intracellular trafficking defects, and ER stress. Thus, mutations of $GABA_A$ receptor subunits have revealed new aspects of the pathomechanisms underlying epilepsy and should facilitate our understanding of epilepsy. Research on the unique pathomechanisms of epilepsy resulting from mutations of $GABA_A$ receptor subunits should open a new avenue for developing new therapies for epilepsy.

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Distinct neurological disorders with *ATP1A3* mutations

Erin L Heinzen, Alexis Arzimanoglou, Allison Brashear, Steven J Clapcote, Fiorella Gurrieri, David B Goldstein, Sigurður H Jóhannesson, Mohamad A Mikati, Brian Neville, Sophie Nicole, Laurie J Ozelius, Hanne Poulsen, Tsveta Schyns, Kathleen J Sweadner, Arn van den Maagdenberg, Bente Vilsen, for the ATP1A3 Working Group

Genetic research has shown that mutations that modify the protein-coding sequence of *ATP1A3*, the gene encoding the α_3 subunit of Na^+/K^+-ATPase, cause both rapid-onset dystonia parkinsonism and alternating hemiplegia of childhood. These discoveries link two clinically distinct neurological diseases to the same gene, however, *ATP1A3* mutations are, with one exception, disease-specific. Although the exact mechanism of how these mutations lead to disease is still unknown, much knowledge has been gained about functional consequences of *ATP1A3* mutations using a range of in-vitro and animal model systems, and the role of Na^+/K^+-ATPases in the brain. Researchers and clinicians are attempting to further characterise neurological manifestations associated with mutations in *ATP1A3*, and to build on the existing molecular knowledge to understand how specific mutations can lead to different diseases.

Introduction

The introduction of next-generation sequencing technology, which allows nearly complete assessment of human exomes and genomes, has significantly boosted gene discovery.[1] These gene discoveries have advanced our knowledge of disease-specific pathophysiology, and have enabled genetic connections to be made between diseases. An example is the recent identification of mutations in *ATP1A3*, a gene previously associated with rapid-onset dystonia parkinsonism (RDP), in alternating hemiplegia of childhood (AHC). This genetic connection between diseases offers unique research opportunities to explore how genetics governs specific clinical phenotypes, and to study the underlying pathophysiology.

Here, we review current knowledge of the roles of ATP1A3 in the brain, as well as the phenotypes associated with mutations in the gene. Finally, we discuss additional research needed to further characterise the full phenotypic spectrum associated with mutations in *ATP1A3* and the associated changes in protein function, to establish possible strategies for the development of new treatments for AHC, RDP, and related disorders.

Roles of Na^+/K^+-ATPases

Na^+/K^+-ATPases are membrane-bound transporters that harness the energy of ATP hydrolysis to move three Na^+ out of the cell in exchange for two K^+ ions moving inwards. The resulting ionic gradients establish membrane potentials that generate electrical impulses and move neurotransmitters and Ca^{2+} across the plasma membrane. Na^+/K^+-ATPases consist of catalytic α, β, and regulatory γ subunits (figure 1). The main role of the α subunit is to bind and transport Na^+ and K^+. There are four α subunits, all encoded by different genes. The α_3 subunit, encoded by *ATP1A3*, is the predominant α subunit expressed in neurons,[2–4] although many neurons also express α_1. The α_3 subunit differs from α_1 in that it has a comparatively low affinity for Na^+ and K^+,[5,6] which enables rapid normalisation of ion gradients after intense neuronal firing. Changes in the activity of Na^+/K^+-ATPases in neurons (predominantly expressing α_3) have physiological consequences. For example, inhibition of Na^+/K^+-ATPases in the thalamus converts neuronal bursting responses to single spike discharges.[7] In the hippocampus, reduction of the activity of Na^+/K^+-ATPase causes interictal epileptiform bursting activity.[8] Furthermore, localisation of α_3 in dendritic spines has been shown to play a part in controlling the size and speed of the small depolarisations caused by fluctuations of intracellular Na^+ that occur during activation of ion-gating neurotransmitter receptors. These fluctuations, known as transients, are summated in dendrites and are the basis of synaptic integration.[9–11]

In addition to its primary function in ion transport, subunits of Na^+/K^+-ATPases have been shown to interact with proteins that assist in localisation of enzymes to the cell membrane, modulate the PI3K, PLCγ, and MAPK signal transduction cascades, and regulate the activity of other transporters and receptors.[12] The protein–protein interactions specific to the α_3 subunit are not fully characterised, but, as an example, the α_3 isoform has been shown in rat neurons to bind specifically to PSD95, a scaffolding protein that organises proteins at the synapse.[10]

ATP1A3 mutations and neurological disorders

In 1999, two large families were identified with several members presenting with RDP.[13,14] Linkage analyses identified the 19q13 locus as the region most likely to harbour mutations associated with disease;[15] a finding that was confirmed in additional families with RDP.[16,17] RDP in all these families was inherited as an autosomal dominant trait with incomplete penetrance. Using a positional cloning approach, investigators identified six different heterozygous missense mutations in *ATP1A3* that co-segregated with the disease phenotype.[18] Since the initial discovery, 11 mutations (nine missense mutations, a 3-bp in-frame deletion, and a 3-bp in-frame insertion; figure 2; table 1[18–34]) have been reported in 20 patients with RDP, 12 of whom had a positive family history of the disease.[17–30,35] Three mutations, one found only in sporadic RDP cases and two found in both sporadic and familial RDP cases, are recurrent, probably because they are located at hypermutable methylated CpG-dinucleotides in *ATP1A3* (table 1).[36]

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Center for Human Genome Variation (E L Heinzen PhD, Prof D B Goldstein PhD), Department of Medicine, Section of Medical Genetics (E L Heinzen), Department of Molecular Genetics and Microbiology (Prof D B Goldstein), Division of Pediatric Neurology (Prof M A Mikati MD), and Department of Neurobiology (Prof M A Mikati), Duke University, School of Medicine, Durham, NC, USA; Epilepsy, Sleep and Pediatric Neurophysiology Department, HFME, University Hospitals of Lyon, and Centre de Recherche en Neurosciences de Lyon, Centre National de la Recherche Scientifique, UMR 5292, INSERM U1028, Lyon, France (A Arzimanoglou MD); Department of Neurology, Wake Forest School of Medicine, Winston Salem, NC, USA (Prof A Brashear MD); School of Biomedical Sciences, University of Leeds, Leeds, UK (S J Clapcote PhD); Istituto di Genetica Medica, Università Cattolica S Cuore, Rome, Italy (Prof F Gurrieri PhD); AHC Federation of Europe and AHC Association of Iceland, Reykjavik, Iceland (S H Jóhannesson); Institute of Child Health, University College London, London, UK (Prof B Neville MB BS); Institut National de la Santé et de la Recherche Médicale, U975, Centre de Recherche de l'Institut du Cerveau et de la Moelle, Paris, France (S Nicole PhD); Centre National de la Recherche Scientifique, UMR7225, Paris, France (S Nicole); Université Pierre et Marie Curie Paris VI, UMRS975, Paris, France (S Nicole); Department of Genetics and Genomic Sciences and Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY, USA (L J Ozelius PhD); Danish Research Institute for Translational Neuroscience, Nordic-EMBL Partnership of Molecular Medicine,

Department of Molecular Biology and Genetics, Aarhus University, and Centre for Membrane Pumps in Cells and Disease—PUMPKIN, Danish National Research Foundation, Aarhus, Denmark (H Poulsen PhD); European Network for Research on Alternating Hemiplegia (ENRAH), Brussels, Belgium (T Schyns PhD); Neurosurgery, Massachusetts General Hospital, Boston, MA, USA (K J Sweadner PhD); Department of Human Genetics and Department of Neurology, Leiden University Medical Centre, Leiden, Netherlands (Prof A van den Maagdenberg PhD); and Department of Biomedicine, Aarhus University, Aarhus, Denmark (Prof B Vilsen DMSc)

Correspondence to: Dr Erin L Heinzen, Levine Science Research Building, Duke University, School of Medicine, Durham, NC 27008, USA
e.heinzen@duke.edu

For the **Exome Variant server** see http://evs.gs.washington.edu/EVS/

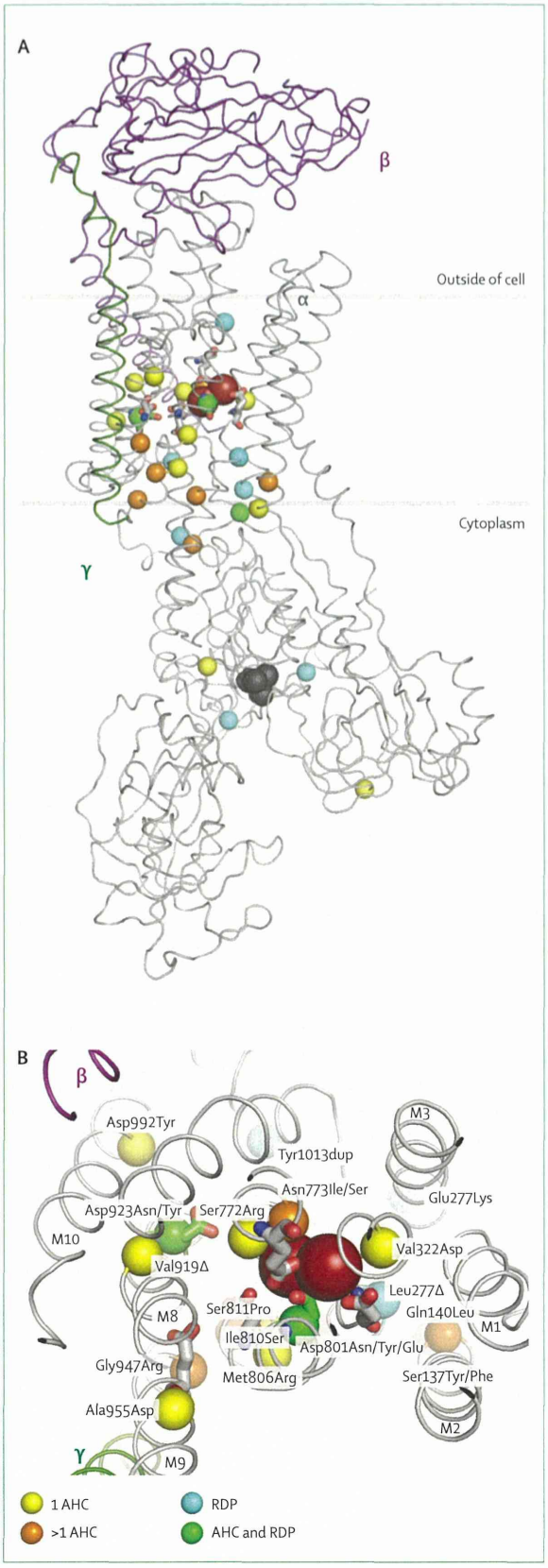


Figure 1: **Structure of the Na^+/K^+-ATPase**
(A) The Na^+/K^+-ATPase in the potassium-occluded state showing K^+ (red spheres), the three protein subunits α (grey lines), β (purple line) and FXYDγ (green line), and phosphorylation, which is mimicked by MgF_4^{2-} (dark grey spheres). Residues reported to be mutated in AHC or RDP, or both, are indicated by spheres at the α carbon, yellow for one case of AHC, orange for more than one case of AHC, cyan for RDP, and green for both AHC and RDP. Key ion binding residues are shown in stick format. (B) A 90° degree rotation in the membrane plane of the representation shown in (A), giving an extracellular view of the ion-binding transmembrane part of the Na^+/K^+-ATPase with disease-causing mutations colour coded as in (A). Two of the ion-binding residues (shown in sticks) have been found to be mutated both in patients with RDP and in those with AHC. Figures made from pdb code 2ZXE. AHC=alternating hemiplegia of childhood. RDP=rapid-onset dystonia parkinsonism.

In 2012, two independent studies, one by an international consortium[31] and one by German researchers,[32] identified de-novo mutations in *ATP1A3* as the cause of AHC. In both studies next-generation sequencing was used to screen the protein-coding portion of the genome of patients with sporadic AHC to look for disease-causing mutations that were absent in their unaffected parents. This approach identified a de-novo *ATP1A3* mutation in each of the ten initial patients screened in the two studies combined, which definitively establishes *ATP1A3* as the first AHC gene.[31,32] This finding was replicated in an independent Japanese study,[33] which found *ATP1A3* mutations in eight of ten patients with AHC. The German study identified mutations in all 24 German patients with AHC.[32] Notably, the international study reported *ATP1A3* mutations in 82 (78%) of 105 patients with AHC,[31] which suggests that some *ATP1A3* mutations might have been missed, that other AHC genes might exist, or that the diagnosis might not always be accurate. 27 different *ATP1A3* mutations have been reported in patients with AHC (figure 2, table 1). Ten mutations have been identified in more than one individual, with one mutation (Asp801Asn) explaining more than 40% of AHC cases with an *ATP1A3* mutation (table 1).

When we consider the location in the ATP1A3 protein sequence of mutations that cause RDP or AHC, an interesting difference emerges. Whereas RDP mutations seem to be spread across the protein, AHC mutations are located almost exclusively in particular regions of the protein (figure 3). The significance of the different mutation patterns in RDP and AHC is currently unknown, but suggests that, unlike in RDP, only specific protein disruptions result in AHC. Additionally, rarely the same aminoacid is mutated in RDP and AHC, but even in these cases the aminoacid substitution is disease-specific (table 1). Only one RDP mutation (Asp923Asn) has also been identified in an unusual case of familial AHC. In this multiplex AHC family, four individuals have the Asp923Asn mutation, including one with a diagnosis of AHC and three with some of the defining symptoms of AHC (see below).[29] This nearly perfect genotype-phenotype correlation, with a nearly non-overlapping set of mutations associated with AHC or RDP, strongly argues for a distinct functional effect of the mutations causing AHC or RDP, which is yet to be elucidated.

Consistent with mutations in *ATP1A3* causing neurodevelopmental diseases, only two polymorphic missense mutations (both with low population frequencies [minor allele frequency <0·1%]) have been reported for *ATP1A3* (figure 2, table 1) in the Exome Variant Server, NHLBI GO Exome Sequencing Project (Seattle, WA, USA). The database houses variants from protein-coding genomes of approximately 6500 individuals who were not identified based on neurodevelopmental or neuropsychiatric disease phenotypes. Evaluating the relationship of the total number of polymorphic functional variants as a function of the total number of variants for each

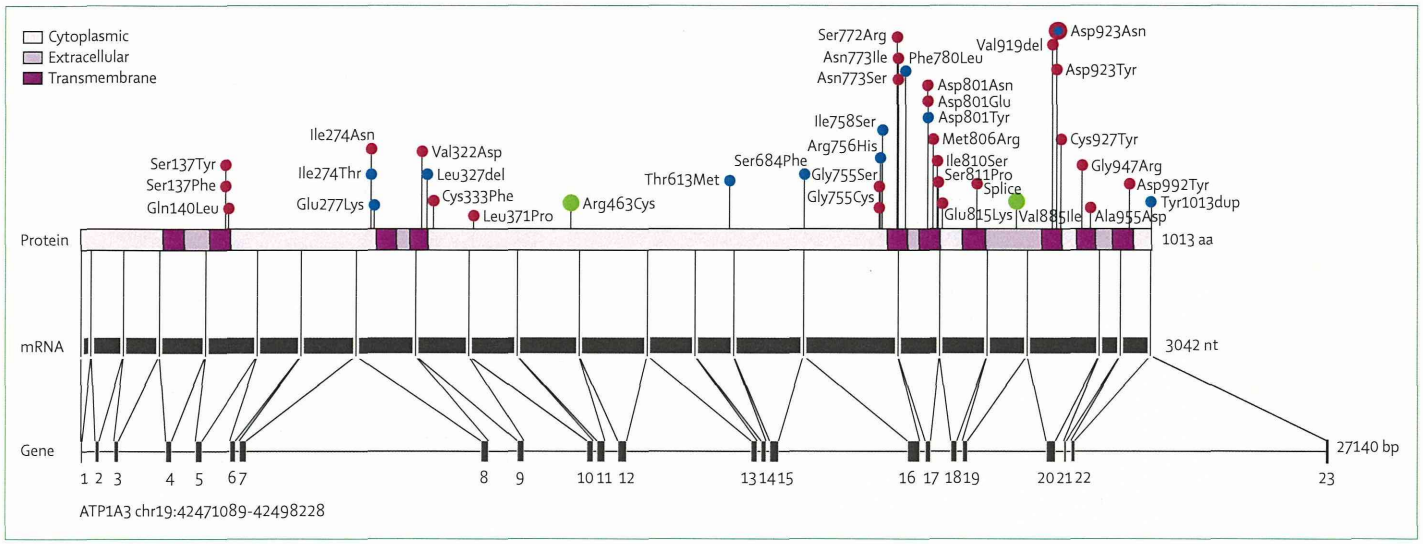


Figure 2: **Location of AHC-causing or RDP-causing mutations in *ATP1A3* gene, mRNA, and protein**
Red dots show AHC-causing mutations and blue dots show RDP-causing mutations. The one mutation shared between disease phenotypes is located at Asp923Asn (blue dot with a red dot inside). Two rare polymorphisms identified in the general population but not associated with a disease at this time are indicated by green dots. AHC=alternating hemiplegia of childhood. RDP=rapid-onset dystonia parkinsonism. aa=aminoacids. nt=nucleotides. bp=basepairs.

sequenced gene in the database indicates that observing two polymorphic functional variants in *ATP1A3* given the total number of variants reported is less that what is expected. Thus, *ATP1A3* is generally intolerant of functional variation in comparison with genome-wide expectations for a gene of its size and mutability, implying that individuals with functional mutations in this gene might be at high risk of developing serious diseases.[37]

Rapid-onset dystonia parkinsonism

Recognition of RDP began in 1993, more than 10 years before *ATP1A3* was identified as the causal gene, when Dobyns and colleagues[14] reported a 15-year-old girl who had abrupt onset of dystonia with prominent dysarthria and dysphagia. The disorder was named RDP because of the abrupt onset of dystonic spasms associated with postural instability and bradykinesia that resemble signs of parkinsonism.[13,38–40] RDP is also sometimes referred to as dystonia 12 (DYT12; OMIM: 128235). *ATP1A3* is the only known RDP gene; however, other RDP genes might exist, as evidenced by one study reporting that, in a select group of 14 patients referred for possible RDP, only three had a mutation in *ATP1A3*.[19]

The clinical presentation of RDP includes three features: its appearance, which often occurs after triggering events such as running, alcohol binges, minor head injuries, overheating, emotional stress, infections, or childbirth;[21] a rapid onset of typically permanent symptoms that develop over hours to days (occasionally even over several weeks); and involuntary movements that are characterised by generalised dystonia with superimposed parkinsonian features (primarily bradykinesia and postural instability without tremor). Many patients present with a rostrocaudal gradient of dystonia and parkinsonism, in the sense that bulbar symptoms are more severe than arm symptoms, and arm symptoms are more severe than leg symptoms. The bulbar and arm symptoms rarely improve after the primary disease onset. A few patients have reported later episodes of abrupt worsening of symptoms that occurred from 1 year to as late as 9 years after the initial onset. One patient, who had transient symptoms after athletic activity, recovered, resumed strenuous athletic activity, and then had permanent onset of fixed symptoms. Not all patients report a recognised trigger, and a few report antecedent periods of cramping. Patients typically lack other disease features such as diurnal fluctuation or episodes of symptoms that are typical of patients with AHC.

A recently published cohort of 26 patients with RDP indicated that 76% had onset of motor symptoms by the age of 25 years.[41] In addition to dystonia, patients can also have non-motor manifestations. Recent work suggests that patients with RDP have an increased prevalence of mood disorders (50%) and psychosis (19%) compared with relatives without an *ATP1A3* mutation.[41] These findings were observed across families with different *ATP1A3* mutations, and are consistent with reports of depression in individuals with *ATP1A3* mutations causing motor problems.[16]

The originally published diagnostic criteria for RDP require a family history, an onset of the disease in teenage years, and prominent bulbar findings.[19] However, as the number of reported cases of RDP increases, these criteria seem too restrictive for several reasons: more than half of patients with RDP lack a positive family history because the disease is caused by de-novo mutations in small, single-patient families; disease onset has been reported in children and adults;[30] and recognition is growing that there are patients in whom RDP can present with atypical

	Disease	Disease inheritance	Number of unrelated patients with the mutation*
410C→T; Ser137Phe	AHC	Sporadic	1
410C→A; Ser137Tyr	AHC	Sporadic	2
419A→T; Gln140Leu	AHC	Sporadic	1
821T→A; Ile274Asn	AHC	Sporadic, familial	2
821T→C; Ile274Thr	RDP	Sporadic	1
829G→A; Glu277Lys	RDP	Sporadic	2
965T→A; Val322Asp	AHC	Sporadic	1
979_981delCTG; Leu327del	RDP	Sporadic	1
998G→T; Cys333Phe	AHC	Sporadic	2
1112T→C; Leu371Pro	AHC	Sporadic	1
1838C→T; Thr613Met	RDP	Sporadic, familial	6
2051C→T; Ser684Phe	RDP	Sporadic	1
2263G→T; Gly755Cys	AHC	Sporadic	2
2263G→A; Gly755Ser	AHC	Sporadic	1
2267G→A; Arg756His	RDP	Sporadic	1
2273T→G; Ile758Ser	RDP	Familial	1
2316C→A; Ser772Arg	AHC	Sporadic	1
2318A→T; Asn773Ile	AHC	Sporadic	1
2318A→G; Asn773Ser	AHC	Sporadic	1
2338T→C; Phe780Leu	RDP	Familial	1
2401G→A; Asp801Asn	AHC	Sporadic	49
2401G→T; Asp801Tyr	RDP	Familial	1
2403T→A; Asp801Glu	AHC	Sporadic	1
2417T→G; Met806Arg	AHC	Sporadic	1
2429T→G; Ile810Ser	AHC	Sporadic	1
2431T→C; Ser811Pro	AHC	Sporadic	4
2443G→A; Glu815Lys	AHC	Sporadic	31
2542+1G→A; splice site	AHC	Sporadic	2
2755_2757delGTC; Val919del	AHC	Sporadic	1
2767G→A; Asp923Asn	RDP, AHC	Sporadic and familial RDP, familial AHC	RDP sporadic, 2; RDP familial, 2; AHC familial, 1
2767G→T; Asp923Tyr	AHC	Sporadic	1
2780G→A; Cys927Tyr	AHC	Sporadic	1
2839G→A; Gly947Arg	AHC	Sporadic	5
2839G→C; Gly947Arg	AHC	Sporadic	2
2864C→A; Ala955Asp	AHC	Sporadic	1
2974G→T; Asp992Tyr	AHC	Sporadic	1
3038_3040dupACT; Tyr1013dup	RDP	Sporadic	1

ATP1A3 mutation coordinates are defined based on UniProt ID P13637 and Consensus CDS ID CCDS12594·1. Mutation 658G→A, Asp220Asn, previously reported as causal by Heinzen and coworkers[31] was later shown to be a rare, inherited mutation, and the disease-causing mutation in this patient was a previously overlooked de-novo Asp801Asn ATP1A3 mutation (unpublished data); thus Asp220Asn is not shown in this table and one additional patient has been counted as having an Asp801Asn mutation. AHC=alternating hemiplegia of childhood. RDP=rapid-onset dystonia parkinsonism. *Compiled from references.[18-34]

Table 1: **Disease-causing *ATP1A3* mutations**

features, including second onsets and unusual, mild-to-moderate improvement after the primary onset of disease symptoms (table 2). Collectively, these findings suggest that the phenotype is broader than originally described in 1993.

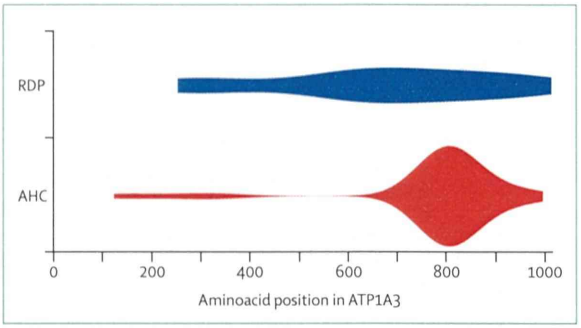


Figure 3: **Density plot showing the distribution of AHC and RDP mutations in *ATP1A3***

Mutations identified to date in 20 patients with RDP (blue) and 118 patients with AHC (red). In general, RDP mutations appear more evenly distributed, whereas AHC mutations are heavily concentrated in particular sites in the protein. AHC=alternating hemiplegia of childhood. RDP=rapid-onset dystonia parkinsonism. Aminoacid positions are based on the ATP1A3 protein defined by UniProt ID P13637.

Drug therapy in RDP is limited; patients are unresponsive to standard drugs for parkinsonism, including levodopa.[14,16,19,20,41] Current treatment is limited to benzodiazepines, which have been reported to provide symptomatic relief in some patients.

Alternating hemiplegia of childhood

Although the first report of AHC was by Verret and Steele in 1971,[60] it was not until 1980 that Krageloh and Aicardi[61] first defined the syndrome. In 1993, the specific clinical criteria to diagnose AHC were proposed.[42] These, named the Aicardi criteria, include seven disease features: (1) paroxysmal episodes of hemiplegia; (2) episodes of bilateral hemiplegia or quadriplegia; (3) other paroxysmal manifestations, such as abnormal eye movements, dystonia, nystagmus, intermittent strabismus, tonic spells, or autonomic disturbance, which can occur during hemiplegia or as isolated events; (4) evidence of permanent neurological dysfunction, which can manifest as intellectual deficiencies, seizures, ataxia, choreoathetosis, developmental delay, or persistent motor deficits such as spastic diplegia or quadriplegia or hypotonia; (5) inducing sleep during a paroxysmal attack might relieve symptoms for a period of time after awakening; (6) first signs of dysfunction occurring before age 18 months; and (7) not being attributed to other disorders. The median age of disease onset of the first paroxysmal event is 3·5 months with a range from the first day of life to 4 years. Developmental delay might not appear until after 12 months. Additional clinical details are summarised in table 2.

Although nearly all cases of AHC are sporadic, some families have an autosomal dominant inheritance of the disorder.[49–51,62] In two families a causal *ATP1A3* mutation was identified,[29,31,62] and in one family a disease-causing *ATP1A2* (encoding the α_2 subunit of the Na^+/K^+-ATPase) mutation was found.[50,51] Consistent with these mutations being highly penetrant, all *ATP1A2* or *ATP1A3* mutation

	AHC	RDP
Age of onset	0–18 months (median 3·5 months), although some isolated cases are reported as late as 4 years	Can occur in children and adults (9 months to 59 years); 76% of patients report symptoms by age 25 years
Inheritance	Typically sporadic; rare cases of familial AHC	Sporadic or familial
Clinical presentation	Paroxysmal symptoms: episodes of hemiplegia, bilateral hemiplegia, or quadriplegia with improvement in sleep and with other paroxysmal abnormalities such as dystonia, tonic spells, epileptic seizures (seizures occur in about 50% of patients and can include focal or generalised tonic, tonic-clonic, or myoclonic seizure types), autonomic changes, or abnormal eye movements (including unilateral [ipsilateral to the hemiplegia] intermittent eye deviation, disconjugate gaze, and pendular nystagmus) often with the following characteristics: common triggers include stress, excitement, extreme heat or cold, water exposure, physical exertion, lighting changes, and foods; variable frequency ranging from multiple episodes per day to one every few months; associated with anarthria, dysphagia, and autonomic disturbances including bradycardia, stridor, bronchospasm, apnoea, dyspnoea, nausea, unilateral or bilateral flushing, hypothermia or hyperthermia; episodes typically remit with sleep, but can return within 10–20 min after waking; episodes of dystonia in concert or separate from episodes of hemiplegia, bilateral hemiplegia, quadriplegia, or abnormal eye movements that are more commonly observed early in the clinical course, typically consist of head turning toward the affected side with eye deviations toward the same side; very brief intense unilateral or bilateral tonic or dystonic attacks with vibratory tremor and pain Interictal symptoms: evidence of developmental delay (including intellectual deficiencies, neuropsychological deficits), mental retardation, and interictal neurological abnormalities such as tone abnormalities, choreoathetosis (spontaneous or movement-induced), or ataxia (sometimes associated with cerebellar atrophy or cerebellar hypometabolism), and persistent motor deficits (such as spastic diplegia or quadriplegia, hypotonia)	Typically abrupt onset of permanent symptoms of generalised dystonia and parkinsonian features including bradykinesia and postural instability Variable progression of symptoms ranging from hours to weeks Diurnal fluctuation or episodes typical of patients with AHC are not seen in RDP Abrupt worsening of symptoms can rarely occur years after initial onset Onset of symptoms typically occurs after running, alcohol binges, minor head injuries, overheating, emotional stress, infections, or childbirth Can be associated with mood disorders and psychosis
Drug therapy	Flunarizine, benzodiazepines	Unresponsive to levodopa

AHC=alternating hemiplegia of childhood. RDP=rapid-onset dystonia parkinsonism.

Table 2: **Proposed diagnostic criteria for AHC and RDP**[13,14,16,19,20,29–31,38–59]

carriers in these families have symptoms of AHC, albeit to varying severity even within families.[29,31,50,51,62] In addition to these unusual familial cases, other patients have atypical presentations that resemble AHC, although currently a role for *ATP1A3* mutation is untested. These include benign nocturnal alternating hemiplegia, which occurs only in boys,[52,53] mild cases with normal cognitive development,[54] cases in which dystonia is the predominant feature,[54] patients who do not have episodes of quadriplegia, patients who have the first signs of the disorder after the age of 18 months,[54,55] patients presenting with neonatal seizures, and patients with status epilepticus with associated long-term atrophy on MRI and residual motor and eye movement abnormalities.[56]

Over the years, patients with AHC have undergone many treatments to alleviate the frequency and severity of hemiplegia, although success has been very limited.[43,44] Flunarizine, a calcium channel blocker, performed best because it seemed to reduce the severity and duration of attacks, at least in some patients.[45–48] Benzodiazepines, which increase the activity of GABA, the major inhibitory neurotransmitter in the CNS, might also have some efficacy either directly or by inducing sleep, which often relieves attacks.[43,44]

Genotype–phenotype correlation

Although the number of patients with AHC or RDP with an identified *ATP1A3* mutation is rapidly growing, definitive phenotypic patterns have not been found for patients with and without mutations, and in patients with recurring *ATP1A3* mutations.[31,32,34] However, one small study[63] evaluating the phenotypes of 35 patients with AHC with *ATP1A3* mutations reports that patients with the Glu815Lys mutation tend to have earlier onset of symptoms, more severe motor and cognitive disabilities, and more often report status epilepticus and respiratory paralysis compared with patients with AHC with other *ATP1A3* mutations. This preliminary finding suggests that genotype–phenotype correlations exist and that additional studies will be needed to further evaluate these patterns in larger sample sizes. The ATP1A3 Working Group is currently analysing genotype–phenotype correlations in 150 patients.

Biological effects of ATP1A3 mutations

In-vitro studies

ATP1A3 protein expression and localisation

Ten RDP and five AHC *ATP1A3* mutations have been investigated at the level of protein expression and cellular localisation of the protein by using heterologous expression systems.[18,31] These studies revealed that for all except two of the tested RDP mutations the ATP1A3 protein expression was reduced, whereas none of the AHC mutations reduced expression of ATP1A3. RDP mutations were not shown to affect the maturation and localisation of the protein in transfected cells;[18] the effect of AHC mutations on ATP1A3 localisation has not yet been studied.

ATP1A3 mutations in platelets and fibroblasts

Platelets and fibroblasts from nine patients with AHC have been screened for differences in protein expression compared with age-matched and sex-matched controls.[64] A consistent increase in the level of activated lysosomal protein cathepsin B was observed in specimens from patients with AHC, which was shown to increase apoptosis. Although the mechanism remains unclear,

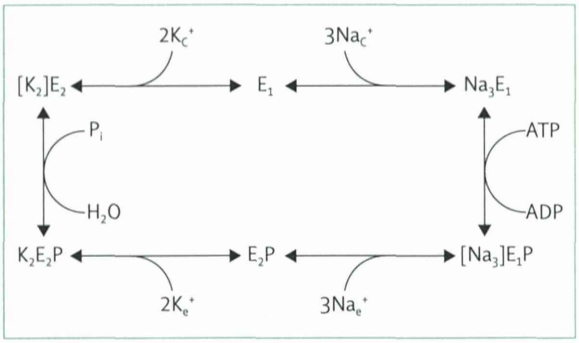


Figure 4: **Post-Albers model[65] for the Na^+/K^+-ATPase reaction cycle**
Reproduced from Toustrup-Jensen and coworkers[66] with permission from the American Society for Biochemistry and Molecular Biology. E_1 and E_2 are major conformational states with preference for binding of Na^+ and K^+, respectively. Cytoplasmic and extracellular ions are indicated by subscripts c and e, respectively. Brackets indicate occlusion of the ions in a cavity in the protein. P indicates the bound phosphate.

this work suggests that similar protein changes might also occur in the brain and could contribute to AHC pathophysiology.

Na^+/K^+-ATPase activity

In the established Post-Albers model for the Na^+/K^+-ATPase transport mechanism (figure 4),[65] in which three cytoplasmic Na^+ ions are exchanged for two extracellular K^+ ions for each ATP being hydrolysed, the E_1 conformation preferentially binds Na^+, whereas E_2 preferentially binds K^+. On the basis of this model, there are several experimental approaches to measure the activity of the Na^+/K^+-ATPase, and specifically the effects of disease-causing mutations in ATP1A3 on the catalytic cycle.

First, using a luminescent kinase assay that measures ADP formed from each catalytic cycle of the Na^+/K^+-ATPase (figure 4) from cells heterologously expressing the wild-type, RDP-causing, or AHC-causing mutant version of the ATP1A3 cDNA, investigators have shown that each mutation reduced the activity of the Na^+/K^+-ATPase.[31] These findings, coupled with the aforementioned protein expression analyses, suggest that mutations that affect Na^+/K^+-ATPase function, but not the amount of Na^+/K^+-ATPase per se, seem to result in the more severe AHC phenotype.

Second, since active Na^+/K^+-ATPase pumps a net charge of +1 out of the cell for each round of the catalytic cycle (figure 4), electrophysiology-based approaches can be used to measure, in a living cell or in a patch of excised membrane, the voltage and ion sensitivity of the pump during steady-state activity. Two disease-causing ATP1A3 mutations have been characterised in detail: Asp801Asn and Asp923Asn. The aspartate located at aminoacid position 801 is highly conserved in Na^+/K^+-ATPases in all investigated species, and is a site that binds either the Na^+ or K^+ ions during the catalytic cycle. Expression of the AHC-causing Asp801Asn mutant in *Xenopus* oocytes showed that the mutation did not generate any measurable pump current, consistent with it being unable to bind K^+ ions.[67] Analysis of the Asp923Asn mutation, which was identified in both patients with RDP[23,25,29,30] and familial AHC,[29] suggested that protonation at this site was crucial for the movement of Na^+ and K^+ across the cell membrane.[68]

Third, during the ion transport process, the Na^+/K^+-ATPase becomes phosphorylated by transfer of the γ-phosphate of ATP to a conserved aspartic acid residue in the P-type ATPase signature sequence (figure 4). By incubating ATP radiolabelled at the γ-phosphate with cell membrane fragments containing the Na^+/K^+-ATPase to quantify this covalent and acid-stable phosphoryl bond, investigators can measure the activity of the Na^+/K^+-ATPase with and without disease-causing mutations.[66,69–71] Because the binding of three Na^+ ions at the cytoplasmic surface of the ATP1A3 protein is needed to activate the enzyme for phosphorylation from ATP, the affinity for Na^+ at these sites can be established by measurement of the Na^+ dependence of phosphorylation, the affinity being defined by the Na^+ concentration giving half maximum phosphorylation. So far, RDP-causing mutations Glu277Lys, Thr613Met, Phe780Leu, +Tyr (an extension of the C-terminus with an extra Tyr residue), and the RDP-causing and AHC-causing mutation Asp923Asn have been characterised with these methods.[24,69–71] Each of the mutations shows striking reductions of Na^+ affinity for activation of phosphorylation. Whereas Na^+ binding from the cytoplasmic side activates phosphorylation from ATP, binding of K^+ from the external side triggers dephosphorylation, thereby stimulating ATP hydrolysis. Therefore, the affinity for external K^+ can be established by study of the K^+ dependence of dephosphorylation or ATPase activity at a fixed Na^+ concentration. Notably, none of the mutants show a reduced affinity for K^+, indicating a selective disturbance of Na^+ binding that is associated with mutations causing RDP and one mutation causing AHC (Asp923Asn).[66,69–71]

The described studies show that a selective reduction of the affinity of the Na^+/K^+-ATPase for cytoplasmic Na^+ without disturbance of K^+ binding is a central feature in RDP. Consequently, increased intracellular Na^+ concentration resulting from the reduced Na^+ affinity could be a key pathogenic factor in RDP.[66] A rise in intracellular Na^+ might result in a secondary increase in intracellular Ca^{2+} via the Na^+/Ca^{2+} exchange system, which can activate signalling cascades triggered by changes in Ca^{2+} concentration. Additionally, a disturbance of the Na^+ gradient could affect the uptake of neurotransmitters such as dopamine and glutamate. Because several AHC-causing mutations target the same residue (Ile274, Asp801, Asp923) as the one mutated in RDP, an essential question to address is whether Na^+ affinity is also typically disturbed in AHC. The affinity for K^+ might also be disturbed in AHC, which could also explain why AHC is at the severe end of the phenotypic spectrum, whereas RDP is at the

mild end. Structural modelling of three AHC-causing mutations (Ile274Asn, Asp801Asn, and Asp923Tyr) and three RDP-causing mutations (Ile274Thr, Asp801Tyr, and Asp923Asn) that affect identical positions in the Na^+/K^+-ATPase α_3 subunit[72] predicted that AHC mutations would bring about structural changes that severely affect efficient K^+ movement along the narrow K^+ access pathway. Instead, RDP-causing mutations seem to have milder structural consequences that are likely to result in a milder impairment of K^+ movement.[73]

Notably, a mutation in the sarcoendoplasmic reticulum Ca^{2+} ATPase, a member of the same type II P-type ATPase family as the Na^+/K^+-ATPase, in *Drosophila melanogaster* causes temperature-sensitive ionic leakage of the transporter.[74] Investigators postulated that a temperature-sensitive gain-of-function mechanism might also underlie the phenotypic consequences of disease-causing mutations in other type II P-type ATPases, including ATP1A3 in AHC and RDP. Although the effect of temperature on the functional effects of AHC-causing and RDP-causing mutations in Na^+/K^+-ATPases is unknown, if correct, this mechanism could explain why environmental triggers such as stress, physical exertion, and temperature changes can lead to symptom onset in patients with RDP or AHC (table 2).

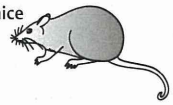
Animal studies

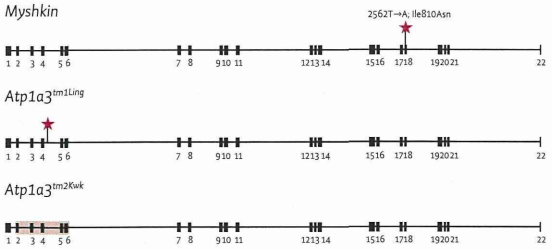
Myshkin mice

Several animal models have been used to study the in-vivo consequences of ATP1A3 modulation (figure 5). The aminoacid sequence identity between the human and mouse Na^+/K^+-ATPase α_3 subunits is about 99%. Heterozygous *Myshkin* (*Atp1a3*$^{Myk/+}$; *Myk*/+) mutant mice have an aminoacid change (Ile810Asn) that affects the identical position to Ile810Ser in the human Na^+/K^+-ATPase α_3 subunit that was identified in a patient with AHC.[31,75] Molecular modelling of Ile810Asn and Ile810Ser showed that both changes bring about similarly severe structural effects on the Na^+/K^+-ATPase α_3 subunit, including the capacity for efficient K^+ movement along the K^+ access pathway.[73] Ile810Asn was generated through *N*-nitroso-*N*-ethylurea (ENU) mutagenesis and results in a normally expressed, but inactive, α_3 protein and a subsequent 36–42% reduction in total Na^+/K^+-ATPase activity (reflecting the combined activity of α_1, α_2, and α_3) in the brain.[75,81]

Heterozygous *Myk*/+ mice have an unsteady, tremulous gait with occasional splaying of the hindlimbs, but without an overt hemiplegia.[73] Phenotypic analysis revealed a range of other abnormalities in *Myk*/+ mice, including reduction in body size, motor deficits in the balance beam and rotarod tests, cognitive deficits in the fear-conditioning

A *Atp1a3* mutant mice





Genetic mutation	NKA α_3 expression	Reduction in brain NKA activity (%)	Homozygote phenotype	Heterozygote phenotype			
				Visible defects	Open field activity	Balance beam performance	Response to stress
Ile810Asn	No change	40%	Perinatal death	Tremor, ataxia, seizures, small body	↑	↓	Seizures
Point mutation in intron 4	↓(60%)	16%	Perinatal death	None	↑	No change	Mild balance deficit (females only)
Deletion of exons 2–6	ND	ND	Perinatal death	None	↑	↑	No change

B *Atpα* mutant *Drosophila*

Allele name	Genetic mutation	AHC α_3 equivalent	NKA α expression	Heterozygote phenotype			
				Locomotor activity	Temperature stress response	Mechanical stress response	Neuropathology
Atpα[CJ10]	Gly744Ser	Gly755Ser	No change	↓	Paralysis	Paralysis	Vacuolar

C *atp1a3a/b* knockdown zebrafish

Knockdown	Reduction in α_3 transcript (%)	60 hpf embryo phenotype		
		Brain ventricles	Spontaneous motility	Touch response
Atp1a3a	62%	Dilation	Abnormal	↓
Atp1a3b	66%	Dilation	Abnormal	↓

Figure 5: **Na^+/K^+-ATPase α_3 genetic animal models**

(A) *Atp1a3* mutant mice. The locations of three mutations in the mouse *Atp1a3* genomic locus are depicted. *Myshkin* mice carry a T→A transversion in exon 18 that results in the substitution of asparagine for isoleucine at position 810 (Ile810Asn).[73,75] *Atp1a3*^{tm1Ling} mice carry a point mutation in intron 4 adjacent to the exon-intron splice site that results in aberrant splicing of the gene, adding 126 bp to the RNA transcript.[75–77] *Atp1a3*^{tm2Kwk} mice carry a STOP-polyA cassette that replaces exons 2–6 in *Atp1a3*.[78] (B) *Atpα* mutant *Drosophila*. *Atpα*^{CJ10} fruit flies carry a G→A transition that results in the substitution of glycine for serine at position 744 (Gly744Ser).[79] Gly744Ser in the *Drosophila* α subunit is equivalent to mutation Gly755Ser in the human α_3 subunit found in a patient with AHC.[31] (C) *atp1a3a/b* knockdown zebrafish. Knockdown of *atp1a3a* or *atp1a3b* RNA transcript by around 65% in 60 hpf embryos had similar phenotypic effects.[80] Red stars show point mutations. NKA=Na^+K^+-ATPase. ↓=lower than wild-type. ↑=greater than wild-type. No change=no change from wild-type. ND=not determined. AHC= alternating hemiplegia of childhood. hpf=hours post-fertilisation.

and conditioned taste aversion tests, neuronal hyperexcitability with spontaneous convulsions, and mania-related behaviours such as increased risk-taking and responsiveness to treatment with lithium and valproic acid.[73,75,81] When subjected to vestibular stress, *Myk*/+ mice have transient tonic attacks and staggering movements that, in a third of mice, develop into tonic-clonic seizures that are accompanied by epileptiform discharges.[75] ^{14}C-2-deoxyglucose imaging of *Myk*/+ mice identified compromised thalamocortical functioning, including a deficit in frontal cortex functioning and reduced thalamocortical functional connectivity.[73] When bred to homozygosity, *Myk/Myk* pups die shortly after birth.[75]

Strategies aimed at increasing Na^+/K^+-ATPase activity have shown some therapeutic effects in *Myk*/+ mice. Transgenic delivery of an additional copy of the wild-type *Atp1a3* gene to the X chromosome increased Na^+/K^+-ATPase α_3 subunit protein expression and whole brain Na^+/K^+-ATPase activity, and reduced the epileptic seizure susceptibility as well as the risk-taking behaviour of *Myk*/+ mice.[75,82] Chronic treatment with rostafuroxin, a compound that antagonises the inhibitory action of ouabain on Na^+/K^+-ATPase,[83] was also found to reduce the risk-taking behaviour of *Myk*/+ mice.[81] Effects of this intervention on the motor and cognitive deficits of *Myk*/+ mice have not yet been established.

Atp1a3^{tm1Ling} *mice*

Heterozygous *Atp1a3*$^{tm1Ling/+}$ mice, which have a point mutation in intron 4 of the *Atp1a3* gene, show a reduction of hippocampal α_3 protein expression of around 60% and a reduction of total brain Na^+/K^+-ATPase activity (of α_1, α_2, and α_3 combined) of around 16%.[75,76,82] Non-stressed (naive) *Atp1a3*$^{tm1Ling/+}$ mice do not have visible neurological defects or restricted growth, but instead show increased locomotor activity in an open field test and deficient spatial learning in the Morris water maze test.[76] After exposure to restraint stress for 5 days, female *Atp1a3*$^{tm1Ling/+}$ mice show mild motor deficits in the balance beam and rotarod tests.[77] *Atp1a3*$^{tm1Ling/+}$ mice exposed to chronic variable stress, consisting of one or two unpredictable mild stressors per day for 6 weeks, have deficits in total brain Na^+/K^+-ATPase activity, sociability, and object recognition memory, as well as increased anxiety and depression-like behaviours, compared with non-stressed *Atp1a3*$^{tm1Ling/+}$ mice.[82] In *Atp1a3* wild-type mice, chronic variable stress also led to depression-like behaviour and reduced sociability, but had no effect on Na^+/K^+-ATPase activity, anxiety, or object recognition memory compared with non-stressed wild-type controls.[82] Homozygous *Atp1a3*^{tm1Ling} pups die shortly after birth.[76]

Atp1a3^{tm2Kwk} *mice*

Heterozygous *Atp1a3*$^{tm2Kwk/+}$ mice have a targeted deletion of *Atp1a3* exons 2–6.[78] *Atp1a3*$^{tm2Kwk/+}$ mice do not show gross morphological defects or apparent histological brain anomalies. Adult male *Atp1a3*$^{tm2Kwk/+}$ mice show increased locomotor activity, both in the home cage and in the open field test. By contrast with *Atp1a3*$^{Myk/+}$ mice, *Atp1a3*$^{tm2Kwk/+}$ mice had improved performance in the balance beam and rotarod tests compared with wild-type mice. *Atp1a3*$^{tm2Kwk/+}$ mice do not develop dystonia spontaneously, nor after various stressors, such as tail suspension, forced swimming, or restraint. Dystonia can be induced pharmacologically[84] in these mice by injection of the neuroexcitatory aminoacid kainate directly into the cerebellum. The response to dystonia induction by kainate injection was increased in *Atp1a3*$^{tm2Kwk/+}$ mice, with a longer duration of sustained dystonia compared with wild-type mice. Electrophysiological studies showed that inhibitory neurotransmission at molecular-layer interneuron Purkinje cell synapses was increased in the cerebellar cortex of *Atp1a3*$^{tm2Kwk/+}$ mice. Homozygous *Atp1a3*^{tm2Kwk} mice show a complete lack of breathing movements and die shortly after birth.[78]

Pharmacological blockade of Na^+/K^+-ATPase α_3

Perfusion of the Na^+/K^+-ATPase inhibitor ouabain into the cerebellum and basal ganglia was found to induce mild dyskinesia in wild-type C57BL/6 mice.[85] When mice were subsequently exposed for 2 h to stress provided in the form of random electric foot shocks in a warm environment (38°C), 70% of the mice developed persistent dystonia and rigidity.[85] These mice show hallmark symptoms of RDP, including dystonia and parkinsonism induced by stress. However, this approach is limited by the similar affinities of the α_2 and α_3 isoforms for ouabain,[86] thus precluding α_3 specificity in this animal model.

Zebrafish

Zebrafish (*Danio rerio*) have two *ATP1A3* orthologues, *atp1a3a* and *atp1a3b*.[87] The paralogous α_{3a} and α_{3b} subunits have aminoacid identities of 95% with each other and 91–92% with the human Na^+/K^+-ATPase α_3 subunit protein sequence. Consistent with mammalian Na^+/K^+-ATPase α_3 subunit protein expression, the transcripts of *atp1a3a* and *atp1a3b* are primarily expressed in the brain, albeit with distinct expression profiles. In 60 h post-fertilisation zebrafish embryos, the *atp1a3a* transcript is widely distributed throughout the brain, whereas distribution of *atp1a3b* mRNA is localised to particular brain structures. Despite having distinct expression profiles, targeted knockdown of *atp1a3a* or *atp1a3b* by morpholino antisense oligonucleotides results in severe brain ventricle dilation in 60 h post-fertilisation embryos, suggesting that both α_3 paralogues are needed for brain ventricle maintenance. The extent of brain ventricle dilation was reduced by co-injection of the mRNA of the knocked-down gene, but *atp1a3b* mRNA did not cross-rescue the phenotype of *atp1a3a*-knockdown embryos. Similarly, *atp1a3a* mRNA did not cross-rescue the phenotype of *atp1a3b*-knockdown embryos. Both morphants display abnormal spontaneous motility and

an abnormal response to tactile stimulation with a needle,[80] suggesting that both α_3 paralogues are needed for embryonic motility.

Drosophila

The gene *Atpα* (FlyBase ID: FBgn0002921) in *Drosophila melanogaster* fruit flies encodes the α subunit of the Na^+/K^+-ATPase, which is orthologous to all vertebrate α subunits, and has aminoacid sequence identities of 76–77% with the α_1, α_2, and α_3 subunits of human beings.[88] Although the *Drosophila Atpα* gene is not a specific orthologue of *ATP1A3*, eight missense mutations, generated through ethylmethanesulfonate mutagenesis at highly conserved aminoacid residues, lead to AHC-relevant phenotypic abnormalities in adult heterozygous flies.[79,89] Flies from any of six lines with mutations (Ser201Leu, Pro262Leu, Ser348Thr, Gly528Ser, Ala588Thr, Gly744Ser) in *Atpα* that were repeatedly knocked to the bottom of a vial using a standard laboratory vortexer showed transient mechanical stress-induced paralysis.[79] Two other mutant lines (Asp981Asn, Glu928Lys) did not show this phenotype when maintained at an ambient temperature of 20–22°C, but showed temperature-sensitive mechanical stress-induced paralysis when maintained at 28°C.[89] When exposed to a temperature of 37–38°C, three mutant lines (Gly744Ser, Asp981Asn, Glu928Lys) showed temperature-sensitive paralysis that was reversed when the ambient temperature was lowered to 20–22°C.[79,89] When the bodyweight of male flies of six of the same mutant lines was measured, only Ser201Leu mutant flies showed a reduction compared with wild-type flies.[79] Western blotting of homogenised fly heads showed that the expression of Na^+/K^+-ATPase α subunit was reduced in two of the *Atpα* mutants (Ser348Thr, Ala588Thr), but unchanged in the other four mutants.[79] Mutations Gly744Ser and Asp981Asn in the *Drosophila* α subunit, which led to temperature-sensitive paralysis, affect equivalent aminoacid residues in the human Na^+/K^+-ATPase α_3 subunit, namely mutations Gly755Cys, Gly755Ser, and Asp992Tyr, which were identified in patients with AHC (table 3).[31,32] All eight missense mutations are homozygous lethal.[79]

	Human α3 equivalent	Temperature-sensitive paralysis	Mechanical stress-induced paralysis	Bodyweight	Protein expression
Ser201Leu	Ser212	No	Yes	↓	No change
Pro262Leu	Pro273	No	Yes	No change	No change
Ser348Thr	Ser359	No	Yes	No change	↓
Gly528Ser	Gly539	No	Yes	No change	No change
Ala588Thr	Ala599	No	Yes	No change	↓
Gly744Ser	Gly755, AHC	Yes	Yes	No change	No change
Asp981Asn	Asp992, AHC	Yes	Mild	ND	No change
Glu982Lys	Asp993	Yes	Mild	ND	No change

Adapted from Ashmore and colleagues.[79] AHC=equivalent aminoacid residue that is substituted in alternating hemiplegia of childhood. Mild=when maintained at 28°C. No change=no change from wild-type. ↓=lower than wild-type. ND=not determined.

Table 3: Drosophila Na^+/K^+-ATPase α subunit mutant phenotypes

Search strategy and selection criteria

References cited in this Review were identified through PubMed searches using the search terms "ATP1A3", "alternating hemiplegia of childhood", "rapid-onset dystonia parkinsonism", and "Na,K-ATPase ", from December, 1993, until January, 2014. Articles were identified through searches of the reference lists of the articles found with the above cited search terms and of the authors' own files. All references used in this Review were published in English, and were selected according to originality and relevance to the content of this Review.

Conclusions

Since the original descriptions of RDP and AHC, substantial work has been done to characterise their clinical presentation and pathophysiology. Through the identification of disease-causing mutations in *ATP1A3*, these two seemingly unrelated diseases are now linked, allowing new opportunities to obtain insight into their biological bases.

We now understand that protein-modifying genetic variations in *ATP1A3* rarely occur in the general population and, when they do, the risk of severe neurological disease is very high. This understanding has led to a new research area, investigating which other diseases might be associated with mutations in *ATP1A3*. One could postulate that *ATP1A3* mutations might also be found in patients with seizures, psychiatric conditions, or other less severe types of dystonia or ataxia. As next-generation sequencing becomes widely used in day-to-day clinical practice, the role of *ATP1A3* mutations in a wider range of phenotypes might become apparent. However, as this genotype–phenotype spectrum is being defined, we can already begin cataloguing *ATP1A3* disease-specific mutations and polymorphic protein-disrupting variants in the general population, to establish molecular and physiological changes associated with these DNA variations in in-vitro and in-vivo test paradigms like those described in this Review. Data from these studies can be used to develop better research models with tiered complexity. We envisage that these studies will include establishing disease models at the transporter level in individual cells, multicellular network models using induced pluripotent cells differentiated into neurons, ex-vivo studies in brain slices to study tissue-level effects, and, finally, evaluations at the organism level to assess in-vivo consequences in animal models. With these model systems we can begin to relate molecular changes to the phenotypic presentations associated with these disorders, including the episodic nature of AHC, how particular stimuli lead to the onset of symptoms, the age-dependent onset of RDP, and the variable effects on organ systems and brain structures that probably underlie the diverse phenotypic presentations.

Importantly, once key biomarkers of disease pathophysiology are identified, we will be able to screen for compounds to rectify the pathophysiological changes associated with *ATP1A3* mutations.

In summary, genetics has illuminated key aspects of disease pathophysiology for both AHC and RDP. Although extensive work is needed to disentangle the complex biology underlying these disorders, we are poised with evolving research approaches to rapidly translate these genetic discoveries to detailed disease pathophysiology, to improve understanding of developmentally mediated and environmentally triggered disease presentation, and ultimately to develop treatments for these debilitating diseases.

Contributors

ELH, AA, AB, SJC, FG, DBG, SHJ, MAM, BN, SN, LJO, HP, TS, KJS, AvdM, and BV compiled relevant information from the literature and wrote the manuscript. The entire ATP1A3 Working Group critiqued and edited the Review and provided scientific and clinical guidance regarding the content.

ATP1A3 Working Group

Alexis Arzimanoglou (University Hospitals of Lyon, Centre National de la Recherche Scientifique, and Institut National de la Santé et de la Recherche Médicale, Lyon, France), Frances M Ashcroft (University of Oxford, Oxford, UK), Allison Brashear (Wake Forest School of Medicine, Winston Salem, NC, USA), Knut Brockmann (University Medical Center, Göttingen, Germany), Jaume Campistol (Hospital Sant Joan de Déu—Barcelona University, Barcelona, Spain), Alessandro Capuano (Bambino Gesù Paediatric Hospital, IRCSS, Rome, Italy), Inês Carrilho (Hospitalar Center of Oporto, Oporto, Portugal), Paul Casaer (University Hospital Gasthuisberg, Leuven, Belgium), Steven J Clapcote (University of Leeds, Leeds, UK), Elisa De Grandis (University of Genoa, Genoa, Italy), Boukje de Vries (Leiden University Medical Centre, Leiden, Netherlands), Michela Di Michele (KU Leuven, Leuven, Belgium), Caroline Dion (Canadian Association for Alternating Hemiplegia, Saint-Mathieu de Beloeil, QC, Canada), Diane Doummar (Hôpital Trousseau, Paris, France), Anja P Einholm (Aarhus University, Aarhus, Denmark), Carmen Fons (Hospital Sant Joan de Déu—Barcelona University. Barcelona, Spain), Filippo Franchini (AHC Federation of Europe, Reykjavík, Iceland, and A.I.S.EA Onlus, Verderio Superiore, Italy), Thomas Friedrich (Technical University of Berlin, Berlin, Germany), Kathleen Freson (KU Leuven, Leuven, Belgium), David C Gadsby (Rockefeller University, New York, NY, USA), Melania Giannotta (IRCCS Istituto delle Scienze Neurologiche di Bologna, Bologna, Italy), David B Goldstein (Duke University, School of Medicine, Durham, NC, USA), Christophe Goubau (KU Leuven and University Hospital Leuven, Leuven, Belgium), Titiana Granata (National Neurological Institute C Besta, Milan, Italy), Fiorella Gurrieri (Università Cattolica S Cuore, Rome, Italy), Erin L Heinzen (Duke University, School of Medicine, Durham, NC, USA), Shinichi Hirose (Fukuoka University, Fukuoka, Japan), Yuki Hitomi (Duke University, School of Medicine, Durham, NC, USA), Rikke Holm (Aarhus University, Aarhus, Denmark), Keiko Ikeda (Hyogo College of Medicine, Hyogo, Japan), Atsushi Ishii (Fukuoka University, Fukuoka, Japan), Sigurður H Jóhannesson (AHC Federation of Europe and AHC Association of Iceland, Reykjavik, Iceland), Kamran Khodakhah (Albert Einstein College of Medicine, New York, NY, USA), Mary D King (Children's University Hospital, Dublin, Ireland), Greer S Kirshenbaum (Mount Sinai Hospital and University of Toronto, Toronto, ON, Canada), Ana Kockhans (AHC-Deutschland eV, Mönchengladbach, Germany), Jan B Koenderink (Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands), Gaetan Lesca (Centre de Recherche en Neurosciences de Lyon, University Hospitals of Lyon, and Claude Bernard Lyon I University, Lyon, France), Karin Lykke-Hartmann (Centre for Membrane Pumps in Cells and Disease—PUMPKIN, Danish National Research Foundation, and Aarhus University, Aarhus, Denmark), Ulrike Maschke (Catholic Hospital St Johann Nepomuk, Erfurt, Germany), Mario R Merida (Stevens-Henager College, Ogden, UT, USA), Mohamad A Mikati (Duke University School of Medicine, Durham, NC, USA), Ralf Müller (AHC-Deutschland eV, Blankenheim, Germany), Giovanni Neri (Università Cattolica S Cuore, Rome, Italy), Brian Neville (University College London, London, UK), Sophie Nicole (Centre de Recherche de l'Institut du Cerveau et de la Moelle, Centre National de la Recherche Scientifique, and Université Pierre et Marie Curie Paris VI, Paris, France), Hang N Nielsen (Aarhus University, Aarhus, Denmark), Poul Nissen (Aarhus University, and Centre for Membrane Pumps in Cells and Disease—PUMPKIN, Danish National Research Foundation, Aarhus, Denmark), Tom O'Brien (Alternating Hemiplegia of Childhood Ireland, Dublin, Ireland), Laurie J Ozelius (Icahn School of Medicine at Mount Sinai, New York, NY, USA), Eleni Panagiotakaki (University Hospitals of Lyon, Lyon, France), Marek Parowicz (Polish AHC Association, Bischofsheim, Poland), Dominique Poncelin (French AHC Organisation, AFHA, St Germain lès Arpajon, France), Hanne Poulsen (Aarhus University, and Centre for Membrane Pumps in Cells and Disease—PUMPKIN, Danish National Research Foundation, Aarhus, Denmark), Sandra P Reyna (University of Utah School of Medicine, Salt Lake City, UT, USA), John C Roder (Mount Sinai Hospital and University of Toronto, Toronto, ON, Canada), Hendrik Rosewich (Georg August University Göttingen, Göttingen, Germany), Masayuki Sasaki (National Center of Neurology and Psychiatry, Tokyo, Japan), Vivien R Schack (Aarhus University, Aarhus, Denmark), Philippe Schyns (European Network for Research on Alternating Hemiplegia [ENRAH], Brussels, Belgium), Tsveta Schyns (ENRAH, Brussels, Belgium), Michela Stagnaro (University of Genoa, Genoa, Italy), Kathleen J Sweadner (Massachusetts General Hospital, Boston, MA, USA), Kathryn J Swoboda (University of Utah School of Medicine, Salt Lake City, UT, USA), Danilo Francesco Tiziano (Università Cattolica S Cuore, Rome, Italy), Mads S Toustrup-Jensen (Aarhus University, Aarhus, Denmark), Arn van den Maagdenberg (Leiden University Medical Centre, Leiden, Netherlands), Albert Vilamala (AHC Federation of Europe, Reykjavík, Iceland, and AHC Association of Spain, AESHA, Barcelona, Spain), Bente Vilsen (Aarhus University, Aarhus, Denmark), Jeff T Wuchich (Cure AHC, Rolesville, NC, USA)

Declaration of interests

AB acts as a consultant for Allergan and Concert. All other authors declare that they have no competing interests.

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