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UPDATE

Axonal neuropathy with neuromyotonia: there is a HINT

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Recessive mutations in the gene encoding the histidine triad nucleotide binding protein 1 (*HINT1*) were recently shown to cause a motor-predominant Charcot–Marie–Tooth neuropathy. About 80% of the patients exhibit neuromyotonia, a striking clinical and electrophysiological hallmark that can help to distinguish this disease and to guide diagnostic screening. HINT1 neuropathy has worldwide distribution and is particularly prevalent in populations inhabiting central and south-eastern Europe. With 12 different mutations identified in more than 60 families, it ranks among the most common subtypes of axonal Charcot–Marie–Tooth neuropathy. This article provides an overview of the present knowledge on HINT1 neuropathy with the aim to increase awareness and spur interest among clinicians and researchers in the field. We propose diagnostic guidelines to recognize and differentiate this entity and suggest treatment strategies to manage common symptoms. As a recent player in the field of hereditary neuropathies, the role of HINT1 in peripheral nerves is unknown and the underlying disease mechanisms are unexplored. We provide a comprehensive overview of the structural and functional characteristics of the HINT1 protein that may guide further studies into the molecular aetiology and treatment strategies of this peculiar Charcot–Marie–Tooth subtype.

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Abbreviations: AMP = adenosine monophosphate; ARS = aminoacyl-tRNA synthetase; CMT = Charcot-Marie-Tooth disease; HIT = histidine triad

Introduction

Hereditary peripheral neuropathies are a clinically and genetically heterogeneous group of disorders, characterized by muscle weakness, wasting and sensory loss, starting in the distal parts of the limbs and slowly progressing in a length-dependent manner (Boerkoel *et al.*, 2002; Patzko and Shy, 2012). In 2012, we identified recessive mutations in the gene encoding the histidine triad nucleotide binding protein 1 (*HINT1*) causing axonal, motor-predominant Charcot–Marie–Tooth (CMT) neuropathy, frequently associated with neuromyotonia (Zimon *et al.*, 2012). *HINT1*

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represents a global cause of CMT, with 79 patients of European, North American and Chinese ancestry identified to date (Zimon *et al.*, 2012; Caetano *et al.*, 2014; Zhao *et al.*, 2014; Boaretto *et al.*, 2015; Jerath *et al.*, 2015; Lassuthova *et al.*, 2015; Rauchenzauner *et al.*, 2016; Veltsista and Chroni, 2016). The frequency of *HINT1* mutations in a heterogeneous cohort of recessive CMT patients is ~10% and rises to 80% when focusing on individuals with axonal neuropathy having the clinical hallmark of neuromyotonia (Zimon *et al.*, 2012, 2015). Thus, HINT1-associated peripheral neuropathy represents a distinct clinical and genetic entity that needs to be differentiated among the numerous subtypes of CMT; and from myotonic dystrophy and the various channelopathies causing non-dystrophic forms of myotonia. This update summarizes the current knowledge on the clinical and electrophysiological aspects of the HINT1 neuropathy, the overlap with other clinical entities, the epidemiology and mutation spectrum, and the structural and functional characteristics of the encoded protein.

Epidemiology

HINT1 neuropathy has a non-random distribution (Fig. 1). The majority of diagnosed individuals are of European origin, a fact attributed to three founder mutations (R37P, C84R, H112N). R37P is the most common among them, displaying a gradient of distribution increasing from west to east in Europe. Forty-eight families described to date carry this mutation, most of them inhabiting or originating from central or south-eastern Europe and Turkey (Fig. 1). The R37P carrier frequency in outbred populations living in this geographic area is as high as 1:67-182, making HINT1 neuropathy one of the most

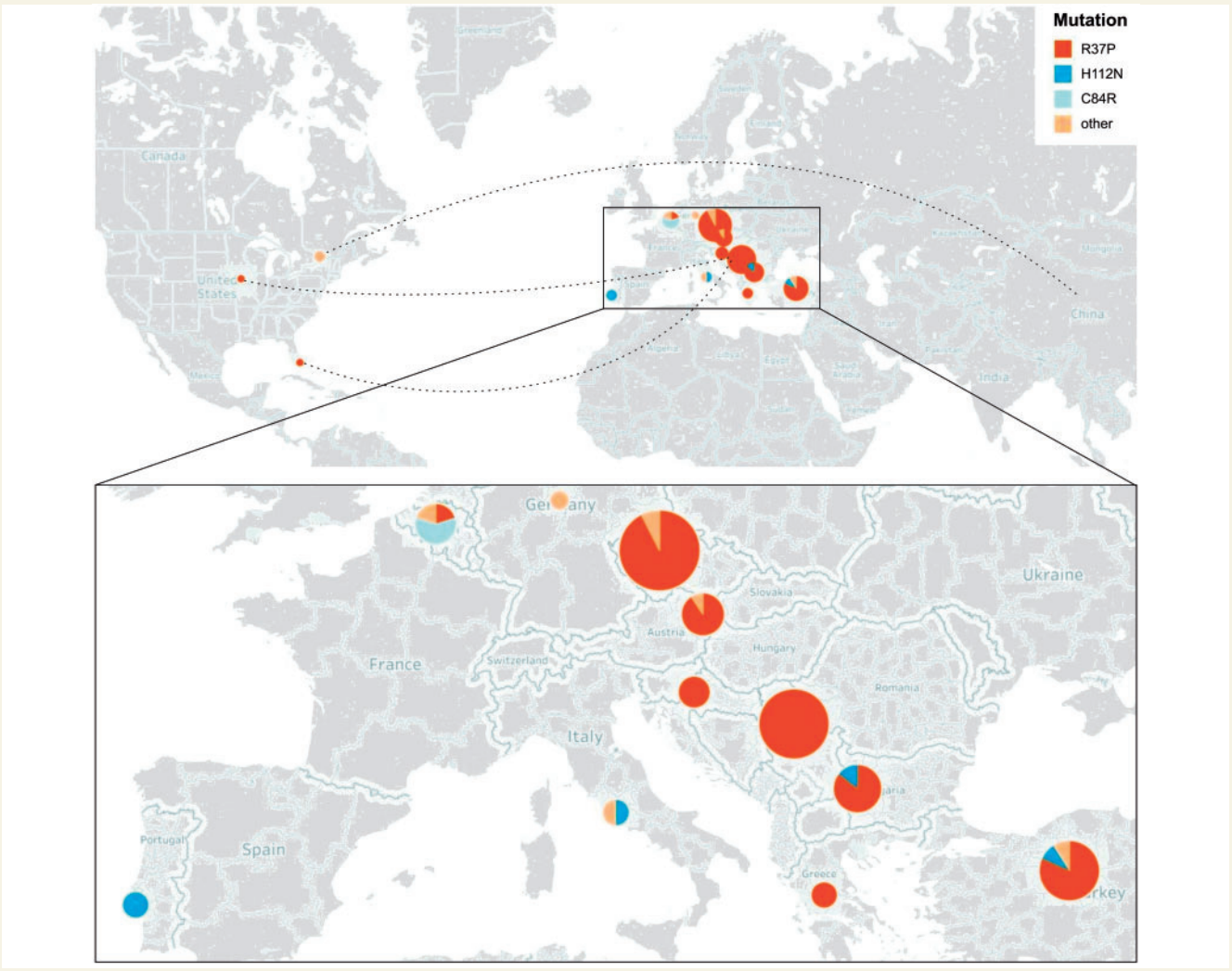


Figure 1 Worldwide distribution of *HINT1* mutations. Pie chart size represents the number of patients identified per country and colours indicate which founder *HINT1* mutations they are carrying. Dashed lines point out the country of origin of the identified patients. Enlarged panel below shows the regions in Europe where most patients are clustered. Note the gradient of distribution for the most common HINT1 mutation (R37P), increasing in central and south-eastern Europe.

common autosomal recessive disorders in this part of the world (Zimon *et al.*, 2012; Lassuthova *et al.*, 2015). The high R37P carrier rate can even lead to 'pseudo-dominant' inheritance of CMT, with affected individuals in two consecutive generations due to the influx of unrelated heterozygous carriers (Jordanova A. and Tournev I., unpublished results). In the Czech population, HINT1 neuropathy is among the most frequent causes of inherited neuropathy, only surpassed by CMT1A/HNPP and mutations in *GJB1* (previously known as *Cx32*) and *MPZ* (Lassuthova *et al.*, 2015). Because 90% of the Czech HINT1 patients carry R37P, genetic diagnosis becomes straightforward. Moreover, the US-based patients homozygous for R37P have central European origin (Zimon *et al.*, 2012; Jerath *et al.*, 2015). H112N is another founder mutation, with five families reported of Italian, Turkish, Bulgarian and (Portuguese) Roma origin. Finally, C84R is present in homozygous or compound heterozygous state in four Belgian families. Overall, the genetic epidemiology suggests that HINT1 neuropathy should be considered in the diagnostic work-up of patients of European descent presenting with axonal CMT.

Clinical features

The phenotype initially related to mutations in *HINT1* encompasses axonal, motor-greater-than-sensory polyneuropathy with an onset mostly in the first decade of life, combined with action neuromyotonia (more pronounced in the hands) and neuromyotonic or myokymic discharges on needle EMG (Zimon *et al.*, 2012; Caetano *et al.*, 2014; Lassuthova *et al.*, 2015; Rauchenzauner *et al.*, 2016). The identification of additional patients extended the clinical spectrum; including a later disease onset (up to 28 years of age) (Zhao *et al.*, 2014), asymmetric gait involvement (Rauchenzauner *et al.*, 2016) or a pure distal motor neuropathy (dHMN) without neuromyotonia (Zhao *et al.*, 2014; Boaretto *et al.*, 2015).

The initial complaints are distal lower limb weakness with gait impairment, combined with muscle stiffness, fasciculations and cramps in hands and legs, worsened by cold. When specifically asked, most patients report difficulties in releasing grip after a strong voluntary hand contraction, dating back from childhood. The disorder is slowly progressive; none of the reported patients lose ambulation until the sixth decade of life. Upon clinical examination, foot/toe extension and flexion weakness to plegia are present in almost all cases (Zimon *et al.*, 2012; Caetano *et al.*, 2014; Boaretto *et al.*, 2015; Lassuthova *et al.*, 2015). Achilles tendon reflexes are diminished to absent, depending on the stage of progression. Upper limbs become involved later in the disease course, usually in the first or second decade. Calf and intrinsic hand and foot muscle wasting is almost always observed to a variable degree (Fig. 2A–E). The hypotrophy of the intrinsic hand muscles, particularly of the hypothenar and thenar eminence is pronounced, leading to flexion contractures of the fingers, even in cases with mild muscle weakness (Fig. 2D and E). Mild distal sensory impairment can be present (Zimon *et al.*, 2012; Caetano *et al.*, 2014; Lassuthova *et al.*, 2015).

Neuromyotonia

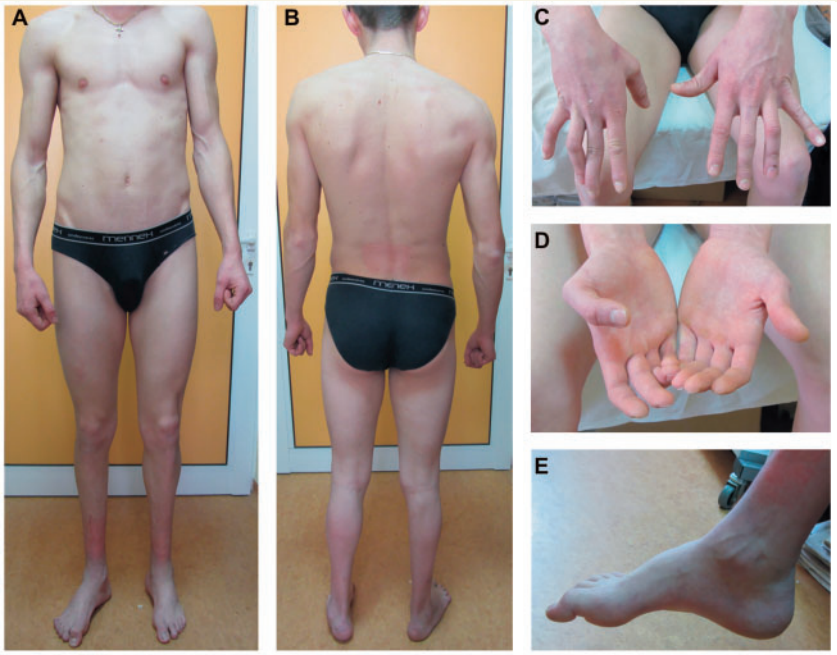
Neuromyotonia is present in 70–80% of patients and is a diagnostic hallmark. It is characterized by spontaneous muscular activity at rest (myokymia), impaired muscle relaxation (pseudomyotonia), and contractures of hands and feet (Maddison, 2006); and can be observed with or without overt peripheral neuropathy (Hahn *et al.*, 1991, 2000). In contrast to myotonia, in which abnormal muscle activity occurs only after voluntary or induced muscle contraction, neuromyotonia results from spontaneously occurring peripheral nerve discharges often accentuated by voluntary muscle contraction (Rauchenzauner *et al.*, 2016). This phenomenon was comprehensively characterized in two sibs of a Canadian family (Hahn *et al.*, 1991), where subsequently *HINT1* mutations were identified (Zimon *et al.*, 2012). The abnormal electrical activity can be enhanced by nerve ischaemia, but not by mechanical or electrical stimulation of the nerve supplying the muscle, thus suggesting that the nerve hyperexcitability is a generalized phenomenon related to a functional or structural abnormality of the axonal membrane. The neuronal origin of neuromyotonia was subsequently proven by regional neuromuscular blockade with curare and nerve block with xylocaine. HINT1 patients display action myotonia (delayed muscle relaxation of the hands after strong flexion of the fingers), while percussion myotonia of the thenar eminence is not typical (Zimon *et al.*, 2012; Caetano *et al.*, 2014; Boaretto *et al.*, 2015; Lassuthova *et al.*, 2015). Unfortunately, the symptoms of peripheral nerve excitability can be easily missed from patients' history or from the neurological examination.

Various types of skeletal deformities are noted in HINT1 patients. Foot deformities (pes cavus, pes equinovarus, pes cavovarus or Achilles tendon shortening) are present in a great proportion of cases (Zimon *et al.*, 2012; Lassuthova *et al.*, 2015). Flexion contractures of the fingers are typical, occurring up to several years after the lower limb involvement (Tournev I., unpublished results). Scoliosis is reported in one-third of the patients (Lassuthova *et al.*, 2015; Jerath *et al.*, 2015).

In some patients, mild-to-moderate elevation of creatine kinase levels is observed (Zimon *et al.*, 2012; Jerath *et al.*, 2015), probably related to the chronic neurogenic muscle atrophy in combination with the neuromyotonia.

Electrophysiology

Electrophysiological studies of peripheral nerves are compatible with axonal polyneuropathy; either motor-and-sensory (42/64; 66%) (Zimon *et al.*, 2012; Lassuthova *et al.*, 2015) or pure motor (22/64; 34%) (Zimon *et al.*, 2012; Zhao *et al.*, 2014; Boaretto *et al.*, 2015). Conduction velocities of motor and sensory fibres are (nearly) normal, while the amplitudes of compound muscle action potential or sensory nerve action potential are decreased. No markers



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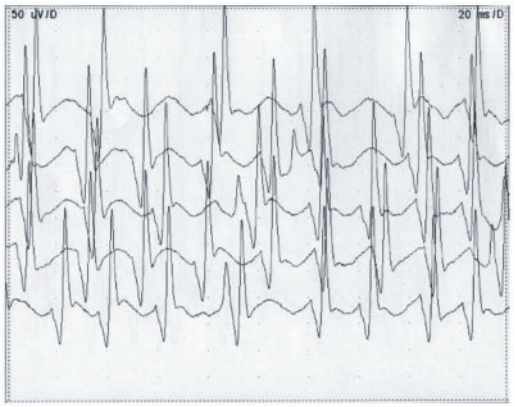

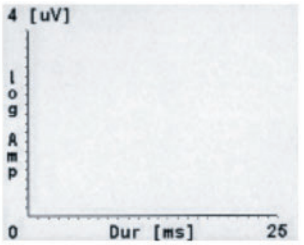

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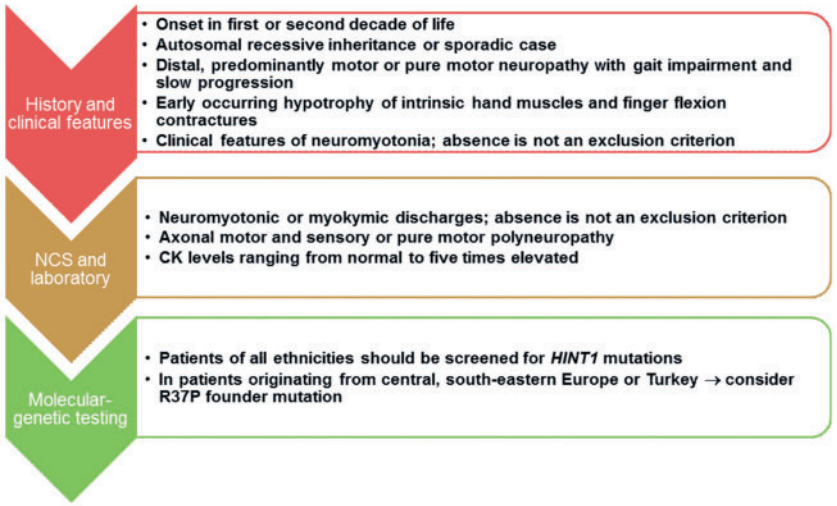

Figure 2 Clinical presentation of *HINT1* patients. (**A–E**) A 29-year-old male patient (genotype R37P/R37P) showing bilateral calf muscle atrophy (**A** and **B**), flexion contractures of the fingers (**C**), intrinsic hand muscle wasting (**D**), and pes cavus (**E**). (**F**) Neuromyotonic discharges, recorded with a concentric needle electrode in the right m. rectus femoris of a 27-year-old female patient (genotype R37P/R37P). (**G**) Diagnostic guidelines for HINT1 neuropathy.

of demyelination (conduction slowing, temporal dispersion or conduction block) are present. Needle EMG shows increased amplitude of motor unit action potentials and reduction of recruitment pattern with temporal summation.

Concentric needle EMG from proximal and distal muscles often displays neuromyotonic discharges (Fig. 2F) occurring spontaneously or provoked by needle movement or muscle contraction (Zimon *et al.*, 2012; Lassuthova *et al.*, 2015). They are characterized by high frequency (150–200 Hz), decrementing, repetitive discharges of a single motor unit with motor unit action potential morphology. Myokymic discharges, representing rhythmic, grouped discharges of the same motor unit, are also observed. The firing frequency within the burst is 2–60 Hz followed by a short period (up to a few seconds) of silence, and then recurrence of the burst at regular intervals (Kucukali *et al.*, 2015). Hyperexcitability and ectopic impulse generation can occur along the whole length of the axons, including the terminal arborizations (Hahn *et al.*, 1991). Although considered an EMG hallmark, neuromyotonic or myokymic discharges are absent in around 20–30% of patients, thus complicating the differential diagnosis (Zimon *et al.*, 2012; Zhao *et al.*, 2014; Boaretto *et al.*, 2015). Moreover, they may occur in the later stages of the disease (Zimon *et al.*, 2012; Caetano *et al.*, 2014; Boaretto *et al.*, 2015; Lassuthova *et al.*, 2015).

Nerve biopsy

The changes observed in the sural nerve of five HINT1 patients are consistent with an axonal neuropathy, even when no clinical features of sensory neuropathy are present (Zimon *et al.*, 2012).

Differential diagnosis

The diagnosis of HINT1-associated hereditary neuropathy requires consideration whether the phenotype is genetic or acquired. Due to the recessive pattern of inheritance this is not always straightforward, especially in sporadic cases. Detailed genealogy, neurological examination, nerve conduction studies and EMG are crucial. Diagnostic guidelines to recognize HINT1 neuropathy are represented in Fig. 2G.

The differential diagnosis includes several acquired and inherited disease entities, associated with abnormal spontaneous muscle/nerve hyperexcitability and/or weakness (Table 1). As neuromyotonia can be absent or under-recognized, other types of hereditary axonal CMT and pure motor neuropathies should be considered (Rossor *et al.*, 2012; Zimon *et al.*, 2012; Zhao *et al.*, 2014).

Treatment strategies

There is no curative treatment for patients with HINT1 neuropathy, therefore regular physical therapy, ankle–foot orthoses and/or special shoes remain mandatory. In the stage of limb deformities, surgical orthopaedic corrections are beneficial. These include soft-tissue procedures (plantar fascia release, tendon release or transfer), osteotomy (metatarsal, midfoot, calcaneal), and joint-stabilizing procedures (triple arthrodesis) (Caetano *et al.*, 2014; Boaretto *et al.*, 2015; Lassuthova *et al.*, 2015; and Tournev I., unpublished results). Additionally, to decrease the symptoms of neuromyotonia and the abnormal spontaneous discharges on EMG, a favourable therapeutic response has been elicited with medications blocking sodium channels, such as antiepileptic drugs (diphenylhydantoin and carbamazepine) (Hahn *et al.*, 1991; Tournev I., unpublished results) and anti-arrhythmics (tocainid) (Hahn *et al.*, 1991).

HINT1 structure and enzymatic activity

HINT1 is a member of the histidine triad (HIT) protein family, sharing a characteristic HIT motif (His-x-His-x-His-x-x, where x is a hydrophobic residue) in the catalytic pocket (Seraphin, 1992; Brenner, 2002). Mammalian HINT1 orthologues are nearly identical, and even though sequence similarity is lower with other eukaryotes, HINT1 function is evolutionary conserved (Bieganowski *et al.*, 2002). The protein is ubiquitous, but highly expressed in brain and spinal cord (Barbier *et al.*, 2007; Liu *et al.*, 2008; Zimon *et al.*, 2012), suggesting its important role in the nervous system.

HINT1 is a globular protein of 13.7 kDa that acts as a homodimer and binds purine nucleosides and nucleotides (Gilmour *et al.*, 1997). Each monomer has a nucleotide-binding cleft containing the HIT motif (Brenner *et al.*, 1997). The nucleotide-contacting residues in this cleft are strictly conserved throughout the HIT superfamily (Brenner *et al.*, 1997), but substrate specificity is dependent on the sequence of the C-terminal loop (Chou *et al.*, 2007). Furthermore, dimerization is required to maintain sufficient catalytic activity (Chou and Wagner, 2007).

The endogenous substrate(s) of HINT1 remain unknown; nevertheless, it appears to be a promiscuous enzyme *in vitro* (Gilmour *et al.*, 1997; Bieganowski *et al.*, 2002; Chou and Wagner, 2007; Ozga *et al.*, 2010; Wang *et al.*, 2012). The general formula of HINT1 substrates is NMP-X [X = -NHR, -OC(O)R, -S] (Krakowiak *et al.*, 2014) (Fig. 3A). HINT1 preferentially hydrolyses adenosine derivatives with a single phosphate, including phosphoramidates (AMP-NH_2, AMP-*N*-ε-lysine, AMP-alanine) and aminoacyl adenylates (see below) (Bieganowski *et al.*, 2002; Wang *et al.*, 2012). Known HINT1 inhibitors are sulfamoyl adenosine and *N*-ethylsulfamyol adenosine (Krakowiak *et al.*, 2004). Crystallographic analysis of HINT1 in complex with artificial substrates has elucidated its catalytic mechanism of action as follows (Lima *et al.*, 1997; Krakowiak *et al.*, 2004; Wang *et al.*, 2012): (i) recognition of the NMP-X substrate: the nucleotide part binds in the HIT nucleotide-binding cleft and, in case the side-chain is an alkylamide, its alkyl portion binds the C-terminal Trp123 residue located across the dimer interface; (ii) nucleophilic attack by the His112 residue on the α-

Table 1 Differential diagnosis of HINT1 neuropathy

Clinical entity	Aetiology	Clinical findings	Electrodiagnostic findings
Acquired disorders			
Isaacs syndrome	VGKC antibodies	Onset predominantly in the mid-40s	Normal sensory and motor NCS, except for after-discharges
		Continuous muscle twitching and myokymia, muscle hypertrophy, weight loss, hyperhidrosis	Neuromyotonic and myokymic discharges, doublets or triplets or multiplets, fasciculation potentials, fibrillation potentials, and cramp discharges, occurring spontaneously or activated by voluntary muscle contraction on needle EMG
		Preserved muscle strength and tendon reflexes	
Morvan syndrome	VGKC antibodies	Similar to Isaacs syndrome plus CNS: encephalopathy, headaches, drowsiness, and hallucinations	Similar to Isaacs syndrome
Cramp fasciculation syndrome	Uncertain	Muscle cramps, exercise intolerance, and muscle twitching	After-discharges on repetitive nerve stimulation and fasciculation potentials on needle EMG
Other	Toxins: lead, silver and gold		Myotonic discharges on needle EMG
Inherited disorders			
Episodic ataxia type 1	Mutations in *KCNA1*	Attacks of generalized ataxia, persistent myokymia	Neuromyotonic and myokymic discharges on needle EMG
Schwartz–Jampel syndrome	Mutations in *HSPG2*	Myotonia, typical facial appearance (blepharophimosis) and skeletal deformities	Myotonic discharges on needle EMG
Rippling muscle disease	Mutations in *CAV3*	Rolling movements of muscles after stretching or percussion	Percussion induced activity
Myotonic dystrophy type 1	Trinucleotide expansion in *DMPK*	Predominant distal muscle weakness, cataracts, cardiac conduction disturbances, cognitive impairment, endocrine disturbances	Rarely neuropathy, secondary to endocrine disturbances
			Myopathic changes and myotonic discharges on needle EMG
Non-dystrophic myotonias	Mutations in *CLNC1* and *SCN4A*	Muscle stiffness as well as pain, weakness and fatigue	Normal NCS and myotonic discharges on needle EMG
Axonal CMT		Slowly progressive muscle weakness, wasting and sensory loss, starting in the distal parts of the limbs, deformities	NCV within normal range or slightly reduced, reduced CMAPs and SNAPs
			Positive waves, polyphasic potentials, or fibrillations on needle EMG
Distal hereditary motor neuropathies		Slowly progressive muscle weakness, wasting, starting in the distal parts of the limbs, deformities	NCV within normal range or slightly reduced, reduced CMAPs
			Positive waves, polyphasic potentials, or fibrillations on needle EMG

CMAPs = compound muscle action potentials; NCS = nerve conduction studies; SNAPs = sensory nerve action potentials; VGKC = voltage-gated potassium channel.

phosphate of the substrate; (iii) formation of a covalent nucleotidyl phospho-HINT1 intermediate (adenylylation); and (iv) hydrolysis of the nucleotide and release of the catalytic products (Fig. 3A).

HINT1 functions

HINT1 hydrolyses aminoacyl adenylates, intermediary products of the charging reaction of tRNAs with their cognate amino acids by aminoacyl-tRNA synthetases (ARS); it was isolated in complexes with lysyl-tRNA synthetase (KARS) and transcription factors (Lee *et al.*, 2004; Lee and Razin, 2005). In the presence of KARS and ATP, HINT1 is adenylated in a lysine-dependent manner, suggesting that the HINT1-AMP formation relies upon the production of lysyl-AMP by KARS (Chou and Wagner, 2007). Similarly, HINT1 reacts with other aminoacyl adenylates (Ala-AMP, Asp-AMP, Met-AMP, His-AMP) produced by their respective cognate (and no other) ARS (Wang *et al.*, 2012). Thus, by hydrolysis of the aminoacyl adenylate intermediate, HINT1 might mediate ARS activity and influence the overall level of tRNA aminoacylation. Intriguingly, like *HINT1*, several *ARS* (*YARS*, *GARS*, *AARS*, *KARS*, *MARS*, *HARS*) are causal genes for CMT (Antonellis *et al.*, 2003; Jordanova *et al.*, 2006; Latour *et al.*, 2010; McLaughlin *et al.*, 2010; Gonzalez *et al.*, 2013; Safka Brozkova *et al.*, 2015). Since they form part of the same functional network, a common pathomechanism could exist, linking HINT1 and ARS jointly to disorders of the peripheral nervous system.

HINT1 desulphurates nucleoside 5'-O-monophosphorothioates (NMPS) *in vivo* (Krakowiak *et al.*, 2014),

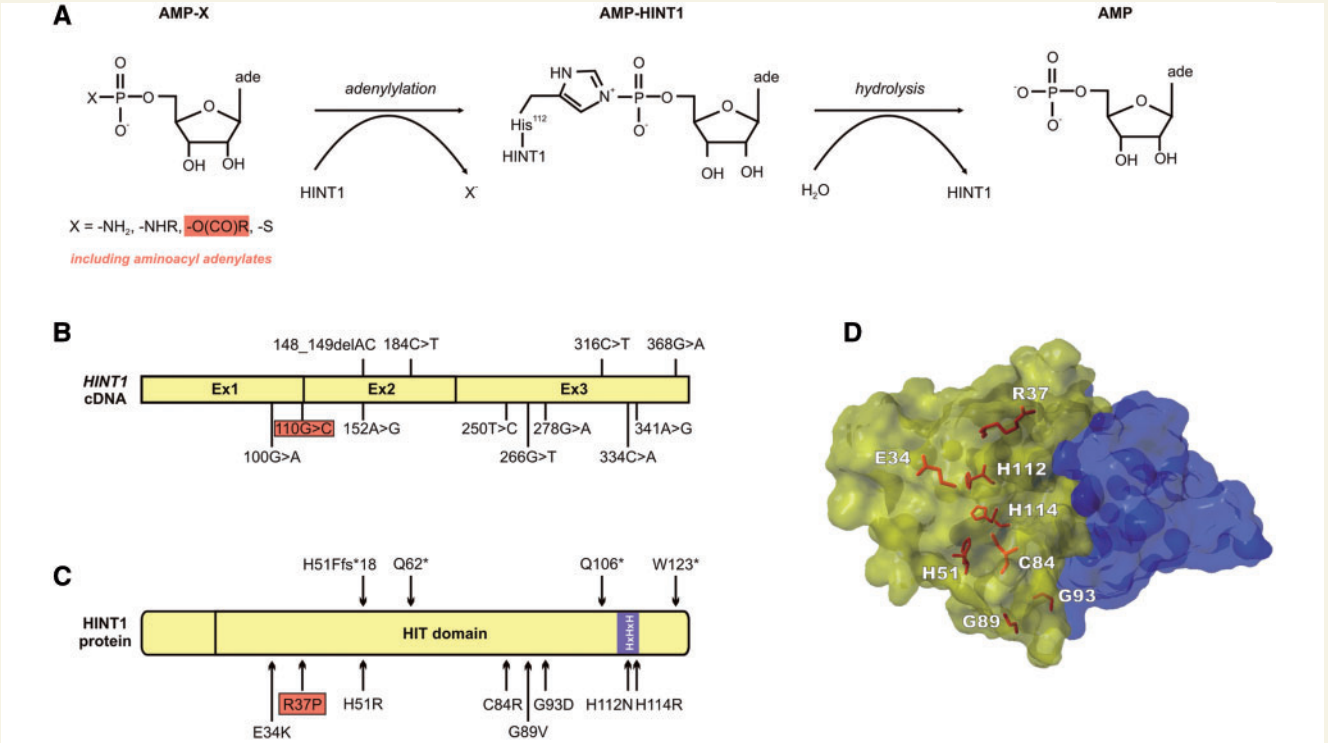

Figure 3 HINT1 structure and known mutations. (**A**) Reaction scheme for cleavage of AMP-linked compounds by the HINT1 enzyme, adapted from Krakowiak *et al.* (2014). (**B** and **C**). Position of the 12 known *HINT1* mutations on the cDNA and protein structures, respectively. (**D**) 3D representation of the HINT1 dimer, highlighting the eight amino acid residues targeted by missense mutations. HINT1 monomers are shown in blue and yellow.

producing free H_2S inside the cell. By influencing the levels of this signalling molecule, HINT1 might regulate physiological processes, like post-translational protein modification, targeting of ATP-sensitive potassium channels and modulation of vascular tone (Koenitzer *et al.*, 2007).

HINT1 acts as a transcriptional suppressor via direct binding to transcription factors. The HINT1–transcription factor complex can be dissociated through the sequestering of HINT1, consequently leading to transcriptional activation. Known sequesters of HINT1 are the diadenosine tetraphosphate (AP_4A), a side-product of KARS activity (Brevet *et al.*, 1982) and the N-terminal intracellular domain of teneurin-1 (TEN1-ICD), a cleaved-off peptide that translocates to the nucleus (Scholer *et al.*, 2015). Basal AP_4A and TEN1-ICD levels thus determine the delicate balance between HINT1-mediated transcriptional repression and activation.

Known transcription factors directly regulated by HINT1 are MITF, USF2, pontin and reptin (Razin *et al.*, 1999; Lee and Razin, 2005; Weiske and Huber, 2005). Independent of its enzymatic activity, HINT1 has an overall repressive effect on the T cell factor (TCF)-4-β-catenin transcriptional activity, neutralizing the activating effect of pontin and strengthening the transcriptional repression by reptin (Weiske and Huber, 2006). Also unrelated to its catalytic activity, HINT1 inhibits the activator protein-1 (AP1) transcription factor by binding to the POSH-JNK2 complex and inhibiting c-Jun phosphorylation (Wang *et al.*, 2007). Finally, HINT1 interacts with the cyclin-dependent kinase 7 (CDK7), part of the TFIIK component of the general transcription factor TFIIH (Keogh *et al.*, 2002). It is presumed that catalysis by HINT1 is a prerequisite for proper formation of the TFIIH complex (Bieganowski *et al.*, 2002).

Loss of HINT1 increases susceptibility to carcinogenesis in mice (Su *et al.*, 2003; Li *et al.*, 2006), suggesting a role as a tumor suppressor. *Hint1*$^{-/-}$ mouse embryonic fibroblasts display an augmented growth rate, spontaneous immortalization and an increased resistance to ionizing radiation (Su *et al.*, 2003). In some cancer cell lines, HINT1 expression is decreased due to epigenetic silencing and its subsequent upregulation then halts cell proliferation, independent of the HINT1 catalytic activity (Wang *et al.*, 2007; Zhang *et al.*, 2009).

HINT1 interacts with the μ-opioid receptor (MOR), the major molecular target for morphine analgesia (Guang *et al.*, 2004; Rodriguez-Munoz *et al.*, 2011). HINT1 functions as an adaptor coupling protein kinase C gamma (PKCγ) to the MOR to downregulate its signalling capacity (Ajit *et al.*, 2007). The role of HINT1 in the MOR pathway is unrelated to its enzymatic activity, as this function does not depend on HINT1 dimerization (Rodriguez-Munoz *et al.*, 2008).

HINT1 is implicated in the regulation of mood and behaviour, suggesting an additional role in the CNS. HINT1 levels are increased in dorsolateral prefrontal cortex of patients with major depression disorder (Martins-de-Souza *et al.*, 2012) and, adversely, decreased in the same brain regions of patients with schizophrenia (Varadarajulu *et al.*, 2012). Furthermore, association studies reveal *HINT1* as a susceptibility gene for schizophrenia (Chen *et al.*, 2008; Kurotaki *et al.*, 2011), bipolar disorder (Elashoff *et al.*, 2007) and nicotine dependence (Jackson *et al.*, 2011; Fang *et al.*, 2014). So far, CMT patients carrying *HINT1* mutations have not been neuropsychiatrically evaluated. Such examinations could help revealing putative common pathomechanisms for disorders of the peripheral and CNS.

CMT mutations cause loss of HINT1 function

The 12 known CMT-causing mutations (Zimon *et al.*, 2012; Zhao *et al.*, 2014; Boaretto *et al.*, 2015; Lassuthova *et al.*, 2015; Rauchenzauner *et al.*, 2016) (Fig. 3B–D) cause loss of functional HINT1 protein, because they: (i) affect residues critical for the catalytic activity of HINT1 (H112N, H114R) (Bieganowski *et al.*, 2002; Ozga *et al.*, 2010); (ii) putatively lead to nonsense-mediated decay of the mutant transcript (H51Ffs*18, Q62*); or (iii) are proven to cause protein instability and subsequent proteasome-mediated degradation (R37P, H51R, C84R, W123*) (Zimon *et al.*, 2012). Five of the mutations (R37P, H51R, C84R, H112N, W123*) were modelled in a yeast strain that is deficient for the orthologous gene, *HNT1* (Zimon *et al.*, 2012). This strain does not grow on synthetic galactose-containing media at 39°C (Bieganowski *et al.*, 2002). Under standard culturing conditions, however, *HNT1* knockout strain is perfectly viable and indistinguishable from the wild-type, indicating that *HNT1* is a non-essential gene. Unlike wild-type human HINT1, the CMT-causing proteins cannot complement the growth phenotype of this strain, thus providing further evidence that loss of functional HINT1 leads to peripheral neuropathy. It is currently unclear which one of the multiple HINT1 functions is mostly affected by the CMT mutations. However, it is likely that this function in dependent on enzymatic activity of the protein, as stable, but catalytically inactive HINT1 versions (e.g. H112N), are capable of causing the neuropathy.

Hint1 knockout mice do not show signs of peripheral neuropathy

Homozygous and heterozygous *Hint1* knockout mice display normal foetal and adult development and appearance. Yet, they are more susceptible to chemically induced carcinogenesis and to spontaneous tumour development on ageing (Su *et al.*, 2003; Li *et al.*, 2006). These findings are indicative of the role of HINT1 as a haploinsufficient tumour suppressor. Additionally, ablation of HINT1 leads to major reprogramming of lipid homeostasis (Beyoglu *et al.*, 2014) likely due to increased proliferative signalling and reduced pro-apoptotic signalling in the liver of *Hint1* knockout mice.

Furthermore, *Hint1*$^{-/-}$ mice exhibit anxiety-related (Varadarajulu *et al.*, 2011; Jackson *et al.*, 2012), aggressive (Dang *et al.*, 2015), and depression-related behaviour (Barbier and Wang, 2009), some of which can be reversed by valproate treatment (Barbier and Wang, 2009). Additionally, the animals respond differently to treatment with CNS-acting compounds, such as morphine (Guang *et al.*, 2004), amphetamine (Barbier *et al.*, 2007), and nicotine. PKCγ protein levels are elevated in *Hint1*$^{-/-}$ mouse brains (Varadarajulu *et al.*, 2011), yet, the PKCγ activation response upon psycho-stimulation with amphetamine is attenuated (Zhang *et al.*, 2015). These combined findings suggest a role for HINT1 and PKCγ in modulating, among others, anxiogenic and stress-coping behaviour in mice.

Intriguingly, unlike in humans, *Hint1*$^{-/-}$ mice do not have overt signs of neuropathy (Seburn *et al.*, 2014). Thorough examination for relevant neurological phenotypes, including motor performance, nerve, muscle and neuromuscular junction anatomy, nerve conduction studies and EMG does not show any evidence of axonal degeneration or neuromyotonia. Mice were aged to more than 1 year and, additionally, they were subjected to external stressors such as low temperature and a potassium channel-blocking agent to provoke neuromyotonia (Shillito *et al.*, 1995); yet all without the appearance of neuropathy-related phenotypes. This finding supports the notion that, similar to yeast, HINT1 is a non-essential gene in mammals and that alternative pathways exist that can functionally complement organismal HINT1 deficiency. This suggests that activation or upregulation of such pathways in patients with HINT1 mutations may provide an attractive route for the development of therapeutic strategies for HINT1 neuropathy.

Conclusion

Recessive, loss-of-function mutations in *HINT1* cause an early-onset, axonal form of motor-predominant peripheral neuropathy, often accompanied by the characteristic feature of neuromyotonia. The considerable prevalence of the disorder, especially in patients of European ancestry, is largely due to the existence of founder mutations, of which R37P is by far the most frequent. Here, we propose guidelines to recognize and differentiate HINT1-related neuropathy and suggest treatment strategies to manage common symptoms.

As a recent player in the field of hereditary neuropathies, the function of HINT1 in peripheral nerves is still completely unexplored. The gene is ubiquitously expressed, playing a role in manifold transcriptional and signalling pathways. Moreover, previous studies have indicated a relation of HINT1 to CNS functioning and pathology, yet, it

was highly unexpected to find this housekeeping gene causing a disorder affecting the peripheral nerves exclusively. The high prevalence and significant burden of the HINT1 neuropathy warrant further investigations into its underlying pathomechanisms, with the aim of finding therapeutic strategies to treat this incurable disorder.

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