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Screening of Human *LPHN3* for Variants With a Potential Impact on ADHD Susceptibility

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Attention deficit hyperactivity disorder (ADHD) is the most common behavioral disorder in childhood, and often has effects detectable into adulthood. Advances in genetic linkage and association analysis have begun to elucidate some of the genetic factors underlying this complex disorder. Recently, we identified *LPHN3*, a novel ADHD susceptibility gene harbored in 4q, and showed that a *LPHN3* common haplotype confers susceptibility to ADHD and predicts effectiveness of stimulant medication. Here we present the mutational analysis of the entire coding region of *LPHN3* in a cohort of 139 ADHD subjects and 52 controls from across the USA. We identified 21 variants, of which 14 have been reported and 7 are novel. These include 5 missense, 8 synonymous, and 8 intronic changes. Interestingly, neither susceptibility nor protective haplotype alleles are associated with obviously significant coding region changes, or canonical splice site alterations, suggesting that non-coding variations determining the quantity and/or quality of *LPHN3* isoforms are the likely contributors to this common behavioral disorder.

Key words: ADHD; latrophilin; behavioral genetics; complex inheritance

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INTRODUCTION

Linkage of ADHD phenotypes to a haplotype block within *LPHN3* has recently been demonstrated in the Paisas from Colombia [Arcos-Burgos et al., 2010] and, subsequently, replicated in additional populations (USA—two independent samples—German, Spain, Norway); however, no readily identifiable pathogenic mutations were found in this population isolate. Suspecting a likely founder effect in this Columbian population, we wanted to determine if sequence variations in *LPHN3* in a more ethnically diverse USA population could bolster its role in ADHD. In addition, this mutational screen might identify additional common sequence variants useful in subsequent linkage and association studies. An ADHD susceptibility haplotype of approximately 325 kb (minimal critical region, MCR) resulted from this analysis, on chromosome 4q13.2. The region of association is located at 62.4–62.7 Mb (UCSC coordinates) encompassing exons 4–19 of the *LPHN3* gene [Arcos-Burgos et al., 2010]. Family based association testing of the Paisa sample suggest that this locus can confer either susceptibility (haplotype frequency of 0.22) or protection from ADHD depending on defined haplotype characteristics with a relative risk of 4.3 between these extremes. Therefore, we set out to attempt to identify the basis of these haplotype differences at the molecular level, beginning with a mutational screen of *LPHN3* in an expanded USA cohort.

Latrotoxin (LTX) is the main component of the black widow spider (*Latrodectus mactans*) venom, which induces massive neurotransmitter release following cell surface binding to receptors of the Neurexin 1a or Latrophilin types. This reagent has been a powerful tool in basic neurobiology to dissect the mechanisms involved in regulating neurotransmitter release [Sudhof, 2001; Ushkaryov et al., 2004]. Latrophilins have been variously referred to as Calcium independent receptors of latrotoxin (CIRL), CIRL/Latrophilin (CL–C/L), or lectomedins (LEC). According to the HUGO nomenclature, the accepted family name is LPHN. The first latrophilin gene, rat Lphn1, was cloned while studying the cell surface receptors for latrotoxin to better understand neurotransmitter physiology [Krasnoperov et al., 1997]. Two additional family

Additional Supporting Information may be found in the online version of this article.

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members, Lphn 2 and 3, were subsequently recognized in rat [Sugita et al., 1998] as well as all higher vertebrates, including humans. Lphn1 has been studied the most extensively due to its high affinity binding to latrotoxin. In contrast, Lphn2 binds the toxin only weakly, and Lphn3 does not bind to any significant degree. Aside from toxin binding, however, the actual physiological roles of Lphn proteins are poorly understood.

The human *LPHN3* gene is located on chromosome 4q13.2, spans approximately 600 kb, and is divided into 24 coding exons. Several mRNA variants reflecting potential alternative splicing has been reported [Ichtchenko et al., 1999; Matsushita et al., 1999]. Expression studies in rat have shown different temporal and spatial patterns of expression among the three described members of the Lphn family. Rat Lphn2 is ubiquitously expressed, whereas Lphn 1 and 3 are prominently expressed in brain—Lphn3 being the most brain-specific [Sugita et al., 1998]. Experiments in rats have shown that Lphn3 is highly expressed during the fetal period, followed by Lphn2, and later by Lphn1 (which seems to be the adult form) [Kreienkamp et al., 2000; Tobaben et al., 2000]. In humans, preliminary results suggest that the pattern of expression of *LPHN3* is also temporally and spatially dynamic such that its expression decreases as the brain matures and ultimately becomes readily detectable only in regions of the brain independently implicated in ADHD pathogenesis [Arcos-Burgos et al., 2010].

The LPHN family belongs to the adhesion family of the seven transmembrane domain superfamily (7TM), also called G-Protein Coupled Receptors (GPCRs) or serpentine receptors [Armbruster and Roth, 2005; Jacoby et al., 2006; Ushkaryov et al., 2008]. LPHN mRNA is translated into a pre-pro-protein that following the removal of the signal peptide, is cleaved in the endoplasmatic reticulum by an endopeptidase recognizing a GPS (GPCR Proteolytic Site) domain that encompasses ~50 residues. As shown in Figure 1, the two resulting fragments, Amino-terminal Fragment (NTF) and Carboxy-terminal Fragment (CTF), reach the membrane independently [Volynski et al., 2004]. Binding of latrotoxin brings the two chains into close proximity. A physiological ligand that would do the same is presently unknown.

Although ADHD is not considered to be a genetic disorder with classical Mendelian inheritance, we embarked in a study of mutational analysis of *LPHN3* in ADHD patients to assess the presence of genetic variants as either potential causative point mutations or, alternatively, predisposing genetic modifiers (risk alleles).

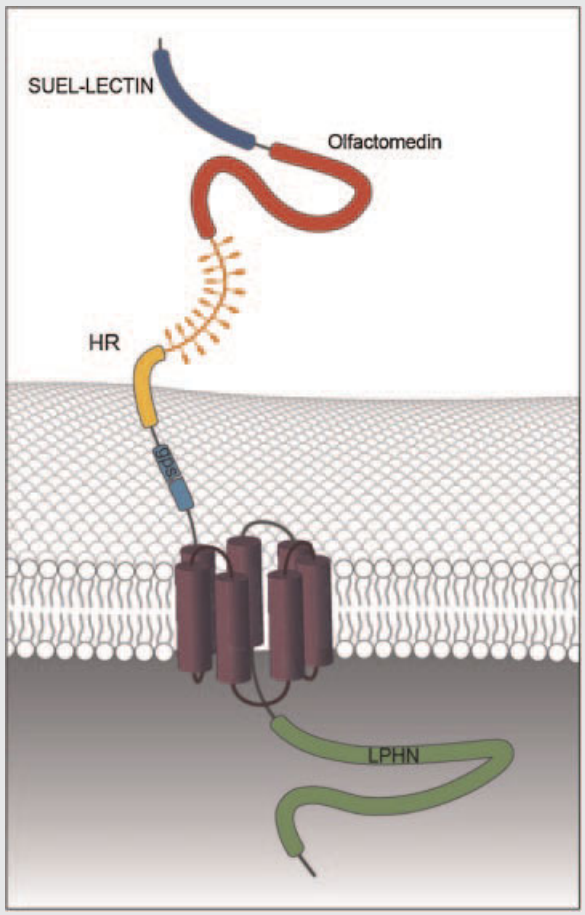

FIG. 1. Schematic representation of the two subunits of the LPHN3 protein, NTF (implicated in cell adhesion) and CTF (a 7TM GPCR). Although both chains are encoded by the same gene, they traffic to the cell membrane independently. A hypothetical ligand (analogous to LTX) may drive the association of the two chains [Arcos-Burgos et al., 2010].

METHODS

Patient Samples and SNP Detection

A total of 139 unrelated patients diagnosed with ADHD [based on DSMIVR criteria; see Palacio et al., 2004; Acosta et al., 2008; Arcos-Burgos et al., 2010] and 52 commercially available normal controls not screened for ADHD were studied. Genomic DNA was extracted from peripheral blood or transformed lymphoblast cell lines by standard methods. All patients with ADHD were recruited into an NHGRI IRB approved research protocol in accordance with their ethical guidelines and supervision.

Mutation detection was performed by PCR-based denaturing high performance liquid chromatograhy (dHPLC) analysis followed by direct sequencing. PCR amplification, dHPLC analysis WAVE™ and WAVEMAKER™ (Transgenomic, Omaha, NE), amplicon purification Qiagen PCR purification kit (Qiagen, Valencia, CA), and DNA sequencing Big Dye™ version 3.1 terminator cycle sequencing on an ABI 3100 (Applied Biosystems, Foster City, CA) instrument were performed according to the manufacturers instructions, essentially as previously described [Schimmenti et al., 2003]. dHPLC has a sensitivity and specificity of over 96% [Xiao and Oefner, 2001].

For this study we used the reference sequence NT_022778.15 from NCBI (which we will refer to as isoform 2—Swissprot Q9HAR2) to determine which exons to examine. This isoform has the greater number of exons (24 exons) and is the proper form based on human mutation nomenclature recommendations (www.hgvs.org/mutnomen). We did not study the 3′UTR region (corresponding to the end of isoform 1) in the USA population by dHPLC; however, the entire exon 24 had previously been sequenced

in nine individuals from the Paisa isolate from the beginning of exon 24 to the polyA addition site without detecting any variations (data not shown). PCR was performed using the primer pairs and conditions listed in Supplementary Table I. Briefly, amplification of genomic DNA was performed in 35 μl reaction volumes, using 60–100 ng of genomic DNA, 200 μM dNTP, 20 pmol of each primer, 1× PCR buffer (Invitrogen, Carlsbad, CA), 0.5× enhancer (Invitrogen), 1.5 mM $MgSO_4$ (Invitrogen), and 2.5 U of AmpliTaq (Applied Biosystems). All reactions were performed in a PTC-225 thermocycler (MJ Research, Waltham, MA). PCR cycling parameters were 95°C for 4 min, followed by 95°C for 30 sec, annealing temp for 30 sec, 72°C for 1 min, for 50 cycles, with a final step of 72°C for 7 min. After amplification, PCR products were denatured at 96°C for 1 min, followed by gradual reannealing to 65°C over a period of 30 min to form homo- and/or heteroduplexes. Products were automatically loaded on a DNA sep column and eluted according to manufacturer's instructions at a flow rate of 1.5 ml/min by mixing buffer A (0.1 mM TEAA) and buffer B (0.1 mM TEAA and 25% acetonitrile) with a Buffer B increase of 10%/min for 2.5 min. Samples were detected by a UV-C system. The oven temperature(s) for optimal heteroduplex separation under partial DNA denaturation were determined for each amplified fragment using the WAVEMaker software (version 4.1) and adjusted according to experience. Because the dHPLC approach is based on the differential retention of homo- and heteroduplex DNA fragments by ion-pair chromatography under conditions of partial heat denaturation, optimal discrimination of double-stranded combinations depends on the temperature at which partial denaturation of heteroduplexes occur. Most amplicons were composed of different melting domains, thus more than one elution temperature was needed to detect the variants. All samples were analyzed by visual inspection and consequently those with clear or suggestive variant peaks were sequenced using the same primers as had been used for PCR.

Isoform Confirmation

Primers flanking the predicted 3.9 kb open reading frame of *LPHN3* isoform 2 (NT_022778.15—Swissprot Q9HAR2) were designed to PCR amplify the transcripts from whole brain cDNA (Clontech, Mountain View, CA); internal primers were designed for bi-directional sequencing of the entire transcript. Isoforms 1 and 2 could be readily separated by agarose gel electrophoresis; visual inspection revealed approximately equal amounts of the two isoforms.

In Silico Analysis

We annotated *LPHN3* using the UCSC Genome Browser custom track capabilities. We included information derived from databases (such as expression information on mRNAs, ESTs, spliced ESTs from NCBI, UCSC, and EMBL; miRNA genes from AceView; miRNA binding sites from UCSC; variation information from dbSNP; domains and motifs from SwissPro, interPro, Pfam, and ProDom).

We then placed the variants in their biological context within our annotation and analyzed if they fell in the following: predicted domains, motifs, glycosylation and/or phosphorylation sites, splice sites (donor, branch point, acceptor), splice site regulators (*AG* nucleotide *E*xclusion *Z*ones: AGEZ; *E*xonic *S*plice-site *E*nhancers using *ESEfinder 2.0*) [Cartegni et al., 2003b], predicted 3′UTR regulatory elements (including miRNAs, miRNA binding sites) and conserved short sequence-stretches: Exact Plus [Antonellis et al., 2006], Vista [Frazer et al., 2004]. Swissprot Q9HAR2 was used as the reference sequence for variant nomenclature.

We performed signal peptide search using SignalP 3.0 [Bendtsen et al., 2004], Eukaryotic Linear Motif (ELM) analysis [Puntervoll et al., 2003] and a Kyte-Doolittle/Hopp-Woods hydropathy plot—as implemented in the Molecular Toolkit/Protein Hydrophobicity Plots (www.vivo.colostate.edu/molkit).

RESULTS

Detection of Sequence Variants

Twenty-four exons, including all intron–exon boundaries, were screened for heterozygous mutations in the USA population sample of ADHD patients and normal controls using dHPLC. Supplementary Table I summarizes the location and nature of the variants observed in this cohort, as well as the Paisa population (denoted by an asterisk). In total, we detected 21 variants out of which 14 have been reported (dbSNP build 130) and 7 are novel. As described in Figure 2, five variants predict non-synonymous aminoacid substitutions, eight are synonymous codon variations, and the remainder are intronic variants. Each sequence variant was analyzed for predicted effects on coding changes, splicing alterations, DNA binding sites, or microRNA binding sites (see Methods Section).

Coding Region Variants

We detected very limited sequence variation in the *LPHN3* gene in this study that would affect the coding structure of the component exons. However, we could easily detect the signature SNPs that distinguish the susceptibility/protective haplotypes in the USA population, as well as the Paisa and German cohorts (specifically: rs10434219, rs9312082, rs2305339, rs734644, rs1397547, and rs1397548; see Table I) indicating that the locus conferring ADHD susceptibility was clearly present in our patient sample set. Superimposed on these common haplotypes, we also detected less common variations that in some cases differed between the Paisa and USA populations. These variants were also evaluated for possible effects despite the fact that they are probably private variants that are more likely to be modifiers, rather than the disease causing mutation.

Multiple sequence alignment analysis of all five missense changes is presented in Figure 3. We carefully examined all coding region changes, irrespective of allele frequency, for their likely effects. No missense changes were detected in known critical domains of the protein. Although an alanine at position 247 is present in most Lphn3 orthologs, this residue is not conserved among the Lphn family. This sequence variant was not detected in the USA population and therefore the p.A247S as well as p.D615N changes may represent either Paisa-specific variants, or simply rare variants in the USA population. Similarly, not only is the p.L928V change

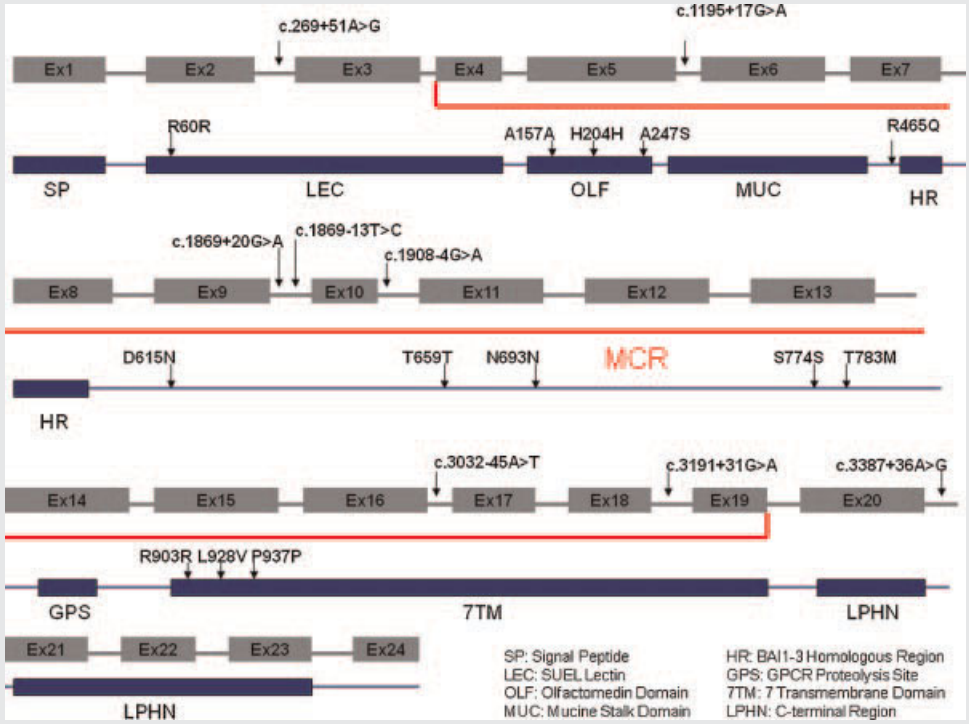

FIG. 2. Schematic diagram of the *LPHN3* exons and their coding potential. The exons (1–24 are depicted as gray rectangles; not drawn to scale) and the relevant parts of the protein domains are in blue, beneath the exons encoding each motif. Note that the minimal critical region (MCR), shown in red, was determined by classical linkage methods in the Paisa population by cross-over events in large families. All sequence variants identified are shown in black (non-coding at gene level above and coding at protein level below).

likely to be biochemically benign, but the minor allele frequency (estimated to be 0.002 based on HapMap data) is so uncommon that it is difficult to attribute a major effect to this missense change. We also note that Leu > Val changes are among the top 15 most frequent aminoacid substitutions (rank position 7) [Ladunga and Smith, 1997]. The p.R465S change is present in both patient and control samples and is located within a linker domain of unknown function and extremely poor conservation. Similarly, the p.T783M variant is located within a poorly conserved motif that has different residues for Lphn1, 2, and 3.

One of the synonymous variants, c.612C>T (p.H204H), lies within a putative exonic splice site enhancer (ESE) predicted to bind SRp40 ($P = 6.32$) and SF2/ASF ($P = 5.26$) (*ESEfinder 2.0*, Cartegni et al. [2003a]). This ESE is in exon 5 that codes for the olfactomedin domain. However, although we can detect a low frequency of exon 4 skipping (~5%) in humans (data not shown; similar to the splicing variants observed in the bovine Lphn family) [Matsushita et al., 1999], the exon 5 splice acceptor is consistently utilized in all transcripts described to date.

Of the 8 intronic variants, two fall in AG exclusion zones (AGEZ) before exons 10 and 11 (IVS9-13T>C and IVS10-4G>A respectively) [Gooding et al., 2006]. Although we have shown that there is evidence of exon 10 being spliced out in isoform 1 of *LPHN3*, neither of these variants would be predicted to affect this common splicing difference.

Isoform Confirmation

We experimentally confirmed the existence of isoform 2 (3.9 kb)—considered to be the reference sequence—containing all 24 exons, and isoform 1 (4.5 kb) which is missing exons 10, 19, and 23 (see Figs. 2 and 4). The absence of exon 23 produces a frameshift that explains the additional 229 aminoacids in the COOH terminal of isoform 1. Examples of this alternative splicing have been well described in the rat and bovine Lphn family and have been suggested to be a general phenomenon in these genes [Sugita et al., 1998]. None of the alternatively spliced exons correspond to known critical domains in the protein (e.g., exons 4, 10, and 19 are small exons that maintain the reading frame).

Isoform Comparison

We annotated the LPHN3 protein by searching for conserved and predicted domains (Figs. 2 and 3) as well as protein motifs in order to analyze the sequence variations. As depicted in Figure 4, of the 11 predicted interaction motifs in isoform 1 (possibly implicated in intracellular signaling due to their location) six are also found in isoform 2. Despite these similarities, there are also unique features attributable to each isoform. In particular, the presence or absence of exon 19 might affect the docking of G proteins or other signaling proteins to the intracellular loop III. Although the functional role of the LPHN domain(s) are poorly characterized, due to the

TABLE I. Sequence Variations in *LPHN3*

Variant	dbSNP	Nucleotide	AA	$Obs_{HET}/Pred_{HET}$	HW_{pval}	Minor, AF	Major, A	HapMap samples	Major, AF (patient/control)
c.180G>A	rs34586911	62281835	R60R*	0.251/0.252	0.494	0.148	A	NA	P
c.269 +51A>G	rs2172802	62281975	Intron*	ND	ND	ND	A	NA	P
c.471G>A	—	62427314	A157A	0.478/0.473	0.392	0.385	G	NA	P/C (1/2)
c.612C>T	rs10434219	62427455	H204H	0.374/0.401	0.405	0.277	T	0.784	P/C (111/50)
c.839G>T	—	62427582	A247S*	ND	ND	ND	G	NA	P
c.1195 +17G>A	rs9312082	62428055	Intron	0.374/0.401	0.405	0.277	A	0.824	P/C (61/31)
c.1394G>A	—	62587257	R465S	0.177/0.168	0.574	0.093	G	NA	P/C (5/4)
c.1670G>A	—	62604203	D615N*	ND	ND	ND	G	NA	P
c.1869 +20G>A	rs12648576	62604249	Intron	ND	NA	NA	G	NA	P/C (62/31)
c.1869 −13T>C	—	62607190	Intron	176/176[a]	ND	ND	T	NA	P/C (139/52)
c.1908 −4G>A	rs2305339	62629320	Intron	0.462/0.429	0.810	0.312	A	0.680	P/C (52/26)
c.1977G>A	rs10013832	62629392	T659T*	ND	ND	ND	G	0.984	P
c.2079T>C	rs734644	62629494	N693N	0.459/0.427	0.820	0.309	C	0.687	P/C (52/26)
c.2322T>C	rs34246405	62641504	S774S	2/178[a]	ND	ND	T	NA	P (2)
c.2348C>T	—	62641530	T783M*	0.324/0.353	1	0.229	C	NA	P
c.2709G>C	rs1397547	62674154	R903R	0.177/0.168	0.574	0.093	C	0.821	P/C (51/29)
c.2782C>G	rs12509110	62674227	L928V	ND	ND	ND	C	0.998	P/C (2/1)
c.2811A>G	rs1397548	62674256	P937P	0.466/0.456	0.753	0.351	A	0.590	P/C (50/51)
c.3032 −45A>T	rs56038622	62690730	Intron	0.006/0.008	1	0.004	A	NA	P (10)
c.3191 +31G>A	rs73823293	62692780	Intron	0.308/0.314	0.0832	0.195	A	NA	P/C (1/2)
c.3387 +36A>G	—	62726130	Intron	1/167[a]	ND	ND	A	NA	P (1)

Variants (apparently) seen only in Paisa study are indicated with an asterisk.
HW, Hardy–Weinberg; AF, allele frequency; AA, amino acid; P, patients; C, controls.[a]Heterozygotes were determined using dHPLC.

frameshift in exon 24 the properties of i1 and i2 are predicted to be quite distinct. For example, the VTSL sequence at the extreme COOH terminus of i1 is a motif that binds to members of the SHANK family. No such sequence is present in i2. Further studies of these isoforms may substantiate or refute the importance of these structural differences.

DISCUSSION

Due to the lack of previous detailed mutational studies of the *LPHN3* gene and the limited information on SNPs reported in this gene, our study confirms the high prevalence of 14 previously described as well as 7 novel sequence variants showing that inactivating mutations in *LPHN3* are unlikely causative of ADHD.

The initial purpose of this study was to identify mutations in *LPHN3* in the USA population that would confirm its role as a candidate gene for ADHD. As was observed in the Paisa population, we detect limited sequence variation in the coding region or splicing junctions. None of the variants that have been detected are predicted to dramatically change the structure of the LPHN3 proteins encoded by either the susceptibility or protective haplotypes.

A secondary goal was to detect variants that would be useful for fine-mapping of the haplotype to refine the locus. This aspect of the study was more informative leading to the definition of a conserved ancestral haplotype that is highly associated with ADHD in multiple populations [Arcos-Burgos et al., 2010]. Thus, the founder effect we suspected at the outset of this study applies not only to the Paisa population, but presumably also to all human populations.

Interestingly, a phylogenetic reconstruction of the haplotypes suggests that the protective allele evolved from the susceptibility allele [see Fig. 5 and Arcos-Burgos et al., 2010]. Unlike the more typical genetic situation, where a normal gene loses some essential function resulting in disease, this argues for a more subtle alteration in gene function. It is difficult to account for ADHD as a loss of function phenotype based on this observation. It is far more likely that a subtle genetic change in *LPHN3* function will ultimately predispose for the disorder and that the lack of dramatic mutations might not be entirely unexpected.

Our mutational study of *LPHN3* has, thus far, failed to detect a molecular difference that would explain these different phenotypes. We conclude that *LPHN3* is not a significant target for new mutation and that screening of the gene for coding region mutations is largely uninformative. Risk estimates for ADHD can instead be inferred from the haplotypes of affected individuals without resorting to attributing the disorder to *LPHN3* mutations per se.

Several scenarios may explain why no obviously causative variants were found in this screen: (1) *LPHN3* is actually not the ADHD susceptibility factor; instead, some other genetic element contained within the MCR is responsible; (2) the genetic lesion has been detected (e.g., a common SNP within the defined haplotype) but its functional effects are sufficiently subtle that we lack the ability to recognize their significance; (3) the genetic lesion affects *LPHN3*, but lies within a non-coding region, thus remains undetected by our analysis; such a variant might affect brain-specific alternative splicing and/or the spatial and temporal expression of the gene or its various isoforms.

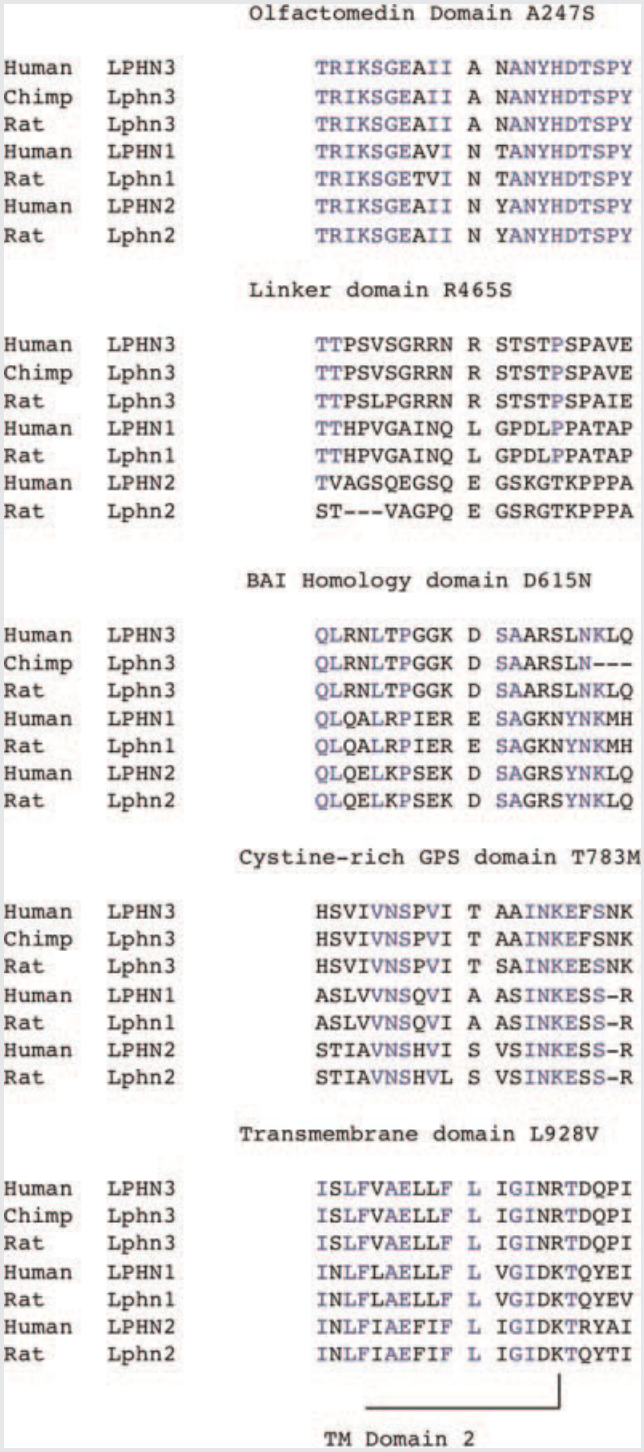

FIG. 3. Multiple sequence alignments of the missense variants. Centered on the missense variant, the flanking primary sequence context and their conservation is presented. An artificial space is included to aid the identification of the variant position. Residues present in at least 5 of the 7 proteins are shown in blue. Note that comparison of the human, chimp, and rat Lphn3 proteins at these motifs reveals few differences. However, when comparisons are made across the rat and human LPHN family, the residues subject to variation are poorly conserved. The exception is L928V within the transmembrane domain 2.

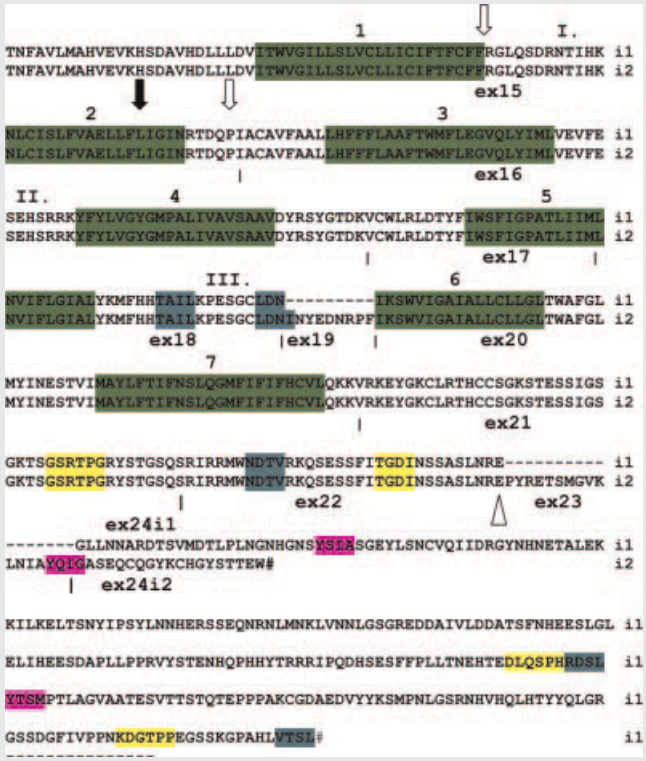

FIG. 4. Alignment of the CTF fragments of isoforms, i1 and i2, emphasizing the effect of alternative splicing on the COOH terminus and the positions of the 11 predicted interaction motifs: Transmembrane domains 1–7 (green); predicted PDZ interaction motifs (light blue); potential phosphorylation dependent interaction motifs (yellow, WW and FHA domains); intracellular loops I–III; and predicted Src domain interaction motif (purple) Intracellular loop III differs between the two isoforms due to the alternatively spliced exon 19 (in frame). The two PDZ domain interaction motifs might not be significantly affected by this difference. The conserved 66 aminoacid stretch of the LPHN domain harbors 3 interaction motifs. Isoform 1 has a longer C-terminal tail due to the out of frame alternative splicing of exon 23, thus five of the motifs are isoform 1 specific. D marks the position of a cryptic exon between exon 22 and 23 that exists in the human genome as a potentially functional exon, similar to the rat gene [Sugita et al., 1998]; however, we have no experimental evidence for its common usage in humans. Synonymous codon variants (white arrows) and non-synonymous changes (black arrow) are shown.

If *LPHN3* is not the gene, another element contained within the defined critical region—unidentified due to limitations of our screening or our incomplete annotation—could be the cause. This scenario cannot be ruled out until the genetic lesion is ultimately identified and fully characterized. However, this is also the least likely explanation due to the absence of detectable alternative candidate genetic elements within the MCR, and the strong correlation between the spatial and temporal expression of the *LPHN3* gene in regions of the brain implicated in ADHD.

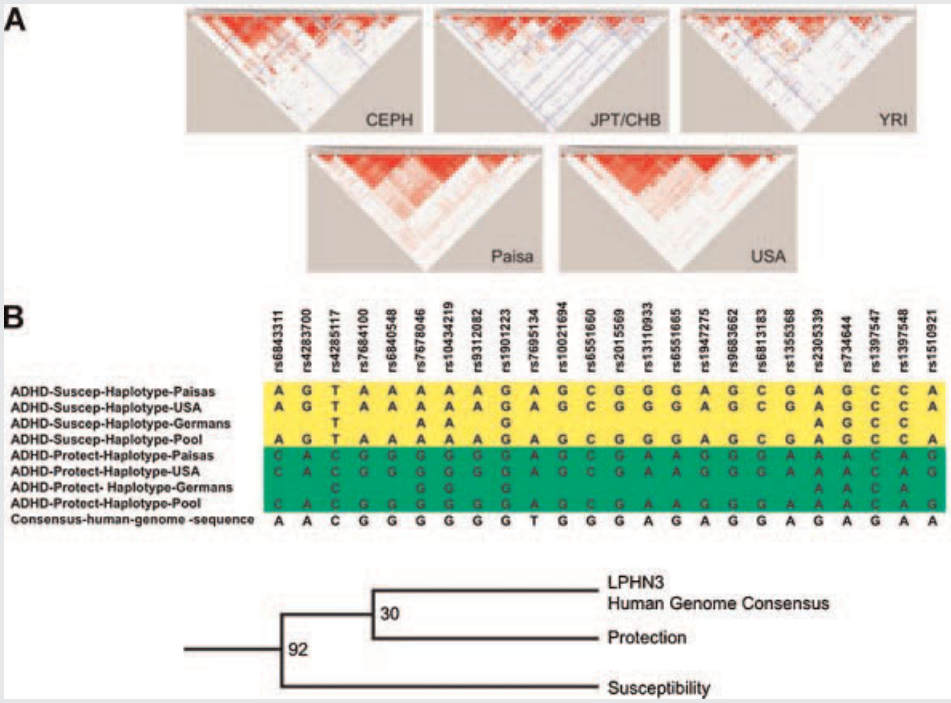

FIG. 5. A: The extent of linkage disequilibrium (LD) detected in multiple populations is shown. B: Alignments of the haplotypes determined those versions detected among human populations supports the evolutionary history of the *LPHN3* gene among human lineages as the susceptibility haplotype preceding that of either the protective or consensus haplotype and being more similar to that of primates [Arcos-Burgos, unpublished data; see also Arcos-Burgos et al., 2010].

The second explanation is that the pathogenetic change has been identified, but remains unrecognized by our analysis. All of the SNPs used to define the various haplotypes were analyzed for their potential effects (data not shown). We have made every effort to analyze all of the known and novel variants using a wide range of in silico tools. However, we cannot exclude this scenario in the absence of functional studies. Since all of the functional studies of the Lphn protein family have depended on the ability to bind LTX, a feature not shared by Lphn3, new strategies to develop functional tests of variant LPHN3 proteins will need to be developed.

The most plausible scenario implicates *LPHN3* as the responsible gene, with qualitative or quantitative expression differences as the cause of the disorder. Our mutational screen was targeted to the 2% of the MCR devoted to coding exons and canonical splice junctions. In addition, there are likely to be hundreds of variants in the MCR that have yet to be identified or characterized. We can reasonably infer that promotor and 3′UTR of the *LPHN3* gene are outside of the MCR and therefore a tissue-specific enhancer seems most likely in this scenario. The assessment of this possibility will require determining the regulatory sequences and factors that control the level of expression of this gene encoded within the remaining 98% of the MCR sequence. Efforts to sequence this region are underway. A major challenge will be to identify physiologically meaningful tissue culture systems that recapitulate the known spatial and temporal brain-specific expression of *LPHN3*.

Finally, structural isoforms (and/or their relative proportions) from differentially spliced transcripts could account for differences between susceptibility and protective alleles. We were able to experimentally confirm the existence of two isoforms (1 and 2) from human brain cDNA. These, or hypothetical ADHD-specific isoforms, might also be contributing to the behavioral trait despite a normal overall gene organization and structure. Isoforms 1 and 2 differ significantly in the predicted functions of the COOH-terminal tail. These different forms might underlie differences in the postulated signal transduction functions predicted for this region. The different number of interaction motifs between the isoforms tends to support this hypothesis. Novel assays will need to be developed to determine the molecular properties of the various isoforms and their signaling functions. A distinct impediment to the development of such assays stems from the absence of a known physiological ligand for LPHN3. A further difficulty is the lack of significant amounts of biologically relevant tissue (e.g., brain or region-specific brain tissue) from affected individuals to quantify differences in the expression of various isoforms.

Application of modern genetic tools has revolutionized the analysis of complex traits, including behavioral genetics. The *LPHN3* locus is the first of several genes by linkage analysis that will ultimately be shown to confer susceptibility to ADHD. Our analysis is consistent with a general trend in behavioral genetics. The susceptibility traits are relatively common in the general population. Obvious mutations are uncommon and difficult to identify; in particular, when based on previous paradigms of dramatic changes in gene expression or function associated with typical Mendelian disorders. Despite quite precise localization of the traits to specific

genetic regions, the responsible lesions are likely to be subtle changes in expression or function. Models of loss of function, or gain of function, may be inadequate to capture the genetic subtlety of these behavioral traits. For example, the locus on chromosome 4q13.2 encodes three different traits: susceptibility, normal and protective. Furthermore, the latter two presumably evolved from the ''disease locus.'' All forms appear to be encoded by a largely normal set of exons. Considerable advances in functional genomics and in the understanding of the complex regulation of gene expression will be required to extend our understanding of these traits at the molecular level.

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