that could be directly placed in the putative ADHD network are indicated in bold.

[b] Analyses were conducted using Ingenuity pathway software (www.ingenuity.com). Significance was determined from a single test p value calculated using the right-tailed Fisher's exact test and taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules linked to the same gene category according to the Ingenuity Knowledge Base. Adjusted significance was determined from multiple test-corrected p values using the Benjamini-Hochberg correction (only categories reaching a corrected statistical significance of <0.05 are shown).

[c] Analyses were conducted using the Biological Network Gene Ontology tool (33). Significance was determined from a single test p value calculated using the hypergeometric test and taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules linked to the same gene ontology term. Adjusted significance was determined from multiple test-corrected p values using the Benjamini-Hochberg correction (only categories reaching a corrected statistical significance of <0.05 are shown).

[d] All significantly enriched gene ontology terms can be further assigned to the molecular function gene ontology subgroup/domain.

ion binding and hexokinase activity were significantly enriched in the 85 ADHD genes (2.94E-03<p<5.43E-03 and p=2.69E-02, respectively) (Table 2).

Further literature analysis of the (putative) function of the proteins encoded by the genes in the enriched BiNGO terms revealed that 21 out of 36 genes in the (calcium) ion binding category and two out of two hexokinase activity genes play a role in neurite outgrowth. Subsequently, we investigated the function of the entire set of 85 genes further and found that a total of 45 of the 85 included genes (indicated in bold in tables, figure legend, and throughout this article) fit into a neurodevelopmental network that is involved in directed neurite outgrowth, which is illustrated in Figure 1. The proposed signaling network links axonal guidance and neuronal cell adhesion proteins at the neuronal cell membrane with downstream acting adaptor proteins and neuronal cytoskeleton/extracellular matrix-associated proteins.

A detailed description of the evidence linking the genes in the network to neurite outgrowth is provided in the data supplement accompanying the online version of this article.

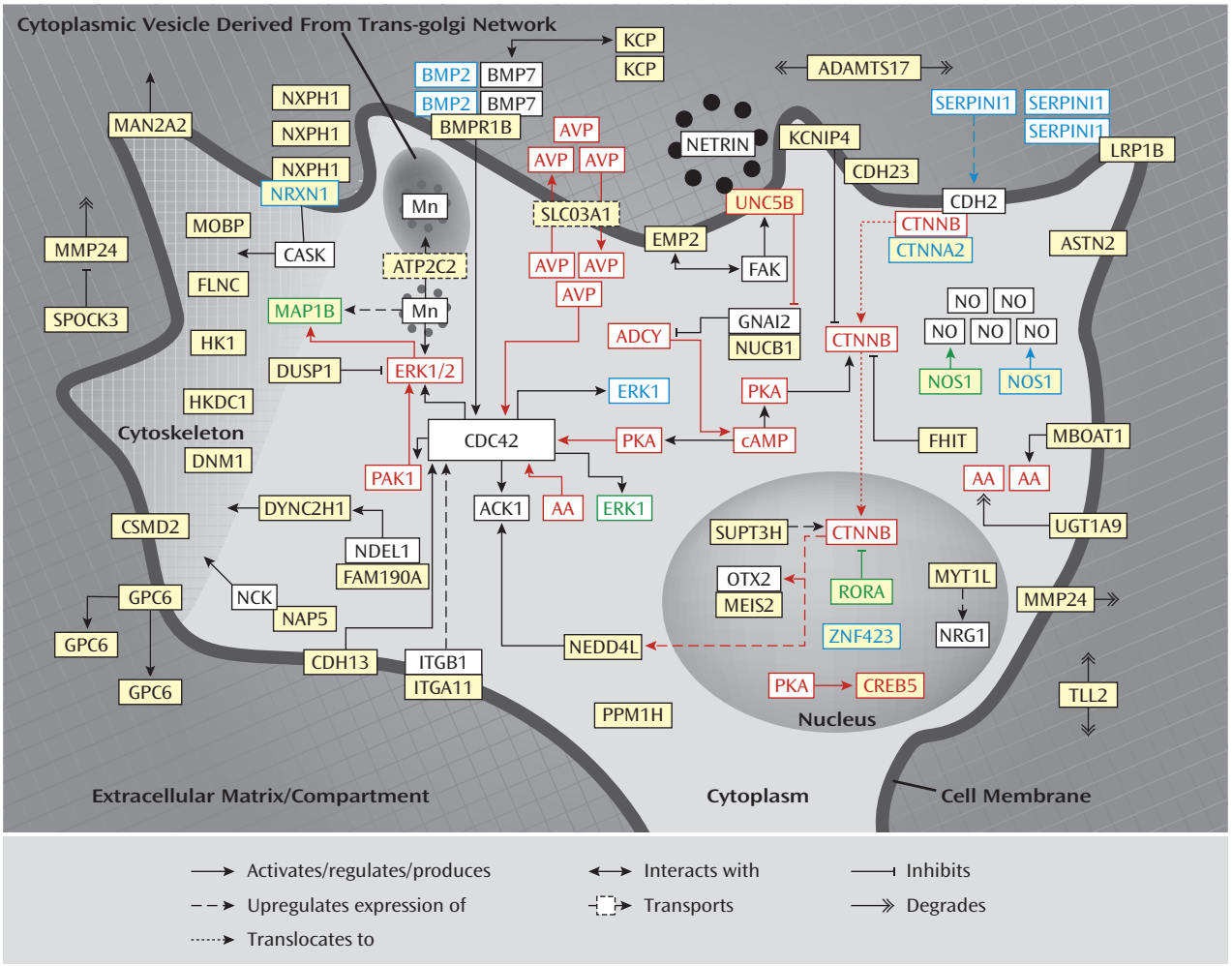
Involvement of the identified network in ADHD is also supported by the finding that several of the identified genes (i.e., ***CTNNA2***, ***NRXN1***, ***NAP5***, *SERPINI1*, ***NOS1***, *ERK1*, ***ZNF423***, ***NEDD4L***, and *BMP2*) are located within copy number variations—and are hence deleted or duplicated—in people with ADHD (20–22, 35–41)(Table 3, Figure 1).

Moreover, partial knockout models of the ***NOS1***, *ERK1*, ***MAP1B***, and ***RORA*** genes in mice provide further evidence of the involvement of these genes in ADHD etiology (Figure 1).

Mice in which the *Nos1* and *Erk1* genes have been knocked out display hyperactive behavior (42, 43), while, in contrast, *Map1b*- (44) and *Rora*-knockout mice (45) show hypoactivity.

Lastly, several proteins from the network appear to be under control of the stimulants methylphenidate and amphetamine that are used to treat ADHD symptoms (4, 5). Both stimulants have been shown to stimulate neurite outgrowth (46, 47) and directly or indirectly regulate the expression and/or function of several genes/proteins implicated in neurite outgrowth, including

FIGURE 1. Schematic Representation of the ADHD Neurodevelopmental Signaling Network for Directed Neurite Outgrowth[a]



[a]The genes/proteins that emerged from the five reported genome-wide association studies for ADHD are indicated in yellow. The proteins encoded by genes found in copy number variations in patients with ADHD are indicated by a blue border. The proteins encoded by genes implicated in the etiology of ADHD through gene knockout studies in mice are indicated by a green border. The genes/proteins of which the expression and/or function is regulated by stimulants are indicated by a red border. Evidence placing the genes/proteins into this network is presented in the data supplement.

a number of genes/proteins from the identified network (Figure 1).

Methylphenidate exposure upregulates the expression of *AVP* (48) and ***CREB5*** (49) (Figure 1). In addition, methylphenidate upregulates the expression of a large number of other genes not directly found in the identified network but functionally implicated in neurite outgrowth (50). Examples of these genes are *MMP14*, *TIMP2*, and *TIMP3*. The *MMP14* gene encodes a neuronal extracellular matrix metalloproteinase, such as **MMP24**, and *TIMP2* and *TIMP3* encode two direct inhibitors of MMP14 (34, 50). Amphetamine upregulates the expression of ***UNC5B*** (51) and *PAK1* (52) and downregulates the expression of *CTNNB* (53). Amphetamine also activates neuronal ERK1 and ERK2 (54) and increases the level of arachidonic acid in neocortical, extrapyramidal, and limbic brain regions (55). Deficiencies or imbalances of arachidonic acid have been shown to be associated with ADHD (56). Lastly, the adenylate cyclase/cAMP/protein kinase A/CREB-signaling cascade (including protein kinase A activating **CREB5**) is also activated by amphetamine (57).

An important potential bias of which to be aware in the analysis of the top findings from the reported GWASs of ADHD is the fact that large genes, which are often brain expressed, may be more likely found as associated with a phenotype in a GWAS as a result of chance, since more SNPs are present in these large genes (58). Indeed, the genes found among the top findings of the ADHD GWASs were considerably larger than the average gene size of the human genome ([Table 1] average size of 85 ADHD genes: 373 kb versus average gene size in human genome: 27 kb [59]).

If large genes are more likely found to be associated with a phenotype of a GWAS because of chance, this would be expected to be the case for GWASs of both nonpsychiatric disorders and ADHD, and therefore we compared the results of our analyses with the Ingenuity and BiNGO software tool results for the 20 top ADHD candidate genes

TABLE 3. Genes Encoding Proteins From the Putative Attention Deficit Hyperactivity Disorder (ADHD) Network and Reported to be Deleted and/or Duplicated in (Genome-Wide) Copy Number Variations Among Individuals With ADHD

Locus	Gene	Copy Number Variant	Phenotype	Additional Comments	Study
2p12	*CTNNA2*[a]	deleted	ADHD	Genome-wide copy number variant analysis	Elia et al. (20)
2p16.3	*NRXN1*[a]	deleted	ADHD	Genome-wide copy number variant analysis	Bradley et al. (35)
2q21.2	*NAP5*[a]	deleted	Attention deficit disorder		Baker et al. (36)
3q26.1	*SERPINI1*	duplicated	ADHD	Genome-wide copy number variant analysis	Elia et al. (20)
3q26.1	*SERPINI1*	deleted	ADHD	Genome-wide copy number variant analysis	Lesch et al. (21)
12q24.22	*NOS1*[a]	duplicated	ADHD		Cappellacci et al. (37)
16p11.2	*ERK1*	duplicated	ADHD	Six/eight unrelated people with ADHD	Shinawi et al. (38)
16p11.2	*ERK1*	duplicated	ADHD	Genome-wide copy number variant analysis	Williams et al. (22)
16p11.2	*ERK1*	deleted	ADHD	Four/15 unrelated people with ADHD	Shinawi et al. (38)
16p11.2	*ERK1*	deleted	ADHD	Genome-wide copy number variant analysis	Williams et al. (22)
16q12.1	*ZNF423*[a]	duplicated	ADHD		van den Berg et al. (39)
18q21.31	*NEDD4L*[a]	duplicated	Attention deficit disorder	Two siblings with attention deficit disorder	Ceccarini et al. (40)
20p12.3	*BMP2*	deleted	Attention deficit disorder		Lalani et al. (41)

[a] One of the six genes from the reported GWAS (Table 1) that could be directly placed in the putative ADHD network.

TABLE 4. Nervous System-Related Gene Functional Categories Significantly Enriched in the Top 20 Candidate Genes From Genome-Wide Association Studies of Attention Deficit Hyperactivity Disorder (ADHD), Diabetes Type I, and Crohn's Disease[a]

Category	Genes	Significance[b]	Adjusted Significance[c]
ADHD			
Neurological disease (13/20 genes)	***ASTN2***, ***ATP2C2***, *C21ORF34*, *C9ORF98*, *CCDC46*, ***CDH13***, ***CSMD2***, *GFOD1*, ***ITGA11***, ***MAP1B***, ***MOBP***, *PRKG1*, ***TLL2***	1.53E-04	7.55E-03
Biogenesis of lamellipodia[d]	***CDH13***	5.49E-03	2.10E-02
Diabetes type I			
Neurological disease (1/20 genes)	*TH*	1.42E-02	3.02E-02
Long-term depression	*INS*	1.30E-03	8.11E-03
Crohn's disease			
Neurological disease (0/20 genes)			

[a] Enrichment analyses were conducted using Ingenuity pathway software (www.ingenuity.com). (Further details are presented in Table 1 of the data supplement accompanying the online version of this article.)
[b] Data were determined using a single test p value calculated with the right-tailed Fisher's exact test and taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules linked to the same gene category according to the Ingenuity Knowledge Base.
[c] Data were determined using multiple test-corrected p values using the Benjamini-Hochberg correction (only categories reaching a corrected statistical significance of <0.05 are shown).
[d] Refers to protrusions of the neuronal growth cone that are formed during the process of neurite outgrowth (60).

with those for the top 20 candidate genes from four published GWASs for diabetes mellitus type I and Crohn's disease (Table 4, Table 5 [also see the data supplement]). This comparison showed that the neurological disease category was not enriched in Crohn's disease and only contained a single gene in diabetes type I (Table 4). In addition, the gene ontology processes that were enriched in the top GWAS findings were clearly different for the three diseases (Table 5).

Discussion

In the present study, we used both bioinformatics tools and manual literature mining to attempt to integrate the top findings of the currently available GWAS data for ADHD. We found that 45 of the 85 top ADHD candidate genes (or approximately 53%) fit into a network that is involved in neurite outgrowth. It should be noted that our decision to only include SNPs within exonic or intronic regions of genes and those with association at p<1.00E-04 is essentially arbitrary. By only including genes with SNPs in exons and introns, we have ignored the possible role of upstream and downstream regulatory sequences (61), which could have increased the number of (potentially relevant) genes to be included in the study. However, we do not expect that this would have altered the general tendency in our results (i.e., the considerable overrepresentation of neurite outgrowth-related genes).

TABLE 5. The Top Five Gene Functional Categories Significantly Enriched in the Top 20 Candidate Genes From Genome-Wide Association Studies of Attention Deficit Hyperactivity Disorder (ADHD), Diabetes Type I, and Crohn's Disease[a]

Category	Genes	Significance[b]	Adjusted Significance[c]
ADHD[d]			
Gene ontology term			
Regulation of chemotaxis	***CDH13,*** *IL16*	3.58E-05	4.40E-03
Regulation of behavior	***CDH13,*** *IL16*	5.25E-05	4.40E-03
Regulation of response to external stimulus	***CDH13,*** *IL16*	2.77E-04	1.32E-02
Integrin complex	***ITGA11,*** *ITGAE*	2.98E-04	1.32E-02
Cell migration	***CDH13,*** *IL16,* ***ITGA11***	6.38E-04	2.34E-02
Diabetes type I[e]			
Gene ontology term			
Growth factor binding	*ERBB3, IL7R, INS*	4.46E-05	2.27E-02
Regulation of secretion	*ERBB3, INS*	3.35E-04	3.54E-02
Anion transport	*C1QTNF6, CFTR, COL1A2*	4.75E-04	3.54E-02
Regulation of insulin receptor signaling	*INS*	9.76E-04	3.54E-02
Tyrosine 3-monooxygenase activity	*TH*	9.76E-04	3.54E-02
Crohn's disease[f]			
Gene ontology term			
Protein kinase cascade	*GCKR, LRRK2, MST1, STAT3, TNFSF15*	5.10E-06	2.28E-03
Intracellular signaling cascade	*GCKR, LRRK2, MST1, NOD2, STAT3, TNFSF15*	6.10E-04	2.42E-02
Cell communication	*ATG16L1, BSN, GCKR, ITLN1, LRRK2, MST1, NOD2, PTPN22, STAT3, TNFSF15*	7.95E-04	2.42E-02
Protein amino acid autophosphorylation	*LRRK2, MST1*	9.08E-04	2.42E-02
Macrophage activation during immune response	*SBNO2*	9.76E-04	2.42E-02

[a] Enrichment analyses were conducted using the Biological Network Gene Ontology tool (33); genes from the reported GWAS (Table 1) encoding proteins that could be directly placed in the putative ADHD network are indicated in bold (further details are presented in Table 1 of the data supplement accompanying the online version of this article).
[b] Data were determined from a single test p value calculated using the hypergeometric test and taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules linked to the same gene ontology term.
[c] Data were determined from multiple test-corrected p values using the Benjamini-Hochberg correction.
[d] The gene ontology terms regulation of chemotaxis, regulation of behavior, regulation of response to external stimulus, and cell migration can be further assigned to the biological process gene ontology subgroup/domain, while integrin complex falls under the cellular component domain.
[e] The gene ontology terms growth factor binding and tyrosine 3-monooxygenase activity can be further assigned to the molecular function gene ontology subgroup/domain, while regulation of secretion, anion transport, and regulation of insulin receptor signaling fall under the biological process domain.
[f] All gene ontology terms can be further assigned to the biological process gene ontology subgroup/domain.

Surprisingly, the enrichment of neurite outgrowth-related genes was not reflected by the finding of gene functional categories and/or gene ontology terms directly related to neurite outgrowth with the bioinformatics tools used in this study (Table 2). This shows the limitations of the currently available bioinformatics software, which is a result of the current incompleteness of the annotation of the human genome.

Therefore, use of bioinformatics tools should always be accompanied by manual and systematic literature and database mining in order to fully understand the processes involved in the etiology of multifactorial disorders. Moreover, the enrichment of neurite outgrowth-related genes in the ADHD GWAS data (53%) should be viewed from the perspective that only 3% (N=576) of all currently annotated human genes (N=18,589) are involved in neurogenesis (gene ontology term: GO:0022008).

Several additional lines of evidence suggest that the identified network provides an important contribution to the etiology of ADHD. Apart from implicating the same genes as the GWASs, these other lines of evidence point to involvement of several other genes (and their corresponding proteins) that were not found (at the p<1.00E-04 level) in the GWASs but that functionally connect the genes from the GWAS findings.

First, nine proteins that act in the identified neurite outgrowth network are encoded by genes that were also found in copy number variations in people with ADHD (20–22, 35–41) (Table 3). Second, knockout mouse models of four network genes reveal ADHD-related behavior (42–45). Third, and most importantly, the expression and/or function of a number of genes/proteins in the identified network is regulated by the stimulants methylphenidate (48–50) and amphetamine (51–55, 57), which are the most commonly used psychopharmacological treatments for ADHD symptoms (4, 5) and directly regulate neurite outgrowth (46, 47). The latter finding might not only increase our understanding of the working mechanism of these drugs but also provide directions for future research into new and more effective psychopharmacological ADHD treatments.

The fact that stimulants regulate neurite outgrowth potentially implicates a number of classic ADHD can-

didate genes in our network. Methylphenidate and amphetamine mediate effects on both dopaminergic and serotonergic neurotransmission in the brain (4, 5, 62). In this respect, the genes encoding the SLC6A3, DRD4, DRD5, SLC6A4, and HTR1B proteins could be putatively linked to the identified network. In addition, the link of these classic candidate genes with the neurite outgrowth network may help to localize the network to dopaminergic and serotonergic brain regions.

The findings from this study fit very well with literature about disturbed brain structure and function in ADHD patients (7–10) and, even more so, with recent reports describing aberrant structural and functional brain connectivity in these patients (11–17). Because differences in brain connectivity have been shown to underlie interindividual variability in complex cognition-related processes, including ADHD (63, 64), relatively minor alterations in neurite outgrowth efficiency or direction may provide a major contribution to the cognitive deficits observed in ADHD.

A possibly important bias in our study may arise from the fact that the brain-expressed genes in the top findings of the ADHD GWASs are substantially larger than the average gene size in the human genome, and seven of the nine very large genes (i.e., genes with a size of >1 Mb) from the list of 85 ADHD genes indeed encode proteins that fit into the proposed ADHD network. It has been argued that the overrepresentation of large genes in the top findings of GWASs might result from bias because one is more likely to find an associated SNP in a larger, usually brain-expressed gene (58). If this is the case, it would be expected that similar results would be found in GWASs of polygenic disorders that are assumed not to primarily originate in the brain, such as diabetes mellitus and Crohn's disease. However, our Ingenuity and BiNGO analyses on the 20 top candidate genes from the GWASs for ADHD, diabetes mellitus type I, and Crohn's disease (Table 4, Table 5) show that this bias does not likely explain the findings for ADHD. In this respect, we would like to submit that the strong enrichment of neurite outgrowth-related genes in the top findings from the five ADHD GWASs, with 45 out of 85 genes fitting into our proposed network, also argues against the fact that these genes are spurious association findings. Nevertheless, we cannot completely exclude the possibility that some of the genes placed in the network still might have been chance findings.

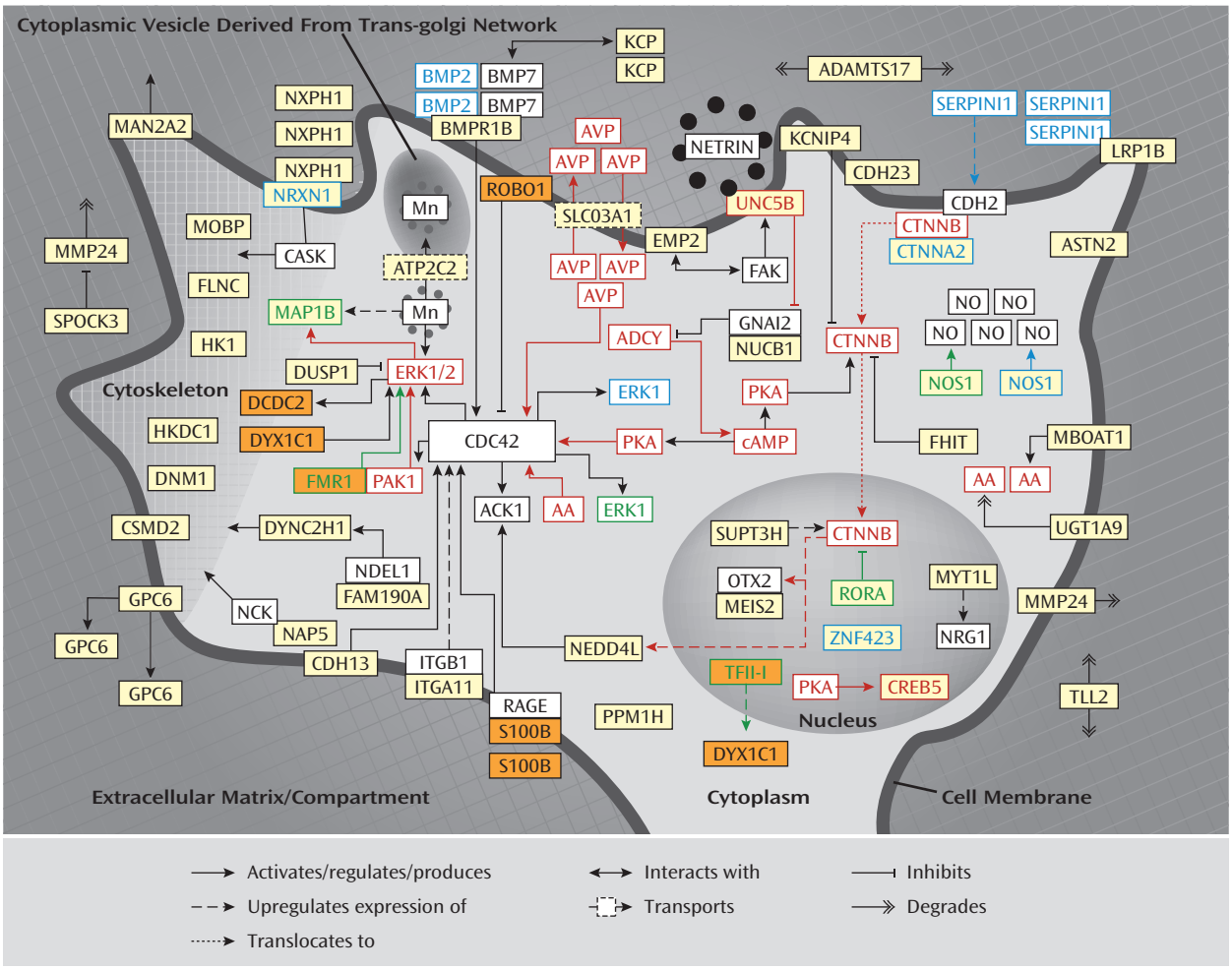
Is the involvement of neurite outgrowth genes specific to the etiology of ADHD? This seems rather unlikely, since we recently also found neurite outgrowth to be implicated in dyslexia (65). Following an approach similar to the one used in the present study, we found that 10 of 14 dyslexia candidate genes reported to date (i.e., *DCDC2, DIP2A, DOCK4, DYX1C1, FMR1, GTF2I, KIAA0319, KIAA0319L, ROBO1, S100B*) fit into a protein network that is involved in neurite outgrowth and neuronal migration. As shown in Figure 2 and explained in further detail in the data supplement, the proteins encoded by six of these genes (i.e., DCDC2, DYX1C1, FMR1, TFII-I [encoded by *GTF2I*], ROBO1, and S100B) fit directly into the identified network for ADHD. It is also interesting to note that decreased serum concentrations of S100B correlate with increasing hyperactivity symptoms in children with ADHD relative to normal comparison subjects (66) and that knockout mouse models of the *FMR1* (67) and *GTF2I* (68) genes show ADHD-like behavior. One possible explanation for the observed genetic overlap between ADHD and dyslexia is that these neurodevelopmental disorders are highly comorbid and genetically correlated with one another (69–71). The same also holds true for autism (72), and recent evidence suggests involvement of neurite outgrowth genes in this neurodevelopmental disorder as well (73). The different functional consequences of disturbed or abnormal neurite outgrowth in the different brain regions that are most affected in ADHD, dyslexia, and autism, respectively, could help to explain why a disturbance of the same neurodevelopmental process could lead to these partially overlapping but still distinct clinical phenotypes.

Several genes that are major players in the identified neurite outgrowth network have not been directly observed in the top findings of the GWASs. An example of such a gene is *ERK1*, which not only has an important function in the network but has also been implicated in ADHD through its location in ADHD-related copy number variations (22, 38) as well as the fact that *Erk1*-knockout mice display hyperactive behavior (43) and that amphetamine directly activates neuronal ERK1 (54). Other examples are *CTNNB* and *CDC42*. These and other genes from the network may be strong candidates for future association studies.

Lastly, our data make a compelling case for the use of polygenic types of association analyses to explain a higher percentage of the heritability of ADHD through the GWAS findings. In this respect, all the individually associated genes from the identified neurite outgrowth network could be fitted simultaneously into one polygenic risk test, as illustrated in the recent study by Yang et al. (74) of all SNPs that have been individually associated in GWASs of variation in human height as well as in two other recent studies applying similar approaches to GWAS data for schizophrenia (75) and functional gene groups for cognitive ability in ADHD (76).

In conclusion, by integrating the top findings of the five GWASs for ADHD with copy number variation data, (psychopharmacologically induced) expression data, and data from animal studies, we have identified a protein signaling network for ADHD that results in directed neurite outgrowth. Systems biology approaches like those used in this study are needed to yield genetic findings that are useful for clinical purposes, such as the prediction of disease prognosis and the identification of new treatment targets and strategies.

FIGURE 2. Schematic Representation of How Six Neurite Outgrowth-Related Dyslexia Candidate Genes Can be Directly Placed in the Signaling Network for ADHD[a]



[a]The six neurite outgrowth-related dyslexia candidate genes (65) that can be placed in the signaling network for ADHD are indicated in orange. The genes/proteins that emerged from the five reported genome-wide association studies for ADHD are indicated in yellow. The proteins encoded by genes found in copy number variations in patients with ADHD are indicated by a blue border. The proteins encoded by genes implicated in the etiology of ADHD through gene knockout studies in mice are indicated by a green border. The genes/proteins of which the expression and/or function is regulated by stimulants are indicated by a red border. Evidence placing the six dyslexia candidate genes/proteins into the ADHD network is presented in the data supplement.

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