Rare exonic deletions implicate the synaptic organizer Gephyrin (*GPHN*) in risk for autism, schizophrenia and seizures

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The *GPHN* gene codes for gephyrin, a key scaffolding protein in the neuronal postsynaptic membrane, responsible for the clustering and localization of glycine and GABA receptors at inhibitory synapses. Gephyrin has well-established functional links with several synaptic proteins that have been implicated in genetic risk for neurodevelopmental disorders such as autism spectrum disorder (ASD), schizophrenia and epilepsy including the neuroligins (NLGN2, NLGN4), the neurexins (NRXN1, NRXN2, NRXN3) and collybistin (ARHGEF9). Moreover, temporal lobe epilepsy has been linked to abnormally spliced GPHN mRNA lacking exons encoding the G-domain of the gephyrin protein, potentially arising due to cellular stress associated with epileptogenesis such as temperature and alkalosis. Here, we present clinical and genomic characterization of six unrelated subjects, with a range of neurodevelopmental diagnoses including ASD, schizophrenia or seizures, who possess rare *de novo* or inherited hemizygous microdeletions overlapping exons of *GPHN* at chromosome 14q23.3. The region of common overlap across the deletions encompasses

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exons 3–5, corresponding to the G-domain of the gephyrin protein. These findings, together with previous reports of homozygous *GPHN* mutations in connection with autosomal recessive molybdenum cofactor deficiency, will aid in clinical genetic interpretation of the *GPHN* mutation spectrum. Our data also add to the accumulating evidence implicating neuronal synaptic gene products as key molecular factors underlying the etiologies of a diverse range of neurodevelopmental conditions.

INTRODUCTION

GPHN encodes the protein gephyrin, a key scaffolding molecule in the neuronal postsynaptic membrane at inhibitory synapses (1,2). After its initial discovery by affinity chromatography experiments as co-purifying with the postsynaptic inhibitory glycine receptor, the protein was named 'gephyrin' from the Greek term for 'bridge', in line with its proposed activity as an intermediary molecule connecting neurotransmitter receptors to the postsynaptic microtubule cytoskeleton (3). Follow-up experiments demonstrated that the clustering and postsynaptic localization of both major types of inhibitory receptors (glycinergic and GABAergic) depend on direct protein–protein interactions of gephyrin with subunits, specifically of the glycine receptor (4) and the GABA$_A$ receptor (5). Gephyrin clustering is essential for GABAergic synapse stability and aberrant *GPHN* expression has been observed in the hippocampus and adjacent neocortex of both patients with temporal lobe epilepsy (TLE) and in a rat model of TLE (6). These findings are in line with known deficits in GABAergic synaptic transmission in TLE (7). One potential molecular explanation for gephyrin's involvement in the epileptic hippocampus could be abnormally spliced forms of RNA produced by cellular stress-induced exon skipping, which can lead to altered or inactive protein (8). However, there have been no reports of genomic *GPHN* mutations in patients with epilepsy.

In addition to its potential involvement in epilepsy, gephyrin has functional links with several synaptic proteins, mutations of which have been reported in a range of neurodevelopmental disorders (Fig. 1 and Supplementary Material, Table S1). Together with collybistin, gephyrin forms complexes with the postsynaptic neuroligin proteins NLGN2 (9) and NLGN4 (10). Gephyrin's specific postsynapatic localization and clustering are mediated by members of the presynaptic neurexin protein family (11). This complex interplay of gephyrin with the neurexins and neuroligins not only attests to its importance in the proper formation and function of synapses, but is also of particular interest given the substantial evidence implicating these trans-synaptic adhesion molecules in genetic risk for a range of neurodevelopmental conditions (12,13), including autism spectrum disorder (ASD) (14–18), schizophrenia (16,19,20) and epilepsy (21). Of specific relevance to gephyrin, rare point mutations in *NLGN2* (20) and *NLGN4* (14) have been identified in patients with schizophrenia and ASD, respectively, while mutations in the collybistin gene (*ARHGEF9*) have been reported in cases with intellectual disability (ID) and epilepsy (22–24). Frameshift mutations truncating *NRXN1* and *NRXN2* in patients with schizophrenia and ASD, respectively, were also observed to abolish the ability of these proteins to induce gephyrin clustering at dendrite contact sites when synaptogenic activity was tested in neuron co-culture assays (17). The SHANK proteins, which act as postsynaptic neuronal

scaffolding proteins in excitatory synapses (a role analogous to that performed by gephyrin in inhibitory synapses), have been implicated by rare mutations in genetic risk for ASD (25–27), schizophrenia (28) and epilepsy (29).

Given the strong evidence supporting *GPHN* as a candidate molecule for involvement in neurodevelopmental disorders, we examined the gene for copy number variations (CNVs) and point mutations in patients from ASD, schizophrenia and seizure disorder case cohorts.

RESULTS

Detection and inheritance testing of rare microdeletions at the GPHN locus

We screened the *GPHN* locus for CNVs using high resolution microarray data from 5384 individuals from ASD, schizophrenia and seizure disorder patient cohorts (Materials and Methods). In five of these patients, we identified hemizygous deletions at 14q23.3 interrupting multiple exons of *GPHN* (Fig. 2 and Table 1). There was also an earlier report of a 183 kb deletion affecting six exons of *GPHN* in a single individual from a cohort of 3391 schizophrenia patients studied by the International Schizophrenia Consortium (30). We were able to obtain additional clinical details for this patient and his family (Family 6 in Figs 2 and 3). Independent experimental validation of the array calls was performed using quantitative PCR (qPCR) or fluorescence *in situ* hybridization (FISH). None of the CNVs detected at other loci in probands from the six families were predicted to be of clinical significance when classified based on American College of Medical Genetics guidelines for interpretation of CNVs (31) (Supplementary Material, Tables S2–S7). Parental testing for CNV status at the *GPHN* locus revealed that the deletions arose *de novo* in three of the probands (Families 1, 3 and 5 in Fig. 3). The deletion in the proband from Family 2 with ASD was inherited from his father, who was reported to have sub-clinical socialization deficits, but no formal diagnosis of ASD. The deletion was not present in the three sisters of the proband. DNA samples were not available from the parents in Family 4 for inheritance testing. The deletion in the proband from Family 6 was inherited from his mother, who was apparently unaffected. DNA samples were not available from the maternal grandmother of the proband, who was reported to have schizophrenia.

Frequency comparison of GPHN deletions in cases and controls

Deletions affecting exons of *GPHN* are extremely rare in the general population. Of the 27 019 population-based control individuals examined for CNVs at this locus (Supplementary Material, Table S8), only three (two from the Study of Addiction: Genetics and Environment (SAGE) control data set and
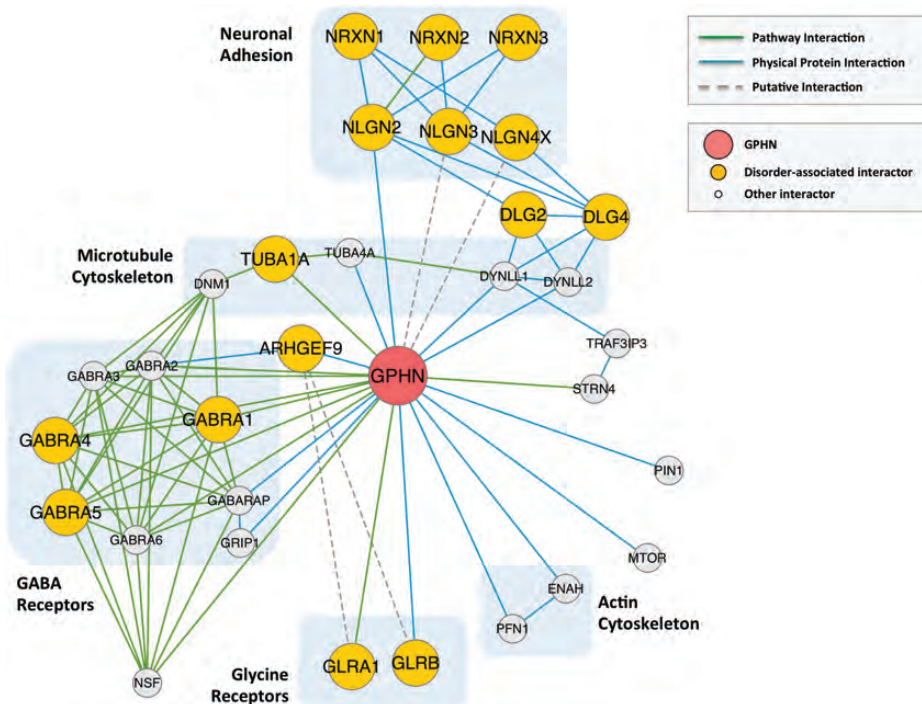
Figure 1. Functional interaction network of Gephyrin. The top 50 interactors of GPHN according to the STRING and GeneMANIA databases were manually filtered and integrated to define the bona-fide GPHN interaction network; the majority of direct interactors were retained. Gephyrin's interactions with other proteins have been reviewed by Tretter *et al.* (1) and Fritschy *et al.* (2). For full names of gene symbols in the figure, see Supplementary Material, Table S1.

one from the International Schizophrenia Consortium control data set) possessed exonic *GPHN* deletions. CNVs present in control individuals might be indicative of variable expression of *GPHN* deletions, lack of rigorous assessment of neuropsychiatric phenotype in the controls, or false–positive calls since DNA was not available from the two SAGE control individuals for experimental validation. Taken together, the frequency of experimentally validated exonic deletions at the *GPHN* locus in cases is significantly greater than that in controls (6/8775 cases versus 3/27 019 controls, respectively; Fisher's exact test two-sided $P = 0.009$). There were no exonic deletions reported at the *GPHN* locus in the Database of Genomic Variants (32).

Clinical features of individuals with exonic GPHN deletions

Family 1 (proband with ASD diagnosis; 357 kb de novo deletion)
The female proband of European ancestry was conceived naturally to a non-consanguineous 35-year-old (yro) mother and 33 yro father. The pregnancy was uncomplicated and the child was delivered at 38 weeks of gestation with a birth weight of 2810 g and length of 47 cm. There are no reported medical or neuropsychiatric conditions in her parents and two older siblings.

During early development, she was noted to be a 'calm' child, with limited movement when laid down. She began walking at 12 months but otherwise had slow motor development and gait issues, which prompted ergotherapy. She also had language delay with no speech at 2 yro, followed by gradual speech development by age 4. She had episodes of echolalia. Up to 6 years of age, she continued to desire swaddling and had hypersensitivity to light and sound associated with self-injury (head banging, tearing hair). Assessment by a psychologist at 13 yro, which included the Autism Diagnostic Observation Schedule (ADOS) and Autism Diagnostic Interview—Revised (ADI-R), resulted in a diagnosis of high-functioning ASD.

She attended kindergarten for 2 years with an early intervention program teacher, followed by regular elementary and secondary school with the aid of an education assistance worker. She completed a secondary school degree with the exception of mathematics.

Upon examination by a pediatrician at 2 yro and again at 4 yro, no dysmorphology was noted. Chromosomal microarray (Affymetrix 6.0) indicated that she had a deletion at cytoband 14q23.3 (chr14:66,274,499–66,631,750 [hg18]), which was not present in the parents (Figs 2 and 3, Family 1).

Family 2 (proband with ASD diagnosis; 319 kb paternally inherited deletion)
The male proband was conceived naturally to a 28 yro mother and 30 yro father. The proband was born vaginally at 35 weeks gestation with a birth weight of 3133 g and length of 50.8 cm. He was treated for respiratory distress in hospital for 9 days following delivery. Coarctation of the aorta and bicuspid aortic valve were noted on echocardiography performed upon re-admittance to the hospital at 18 days of life, secondary to continued respiratory distress. He had an end-to-end anastomosis procedure at 1 month followed by a coarctation repair with patch annuloplasty at 4 months. Post-treatment echocardiograms have been normal and he remains asymptomatic.
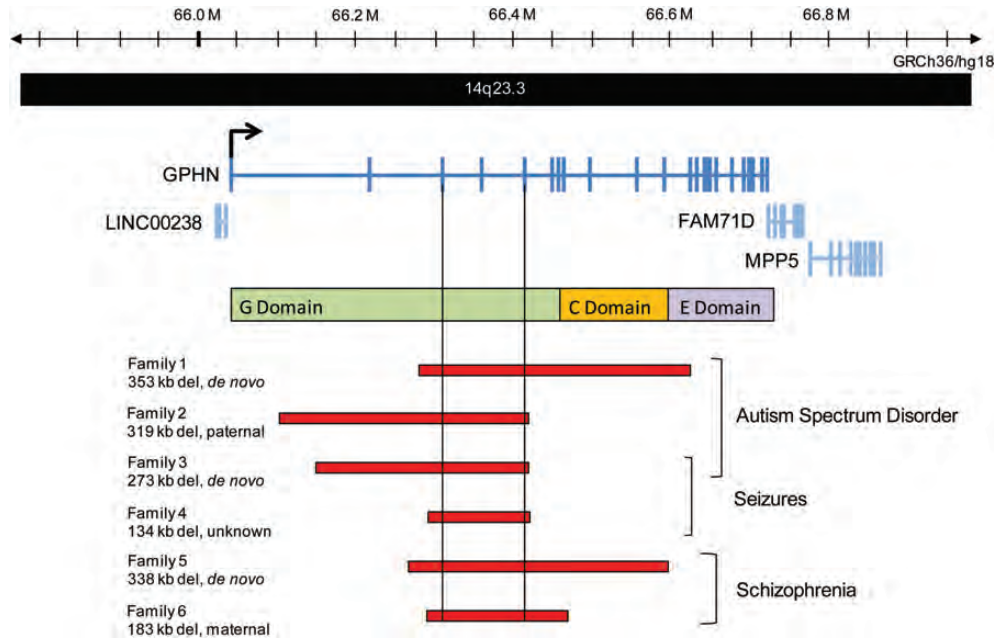
Figure 2. Genomic locations of *GPHN* deletions described in this study. Red bars represent the hemizygous exonic *GPHN* deletions at 14q23.3 detected in the index probands of the six families presented in this paper. Information about transcript isoforms and genomic co-ordinates corresponds to human genome Build 36 (hg18). The common region of overlap of the six deletions encompasses exons 3–5, which correspond to the G-domain of the gephyrin protein. Coordinates (hg18) of the deletions observed in control individuals are chr14:66,055,080–66,882,227, chr14:66,153,552–66,267,833 and chr14:66,287,336–66,355,274.

Table 1. Genetic and phenotypic details of probands with GPHN deletions

Family (proband sex)	Genotype *GPHN* deletion extent, hg18 (size)	Exons affected	Inheritance	Phenotype Diagnosis	Dysmorphic features	Speech delay	Motor delay	Other
Family 1 (female)	chr14:66,274,499–66,631,750 (357 kb)	3–12	*De novo*	ASD	N	Y	Y	–
Family 2 (male)	chr14:66,102,556–66,421,440 (319 kb)	2–5	Paternal	ASD	N	N	N	Coarctation of aorta
Family 3 (male)	chr14:66,148,602–66,421,440 (273 kb)	2–5	*De novo*	ASD and seizures	N/A	Y	N	Intellectual disability
Family 4 (male)	chr14:66,287,215–66,421,440 (134 kb)	3–5	Unknown	Seizures	N/A	N/A	N/A	–
Family 5 (male)	chr14:66,267,488–66,605,185 (338 kb)	3-11	*De novo*	Schizophrenia	N	N	N	–
Family 6 (male)	chr14:66,287,324–66,470,381 (183 kb)	3–8	Maternal	Schizophrenia	N/A	N	N	Learning difficulties

ASD, autism spectrum disorder; N/A, not assessed.

He exhibited mild global developmental delay early in life, with walking at 16 months and speech emergence at 15–16 months. By the age of 3 years, he could speak in two word sentences. At age 6, he was diagnosed with ASD by a child psychologist. In particular, he had difficulties with socialization and repetitive behaviors (specifically throat clearing and tics). The proband experienced academic difficulties and required a modified program in preschool and kindergarten. By 9 years of age, he had graduated from this program and received no additional special education.

Upon examination by a clinical geneticist at 6 years 8 months, no significant dysmorphology was noted. Chromosomal microarray (Agilent 44K) indicated he had a deletion at cytoband 14q23.3 (chr14:66,102,556–66,421,440 [hg18]), which was paternally inherited, but not present in his siblings (Figs 2 and 3, Family 2).

The proband is the third of four children of non-consanguineous parents (Fig. 3, Family 2). He has one sister with anxiety, another sister with speech delay and hyperactivity, a third sister with head-banging behaviors and a maternal half-brother with bipolar disorder. His mother has been diagnosed with depression and anxiety and was noted to have speech difficulties in school. In the maternal extended family, there are individuals with additional psychological concerns. The father is apparently healthy with normal intelligence, but has some challenges with socialization. There is significant psychiatric illness in the paternal extended family.

Family 3 (proband with diagnosis of ASD and seizures; 273 kb de novo *deletion)*
The male proband was conceived naturally to a 36 yro mother and 40 yro father. The pregnancy was uncomplicated and the proband was delivered naturally with a birth weight of 3714 g and length of 48 cm. He crawled at 7 months and walked at 15 months. Developmental delay was first noticed by parents at age 2, 2 months after a serious encephalitic illness that required hospitalization for 3 days. Cyclical seizures began at the time of this illness and recurred until age 6 and
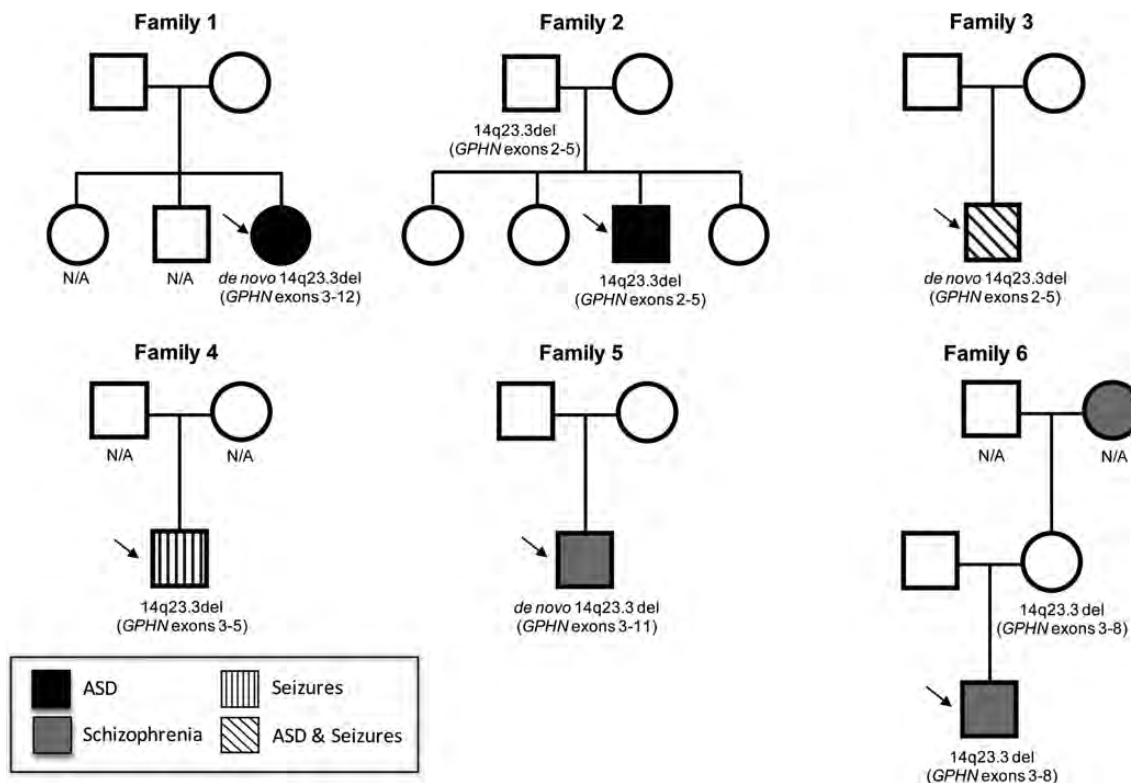
Figure 3. Pedigrees of probands with *GPHN* deletions. Squares and circles denote males and females, respectively. Arrow indicates the index proband in each family. 'N/A' represents individuals from whom DNA was not available for testing. All other individuals were assayed for deletions at the *GPHN* locus using microarray, FISH or qPCR.

subsequently he remained seizure free without anti-convulsant treatment. He exhibited speech and language delay and did not talk until approximately age 4. He was diagnosed with ASD (pervasive developmental disorder) at age 4 by a child psychologist on the basis of behavioral assessments provided by parents, teachers and therapists. He was diagnosed with ID a few years later by a school psychologist. He continues to have behavioral issues including anxiety, OCD, tics and impulsive, sometimes aggressive behaviors. He talks to himself frequently, and has trouble concentrating and sitting still.

The proband attended early intervention therapy from age 3–6. He has received special education and/or tutoring throughout school as well as speech therapy and occupational therapy. He is currently in tenth grade. He has had physical therapy in the past for toe walking.

The proband tested negative for fragile X and had normal results from magnetic resonance imaging and computed tomography brain scans and electroencephalography. At 15 years of age, he was referred for further genetic testing. Chromosomal microarray (Agilent 180K) indicated he had a deletion at cytoband 14q23.3 (chr14:66,148,602–66,421,440 [hg18]) (Fig. 2, Family 3). Neither parent had the deletion when tested by FISH (Fig. 3, Family 3). After detection of the *de novo* hemizygous exonic deletion in the proband, clinical metabolic testing for molybdenum cofactor (MoCo) deficiency was performed and the results were negative (Supplementary Material, Table S9). The testing measured urine levels of uric acid, xanthine, hypoxanthine and uracil and serum levels of molybdenum and uric acid.

Family 4 (proband with diagnosis of seizures; 134 kb deletion)
The male proband was examined at 5 years of age for seizures of unknown type or duration. Clinical chromosomal microarray (Agilent 44K) indicated he had a deletion at cytoband 14q23.3 (chr14:66,287,215–66,421,440 [hg18]) (Fig. 2, Family 4). This individual was lost to follow-up and detailed clinical information was not obtained. Samples from the parents were not available for testing (Fig. 3, Family 4).

Family 5 (proband with schizophrenia diagnosis; 338 kb de novo deletion)
The male proband was conceived naturally to non-consanguineous parents, a 29 yro mother and 36 yro father of European ancestry. There was no evidence of developmental delay or autistic features. The proband completed tenth grade with no reported difficulties, leaving school at age 17 years to work.

At 23 years of age, he began to have increasing anxiety, preoccupation and paranoia that responded well to a few months of treatment with chlorpromazine. At age 24 years, he was admitted to a hospital for schizophrenia. Neuropsychological testing using the Wechsler Adult Intelligence Scale (WAIS) revealed a Full Scale IQ of 92. He improved with electroconvulsive therapy, and was discharged after 2 months. Following two additional hospitalizations, he has been relatively stable on a standard antipsychotic medication regimen with an adjuvant antidepressant for many years. A detailed psychiatric assessment, including use of a modified version of Structured Clinical Interviews for DSM-III-R for Axis I disorders,

confirmed a research diagnosis of chronic schizophrenia (33,34). He has no history of seizures and there is no known history of neuropsychiatric illness or seizures in his parents or siblings. There is a significant history of schizophrenia in the paternal extended family.

There was no significant dysmorphology noted on examination as an adult by a psychiatric geneticist. Research chromosomal microarray (Affymetrix 6.0) indicated he had a deletion at cytoband 14q23.3 (chr14:66,267,488–66,605,185 [hg18]) (Fig. 2, Family 5). Neither parent had the deletion when tested by Affymetrix 6.0 microarray and qPCR (Fig. 3, Family 5).

Family 6 (proband with schizophrenia diagnosis; 183 kb maternally inherited deletion)
The male proband of European ancestry was recruited into the International Schizophrenia Consortium (ISC) study (30) at age 26 yro. He had normal early milestones. During his schooling, he had poor marks and left early after 8 years of education. The first onset of psychiatric symptoms started at 21 years with changes in his behavior, 3 months before he attended the National Service. After finishing the service, he appeared withdrawn, talked little and claimed that 'things were different' and that his genitals were changing. At age 22, he was seen by a psychiatrist after reporting he heard voices laughing at him and people were looking at him. He believed that he was being poisoned. He was subsequently treated with long-acting injectable antipsychotic medication for the 3 years prior to recruitment into the ISC study. He remained in a chronic condition, with similar delusions, anhedonia (loss of interest in pleasurable activities) and severe lethargy.

Chromosomal microarray testing (Affymetrix 6.0) as part of the ISC study indicated that he had a deletion at cytoband 14q23.3 (chr14:66,287,324–66,470,381 [hg18]) (Fig. 2, Family 6). Microarray testing showed the deletion to be present in the mother, who was reported to be unaffected (Fig. 3, Family 6). DNA was not available for testing from the proband's maternal grandmother, who reportedly also had schizophrenia.

GPHN sequencing and mutation screening

To search for smaller sequence-level point or insertion/deletion (indel) mutations in *GPHN*, we examined all coding exons and intron–exon splice sites of the gene in 558 unrelated ASD and 95 unrelated schizophrenia cases using Sanger and next-generation exome sequencing approaches. Missense coding variants were identified at six positions (Supplementary Material, Tables S10 and S11), three of which (c.2041(A>C) [p.Gln307Pro], c.2853(G>A) [p.Gly578Ser] and c.3210(G>A) [p.Asp697Asn]) were not found in the NCBI database of known SNPs (dbSNP build 137). Each of these variants occurred in single ASD cases. The glutamine (hg18 chr14:66,501,752), glycine (hg18 chr14:66,658,831) and aspartate (hg18 chr14:66,716,057) at these positions were conserved across all 15 species with sequence data reported in the UCSC genome browser and were not observed in exome sequence data from > 5000 individuals from the NIH National Heart, Lung and Blood Institute (NHLBI) project (http://evs.gs.washington.edu/EVS/). These changes

were also predicted to be likely damaging by the Polyphen and/or SIFT software programs. However, the clinical relevance of these changes is unclear given their presence in unaffected relatives—in the father of the proband with Gln307Pro, in the father and brother of the proband with Gly578Ser, and in the grandmother of the proband with Asp697Asn. DNA was not available for testing from the parents of the ASD proband with the Asp697Asn variant.

DISCUSSION

Here, we present clinical and molecular characterization of six unrelated index cases possessing rare *de novo* or inherited microdeletions overlapping the *GPHN* gene at 14q23.3. Perhaps the most compelling evidence for the pathogenicity of the deletions is the fact that they arose as de novo events in the probands in three of the five families for whom DNA from both parents was available for inheritance testing. The deletions in the probands in Families 2 and 6 were inherited from a parent with sub-clinical socialization deficits and an unaffected parent, respectively, suggesting variable expression of structural mutations at this locus.

To our knowledge, this is the first report of hemizygous deletions at the *GPHN* locus in connection with ASD or seizure phenotypes. The deletions in probands from Families 5 and 6 add to previous evidence supporting the involvement of *GPHN* in genetic risk for schizophrenia. The *GPHN* locus was one of the top ranking genetic loci at which runs of homozygosity were significantly over-represented in cases versus controls in a study examining highly penetrant risk loci in schizophrenia (35). In addition, the statistical method Gene Relationships Among Implicated Loci (GRAIL) employed by Raychaudhuri *et al.* pinpointed *GPHN* as a key gene highly likely to play an etiological role in schizophrenia based on its functional relatedness to other genes implicated in risk for the disorder (36).

The common region of overlap across the six deletions encompasses exons 3–5 (Fig. 2), corresponding to the coding segment of the G domain of the gephyrin protein. G-domain trimerization is vital for the formation of the hexagonal gephyrin oligomer scaffolds required for stable GABA receptor clustering in postsynaptic inhibitory neurons (2). Interestingly, abnormally spliced gephyrin mRNA (with no corresponding genomic mutation), lacking several exons corresponding to the G domain, has been isolated in the hippocampus of patients with TLE (8). Due to the missing G-domain exons, these aberrant protein variants were no longer able to form trimers and were observed to act in a dominant-negative manner by interacting with normally spliced gephyrin and impairing its function, as observed by depleted GABA receptor cluster density and reduced GABAergic postsynaptic current amplitudes (8). The authors of this study speculated that cellular stressors such as elevated temperature or alkalosis, which can cause or arise from seizure activity, respectively, might induce exon skipping in GPHN mRNA leading to a dominant-negative effect (8). This type of 'environmentally' induced mRNA mutation might have an equivalent functional impact to dosage effects of a hemizygous genomic deletion of the underlying *GPHN* locus (as is seen in our six families). However, the situation *in vivo* may

be more complex than that observed *in vitro* due to the multiple isoforms and splice variants of the gene, differences in gephyrin expression across various synapse types and factors affecting the expression levels of the non-deleted allele. Given the high prevalence of epilepsy in ASD (>21% in ASD individuals with an ID and 8% in those without ID) (37), our findings call attention to considering models (38) whereby the effects of seizures may contribute to expression of ASD, instead of being interpreted as associated medical comorbidities.

In addition to its synaptic function in the central nervous system, gephyrin has a more wide-ranging role in peripheral tissues in the synthesis of MoCo (39,40), the catalytically active center of molybdoenzymes (class of molybdenum containing enzymes), whose function is essential for the survival of almost all life forms. Gephyrin exhibits extensive sequence conservation across different species ranging from bacteria to humans, attesting to the strong evolutionary pressure preserving MoCo synthesis (40). To date, there have been two reports of homozygous *GPHN* mutations in humans, both in connection with MoCo deficiency, an extremely rare and sometimes lethal autosomal recessive metabolic disease characterized by untreatable neonatal seizures with opisthotonos, hypotonia, feeding difficulties, facial dysmorphism and ID. Reiss *et al.* (41) identified homozygous deletions, which eliminated exons 2 and 3 resulting in a frameshift after only 21 codons of normal coding sequence and complete loss of protein function, in the last of three affected infants born to Danish first-cousin parents. All three infants exhibited symptoms of severe MoCo deficiency and died within a month after birth. While the vast majority of cases of MoCo deficiency are caused by point mutations in one of two other related genes involved in MoCo biosynthesis, *MOCS1* and *MOCS2* (42), there has been one other case linked to a homozygous point mutation in *GPHN* (43). This D580A missense change was recently reported in a female child (of first cousin parents) of Algerian origin who exhibited a milder MoCo deficiency phenotype when compared with the patients carrying the *GPHN* homozygous deletion (43). The parents, who were carriers of hemizygous *GPHN* mutations, did not exhibit any MoCo deficiency symptoms in either of the two families. We have attempted to see if these parents might have a neuropsychiatric phenotype, but were unable to obtain information.

The severe symptoms of *GPHN* null mutations in humans are consistent with phenotypes observed by Feng *et al.* (39) in geph$^{-/-}$ knockout mice embryos that were born in expected numbers without apparent physical abnormalities but died within 1 day of birth. Knockout neonates had breathing difficulties and a heightened startle response, becoming rigid and exhibiting a hyperextended posture when lightly touched. They also had other phenotypic traits similar to human MoCo deficiency patients including myoclonus and feeding difficulties. The authors demonstrated that gephyrin was essential for both glycine receptor clustering and MoCo synthesis. While heterozygous mutant (geph$^{+/-}$) mice were apparently phenotypically normal (39), there is some evidence from other studies for subtle gene dosage effects. The concentration of gephyrin protein was reduced by 50% in geph$^{+/-}$ mice (44), and reductions in GABA$_A$ receptor clustering have been observed *in vitro* (but not *in vivo*) in neuronal cultures derived from geph$^{+/-}$ animals (45). RNAi experiments

that reduced gephyrin expression by ∼50% in mouse hippocampal neurons observed significant reduction in GABA receptor cluster density (46). These findings hint at potential disturbances in synaptic homeostasis in hemizygous *GPHN* mutation carriers. Thus while homozygous *GPHN* mutations abolishing both the synaptic and molybdenum cofactor biosynthetic activity of the protein result in the severe metabolic phenotype of MoCo deficiency, hemizygous deletions reported in this study presumably affect only the synaptic functions of the protein, as can be inferred from the absence of MoCo deficiency symptoms in our patients and in previously reported individuals with such mutations (41).

Our data add to the accumulating evidence highlighting the role of dysfunctional inhibitory signaling with ASD etiology (47). *GPHN* joins the growing list of genetic risk loci evidently shared between ASD and schizophrenia. This intriguing cross-disorder genetic overlap includes reports of very large (>500 kb) rare deletions and/or duplications in both ASD and schizophrenia patients at regions such as 1q21.1 (48,49), 3q29 (50,51), 15q13.3 (49,52), 16p11.2 (53–56), 16p13.11 (57,58), 17q12 (59) and 22q11.2 (60,61). Each of these syndromic microdeletions and microduplications overlaps several genes and the varying neurocognitive traits observed in connection with them could be a consequence of aberrant dosage of any or all of these genes. Higher resolution microarray and sequencing scans have detected smaller ultra-rare single gene CNVs and/or truncating point mutations affecting certain specific genes in both schizophrenia and ASD patient cohorts, such as *NRXN1* (15,19), *SHANK3* (27,28), *CNTNAP2* (62,63), *ASTN2* (64–66), *A2BP1* (67,68), *DPP6* (54,68,69), *DPYD* (70,71), *ZNF804A* (72–74), *GRIN2B* (74,75), *EHMT1* (74,76) and *CSMD3* (77,78) among others. Since several of these genes have crucial roles in the formation and proper function of neuronal synapses, the disruption of complexes and interacting proteins within the synapse proteome is increasingly being recognized as a key etiologic factor in both disorders (16,79–83). Recent research has also highlighted the involvement of CNVs in epilepsy at several of the loci first implicated in ASD and schizophrenia, including 15q13.3, 16p13.11 and 15q11.2 as well as at individual genes such as *CNTNAP2*, *NRXN1* and *SHANK3* (21,29,84,85).

Our finding of different disease states arising from deletions of the same gene is in line with previous reports of several other genetic loci being involved in genetic risk for a range of neurodevelopmental disorders including autism, schizophrenia and epilepsy. For example, these observations of pleiotropic effects of mutations at the same gene are also seen in connection with mutations in pre-synaptic neurexin and in the post-synaptic SHANK scaffolding proteins. Duong *et al.* (21) recently reported variable penetrance and pleiotropy of *NRXN1* mutations within a single family—individuals with hemizygous deletions and point mutations had differing neurodevelopmental phenotypes ranging from ASD and epilepsy to schizophrenia and psychotic disorder. As with these and other CNVs, the variable expression and penetrance of the neurodevelopmental disorder phenotypes associated with structural mutations at the *GPHN* locus could be modulated by other risk alleles, protective genetic modifiers and/or environmental factors. Differences among the translational products, expression levels and stabilities of the pathogenic gephyrin forms, together with the multiple

types of inhibitory receptors potentially affected downstream, could also contribute to the range of observed disorder phenotypes.

Although ASD and schizophrenia exhibit important differences in core phenotypes, age of onset and treatment regimens, there is evidence for a certain degree of overlap of clinical traits (86–89), consistent with the observations of shared genetic risk. The clinical link to ASD is perhaps strongest in childhood-onset schizophrenia (COS) with several studies reporting ASD traits predating the development of psychosis in a sizeable subset of patients with COS (86). There is converging evidence of similar neuronal functional deficits and connectivity impairments being involved in the etiology of both disorders (87). Epidemiological studies have highlighted an increased ASD risk in children with a familial history of schizophrenia (90,91). There is also emerging evidence of comorbidity and functional links between epilepsy and ASD (37,92), and between epilepsy and schizophrenia (93).

Our results enhance understanding of the *GPHN* mutation spectrum and will aid in the interpretation of clinical genetic testing in neurodevelopmental disorders. The molecular findings and clinical case descriptions provided here will serve as a foundation for future studies elucidating a more comprehensive correlation of DNA, mRNA and protein variants at this locus with the resulting neurodevelopmental clinical outcomes. Building from the work of Forstera *et al*. (8), our findings potentially link genomic CNV dosage effects and epilepsy-induced mRNA dysregulation to *GPHN*, possibly providing a sought after model to explore gene–environment interactions in neuropsychiatric disease.

MATERIALS AND METHODS

Study subjects

The patient cohort inspected for *GPHN* deletions in this study was composed of 1158 Canadian patients with ASD, 72 Austrian patients with ASD, 450 Canadian patients with schizophrenia and a clinical data set of 3704 individuals with primary diagnoses of ASD and/or seizure disorders that were referred to for clinical microarray testing at the Mayo Clinic cytogenetics laboratory. The Canadian and Austrian ASD patients met criteria for ASD diagnosis on the basis of the Autism Diagnostic Interview-Revised (ADI-R) and/or the Autism Diagnostic Observation Schedule (ADOS) (94). The Canadian ASD cohort was recruited as described previously (25). The Canadian schizophrenia sample is composed of patients from two cohorts: a previous genome-wide linkage study involving extended pedigrees of northern European ancestry in which schizophrenic illness appeared to be segregating in a unilineal, autosomal dominant-like manner (34) and a community-based catchment population of schizophrenia (95). The comprehensive clinical assessments performed on these cohorts are described in detail elsewhere (33). All samples were approved for inclusion in the study by the appropriate local research ethics boards. The *GPHN* region was inspected for CNVs in microarray data analyzed by our group (54,66,69,96) from 21 345 population-based adult controls genotyped with high-resolution arrays (Supplementary Material, Table S8) and published data from 2493 controls

genotyped at the University of Washington (97) and from 3181 controls analyzed by the International Schizophrenia Consortium (30).

Microarray genotyping and CNV analysis

DNA samples from probands of Families 2, 3 and 4 were genotyped at the Mayo Clinic employing either the Agilent ISCA 44 K or 180 K microarrays (98). CNV analysis was performed using the DNA Analytics software (Agilent) with clinically validated settings. CNVs were assessed for reporting that involved at least four consecutive probes for the 44 K array and at least five probes for the 180 K array. For the proband of Family 1 and the proband and parents of Family 5, genotyping was performed at The Centre for Applied Genomics (Toronto, Canada) using the Affymetrix GeneChip SNP 6.0 following standard protocols provided by the manufacturer. CNV calling was performed using a previously described multi-algorithm approach (66,99) incorporating Birdsuite, iPattern & Genotyping Console. Subsequent analyses focused on those CNVs spanning five or more array probes and detected by at least two algorithms. We have shown previously that such variants exhibit very high positive validation rates ($> 95\%$) using independent experimental approaches such as qPCR and FISH (54,66,69,100). Genotyping and CNV analysis of DNA samples from Family 6 (using the Affymetrix 6.0 platform) were performed as part of the International Schizophrenia Consortium study as described elsewhere (30).

CNV validation and inheritance testing

Validation of deletions in probands from Families 2, 3 and 4, and in their family members from whom DNA was available was conducted by FISH. CNV validation in probands from Families 1, 5 and 6, and in their family members from whom DNA was available was performed using SYBR-Green-based real-time qPCR, for which two independent primer pairs were designed at the *GPHN* locus and at the *FOXP2* locus on chromosome 7 as a diploid control. The parentage of the proband in Family 5 with the *de novo GPHN* deletion was confirmed by calculating the Mendelian error rate of Affymetrix SNP 6.0 array genotypes using the PLINK tool set (101). Parentage of the proband with a *de novo* deletion in Family 1 was confirmed using the AmpFlSTR Identifiler PCR Amplification Kit (Life Technologies, Foster City, CA, USA) using the manufacturer's instructions. Briefly, the kit employs a short tandem repeat multiplex assay that amplifies 15 tetranucleotide repeat loci and the Amelogenin gender determining marker in a single PCR reaction. Samples were run on an AB 3100 Genetic Analyzer using POP4, dye set G5 and analyzed in GeneMapper v3.7.

Sequencing and mutation screening methods

All 23 coding exons of *GPHN* isoform 1 (NM_020806.4) and the intron–exon splice sites were sequenced in 280 unrelated Canadian probands with ASD and 95 probands with schizophrenia using standard PCR-based Sanger sequencing. Primers were designed using Primer3 software v. 0.4.0 (http:// frodo.wi.mit.edu/primer3). All primers used for Sanger sequencing are listed in Supplementary Material, Table S12.

Sequencing of amplified products was performed using the ABI 3730XL capillary sequencer (Applied Biosystems), followed by variant detection utilizing the SeqScape software from Applied Biosystems.

The *GPHN* locus was also examined for coding sequence variants in next-generation exome sequencing data from 278 additional Canadian ASD probands, distinct from those screened by Sanger sequencing. After target enrichment utilizing the Agilent SureSelect V3 50 Mb Human All Exon kit (Agilent Technologies, Santa Clara, CA, USA), paired-end sequencing was conducted on Life Technologies SOLiD4 and SOLiD5500XL (Life Technologies) platforms. Protocols for sequencing and target capture followed specifications from the manufacturers. The generated paired end reads were mapped to the reference human genome (UCSC hg19 build) using BFAST. MarkDuplicates (Picard tools version 1.35; http:// picard.sourceforge.net) was used to remove duplicate paired end reads and the subsequent alignments were refined using local realignment in colorspace implemented in SRMA version 0.1.15. GATK version1.1.28 was used for the calling of indels and SNPs.

Those novel non-synonymous variants detected by Sanger and NGS exome sequencing, which were not previously reported in the SNP database (dbSNP) build 137, were validated bi-directionally using Sanger re-sequencing in the case and in samples from both parents, when available.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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