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PNPLA6 mutations cause Boucher-Neuhäuser and Gordon Holmes syndromes as part of a broad neurodegenerative spectrum

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Boucher-Neuhäuser and Gordon Holmes syndromes are clinical syndromes defined by early-onset ataxia and hypogonadism plus chorioretinal dystrophy (Boucher-Neuhäuser syndrome) or brisk reflexes (Gordon Holmes syndrome). Here we uncover the genetic basis of these two syndromes, demonstrating that both clinically distinct entities are allelic for recessive mutations in the gene

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***PNPLA6*. In five of seven Boucher-Neuhäuser syndrome/Gordon Holmes syndrome families, we identified nine rare conserved and damaging mutations by applying whole exome sequencing. Further, by dissecting the complex clinical presentation of Boucher-Neuhäuser syndrome and Gordon Holmes syndrome into its neurological system components, we set out to analyse an additional 538 exomes from families with ataxia (with and without hypogonadism), pure and complex hereditary spastic paraplegia, and Charcot–Marie–Tooth disease type 2. We identified four additional *PNPLA6* mutations in spastic ataxia and hereditary spastic paraplegia families, revealing that Boucher-Neuhäuser and Gordon Holmes syndromes in fact represent phenotypic clusters on a spectrum of neurodegenerative diseases caused by mutations in *PNPLA6*. Structural analysis indicates that the majority of mutations falls in the C-terminal phospholipid esterase domain and likely inhibits the catalytic activity of PNPLA6, which provides the precursor for biosynthesis of the neurotransmitter acetylcholine. Our findings show that *PNPLA6* influences a manifold of neuronal systems, from the retina to the cerebellum, upper and lower motor neurons and the neuroendocrine system, with damage of this protein causing an extraordinarily broad continuous spectrum of associated neurodegenerative disease.**

Keywords: ataxia; recessive ataxia; hypogonadism; retinal degeneration; spastic ataxia; early onset ataxia; spasticity; genetics; hereditary spastic paraplegia

Abbreviations: ARCA = autosomal recessive cerebellar ataxia; CMT = Charcot–Marie–Tooth disease; EST = phospholipid esterase domain

Introduction

Autosomal recessive cerebellar ataxias (ARCAs) are a clinically and genetically heterogeneous group of spinocerebellar diseases, often associated with additional non-cerebellar features (Fogel and Perlman, 2007; Anheim *et al.*, 2012). Two clinically defined syndromes combine early-onset ARCA with hypogonadotropic hypogonadism: (i) Boucher-Neuhäuser syndrome (MIM 215470), which is additionally associated with chorioretinal dystrophy (Boucher and Gibberd, 1969; Neuhauser and Opitz, 1975; Limber *et al.*, 1989); and (ii) Gordon Holmes syndrome (MIM 212840), which is additionally associated with brisk reflexes (Holmes, 1907). Despite various descriptions of familial occurrence, the genetic basis of these syndromes has remained elusive. Moreover, it has remained unclear whether these syndromes present clinically and genetically distinct entities or, alternatively, phenotypic clusters on a phenotypic continuum of neurodegenerative diseases caused by mutations in the same gene.

Here we demonstrate that Boucher-Neuhäuser and Gordon Holmes syndromes are indeed allelic diseases and reveal the major disease gene for these clinical disease entities. By using the significant genetic variants identified in Boucher-Neuhäuser and Gordon Holmes syndrome families as a seed we also analysed >500 exomes from patients with hereditary ataxia and/or spasticity syndromes and establish that variants in the new Boucher-Neuhäuser syndrome/Gordon Holmes syndrome disease gene are not specific to these particular hypogonadism syndromes, but rather cause these presentations as part of a wider spectrum of neurodegenerative disease.

Materials and methods

Whole exome sequencing of index patients with Boucher-Neuhäuser syndrome and Gordon Holmes syndrome

Whole exome sequencing was performed on two index patients with familial Boucher-Neuhäuser syndrome and one index patient with sporadic Gordon Holmes syndrome. Informed consent was obtained from all individuals and the Institutional Review Boards of the participating medical centres approved the study. The SureSelect Human All Exon 50 Mb kit (Agilent) was used for in-solution enrichment and exome sequencing was performed using the HiSeq2000 instrument (Illumina). Paired-end reads of 100-bp length were produced. BWA and GATK software packages (Li and Durbin, 2009; McKenna *et al.*, 2010; DePristo *et al.*, 2011) were used to align sequence reads to the reference and call variant positions, respectively. All data were then annotated and imported into GEnomes Management Application (GEM.app), a web-based tool for next generation sequencing data analysis (Gonzalez *et al.*, 2013) (genomics.med.miami.edu). An average of 73 609 687 sequence reads was produced per sample, 98.8% of which could be aligned to the targeted sequence. Mean coverage was 69-fold; 71% of the targeted sequence was covered by at least 20 reads.

Assuming a common cause for Boucher-Neuhäuser and Gordon Holmes syndromes, we used the GEM.app analysis module 'Genes Across Families' to filter for non-synonymous homozygous or compound heterozygous variants, with low frequency in public databases (minor allele frequency in dbSNP137 and NHLBI ESP6500 < 0.5%), moderate conservation (GERP score > 2 OR PhastCons score > 0.6) and moderate genotype quality (GATK quality index > 30 and genotype quality GQ > 30) in genes shared across the three families. Only one gene, *PNPLA6* (NCBI reference NM_001166111.1), remained as a candidate gene between the Boucher-Neuhäuser and Gordon Holmes syndrome index patients with segregating variants that were conserved, rare, and predicted to be damaging by at least three different *in silico* algorithms (MutationTaster; MutationAssesor; Likelihood Ratio Test; and PolyPhen2).

Sanger sequencing of the candidate gene in additional Boucher-Neuhäuser syndrome families

To confirm the significance of *PNPLA6* mutations in the pathogenesis of ataxia-hypogonadism syndromes, we screened the *PNPLA6* gene in index patients from four additional Boucher-Neuhäuser syndrome families by conventional Sanger sequencing. Oligonucleotide sequences are available upon request.

PNPLA6 seed analysis in more than 500 exomes of index patients with ataxia, hereditary spastic paraplegia and Charcot–Marie–Tooth disease

To explore the possibility that Boucher-Neuhäuser and Gordon Holmes syndromes are clusters on a wide spectrum of neurodegenerative disease, *PNPLA6* was used as a seed in the analysis of exome data from 538 unrelated patients with the following non-Boucher-Neuhäuser syndrome/Gordon Holmes syndrome neurodegenerative diseases: non-Boucher-Neuhäuser syndrome/Gordon Holmes syndrome early-onset ataxia (age of onset <30 years) ($n = 67$), non-Boucher-Neuhäuser syndrome/Gordon Holmes syndrome complex hereditary spastic paraplegia ($n = 144$), pure hereditary spastic paraplegia ($n = 192$) and Charcot–Marie–Tooth disease (CMT) type 2 compatible with recessive disease (CMT2; $n = 135$). In addition, we screened for *PNPLA6* mutations in 1637 additional whole exomes from a wide range of (non-ataxia, non-hereditary spastic paraplegia, non-CMT2) neurological phenotypes. The latter were used as a control cohort to scrutinize the possibility that recessive mutations observed in our target cohort are just a result of genetic variability of the *PNPLA6* gene with common occurrence of unique or rare variants not related to the disease.

Structural models of wild-type and mutant *PNPLA6* domains

Structural models of various wild-type and mutant domains of human PNPLA6 (UniProt# Q8IY17-4) were built using the MODELLER software based on homology modelling (Marti-Renom *et al.*, 2000). Briefly, the structural model of wild-type phospholipid esterase domain (EST), which represents the site of location of Ser1045Leu, Thr1058Ile, Phe1066Ser, Val1100Gly, Val1110Met and Pro1122Leu mutations, was constructed using the crystal structure of the homologous EST catalytic domain of the plant patatin PAT17 (PDB# 1OXW) (Rydel *et al.*, 2003). The structural models of various wild-type and mutant CNB1/2 domains in complex with cyclic adenine monophosphate were obtained using the crystal structure of the homologous CNB2 domain of the regulatory alpha-subunit of cyclic adenine monophosphate-dependent protein kinase A bound to cyclic adenine monophosphate (PDB# 1RGS) (Su *et al.*, 1995). In each case, a total of 100 atomic models were calculated and the structures with the lowest energies, as judged by the MODELLER Objective Function, were selected for further analysis. The atomic models were rendered using RIBBONS (Carson, 1991).

Clinical assessment

All index patients carrying two pathogenic PNPLA6 variants as well as their affected siblings received a systematic clinical assessment for disturbances in multiple neurological systems (Table 1). In all subjects, routine MRI was performed.

Results

PNPLA6 causes Boucher-Neuhäuser syndrome and Gordon Holmes syndrome

By intersecting the identified variants in whole exomes from two index patients with Boucher-Neuhäuser syndrome and one index patient with Gordon Holmes syndrome under recessive inheritance models, only *PNPLA6* remained as a candidate gene. All variants identified in these cases were conserved, had low frequency in the general population, and were predicted to be damaging by at least three different *in silico* algorithms (Table 2; for coverage see Supplementary Fig. 5). We identified a homozygous missense mutation (c.[3173C > T];[3173C > T], p.[Thr1058Ile];[Thr1058Ile]) in Family IHG25190, compound heterozygous splice/missense mutations in Family ARCA_05 (c.[2212-1G > C];[3328G > A], p.[Val738Glnfs*98];[Val1110Met]), and compound heterozygous frameshift/missense mutations (c.[3084_3085insGCCA](;)[4084C > G], p.[Arg1031Glufs*38](;)[Arg1362Gly]) in Family IHG25330. The splice mutation in Family ARCA_05 destroys a known splice acceptor site in intron 19-20, most likely resulting in skipping of exon 20 (76 bp) and a consecutive shift of the reading frame; *in silico* analysis did not indicate activation of cryptic splice sites (Divina *et al.*, 2009) (Table 2; for pedigrees and electropherograms see Supplementary Figs 1 and 2).

To confirm the significance of *PNPLA6* mutations in the pathogenesis of ataxia-hypogonadism syndromes, index patients from four additional independent Boucher-Neuhäuser syndrome families were screened for *PNPLA6* mutations by conventional Sanger sequencing. We identified two additional Boucher-Neuhäuser syndrome families with *PNPLA6* mutations: (Family IHG25357: c.[3134C > T]; [3365C > T], p.[Ser1045Leu]; [Pro1122Leu]; and Family IHG25353: c.[1732G > T]; [3197T > C], p.[Gly578Trp]; [Phe1066Ser]) (Table 2; for pedigrees and electropherograms, see Supplementary Figs 1 and 2).

PNPLA6 causes a wide spectrum of neurodegenerative disease

To explore the possibility that Boucher-Neuhäuser syndrome and Gordon Holmes syndrome are only clusters on a wider spectrum of neurodegenerative disease, *PNPLA6* was used as a 'seed' in the analysis of exome data from 538 unrelated patients with non-Boucher-Neuhäuser syndrome/Gordon Holmes syndrome neurodegenerative diseases (non-Boucher-Neuhäuser syndrome/Gordon Holmes syndrome early-onset ataxia; non-Boucher-Neuhäuser syndrome/Gordon Holmes syndrome hereditary spastic paraplegia; CMT type 2) where family history was compatible with recessive disease. This analysis identified a spastic ataxia patient (Subject IHG26117), who carried significant compound heterozygous changes (c.[3084_3085insGCCA]; [3299T > G], p.[Arg1031Glufs*38]; [Val1100Gly]) and a case with hereditary spastic paraplegia and mild motor neuropathy (Subject IHG26041) with compound heterozygous variants (c.[787G > A]; [2519G > A], p.[Val263Ile]; [Gly840Glu]) in *PNPLA6* (Table 2).

In total, we report seven families from five different countries with significant novel mutations in *PNPLA6*. These variants co-segregated with the disease in all families where family members were available (Supplementary Fig. 1). All affected base pairs are highly conserved across species (Fig. 1B) and were absent or had low minor allele frequency in GEM.app (2175 exomes), dbSNP137, and NHLBI ESP (6500 exomes) (Table 2). Remarkably, in 1637 additional whole exomes from a wide

Table 1 Clinical features of PNPLA6 patients

Family identifier	Subject	Origin	Gender	Phenotypic syndrome	Age of onset of first symptom (years)	Age at last examination (years)	Gait ataxia	Hypogonadotropic hypogonadism	Chorioretinal dystrophy	Spasticity LL/extensor plantar response	Distal muscle wasting	Tendon reflexes PTR/ATR	Cognitive impairment	Imaging
IHG25190	1	B	F	BNS	2, visual impairment	56	+, since age 6 years	+ (including primary amenorrhoea)	+	−/−	+	↓/↓	Mild	Atrophy cerebellum and pons; small pituitary
	2	B	M	BNS	3, visual impairment	55	+, since age 6 years	+	+	−/−	+	↓/↓	Mild	Atrophy cerebellum and pons, small pituitary
	3	B	F	BNS	1, visual impairment and ataxia	53	+, since age 7 years	+ (including primary amenorrhoea)	+	−/−	+	↓/↓	Mild	Atrophy cerebellum and pons, small pituitary
	4	B	F	BNS	2–3, visual impairment	48	+, since age 7 years	+ (including primary amenorrhoea)	+	−/−	+	↓/↓	Mild	Atrophy cerebellum and pons, small pituitary
ARCA-05	1	I	F	BNS	12, visual impairment	44	+, since age 27	+ (including primary amenorrhoea)	+	+/+	+	↑/Ø	Mild	Atrophy cerebellum
	2	I	F	BNS without retinal dystrophy	6, gait ataxia	42	+, since age 6	+ (including primary amenorrhoea)	−	+/+	−	n/Ø	Mild	Atrophy cerebellum
IHG25353	1	B	M	BNS	6, gait ataxia	61	+, since age 6	+	+	−/−	−	↑/↓	N	Atrophy cerebellum
	2	B	M	BNS	6, gait ataxia	57	+, since age 6	+	+	+/−	−	↑/↓	N	Atrophy cerebellum
IHG25357	1	V	M	BNS	14, delayed puberty; visual impairment	26	+, since age 20	+	+	−/−	−	n	N	Atrophy cerebellum
IHG25330	1	F	M	GHS	14, delayed puberty	63	+, since age 30	+	−	+/−	−	↑/↑	N	Atrophy cerebellum; empty sella
IHG26041	1	G	F	HSP	20, spasticity	54	-	−	−	+/+	−	↑/↑	N	N
IHG26117	1	G	M	spastic ataxia	4, gait ataxia	48	+, since age 4	−	−	+/+	−	↑/↑	N	Atrophy cerebellum

M = male; F = female; N = normal; B = Brazilian; I = Italian; F = French; G = German; V = Venezuela; BNS = Boucher-Neuhäuser syndrome; GHS = Gordon-Holmes syndrome; HSP = hereditary spastic paralegia; Ø = absent; LL = lower limbs; PTR = patellar tendon reflex; ATR = achilles tendon reflex.
Cognitive impairment was rated by clinical impression.

Table 2 *PNPLA6* mutations identified in this study

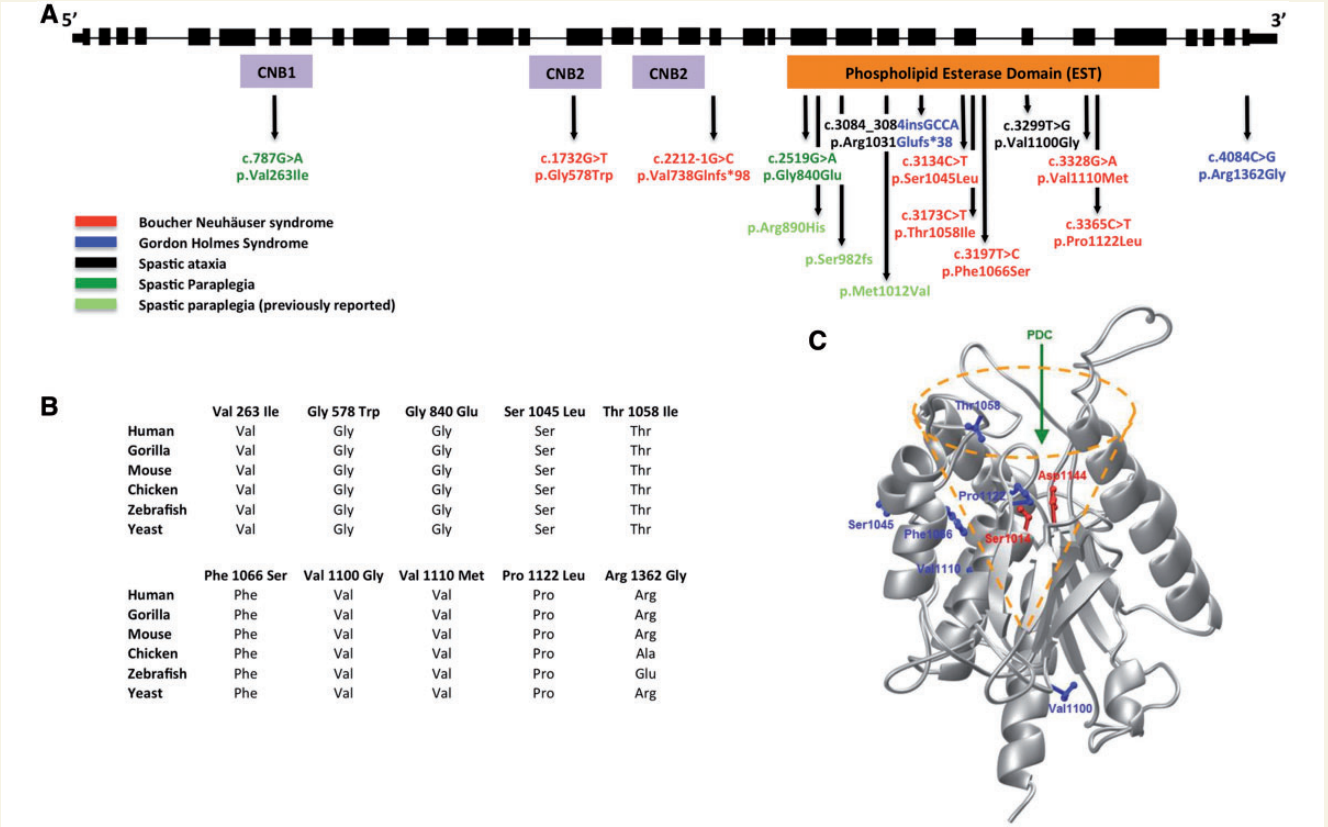
Family ID	Phenotype	cDNA change	Protein change	GVS function	GERP	PhastCons	phyloP	dbSNP137 MAF	NHLBI EVS MAF	GEM. appMAF	MT	MA	LRT	PP2	SIFT
ARCA_05	BNS	c.2212-1G > C	p.Val738Glnfs*98	Splice-site	4.8	0.83	2.20	np	np	np					
ARCA_05	BNS	c.3328G > A	p.Val1110Met	Missense	3.93	0.994	2.04	np	np	np	D	L	N	D	0
IHG26117	sATX	c.3084_3085insGCCA	p.Arg1031Glufs*38	Frameshift	4.04	1	−0.07	np	0.00032	0.00033					
IHG26117	sATX	c.3299T > G	p.Val1100Gly	Missense	4.97	1	1.88	np	np	0.00033	D	H	D	D	0
IHG26041	HSP	c.787G > A	p.Val263Ile	Missense	5.2	0.998	2.44	np	np	0.00033	D	L	D	P	0.06
IHG26041	HSP	c.2519G > A	p.Gly840Glu	Missense	4.66	1	2.59	np	np	0.00033	D	M	D	P	0.01
IHG25357	BNS	c.3134C > T	p.Ser1045Leu	Missense	4.98	0.661	2.32	np	np	np	D	M	D	D	0
IHG25357	BNS	c.3365C > T	p.Pro1122Leu	Missense	4.09	0.996	2.12	np	np	np	D	H	U	D	0
IHG25353	BNS	c.1732G > T	p.Gly578Trp	Missense	5.17	0.939	2.41	np	np	np	D	H	D	D	0
IHG25353	BNS	c.3197T > C	p.Phe1066Ser	Missense	5.1	1	1.93	np	np	np	D	M	D	D	0
IHG25330	GHS	c.3084_3085insGCCA	p.Arg1031Glufs*38	Frameshift	4.04	1	−0.07	np	0.00032	0.00033					
IHG25330	GHS	c.4084C > G	p.Arg1362Gly	Missense	2.22	0.039	1.11	np	np	0.00033	N	N	N	B	0.28
IHG25190	BNS	c.3173C > T	p.Thr1058Ile	Missense	4.98	1	2.32	np	np	0.00066	D	M	D	D	0.02

BNS = Boucher-Neuhäuser syndrome; sATX = sporadic ataxia; HSP = hereditary spastic paraplegia; GHS = Gordon Holmes syndrome; MAF = minor allele frequency; MT = MutationTaster; MA = MutationAssessor; LRT = Likelihood Ratio Test; PP2 = PolyPhen2; SIFT = Sorting Tolerant From Intolerant; D = damaging; L = low probability of damaging effect; H = high probability of damaging effect; B = benign; M = medium probability of damaging effect; N = neutral; P = probably damaging; U = unknown; np = not present.
GERP = Genomic Evolutionary Rate Profiling; GVS = Genome Variant Server.
SIFT scores < 0.05 represent damaging effect.

range of neurological phenotypes no additional homozygous or compound heterozygous changes were present in *PNPLA6* under relaxed filter conditions (minor allele frequency in Gem.app, dbSNP137 and NHLBI ESP 6500 exomes <3%, low quality scores, low conservation scores) (Fisher's exact test, two-tailed, $P = 0.0001$).

Structural *in silico* protein modelling

The majority of identified *PNPLA6* mutations clustered within a short stretch of <100 residues in the EST domain. This domain has been shown to de-esterify phosphatidylcholine, a major component of biological membranes, into its constituent fatty acids and glycerophosphocholine (Strickland *et al.*, 1995; Atkins *et al.*, 2002; van Tienhoven *et al.*, 2002; Zaccheo *et al.*, 2004) (Fig. 1A). Glycerophosphocholine serves as a precursor for the biosynthesis of acetylcholine, a key neurotransmitter involved in mediating cellular signalling in the nervous system. On the CNB1-CNB2-CNB3-EST modular architecture of *PNPLA6* (Fig. 1A), we observe that mutations from residues Gly840–Ser1031 associated with spasticity (spastic paraplegia and spastic ataxia) largely cluster at the N-terminal side of the EST domain. Towards the C-terminal end of the EST domain, a second mutational cluster between residues Ser1031–Pro1122 causes cerebellar ataxia and/or hypogonadism (Boucher-Neuhäuser syndrome, Gordon Holmes syndrome, spastic ataxia). Next, we built structural models of CNB1/2 and EST domains using the crystal structures of the homologous CNB2 domain of the regulatory alpha-subunit of cyclic adenine monophosphate-dependent protein kinase A bound to cyclic adenine monophosphate (Protein Data Bank #1RGS) (Su *et al.*, 1995) and the homologous EST catalytic domain of the plant patatin PAT17 (Protein Data Bank #1OXW) (Rydel *et al.*, 2003) (Fig. 1C). An extensive description of these studies is provided in Supplementary Figs 3 and 4. Our structural models reveal that the catalytic centre of the EST domain adopts a funnel-like shape that constitutes the entry route for the phosphatidylcholine substrate, where the active site Ser1014/Asp1144 catalytic dyad is located deep at the base of the funnel. The EST missense mutations may result in misalignment of active site residues Ser1014/Asp1144 so as to compromise its enzymatic function. It is also highly probable that mutations such as Thr1058Ile and Pro1122Leu may block the entry of phosphatidylcholine substrate to the active site residues located deep at the base of the catalytic funnel. Finally, although the highly conserved Val1100Gly mutation is located at a distal site away from the catalytic centre, it may also indirectly affect protein dynamics that could ultimately shut down the enzymatic activity of PNPLA6. Additionally, two changes were located in the CNB1 and 2 domains (p.Val263Ile, p.Gly578Trp) (Fig. 1A). Our analysis suggests that these two mutations will likely compromise the ability of one or both regulatory CNB1/2 domains to bind cyclic nucleotide-monophosphate binding domains (cNMPs) and their failure to undergo a conformational change in response to changes in intracellular concentration of cNMPs would thus keep the EST domain in an auto-inhibited state (Supplementary Fig. 4).



	Val 263 Ile	Gly 578 Trp	Gly 840 Glu	Ser 1045 Leu	Thr 1058 Ile
Human	Val	Gly	Gly	Ser	Thr
Gorilla	Val	Gly	Gly	Ser	Thr
Mouse	Val	Gly	Gly	Ser	Thr
Chicken	Val	Gly	Gly	Ser	Thr
Zebrafish	Val	Gly	Gly	Ser	Thr
Yeast	Val	Gly	Gly	Ser	Thr

	Phe 1066 Ser	Val 1100 Gly	Val 1110 Met	Pro 1122 Leu	Arg 1362 Gly
Human	Phe	Val	Val	Pro	Arg
Gorilla	Phe	Val	Val	Pro	Arg
Mouse	Phe	Val	Val	Pro	Arg
Chicken	Phe	Val	Val	Pro	Ala
Zebrafish	Phe	Val	Val	Pro	Glu
Yeast	Phe	Val	Val	Pro	Arg

Figure 1 Representation of identified *PNPLA6* mutations and structural analysis of their effect on the PNPLA6 protein. (**A**) Schematic of the exon-intron arrangement of *PNPLA6* (NCBI reference NM_001166111.1), with positions of mutations identified in five families. Exons are indicated as black boxes. CNB1/2 and the phospholipid esterase functional domains are indicated by purple and orange boxes, respectively. The mutations are indicated and colour-coded by phenotype observed. (**B**) The inter-species conservation of the amino acids affected by missense mutations identified in this study. (**C**) Structural model of EST domain (grey) of human *PNPLA6*. The side chain moieties of active site residues (Ser1014/Asp1144) and those identified here to be prone to mutations (Ser1045/Thr1058/Phe1066/Val1100/Val110/Pro1122) are coloured red and blue, respectively. The funnel-shaped catalytic centre of the EST domain—where the active site residues Ser1014/Asp1144 are located deep at the base of the funnel whereas the rim of the funnel represents the entry route for the phosphatidylcholine (PDC)_substrate (green arrow)—is represented by yellow dashed lines. Note that the structural model of EST domain was built using the crystal structure of the homologous EST catalytic domain of the plant patatin PAT17 (PDB# 1OXW) (Rydel *et al.*, 2003) in MODELLER (Marti-Renom *et al.*, 2000). A total of 100 atomic models were calculated and the structure with the lowest energy, as judged by the MODELLER Objective Function, was selected for further analysis. The structural model was rendered using RIBBONS (Carson, 1991) (for a larger version and an additional rotation perspective, see Supplementary Fig. 3).

Clinical findings

We aggregated clinical data from 12 affected subjects belonging to seven families. All four index subjects with Boucher-Neuhäuser syndrome presented with the classical triad: visual impairment, ataxia and delayed puberty (Fig. 2B–D). Symptoms in the individuals with Boucher-Neuhäuser syndrome started before the age of 8 years and were slowly progressive in all affected individuals with variable progression rates both between and within families, leading to wheelchair-dependency in the most severely affected subjects. In-line with the original publications of Boucher-Neuhäuser and Gordon Holmes syndromes (Holmes, 1907; Neuhauser and Opitz, 1975), two of four subjects with Boucher-Neuhäuser syndrome as well as the subject with Gordon Holmes syndrome showed clinical signs of upper motor neuron disease (spasticity, positive extensor plantar reflex and/or brisk reflexes), including a spastic Boucher-Neuhäuser syndrome phenotype (Family ARCA_05). Achilles tendon reflexes were reduced or absent in all subjects with Boucher-Neuhäuser syndrome, providing clinical evidence that peripheral neuropathy is commonly associated with Boucher-Neuhäuser syndrome in our subjects. Neuropathy was of sensorimotor axonal type in those subjects with Boucher-Neuhäuser syndrome where nerve conduction studies were available. Affected siblings within Boucher-Neuhäuser syndrome families showed broadly similar phenotypes and age of onset (Table 1). However, the initial symptom of disease differed between and within Boucher-Neuhäuser syndrome families: whereas disease started with visual impairments in some subjects with Boucher-Neuhäuser syndrome, it presented with gait ataxia or delayed puberty in others (Table 1). Moreover, in Boucher-Neuhäuser syndrome Family ARCA_05 one of the affected siblings (Subject 2) did not show evidence for chorioretinal dystrophy

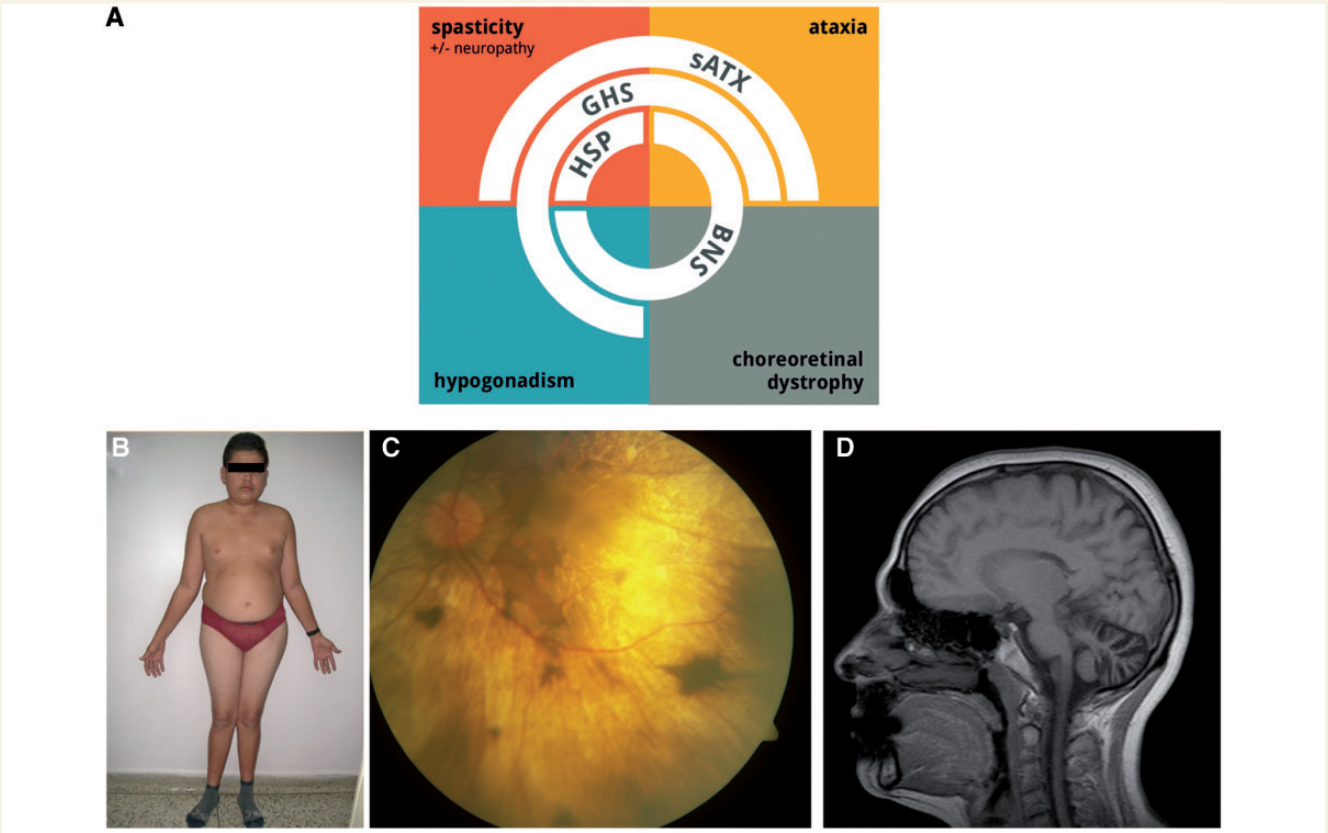


Figure 2 The continuous spectrum of PNPLA6-associated disease (**A**) and the classical clinical trias of the Boucher-Neuhäuser syndrome (**B–D**). (**A**) The clinical spectrum of *PNPLA6* mutations unfolds along four different neurological key features: ataxia, chorioretinal dystrophy, hypogonadotropic hypogonadism and motor neuron disease (upper motor neuron disease with or without additional lower motor neuropathy). Accordingly, Boucher-Neuhäuser syndrome (BNS) and Gordon Holmes syndrome (GHS) are not distinct entities, but clusters on a continuous spectrum of PNPLA6-associated disease, extending from Boucher-Neuhäuser syndrome via Gordon Holmes syndrome to spastic ataxia (sATX) and pure hereditary spastic paraplegia (HSP). The phenotype of complicated hereditary spastic paraplegia, which has been considered the most prominent phenotype of PNPLA6 so far (Rainier *et al.*, 2008), is only the 'tip of the iceberg' of this broad disease spectrum. (**B**) Photograph of the full body of a male patient with Boucher-Neuhäuser syndrome (Subject IHG 25357) at age 26 years illustrating incomplete secondary sex characteristics with lack of body hair and gynecomastia. (**C**) Exemplary fundus photography of the Boucher-Neuhäuser syndrome Patient ARCA_05 showing chorioretinal degeneration, characterized by diffuse atrophy of choroidal vessels and the retinal pigment epithelium with pigment clumps. The optic nerve shows no signs of atrophy. (**D**) Sagittal T_2-weighted MRI of this subject with Boucher-Neuhäuser syndrome shows marked cerebellar atrophy.

indicating this as a non-obligate feature. This is further supported by the findings that the cases with hereditary spastic paraplegia and spastic ataxia showed neither retinal dystrophy nor hypogonadism (Table 1), thus demonstrating that hypogonadism is not an obligate feature of disease. Disease in the cases with hereditary spastic paraplegia and spastic ataxia also started before the age of 20 years, corroborating the notion that early-onset neurodegeneration is a common denominator across all affected subjects.

Discussion

To date, the genetic basis of Boucher-Neuhäuser and Gordon Holmes syndromes has not been identified. Here we use exome sequencing to uncover the genetic cause of these two clinical entities and demonstrate that they are allelic diseases, both caused by recessive *PNPLA6* mutations. PNPLA6 seems to be a major cause of Boucher-Neuhäuser syndrome with four of six Boucher-Neuhäuser syndrome families carrying *PNPLA6* mutations. As not all subjects with Boucher-Neuhäuser syndrome carried *PNPLA6* mutations, the genetic basis of Boucher-Neuhäuser syndrome might either be heterogeneous or caused by *PNPLA6* mutations not detected by routine sequencing procedures (e.g. deletions or intronic mutations). Further heterogeneity of ataxia-hypogonadism syndromes is also supported by the recent observation that mutations in *RNF216* and the combination of mutations in *RNF216* and *OTUD4* (Margolin *et al.*, 2013) cause ataxia-hypogonadism syndromes complicated by dementia. In contrast to RNF216/OTUD4-associated ataxia-hypogonadism, Boucher-Neuhäuser syndrome and Gordon Holmes syndrome are not typically accompanied by dementia.

Complicated hereditary spastic paraplegia has been considered the prominent phenotype of *PNPLA6* thus far (Rainier *et al.*, 2008). However, here we show that complicated hereditary spastic paraplegia represents only a relatively special case along a multidimensional *PNPLA6*-associated spectrum of neurodegenerative disorders. This spectrum includes at least four clinical key features: ataxia, motor neuron disease (upper motor neuron disease with or without additional lower motor neuropathy), hypogonadism and chorioretinal dystrophy (Fig. 2A). Although these clinical features appear to be frequent in *PNPLA6* disease none of them is an obligate feature of the disease (Table 1 and Fig. 2A). We postulate that new phenotypic combinations on this *PNPLA6* associated disease spectrum will be identified as more patients representing broader phenotypes will be screened for mutations (Fig. 2A). PNPLA6 therefore appears to be another representative of a growing number of disease-related proteins that have been shown to cause a spectrum or even a multitude of seemingly unrelated phenotypic expressions (Ho and Lammerding, 2012; Nilius and Voets, 2013). Driven by the more comprehensive screening capabilities of next-generation sequencing-based approaches, we will likely uncover a more complex structure of phenotype/genotype correlations in the coming years. On a functional level, proteins like PNPLA6, on which different phenotypes converge, may represent functional platforms or hubs that connect a diversity of pathways with multiple biological functions. These 'hub proteins', intersection nodes in phenotypic as well as cellular disease networks, might offer promising opportunities for therapeutic intervention for a number of diseases.

PNPLA6 belongs to a protein family of nine patatin-like phospholipase domain-containing proteins. The phospholipid esterase domain (EST) is altered by intoxication of organophosphorous compounds (Rainier *et al.*, 2008; Richardson *et al.*, 2013). These compounds are used in industry, agriculture, suicide attempts, chemical warfare, terrorist incidents (1995 Tokyo subway incident) and adulterated alcoholic beverages (Jamaica 'Ginger Jake' during the Prohibition era) (Rainier *et al.*, 2008; Richardson *et al.*, 2013). Structural analysis of the various *PNPLA6* mutations indicated that all mutations identified here harbour the potential to seriously affect the enzymatic activity of the EST domain. Based on current knowledge there are two main pathways of action for PNPLA6. Firstly, PNPLA6 de-esterifies phosphatidylcholine, a major component of biological membranes, into its constituent fatty acids and glycerophosphocholine (Strickland *et al.*, 1995; Atkins *et al.*, 2002; van Tienhoven *et al.*, 2002; Zaccheo *et al.*, 2004). Glycerophosphocholine serves as a precursor for the biosynthesis of acetylcholine, a key neurotransmitter involved in mediating cellular signalling in the nervous system. This may lead to disturbance of development and maintenance of synaptic connections in a variety of neuronal networks. Secondly, PNPLA6—which possesses lysophospholipase activity (van Tienhoven *et al.*, 2002)—has recently been suggested to catalyse 2-arachidonoyl lysophosphatidylinositol, which brings it into close functional relationship with other hereditary spastic paraplegia genes (*DDHD1*/SPG28, *CYP2U1*/SPG49) (Tesson *et al.*, 2012). Thus, PNPLA6 disease might need to be added to this rapidly increasing list of genes involved in lipid metabolism associated with neurodegenerative disease (Schuurs-Hoeijmakers *et al.*, 2012; Tesson *et al.*, 2012; Boukhris *et al.*, 2013; Martin *et al.*, 2013). Our PNPLA6 findings demonstrate that the phenotypic spectrum of these neurodegenerative phospholipid diseases is much broader than previously thought, extending from Boucher-Neuhäuser syndrome/Gordon Holmes syndrome to spastic ataxia and hereditary spastic paraplegia with or without motor neuropathy.

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Supplementary material

Supplementary material is available at *Brain* online.

References

Anheim M, Tranchant C, Koenig M. The autosomal recessive cerebellar ataxias. N Engl J Med 2012; 366: 636–46.

Atkins J, Luthjens LH, Hom ML, Glynn P. Monomers of the catalytic domain of human neuropathy target esterase are active in the presence of phospholipid. Biochem J 2002; 361 (Pt 1): 119–23.

Boucher BJ, Gibberd FB. Familial ataxia, hypogonadism and retinal degeneration. Acta Neurol Scand 1969; 45: 507–10.

Boukhris A, Schule R, Loureiro JL, Lourenco CM, Mundwiller E, Gonzalez MA, et al. Alteration of ganglioside biosynthesis responsible for complex hereditary spastic paraplegia. Am J Hum Genet 2013; 93: 118–23.

Carson M. Ribbons 2.0. J Appl Crystallogr 1991; 24: 958–61.

DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011; 43: 491–8.

Divina P, Kvitkovicova A, Buratti E, Vorechovsky I. Ab initio prediction of mutation-induced cryptic splice-site activation and exon skipping. Eur J Hum Genet 2009; 17: 759–65.

Fogel BL, Perlman S. Clinical features and molecular genetics of autosomal recessive cerebellar ataxias. Lancet Neurol 2007; 6: 245–57.

Gonzalez MA, Lebrigio RF, Van Booven D, Ulloa RH, Powell E, Speziani F, et al. GEnomes Management application (GEM.app): a new software tool for large-scale collaborative genome analysis. Hum Mutat 2013; 34: 842–6.

Ho CY, Lammerding J. Lamins at a glance. J Cell Sci 2012; 125 (Pt 9): 2087–93.

Holmes G. A form of familial degeneration of the cerebellum. Brain 1907; 30: 466–89.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009; 25: 1754–60.

Limber ER, Bresnick GH, Lebovitz RM, Appen RE, Gilbert-Barness EF, Pauli RM. Spinocerebellar ataxia, hypogonadotropic hypogonadism, and choroidal dystrophy (Boucher-Neuhauser syndrome). Am J Med Genet 1989; 33: 409–14.

Margolin DH, Kousi M, Chan YM, Lim ET, Schmahmann JD, Hadjivassiliou M, et al. Ataxia, dementia, and hypogonadotropism caused by disordered ubiquitination. N Engl J Med 2013; 368: 1992–2003.

Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F, Sali A. Comparative protein structure modeling of genes and genomes. Annu Rev Biophys Biomol Struct 2000; 29: 291–325.

Martin E, Schule R, Smets K, Rastetter A, Boukhris A, Loureiro JL, et al. Loss of function of glucocerebrosidase GBA2 is responsible for motor neuron defects in hereditary spastic paraplegia. Am J Hum Genet 2013; 92: 238–44.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20: 1297–303.

Neuhauser G, Opitz JM. Autosomal recessive syndrome of cerebellar ataxia and hypogonadotropic hypogonadism. Clin Genet 1975; 7: 426–34.

Nilius B, Voets T. The puzzle of TRPV4 channelopathies. EMBO Rep 2013; 14: 152–63.

Rainier S, Bui M, Mark E, Thomas D, Tokarz D, Ming L, et al. Neuropathy target esterase gene mutations cause motor neuron disease. Am J Hum Genet 2008; 82: 780–5.

Richardson RJ, Hein ND, Wijeyesakere SJ, Fink JK, Makhaeva GF. Neuropathy target esterase (NTE): overview and future. Chem Biol Interact 2013; 203: 238–44.

Rydel TJ, Williams JM, Krieger E, Moshiri F, Stallings WC, Brown SM, et al. The crystal structure, mutagenesis, and activity studies reveal that patatin is a lipid acyl hydrolase with a Ser-Asp catalytic dyad. Biochemistry 2003; 42: 6696–708.

Schuurs-Hoeijmakers JH, Geraghty MT, Kamsteeg EJ, Ben-Salem S, de Bot ST, Nijhof B, et al. Mutations in DDHD2, encoding an intracellular phospholipase A(1), cause a recessive form of complex hereditary spastic paraplegia. Am J Hum Genet 2012; 91: 1073–81.

Strickland JA, Orr GL, Walsh TA. Inhibition of diabrotica larval growth by patatin, the lipid acyl hydrolase from potato tubers. Plant Physiol 1995; 109: 667–74.

Su Y, Dostmann WR, Herberg FW, Durick K, Xuong NH, Ten Eyck L, et al. Regulatory subunit of protein kinase A: structure of deletion mutant with cAMP binding domains. Science 1995; 269: 807–13.

Tesson C, Nawara M, Salih MA, Rossignol R, Zaki MS, Al Balwi M, et al. Alteration of fatty-acid-metabolizing enzymes affects mitochondrial form and function in hereditary spastic paraplegia. Am J Hum Genet 2012; 91: 1051–64.

van Tienhoven M, Atkins J, Li Y, Glynn P. Human neuropathy target esterase catalyzes hydrolysis of membrane lipids. J Biol Chem 2002; 277: 20942–8.

Zaccheo O, Dinsdale D, Meacock PA, Glynn P. Neuropathy target esterase and its yeast homologue degrade phosphatidylcholine to glycerophosphocholine in living cells. J Biol Chem 2004; 279: 24024–33.