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REVIEW

Genetically altered animal models for *ATP1A3*-related disorders

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ABSTRACT

Within the past 20 years, particularly with the advent of exome sequencing technologies, autosomal dominant and *de novo* mutations in the gene encoding the neurone-specific α_3 subunit of the Na^+,K^+-ATPase (NKA α_3) pump, *ATP1A3*, have been identified as the cause of a phenotypic continuum of rare neurological disorders. These allelic disorders of *ATP1A3* include (in approximate order of severity/disability and onset in childhood development): polymicrogyria; alternating hemiplegia of childhood; cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss syndrome; relapsing encephalopathy with cerebellar ataxia; and rapid-onset dystonia-parkinsonism. Some patients present intermediate, atypical or combined phenotypes. As these disorders are currently difficult to treat, there is an unmet need for more effective therapies. The molecular mechanisms through which mutations in *ATP1A3* result in a broad range of neurological symptoms are poorly understood. However, *in vivo* comparative studies using genetically altered model organisms can provide insight into the biological consequences of the disease-causing mutations in NKA α_3. Herein, we review the existing mouse, zebrafish, *Drosophila* and *Caenorhabditis elegans* models used to study *ATP1A3*-related disorders, and discuss their potential contribution towards the understanding of disease mechanisms and development of novel therapeutics.

KEY WORDS: *ATP1A3*, Na^+,K^+-ATPase α_3, Neurological disorders, Animal models

Introduction

Na^+,K^+-ATPase structure and function

The sodium-potassium pump, Na^+,K^+-ATPase (NKA), actively transports three Na^+ ions out of the cell and two K^+ ions into the cell against their concentration gradients, utilising energy derived from adenosine 5′-triphosphate (ATP) hydrolysis (Kaplan, 2002). The NKA belongs to the P-type ATPase family, so called because the ATPase cycle (known as the 'Albers-Post' cycle) is facilitated by the phosphorylation (hence 'P') and dephosphorylation of an aspartate residue (Fig. 1) (Albers, 1967; Post et al., 1972). During the cycle, the pump adopts two major conformations, the inward-facing E_1 state and the outward-facing E_2 state, which preferentially bind to Na^+ and K^+, respectively (Morth et al., 2007; Kanai et al., 2013). The electrochemical ion gradients generated and maintained by the NKA are essential for restoring resting membrane potentials, initiating action potentials and signalling by neurotransmitters (Holm et al., 2016b). Given the importance of NKA function in these cellular processes, it is unsurprising that the pump consumes ∼50% of the energy in the central nervous system (CNS) (Harris et al., 2012).

The NKA consists of two obligatory subunits, a large catalytic α-subunit and a smaller glycosylated auxiliary β-subunit, and occasionally a tissue-specific regulatory γ-subunit from the FXYD protein family that interacts with the αβ heterodimer (Kanai et al., 2013; Kaplan, 2002; Morth et al., 2007). The α-subunit consists of three cytoplasmic domains [nucleotide binding (N), phosphorylation (P) and actuator (A)] and ten transmembrane helices (M1-M10). Mammals express four distinct α-isoforms: α_1 is expressed ubiquitously; α_2 is expressed in glial cells (McGrail et al., 1991), the heart (Zahler et al., 1992) and muscles (Hundal et al., 1992); α_3 is expressed in neurones (McLean et al., 2009; Shamraj and Lingrel, 1994) and the heart (Zahler et al., 1992); and α_4 is expressed in testis (Woo et al., 2000). NKA α_3 plays an important role in the rapid restoration of basal intracellular Na^+ concentrations after high neuronal activity (Azarias et al., 2013). The β-subunit, which is required for NKA maturation, transportation of the αβ complex to the plasma membrane and stabilisation of the E_2 state, has three isoforms: β_1, β_2 and β_3 (Geering et al., 1996; Geering, 2001; McDonough et al., 1990; Lutsenko and Kaplan, 1993). Although the γ-subunit is not necessary for NKA function (Jones et al., 2005), it modulates the kinetic properties of the enzyme to adapt to the physiological needs of various tissue types (Mishra et al., 2011).

Human NKA α_3 mutations

The link between heterozygous missense mutations in the *ATP1A3* gene and the manifestation of rare neurological disorders was first established in 2004 (de Carvalho Aguiar et al., 2004). The human *ATP1A3* gene, comprising 23 exons on chromosome 19q13.2, has alternatively spliced transcript variants encoding three isoforms: variant 1/isoform 1 [3551 bp/1013 amino acids (aa); NM_152296] is the predominant transcript; variant 2/isoform 2 (3466 bp/1024 aa; NM_001256213) has an alternate 5′ exon, resulting in a longer N-terminus; and variant 3/isoform 3 (3590 bp/1026 aa; NM_001256214) has an additional in-frame segment in the 5′ coding region, resulting in a longer protein (Brown et al., 2015). The distribution of, and functional differences between, the *ATP1A3* transcript variants are unknown.

ATP1A3-related disorders represent a phenotypic continuum, as different non-synonymous mutations in *ATP1A3* result in a spectrum of clinical syndromes with overlapping symptoms that range broadly in severity, age of onset and progression (Sweney et al., 2015) (Table 1). Mutations causing neonate-onset disorders, such as polymicrogyria (PMG) and alternating hemiplegia of childhood (AHC), are considered to be the most severe (Sweadner et al., 2019). Disorders with onset later in childhood include cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss (CAPOS) syndrome and relapsing encephalopathy with cerebellar ataxia (RECA), whereas disorders with onset in adolescence or adulthood include rapid-onset dystonia-parkinsonism (RDP). Some

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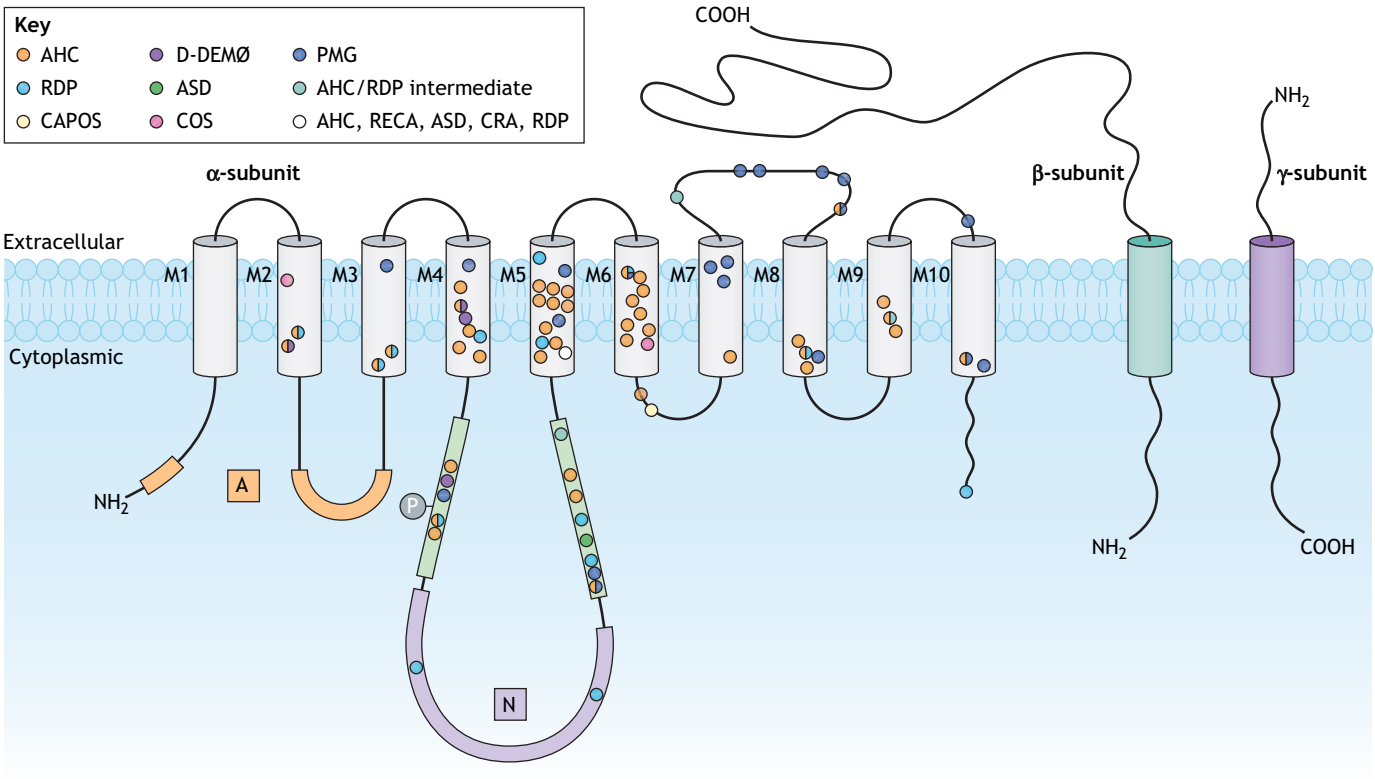

Fig. 1. Location of *ATP1A3*-related disorder mutations. Schematic of the NKA α3 protein with the cytoplasmic domains (A, N and P), ten transmembrane helices (M1-M10) and extracellular loops. Each circle represents the location of an amino acid residue mutated in *ATP1A3*-related disorders. Different colours represent different disorders. The white circle in M5 indicates R756, mutated in multiple disorders. The encircled 'P' indicates the transient phosphorylation of the P-domain aspartate D369. AHC, alternating hemiplegia of childhood; ASD, autism spectrum disorder; CAPOS, cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss; COS, childhood-onset schizophrenia; CRA, childhood rapid-onset ataxia; D-DEMØ, dystonia, dysmorphism of the face, encephalopathy with developmental delay, brain MRI abnormalities always including cerebellar hypoplasia, no hemiplegia (Ø); NKA, Na^+,K^+-ATPase; PMG, polymicrogyria; RDP, rapid-onset dystonia-parkinsonism; RECA, relapsing encephalopathy with cerebellar ataxia.

patients present intermediate, atypical or combined phenotypes (Rosewich et al., 2014a; Termsarasab et al., 2015; Sival et al., 2018), a number of which have been given new names, such as D-DEMØ. However, Sival et al. (2018) have cautioned that the assignment of overly exact phenotype-genotype relationships could promote inaccurate interpretation of the overlapping phenotypes within the spectrum of *ATP1A3*-related disorders.

The positions of mutations within the NKA α_3 protein (Fig. 1; Tables S1 and S2) have been correlated with phenotypic severity, following the observation that a majority of AHC mutations (~70%) cluster within and around the ion-binding sites (in M4-M6) relative to mutations associated with milder phenotypes (Sweadner et al., 2019). Pathogenicity studies have shown that *ATP1A3* mutations result in a reduction in catalytic activity and a failure to generate pump current, resulting from a variety of causes, including reduced protein expression, reduced Na^+ or K^+ affinity, unusually strong inhibition by K^+ or an inability of the pump to undergo conformational change (de Carvalho Aguiar et al., 2004; Heinzen et al., 2012; Holm et al., 2016b; Kirshenbaum et al., 2013; Roenn et al., 2019; Simmons et al., 2018; Toustrup-Jensen et al., 2014; Tranebjærg et al., 2018; Weigand et al., 2014).

ATP1A3-related disorders

RDP

RDP [DYT12; Online Mendelian Inheritance in Man (OMIM) 128235] was the first disorder identified as being caused by mutations in *ATP1A3* (de Carvalho Aguiar et al., 2004). It is characterised by the sudden onset of dystonia with parkinsonism features and bulbar symptoms, initially affecting the arms or legs in the majority of RDP patients (Haq et al., 2019). The onset of symptoms most commonly occurs in young adulthood and is triggered by stressors including exercise, fever, heat, childbirth and psychological stress (Dobyns et al., 1993; Brashear et al., 2007). Levels of homovanillic acid, a dopamine metabolite, in cerebrospinal fluid (CSF) are diminished in some patients (Brashear et al., 1998), but neuroimaging and pathology do not suggest nigral degeneration (Brashear et al., 1999; Oblak et al., 2014), and dopaminergic medication does not improve symptoms (Brashear et al., 2007). Neuropathology associated with RDP includes atrophy and neuronal loss in parts of the basal ganglia system, brainstem and cerebellum involved in the regulation of movement, motor coordination and motor learning (Oblak et al., 2014).

AHC

AHC (OMIM 614820) is an infantile-onset (<18 months) and the most common *ATP1A3*-related disorder, with a reported prevalence of between 1 in 1,000,000 (Neville and Ninian, 2007) and 1 in 100,000 children (Hoei-Hansen et al., 2014). It is characterised by paroxysmal episodes of hemiplegia involving one or both sides of the body, with variable clinical features such as abnormal ocular movements, choreoathetosis and dystonia, and resolution of symptoms with sleep or occlusion of the eyes (Bourgeois et al., 1993; Panagiotakaki et al., 2010; Rosewich et al., 2014a; Verret and Steele, 1971). Like RDP, paroxysmal episodes are often

Table 1. *ATP1A3*-related disorders: onset, inheritance, clinical features, treatments and most common mutations

Primary diagnosis	Age at onset	Inheritance	Prevalence	Clinical features	Most common mutations	References
D-DEMØ	Neonatal	*De novo*	Unknown (4 cases)	Dystonia; face dysmorphism; encephalopathy; developmental delay; cerebellar hypoplasia	Q140H, G325D, E324G, T360R	Prange et al., 2020
EIEE	<10 weeks	*De novo*	Unknown (3 cases)	Early-life epilepsy; shortened lifespan; recurrent apnoea; developmental delay; microcephaly	G358V, I363N, L924P	Paciorkowski et al., 2015; Arystarkhova et al., 2019
PMG	<5 months	*De novo*; very rarely familial	Unknown	PMG; epilepsy; developmental delay	F857del, D992del	Miyatake et al., 2021; Smith et al., 2021; Vetro et al., 2021
AHC	<18 months	*De novo*; very rarely familial	1/1,000,000[1]-1/100,000[2]	Paroxysmal episodes of hemiplegia or dystonia; nystagmus; epilepsy[3]; ataxia; developmental delay; cognitive impairment; episodic symptom relief upon sleep	D801N, E815K, G947R	Bourgeois et al., 1993; Heinzen et al., 2012; Ito et al., 2018; Jasien et al., 2019; Panagiotakaki et al., 2010; Rosewich et al., 2012; Schirinzi et al., 2018a; Severino et al., 2020; Verret and Steele, 1971
CRA	13-30 months	*De novo* or familial	Unknown (3 cases)	Febrile illness-induced rapid-onset persistent ataxia	R756C, E818K	Schirinzi et al., 2018b
CAPOS syndrome	6 months to 7 years	*De novo* or familial	<1/10,00,000	Febrile illness-induced cerebellar ataxic encephalopathy; areflexia; pes cavus[4]; bilateral optic atrophy; sensorineural hearing loss	E818K	Demos et al., 2014; Duat Rodriguez et al., 2017; Heimer et al., 2015; Maas et al., 2016; Potic et al., 2015; Rosewich et al., 2014c; Stenshorne et al., 2019
RECA	8 months to 5.5 years	*De novo* or familial	Unknown	Febrile illness-induced relapsing encephalopathy; hypotonia; cerebellar ataxia; dysarthria	R756C, R756L	Dard et al., 2015; Hully et al., 2017; Nakamura et al., 2018; Sabouraud et al., 2019; Yano et al., 2017
ASD	<9 years	*De novo*	Unknown (2 cases)	ASD with fatigue and hypersomnia or dyskinesia	A681T, R756C	Takata et al., 2018; Torres et al., 2018
COS	<13 years	*De novo* or familial	Unknown (2 cases)	Hallucinations; aggression; depression; delusion of persecution	V129M, A813V	Chaumette et al., 2020; Smedemark-Margulies et al., 2016
ARA	21 years	*De novo*	Unknown (1 case)	Ataxia; bradykinesia; dysarthria and drooling; hand tremor; cognitive impairment; cerebellar degeneration	G316S	Sweadner et al., 2016
RDP	9 months to 55 years	*De novo* or familial	<1/1,000,000	Abrupt onset of dystonia; parkinsonism; bulbar symptoms; triggered by stress; seizures[4]; anatomical brain abnormalities	E277K, T613M, D801Y, D923N	Brashear et al., 2012a; Cook et al., 2014; de Carvalho Aguiar et al., 2004; Dobyns et al., 1993; Haq et al., 2019; Oblak et al., 2014
adCORD	23 years	Familial	Unknown (2 cases)	Deterioration of photoreceptors; progressive vision loss; abnormal colour vision	D591V	Zhou et al., 2020

[1]Neville and Ninian (2007); [2]Hoei-Hansen et al. (2014); [3]~50% of cases; [4]~30% of cases. adCORD, autosomal-dominant cone-rod dystrophy; AHC, alternating hemiplegia of childhood; ARA, adult rapid-onset ataxia; ASD, autism spectrum disorder; CAPOS, cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss; COS, childhood-onset schizophrenia; CRA, childhood rapid-onset ataxia; D-DEMØ, dystonia, dysmorphism of the face, encephalopathy with developmental delay, brain MRI abnormalities always including cerebellar hypoplasia, no hemiplegia (Ø); EIEE, early infantile epileptic encephalopathy; PMG, polymicrogyria; RDP, rapid-onset dystonia-parkinsonism; RECA, relapsing encephalopathy with cerebellar ataxia.

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triggered by environmental or physiological stressors (Sweney et al., 2009).

Epileptic seizures occur in ∼50% of AHC patients (Panagiotakaki et al., 2010; Uchitel et al., 2019), and at least 94% of AHC patients exhibit cognitive impairment (Panagiotakaki et al., 2010; Sweney et al., 2009). Among 34 AHC patients evaluated with the Social Responsiveness Scale, 79% showed impaired social skills involving multiple domains and 26% were subsequently diagnosed with comorbid autism spectrum disorder (ASD) (Uchitel et al., 2020). A study of 12 AHC patients revealed reduced total brain and white matter volumes, and a negative correlation between the volume of cerebellar grey matter and severity of ataxia and disability (Severino et al., 2020). Among 87 patients with AHC, 60% exhibited resting electrocardiogram (ECG) abnormalities (Balestrini et al., 2020). Whether AHC is a progressive disorder remains unclear, but a study of 94 patients with AHC detected mild worsening of motor and intellectual disability with age (Uchitel et al., 2021), while abrupt stepwise deterioration (Sasaki et al., 2014) and progressive brain atrophy (Saito et al., 1998; Sasaki et al., 2017) in AHC patients have also been reported. The most common AHC-causing mutations in *ATP1A3* are D801N, E815K and G947R (Table S2), of which E815K has the most severe phenotype (Sasaki et al., 2014; Yang et al., 2014).

CAPOS syndrome

CAPOS syndrome (OMIM 601338) patients experience early-onset (<5 years of age) febrile illness-induced relapsing episodes of cerebellar ataxia, usually associated with progressive optic atrophy and sensorineural hearing loss, generalised hypotonia and areflexia. Pes cavus (a foot deformity) affects ∼30% of CAPOS patients (Duat Rodriguez et al., 2017), and cases without pes cavus are sometimes referred to as CAOS (Heimer et al., 2015). CAPOS begins with one to three fever-induced acute episodes of ataxic encephalopathy, from which patients usually recover over days to months, and slow disease progression thereafter (Duat Rodriguez et al., 2017). In CAPOS patients, cochlear outer hair cell activity is preserved, but auditory brainstem responses are grossly abnormal (Tranebjærg et al., 2018). All of the reported cases of CAPOS, to date, are heterozygous for a single recurrent missense mutation, E818K (Demos et al., 2014; Duat Rodriguez et al., 2017; Han et al., 2017; Heimer et al., 2015; Maas et al., 2016; Potic et al., 2015; Rosewich et al., 2014c; Stenshorne et al., 2019). Some CAPOS patients exhibit mild symptoms, resulting in recovery with minimal residual ataxia (Demos et al., 2014), although one subject with a *de novo* E818K mutation exhibited only sensorineural hearing loss that began when she was a teenager (Han et al., 2017).

PMG

PMG is the most common developmental malformation of the cerebral cortex, characterised by abnormal folding and laminar organisation. Recently, *de novo* and familial mutations in *ATP1A3* were found in patients affected by a severe form of PMG characterised by epilepsy and a global developmental delay (Miyatake et al., 2021; Smith et al., 2021; Vetro et al., 2021). PMG mutations include D801N, the most common mutation in AHC (Panagiotakaki et al., 2015), and L924P, which is also observed in early infantile epileptic encephalopathy (EIEE) (Arystarkhova et al., 2019). A human *ATP1A3* complementary DNA (cDNA) construct harbouring the mutation D992del, observed in two patients with severe PMG, was found to impair radial neuronal migration in the cerebral cortex of C57BL/6 mice at embryonic day (E)18.5, after being introduced into the ventricles on E14.5 by *in utero* electroporation (Miyatake et al., 2021), suggesting that *ATP1A3*^{D992del} causes defects in cortical architecture.

RECA

RECA is characterised by recurrent neurological decompensation episodes triggered by febrile illness, leading to encephalopathy with acute cerebellar ataxia and occasionally chorea, dystonia, dysarthria, mutism and dysphagia (Dard et al., 2015; Hully et al., 2017; Nakamura et al., 2018; Sabouraud et al., 2019). To date, all RECA cases are associated with substitutions of arginine 756 (R756H, R756C) (Dard et al., 2015; Hully et al., 2017; Jaffer et al., 2017; Nakamura et al., 2018; Nicita et al., 2016; Sabouraud et al., 2019). A very similar *ATP1A3*-related disorder, febrile-induced paroxysmal weakness and encephalopathy (FIPWE), is also caused by mutations at R756 (Yano et al., 2017). Owing to the genetic and phenotypic overlaps between FIPWE and RECA (Sabouraud et al., 2019), in this Review, we ascribe the term RECA to all FIPWE cases.

Autosomal-dominant cone-rod dystrophy

Autosomal-dominant cone-rod dystrophy (adCORD) is an inherited retinal degenerative disorder characterised by progressive deterioration of cone and rod photoreceptors. Affected individuals typically present with progressive loss of vision, reduced visual acuity and problems with colour vision from the age of 12 (Thiadens et al., 2012). The *ATP1A3* mutation D591V has been identified in a single family affected by a form of adCORD (Zhou et al., 2020).

Other *ATP1A3*-related clinical phenotypes

Mutations in *ATP1A3* have also been identified in individuals presenting with other rare clinical phenotypes. D-DEMØ, observed in four patients, consists of dystonia, dysmorphism of the face, encephalopathy with developmental delay and magnetic resonance imaging (MRI) brain abnormalities (cerebellar hypoplasia) (Prange et al., 2020). EIEE, observed in three patients, is characterised by seizures beginning within the first few days after birth, resulting in the death of two patients in infancy (Paciorkowski et al., 2015; Arystarkhova et al., 2019). Childhood rapid-onset ataxia (CRA), observed in three patients, is characterised by the presentation of ataxic features following a febrile episode during infancy (<36 months); two patients have the R756C mutation associated with RECA and the third has the E818K mutation associated with CAPOS syndrome (Schirinzi et al., 2018b). Adult rapid-onset ataxia (ARA) has been observed in one patient, with a *de novo* G316S mutation, in whom difficulties with balance and gait emerged at 21 years (Sweadner et al., 2016). *ATP1A3* mutations have also been identified in individuals with forms of ASD (Takata et al., 2018; Torres et al., 2018; Chaumette et al., 2020) and childhood-onset schizophrenia (COS) (Smedemark-Margulies et al., 2016; Chaumette et al., 2020).

Animal models of *ATP1A3*-related neurological disorders

Mouse

Mouse models are valuable tools to study *ATP1A3*-related disorders, owing to their high genomic similarity to humans. The mouse orthologue, *Atp1a3*, comprising 23 exons on chromosome 7, has 99.6% protein and 90.8% DNA identity relative to human *ATP1A3* (NCBI HomoloGene 113729) (Table 2). *Atp1a3* has alternatively spliced transcript variants encoding two isoforms: variant 1/isoform 1 (3615 bp/1026 aa; NM_001374627) and variant 2/isoform 2 (3609 bp/1013 aa; NM_001290469). Several mouse models to study the *in vivo* consequences of NKA α_3 mutations have

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Table 2. *ATP1A3* orthology between human, mouse, zebrafish, *C. elegans* and *Drosophila*

Species	Gene symbol	Chromosomal location	% protein homology	% DNA homology
Human (*H. sapiens*)	*ATP1A3*	19q13.2	100	100
Mouse (*M. musculus*)	*Atp1a3*	7	99.6	90.8
Zebrafish (*D. rerio*)	*atp1a3a*	19	90.0	81.0
	atp1a3b	16	91.6	79.5
D. melanogaster	*Atpα*	3R	76.3	71.7
C. elegans	*eat-6*	V	73.5	67.0

Shown are the percentage protein and percentage DNA homology of orthologues relative to human *ATP1A3* (NCBI HomoloGene 113729; https://www.ncbi.nlm.nih.gov/homologene/113729).

been published to date (Table 3). The seven behavioural tests most commonly used to assess mouse models of *ATP1A3*-related disorders are briefly described in Box 1.

Atp1a3$^{tm1/Ling}$

The *Atp1a3*$^{tm1/Ling}$ model [Alpha3^{-}; Mouse Genome Informatics (MGI) 3696954] was the first-described mouse line harbouring a mutation of the *Atp1a3* gene (Moseley et al., 2007). The line was generated via homologous recombination in 129P2/OlaHsd-derived (mEMS32) embryonic stem (ES) cells that introduced a point mutation into *Atp1a3* intron 4 adjacent to the exon-intron splice site, resulting in aberrant splicing, adding 126 bp to the transcript. Homozygous *Atp1a3*$^{tm1/Ling}$ mice die neonatally and show a very low level of NKA α_3 mRNA in the foetal brain compared with wild-type (WT) mice, such that *Atp1a3*$^{tm1/Ling}$ is not a complete null. Heterozygous mice (*Atp1a3*$^{tm1/Ling/+}$) have a ∼60% reduction in NKA α_3 protein levels in the hippocampus (Moseley et al., 2007) and a 16% reduction in brain NKA activity (contributed by α_1+α_2+α_3 subunits) (Clapcote et al., 2009).

Atp1a3$^{tm1/Ling/+}$ mice with a 129P2/OlaHsd×Black Swiss F_2 genetic background displayed increased locomotor activity in the open field and after administration of methamphetamine (a drug that elevates levels of extracellular monoamine neurotransmitters in the brain), as well as spatial learning and memory deficits in the Morris water maze (Moseley et al., 2007). Restraint-stress induced motor deficits in female *Atp1a3*$^{tm1/Ling/+}$ mice in the beam-walking and rotarod tests, but non-stressed females were unaffected (DeAndrade et al., 2011). Grip strength in stressed *Atp1a3*$^{tm1/Ling/+}$ mice was intact, suggesting that motor deficits were not of a muscular origin. Male *Atp1a3*$^{tm1/Ling/+}$ mice did not display restraint stress-induced motor deficits (DeAndrade et al., 2011), indicating a sex-dependent effect.

Atp1a3$^{tm1/Ling/+}$ mice with a C57BL/6NCrl congenic background were exposed to chronic variable stress (CVS) by subjecting them to unpredictable mild stressors daily for 6 weeks (Kirshenbaum et al., 2011b). CVS-treated *Atp1a3*$^{tm1/Ling/+}$ mice displayed 33% reduced brain NKA activity (versus 16% reduction in naïve mice), which was associated with increased anxiety in the elevated plus maze, impaired long-term memory in the novel object recognition test and reduced preference for social novelty compared with CVS-treated WT mice. This demonstrates that the interaction of environmental stress and NKA α_3 mutation can have a negative impact on brain NKA activity, with consequent effects on behaviour. CVS-treated *Atp1a3*$^{tm1/Ling/+}$ and WT mice both exhibited despair-like behaviour in the tail suspension test, and anhedonia in the sucrose preference test. No sex differences were reported (Kirshenbaum et al., 2011b).

Atp1a3^{tm2Kwk}

The *Atp1a3*^{tm2Kwk} mouse model (MGI 5572809) was generated via homologous recombination in C57BL/6NCrl×CBA/J F_1-derived (TT2) ES cells by replacing *Atp1a3* exons 2-6 with a loxP-flanked *Atp1a3*-enhanced green fluorescent protein (EGFP) fusion protein, which was subsequently deleted by Cre-mediated recombination (Ikeda et al., 2013). Homozygous *Atp1a3*^{tm2Kwk} mice die neonatally, owing to a complete lack of breathing movements. Heterozygous (*Atp1a3*$^{tm2Kwk/+}$) mice with a C57BL/6JJcl congenic background did not exhibit obvious motor deficits, spontaneously or after exposure to stressors (tail suspension, forced swimming or restraint stress). *Atp1a3*$^{tm2Kwk/+}$ mice did, however, display increased locomotor activity in the open field, and performed significantly better than WT mice in the beam-walking and rotarod tests. Injection of kainic acid (KA; a convulsant) into the cerebellum, a brain region important in motor control, induced dystonia in both *Atp1a3*$^{tm2Kwk/+}$ and WT mice, but heterozygotes had a longer dystonic response and recovery time (Ikeda et al., 2013). Ikeda et al. (2013) also observed altered neurotransmission across inhibitory synapses in *Atp1a3*$^{tm2Kwk/+}$ mice, suggesting that perturbed inhibitory circuits may have a potential role in the manifestation of dystonia.

In an evaluation of social behaviours, *Atp1a3*$^{tm2Kwk/+}$ mice showed a lower degree of social interaction, a lower frequency of barbering (removal of whiskers and facial hair) and a lower hierarchical rank (a reflection of social dominance) within a mixed-genotype cage relative to WT mice (Sugimoto et al., 2018).

Levels of ascorbic acid (vitamin C; a vital antioxidant) were found to be decreased in the basal ganglia and cerebellum of *Atp1a3*$^{tm2Kwk/+}$ mice, and were decreased further in *Atp1a3*$^{tm2Kwk/+}$ dams within 12 h of giving birth, compared with WT controls (Ikeda et al., 2021). Perinatal *Atp1a3*$^{tm2Kwk/+}$ dams also displayed transient dystonic episodes (Ikeda et al., 2021), paralleling the worsening of symptoms observed in CAPOS syndrome during pregnancy and in the immediate postpartum period, when maternal body temperature is raised (Chang et al., 2018). Thus, although the *Atp1a3*$^{tm1/Ling}$ and *Atp1a3*^{tm2Kwk} models do not replicate specific mutations found in *ATP1A3*-related disorders, they have both demonstrated utility for investigating trigger-induced symptoms.

Myshkin

The *Myshkin* mouse model (*Atp1a3*^{Myk}; MGI 4356167) harbouring mutation I810N in *Atp1a3* exon 18 was originally identified as a phenodeviant C57BL/6J×129S1/SvImJ F_1 mouse with an altered phenotype in an N-nitroso-N-ethylurea (ENU; a chemical mutagen) mutagenesis screen (Clapcote et al., 2009). The I810N mutation has been observed in three AHC patients, including one with comorbid ASD, all of whom presented with typical AHC symptoms and epilepsy (Panagiotakaki et al., 2015; Yang et al., 2014, 2015). Homozygous *Myshkin* mice die neonatally and show a 56% reduction in brain NKA activity at E18.5 compared with WT littermates. Adult heterozygous *Myshkin* (*Myk*/+) mice backcrossed 12 generations (N_{12}) to the C57BL/6NCr strain have a 42% reduction in NKA enzyme activity in the adult brain and a 16-18%

Table 3. Summary of genetically altered mouse models of *ATP1A3*-related disorders

Mouse model	Mutation	Human disorder	Hom phenotype	Genetic background	NKA α in Het brain	*In vivo* phenotypic test differences in Het versus WT	Spontaneous seizures	References
Atp1a3^{tm1Ling} (*Alpha3*$^{-}$; MGI 3696954)	Point mutation in *Atp1a3* intron 4 (targeted knock-in)		Neonatal death	129P2/OlaHsd×Black Swiss F_2	α_3 protein: ∼60% ↓ α_1 protein: ∼35% ↑	Open field ambulation ↑ Methamphetamine response ↑ Water maze: hidden platform latency ↑ Beam: slips ↑ (restraint ♀) Rotarod: fall latency ↓ (restraint ♀)	No	DeAndrade et al., 2011; Moseley et al., 2007
				C57BL/6NCr congenic	NKA activity: 16% ↓ (naïve) 33% ↓ (CVS)	TST immobility ↑ (CVS) EPM open arms ↓ (CVS) Novel object recognition ↓ (CVS) Social approach ↓ (CVS)	No	Clapcote et al., 2009; Kirshenbaum et al., 2011b
Atp1a3^{tm2Kwk} (MGI 5572809)	Deletion of *Atp1a3* exons 2-6 (targeted knockout)		Neonatal death	C57BL/6JJcl congenic	Unknown	Home cage ambulation ↑ Open field ambulation ↑ Beam: traversal time ↓ Rotarod: fall latency ↑ Cerebellar KA response ↑	No	Ikeda et al., 2013
Atp1a3^{D801Y} (alpha3^{D801Y}; *Atp1a3*^{tm1Klh}; MGI 6163502)	D801Y (targeted knock-in)	RDP/mild AHC	Neonatal death	C57BL/6JRj congenic	α_3 protein: 15% ↓ α_1 protein: 33% ↑	Open field ambulation ↑ EPM open arms ↑ Barnes maze ↓ Passive avoidance ↓ Beam: traversal time and slips ↑ Vertical rope climbing ↓ Parallel rod floor ↓ PTZ-induced Sz threshold ↓ Dystonic response to hypothermia ↑	No	Holm et al., 2016a; Isaksen et al., 2017
Myshkin (*Atp1a3*^{Myk}; MGI 4356167)	I810N (ENU induced)	AHC	Neonatal death	C57BL/6NCr congenic	NKA activity: 42% ↓ (adult) 34% ↓ (E18)	Body weight ↓ Vestibular stress-induced Sz ↑ Hole-board nose-pokes ↑ Open field ambulation ↑ D-amphetamine response ↑ EPM open arms ↑ LDB light side ↑ Sucrose preference ↑ FST immobility ↓ PPI ↓ Startle habituation ↓ Contextual fear conditioning ↓ Conditioned taste aversion ↓ Novel object recognition ↓ T-maze habituation ↓ Social recognition ↓ Water maze: visible platform latency ↑ Tremor amplitude ↑ Hindlimb gait abnormalities ↑ Hindlimb clasping ↑ Beam: traversal time and slips ↑ Rotarod: fall latency ↓	Yes	Clapcote et al., 2009; Kirshenbaum et al., 2011a, 2013, 2015, 2016b

Continued

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Table 3. Continued

Mouse model	Mutation	Human disorder	Hom phenotype	Genetic background	NKA α in Het brain	*In vivo* phenotypic test differences in Het versus WT	Spontaneous seizures	References
						Social approach ↓ Nest building ↓ Pup retrieval ↓ (dams) Sleep duration ↓ Circadian period ↑		
Mashlool (Mashl; *Atp1a3*^{tm1Ute}; MGI 6162645)	D801N (targeted knock-in)	AHC	Unknown	C57BL/6J backcross	Unknown	Body weight ↓ (♂) Flurothyl-induced Sz threshold ↓ Kindling-induced Sz threshold ↓ Cold water-induced Sz ↑ Open field ambulation ↑ (0-5 min) Novel object recognition ↓ Water maze: visible platform latency ↑ Tremor amplitude ↑ Hindlimb gait abnormalities ↑ Hindlimb clasping ↑ Beam: traversal time and slips ↑ Hot plate (pain) response ↓ ECG abnormalities ↑	Yes	Balestrini et al., 2020; Hunanyan et al., 2015; Uchitel et al., 2021
Matoub (Matb; *Atp1a3*^{E815K}; *Atp1a3*^{tm1Mika}; MGI 6197036)	E815K (targeted knock-in)	AHC	Unknown	C57BL/6J N_4 backcross	Unknown	Body weight ↓ (♀) Open field ambulation ↓ (0-2 min) Novel object recognition ↓ Rotarod: fall latency ↓ Beam: traversal time and slips ↑ Hindlimb gait abnormalities ↑ Hindlimb clasping ↑ FST immobility ↑ Vestibular stimulation response ↑ Warm water response ↑ Kindling-induced Sz threshold ↓	Yes	Helseth et al., 2018
ROSA26-*ATP1A3*^{D591V} [*Gt(ROSA)26Sor*$^{tm1(CAG\text{-}ATP1A3\text{-}D591V,\text{-}EGFP)}$]	D591V (ROSA26 targeted knock-in)	adCORD	Unknown	C57BL/6	Unknown	Maximal ERG responses ↓	No	Zhou et al., 2020

↑, significant increase; ↓, significant decrease; ♀, females; ♂, males; AHC, alternating hemiplegia of childhood; CVS, post-chronic variable stress; ECG, electrocardiogram; EPM, elevated plus maze; ERG, electroretinogram; FST, forced-swim test; Het, heterozygous; Hom, homozygous; KA, kainic acid; LDB, light-dark box; MGI, Mouse Genome Informatics; NKA, Na^+,K^+-ATPase; PPI, prepulse inhibition; PTZ, pentylenetetrazole; RDP, rapid-onset dystonia-parkinsonism; Sz, seizure(s); TST, tail suspension test; WT, wild type.

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Box 1. Behavioural tests in mouse models of *ATP1A3*-related disorders

Learning and memory

Morris water maze: the hidden platform (spatial) version is a test of spatial learning and memory, in which the mouse is placed in a large circular tub of water and required to use extramaze visual cues to navigate to a submerged escape platform. The visible platform version is sometimes used as a non-spatial control task, in which the escape platform is indicated by a marker above the water surface.

Novel object recognition: the mouse is presented with two identical objects during the training session, and then one of the two objects is replaced by a novel object during a test session. The amount of time taken exploring the novel object provides a measure of recognition memory.

Motor function and locomotor activity

Beam-walking: the goal of the balance beam test is for the mouse to stay upright and walk across an elevated narrow beam to a safe platform. The number of errors (foot slips) and latency to cross the beam are recorded as measures of motor skills and balance.

Gait analysis: the fore and hind paws of the mouse are coated with two colours of non-toxic paint, and then the mouse is allowed to walk along a narrow, paper-covered runway. The width and length of the fore stride and hind stride are measured from the resulting pawprint patterns.

Hindlimb clasping: the mouse is suspended by the tail for 30 s and observed for hindlimb clasping (retraction of either or both hindlimbs into the body and toward the midline), a behaviour indicative of general neurological dysfunction.

Open field: the open field consists of a square wall-enclosed arena into which the mouse is placed and allowed to move about freely for 10-60 min while being recorded by an overhead camera. The test is used to determine various parameters of locomotor activity (e.g. ambulation) and exploration habits (e.g. time at periphery versus centre).

Rotarod: the mouse is placed on a horizontally oriented rod that begins rotating at a constant or accelerating speed. The latency to fall onto a platform below, and the speed of rod rotation upon falling, are recorded as measures of balance and coordination.

reduction in body weight compared with WT mice (Clapcote et al., 2009). *Myk*/+ mice exhibit an elevated metabolic rate, a visible whole-body tremor and a broad-based gait (Kirshenbaum et al., 2013; Timothy et al., 2018). 2-Deoxy-D-glucose imaging identified a deficit in frontal cortex functioning (hypofrontality) in the *Myk*/+ mouse brain, directly mirroring that reported in AHC (Sasaki et al., 2009; Sweney et al., 2009), along with reduced thalamocortical functional connectivity (Kirshenbaum et al., 2013).

Myk/+ mice exhibit spontaneous and vestibular stress-induced seizures, medial temporal sclerosis, sleep abnormalities, and a variety of motor, cognitive, social and other behavioural deficits (Table 3) (Clapcote et al., 2009; Kirshenbaum et al., 2011a, 2013, 2015, 2016b) consistent with the multiple comorbidities in AHC (Jasien et al., 2019; Kansagra et al., 2019; Neville and Ninan, 2007). In hippocampal slices from *Myk*/+ mice, the CA3-CA1 hippocampal pathway showed normal levels of synaptic transmission, plasticity and excitability under basal conditions, but became hyperexcitable after high-frequency synaptic activity (Clapcote et al., 2009), such as that precipitated by stress, consistent with the role of NKA α_3 in the rapid extrusion of Na^+ after high neuronal activity (Azarias et al., 2013).

The seizures of *Myk*/+ mice are occasionally fatal, but their severity was reduced by valproate (an anticonvulsant), and they were prevented by inheritance of a WT *Atp1a3* cDNA transgene, conferring a 16% increase in brain NKA activity (Clapcote et al., 2009). The WT *Atp1a3* cDNA also rescued *Myshkin* body weight, motor dysfunction, contextual fear conditioning and mania-like behaviours (greater risk-taking and impulsivity) (Kirshenbaum et al., 2011a, 2016a). Attenuation of mania-like behaviours was also observed following treatment with lithium and valproate (mood-stabilisers), SL327 (an ERK inhibitor) and rostafuroxin (an antagonist of the NKA blocker ouabain) (Kirshenbaum et al., 2011a).

Compared with N_{12} C57BL/6NCr animals, *Myk*/+ mice backcrossed 20 generations to C57BL/6NCr (N_{20}) showed a significantly lower reduction in adult brain NKA activity (36% versus 42%) and did not exhibit seizure activity in electrocorticography recordings when subjected to vestibular stress (Kirshenbaum et al., 2011a). This demonstration of phenotypic modification by the genetic background is supported by the observation that two crosses (N_2) to the seizure-susceptible FVB/NCr strain (Goelz et al., 1998) resulted in significant levels of focal reactive astrogliosis and microglial activation in the hippocampus that were not observed in N_{12} C57BL/6NCr *Myk*/+ mice (Clapcote et al., 2009).

Atp1a3^{D801Y}

The *Atp1a3*^{D801Y} mouse model (alpha3^{D801Y}; *Atp1a3*^{tm1Klh}; MGI 6163502) was generated via homologous recombination in 129S1/SvImJ-derived (CJ7) ES cells that replaced *Atp1a3* exon 17 with a modified exon 17 harbouring the D801Y mutation (Holm et al., 2016a). A variety of mutations affecting the aspartic acid at position 801 (D801) can cause RDP, AHC or PMG. D801E and D801V are AHC-causing mutations (Panagiotakaki et al., 2015), whereas D801N can cause AHC and PMG (Panagiotakaki et al., 2015; Vetro et al., 2021), and D801Y can cause RDP and AHC (de Carvalho Aguiar et al., 2004; Viollet et al., 2015). Homozygous *Atp1a3*^{D801Y} mice die neonatally. Heterozygous (*Atp1a3*$^{D801Y/+}$) mice with a C57BL/6JRj congenic background have a 15% reduction in total NKA α3 protein levels in the brain (Holm et al., 2016a).

Atp1a3$^{D801Y/+}$ mice display increased locomotor activity in the open field, a lower threshold for seizures induced by pentylenetetrazole (PTZ; a convulsant) and learning and memory impairments in the Barnes maze and passive avoidance test, of which the latter was rescued by administration of clonazepam (a tranquiliser that enhances GABAergic inhibition) (Holm et al., 2016a). Although gait, posture and grip strength are not significantly different in *Atp1a3*$^{D801Y/+}$ mice, beam-walking, vertical rope climbing and parallel rod floor tests revealed moderate motor deficits (Isaksen et al., 2017). *Atp1a3*$^{D801Y/+}$ mice also display hypothermia-induced dystonia after cold water (5-10°C) swimming for 4 min or cold environment (−20°C) exposure, consistent with dystonic episodes triggered by temperature changes in RDP patients (de Carvalho Aguiar et al., 2004; Isaksen et al., 2017). Other stressors, including forced swimming in warm (35°C) water, high temperature (43°C) for 15 min, restraint stress and foot shocks, do not induce dystonia in *Atp1a3*$^{D801Y/+}$ mice (Isaksen et al., 2017).

Mashlool

The Mashlool mouse model (Mashl; *Atp1a3*^{tm1Ute}; MGI 6162645) was generated via homologous recombination in 129S1/SvImJ×129X1/SvJ F_1-derived (R1) ES cells that replaced *Atp1a3* exon 17 with a modified exon 17 harbouring the D801N mutation

(Hunanyan et al., 2015). D801N is the mutation most commonly observed in humans with AHC (Table S2). *In vitro* studies involving heterologous overexpression of *ATP1A3* have shown that D801N does not affect protein levels but reduces ATPase activity by 54-80% (Heinzen et al., 2012). Heterozygous Mashlool (Mashl/+) mice with a C57BL/6J backcross background exhibit reduced body weight in males (by ∼11%) but not females (Hunanyan et al., 2015).

In the open field, Mashl/+ mice display increased locomotor activity and time at the centre of the arena during the first 5 min but not thereafter (Hunanyan et al., 2015). Mashl/+ mice also display impaired long-term memory in the novel object recognition test and motor dysfunction in the gait, beam-walking and hindlimb clasping tests. During these experiments, 40% of Mashl/+ mice exhibited transient (∼90-s duration) hemiplegia or hemiparesis, replicating a core symptom of AHC. Mashl/+ mice also exhibit a lower threshold for seizures induced by amygdala kindling (focal electrical stimulation) and flurothyl (a convulsant). Spontaneous recurrent and occasionally fatal seizures were also observed in both kindled and non-kindled mutant mice (Hunanyan et al., 2015).

Resting ECGs in Mashl/+ mice revealed an increased heart rate and intracardiac conduction delay compared with WT mice (Balestrini et al., 2020). After injection of KA into the amygdala, Mashl/+ mice show ECG abnormalities in response to seizure activity (Balestrini et al., 2020). Compared with young (5-week-old) Mashl/+ mice, adult (17-week-old) Mashl/+ mice exhibit an inferior beam-walking performance and an increased severity of seizures and mortality induced by cold water (5-10°C) swimming for 4 min (Uchitel et al., 2021), suggestive of disease progression.

In hippocampal slices from Mashl/+ mice, the CA3-CA1 hippocampal pathway shows hyperexcitable responses to repetitive high-frequency stimulation (Hunanyan et al., 2015), similar to those observed in *Myk*/+ mice (Clapcote et al., 2009). This increased hippocampal excitability is due to decreased $GABA_A$ receptor-mediated inhibition, consistent with a reduced number of parvalbumin-expressing inhibitory interneurones in the hippocampal CA1 in Mashl/+ mice (Hunanyan et al., 2018). The application of extracellular potassium induces greater spreading depression (a transient wave of depolarisation) in Mashl/+ slices than in WT slices (Hunanyan et al., 2015), consistent with previous work demonstrating prolonged spreading depression in response to ouabain (Haglund and Schwartzkroin, 1990).

An adeno-associated virus serotype 9 (AAV9) vector expressing human *ATP1A3* cDNA under the control of a human *SYN1* (neurone-specific) promoter, which had been shown to increase brain NKA activity in WT mice, was injected into the CSF, via the cerebral ventricles and cisterna magna, of Mashl/+ mice on postnatal day (P)10 (Hunanyan et al., 2021). This exogenous delivery of WT *ATP1A3* to Mashl/+ mice resulted in a reduced occurrence of hemiplegic but not dystonic episodes induced by cold water swimming at P40, improvement in beam-walking performance at P40 and P70 (10 weeks) and prolonged survival compared with untreated Mashl/+ mice up to 10 weeks of age. Although other behavioural tests did not reveal differences in treated Mashl/+ mice (Hunanyan et al., 2021), these results demonstrate that gene therapy can ameliorate some of the manifestations of AHC in the Mashlool model.

Matoub

The Matoub mouse model (Matb; *Atp1a3*^{E815K}; *Atp1a3*^{tm1Mika}; MGI 6197036) was generated via homologous recombination in 129S1/SvImJ×129X1/SvJ F_1-derived (R1) ES cells by replacing *Atp1a3* exon 18 with a modified exon 18 harbouring the E815K mutation (Helseth et al., 2018). E815K is the second most common mutation in patients with AHC (Table S2) and has a more severe phenotype than that of D801N and G947R heterozygotes. E815K heterozygous AHC patients have an earlier onset of disease and seizures, more frequent status epilepticus, plegic attacks and autonomic dysfunction, and more severe cognitive and motor deficits (Ishii et al., 2013; Panagiotakaki et al., 2015; Sasaki et al., 2014; Viollet et al., 2015; Yang et al., 2014).

Heterozygous (Matb/+) mice with a C57BL/6J N_4 backcross background manifest episodes of hemiplegia or seizures around the time of weaning at 3-4 weeks of age. Female, but not male, Matb/+ mice exhibit reduced body weight (by ∼29%). Within a cohort of mice monitored up to 12 months, Matb/+ individuals showed increased mortality, with ∼33% dying during a witnessed seizure and none surviving beyond 9 months of age. Matb/+ mice also displayed impaired long-term memory in the novel object recognition test and motor dysfunction in the gait, rotarod, beam-walking and hindlimb clasping tests (Helseth et al., 2018).

After being subjected to 1 min of forced swimming in 40°C water, warmer than the body temperature associated with a febrile state in humans (37.5-39°C) (Del Bene, 1990), 75% of Matb/+ mice displayed an episode of hemiplegia. The duration of the hemiplegia was shortened by treatment with flunarizine (Helseth et al., 2018), a calcium channel blocker that reduces the severity of hemiplegic attacks in some AHC patients (Sasaki et al., 2001; Ito et al., 2018). However, retesting of Matb/+ mice 35 and 98 days after the last injection indicated no long-term beneficial effect of flunarizine treatment (Helseth et al., 2018).

ROSA26-*ATP1A3*^{D591V}

The ROSA26-*ATP1A3*^{D591V} mouse model [*Gt(ROSA)26Sor*$^{tm1(CAG\text{-}ATP1A3\text{-}D591V,\text{-}EGFP)}$], carrying the mutation D591V in *ATP1A3* identified in adCORD, was generated via homologous recombination in C57BL/6-derived ES cells (Zhou et al., 2020). However, the endogenous mouse *Atp1a3* gene was left intact. Instead, a human *ATP1A3* cDNA harbouring D591V, under the control of the CMV early enhancer/chicken β-actin (CAG) promoter, was introduced into the mouse *Gt(ROSA)26Sor* locus (Zhou et al., 2020), which is a widely used site for the integration of transgenes. The CAG promotor drives strong and ubiquitous gene expression, in contrast to the *Atp1a3* promoter that drives selective expression in neurones.

Heterozygous ROSA26-*ATP1A3*$^{D591V/+}$ mice with a C57BL/6 genetic background had unaltered electroretinogram (ERG) responses and retinal cell morphology at 3 months of age (young adult), but maximal ERG responses were decreased at 12 months, indicative of cone and rod dysfunction (Zhou et al., 2020). ROSA26-*ATP1A3*$^{D591V/+}$ mice thus had a comparatively later onset and less severe retinal dysfunction than that exhibited by D591V heterozygous adCORD patients (Zhou et al., 2020). This could be because, unlike D591V/+ heterozygous adCORD patients, both copies of the NKA α3 gene are intact in ROSA26-*ATP1A3*$^{D591V/+}$ mice.

Zebrafish (*Danio rerio*)

Zebrafish have two *ATP1A3* orthologues, *atp1a3a* and *atp1a3b* (Rajarao et al., 2001), which have a 94.5% amino acid sequence identity with each other. Moreover, the proteins are 90.0% (*atp1a3a*) and 91.6% (*atp1a3b*), and the DNA is 81.0% (*atp1a3a*) and 79.5% (*atp1a3b*), identical relative to human *ATP1A3* (NCBI HomoloGene 113729) (Table 2). Similar to mammalian NKA α_3, *atp1a3a* and *atp1a3b* are predominantly

expressed in the brain (Doğanlı et al., 2013). Zebrafish embryos serve as a promising bridge model between *in vitro* and *in vivo* research, with the potential to replace full-grown animals in experimentation. Expression of the *atp1a3a* transcript is highest in zebrafish embryos at 60 h post-fertilisation (hfp) and is found in various CNS structures, including the epiphysis, tegmentum, tectum, cerebellum, cranial ganglia, hindbrain and spinal cord (Doğanlı et al., 2013). By contrast, expression of the *atp1a3b* transcript is observed in both 60 hpf zebrafish embryos and adults. Within embryos, *atp1a3b* is localised to similar CNS regions as *atp1a3a*, excluding the tectum and posterior spinal cord (Doğanlı et al., 2013).

Splice-blocking morpholino antisense oligonucleotides (SP-MOs) and translation-blocking morpholino antisense oligonucleotides (MOs), which cause mRNA knockdown (KD) by nonsense-mediated mRNA decay or inhibition of protein synthesis, respectively, have facilitated the study of NKA α_3 in zebrafish embryos (Doğanlı et al., 2013). The majority of embryos injected with *atp1a3a*-SP-MO or *atp1a3b*-SP-MO exhibit a reduced level of *atp1a3a* (~62%) or *atp1a3b* (~66%) transcript and show severe hydrocephalus as a consequence of CSF build-up within the brain ventricle (Doğanlı et al., 2013). Meanwhile, the majority of *atp1a3a*-MO- and *atp1a3b*-MO-injected embryos showed a mild hydrocephalus phenotype. This phenotype was rescued in a significant proportion of KD embryos by co-injection of WT *atp1a3a* mRNA (but not WT *atp1a3b* mRNA) in *atp1a3a* KD embryos, and WT *atp1a3b* mRNA (but not WT *atp1a3a* mRNA) in *atp1a3b* KD embryos (Doğanlı et al., 2013). Although hydrocephalus is not typically observed in patients with *ATP1A3*-related disorders, this zebrafish finding raises the possibility that NKA α_3 is involved in regulating brain volume (Doğanlı et al., 2013). Zebrafish embryos may thus provide insight into the smaller total brain volume observed in both AHC and EIEE patients (Paciorkowski et al., 2015; Severino et al., 2020). In response to tactile stimulation of the embryo trunk, *atp1a3a* KD embryos and *atp1a3b* KD embryos (60 hpf) both display brief distance recoils and occasional convulsions, which are delayed in *atp1a3b* KD embryos, in contrast to the burst swimming exhibited by control embryos (Doğanlı et al., 2013). These observations underline the importance of NKA α_3 in motor control.

Fruit fly (*Drosophila melanogaster*)

The *D. melanogaster ATPalpha* (*Atpα*) gene (FlyBase ID FBgn0002921) is responsible for encoding the Na^+ pump α subunit, which is orthologous to all four α-subunits expressed in mammals, such that *Atpα* is not a specific one-to-one orthologue of *ATP1A3*. The catalytic α-subunit in the fruit fly is expressed ubiquitously, unlike the selective expression of *ATP1A3* in neurones, and has a protein identity of 76.3% and a DNA identity of 71.7% relative to human *ATP1A3* (NCBI HomoloGene 113729) (Table 2). All the residues known to be mutated in *ATP1A3*-related disorders, apart from Q895, are conserved in the *Drosophila* α subunit (Tables S1 and S2). Mutations in *Atpα*, affecting highly conserved amino acid residues, have been generated through ethylmethanesulfonate (EMS) mutagenesis. The *Drosophila* mutant $Atp\alpha^{CJ10}$ (*CJ10*) carries an amino acid change (G744S) affecting glycine 744 in *Atpα*, which is conserved as glycine 755 in *ATP1A3* and mutated to G755A, G755C, G755S and G755V in patients with AHC (Sasaki et al., 2014; Viollet et al., 2015). The *Drosophila* mutant $Atp\alpha^{DTS2}$ (*DTS2*) carries an amino acid change (D981N) affecting aspartic acid 981 in *Atpα* (Ashmore et al., 2009), which is conserved as aspartic acid 992 in *ATP1A3* and deleted (D992del) and duplicated (D992dup) in PMG (Table S1) and mutated to D992Y in AHC (Table S2).

Atpα mutations at both G744 and D981 are homozygous lethal but lead to AHC-relevant phenotypes in heterozygous adults (Palladino et al., 2003; Ashmore et al., 2009). Heterozygous *CJ10* (*CJ10*/+; G744S) flies exhibit deficits in total locomotor activity, total time active and locomotor activity following startle stimulation compared with age-matched WT controls (Ashmore et al., 2009). Following mechanical disturbance via vortexing, *CJ10*/+ flies show an age-dependent mechanical stress-induced paralysis, in which the fly lies on its back with little effective movement of the legs and wings, with a longer duration in older flies (30 days post-eclosion) than younger flies (3 and 10 days post-eclosion) (Ashmore et al., 2009). Heterozygous *DTS2* (*DTS2*/+; D981N) flies display an age-dependent decrement in locomotor activity, becoming sedentary, with a premature loss of walking activity and flight ability (Palladino et al., 2003). *DTS2*/+ flies also exhibit mechanical stress-induced paralysis, but only when maintained at an elevated temperature (28°C), rather than room temperature (20-22°C) (Palladino et al., 2003). Upon acute exposure to an even higher ambient temperature (37-38°C), *CJ10*/+ and *DTS2*/+ flies exhibit a reversible heat-induced paralysis (Palladino et al., 2003; Ashmore et al., 2009), paralleling the heat sensitivity of symptoms in *ATP1A3*-related disorders.

Consistent with the severity of the age-dependent stress-sensitive locomotor impairments observed in *CJ10*/+ and *DTS2*/+ flies, comparisons of the time required to reach 50% survivorship on their respective lifespan curves (median lifespan) relative to WT flies reveal that both *Atpα* mutants are comparatively short lived (Palladino et al., 2003; Ashmore et al., 2009). This shortened lifespan and the progressive loss of motor activity exhibited by *CJ10*/+ and *DTS2*/+ flies are consistent with the phenotypes exhibited by other *Drosophila* mutants known to be associated with neurodegeneration (Min and Benzer, 1997). Histological analysis revealed no evidence of neurodegeneration in young *Atpα* mutants, but *CJ10*/+ and *DTS2*/+ flies aged to median lifespan exhibited loss of brain tissue, indicated by the accumulation of vacuolar and spongiform-like neuropathology in the central brain and optic lobe, in contrast to the minimal neuropathology seen in aged WT controls (Palladino et al., 2003; Ashmore et al., 2009). Thus, neurodegeneration in these *Atpα* mutants appears to be age dependent, paralleling the progressive brain atrophy observed in AHC patients (Saito et al., 1998; Sasaki et al., 2017).

In a genome-wide screen for novel modifier loci, using 358 deficiency strains, each having a unique deletion of a segment of the genome, Talsma et al. (2014) identified 29 loci that modified the mechanical stress-induced paralysis of *CJ10*/+ flies at 15 days post-eclosion. Within these loci, classical mutants and transgenic RNA interference were used to identify 33 genes that, when disrupted, suppressed (32 genes) or enhanced (one gene) the paralysis phenotype. Suppressors include *Ppk11*, *Ppk21* and *Ppk24*, which encode ligand-gated Na^+ channels (Talsma et al., 2014). Those suppressor genes that encode proteins expressed in the adult fly offer proteins/pathways that could be viable targets for the development of new drugs for AHC and other *ATP1A3*-related disorders.

A limitation of *Drosophila Atpα* mutants for modelling *ATP1A3*-related disorders is that their NKA α-subunit dysfunction is not restricted to neurones. An alternative, transgenic approach would employ the Gal4-UAS system (Brand and Perrimon, 1993) to generate flies with neurone-specific expression of a human mutant *ATP1A3* cDNA construct. Using the Gal4-UAS system, Paul et al.

(2007) demonstrated that ubiquitous expression of rat *Atp1a1* cDNA could prevent the homozygous embryonic lethality of the *Drosophila* mutant $Atp\alpha^{DTS2R3}$, with amino acid changes E39A and L346F (Palladino et al., 2003), indicating that rat NKA α_1 can form a functional αβ complex in the fly context. Similarly, Gal4-UAS transgenic flies with expression of mutant (D369N) *Atpα* restricted to NKA β_2 subunit (*nrv2*)-positive cells showed increased sensitivity to 1 mM ouabain compared with transgenic flies expressing WT *Atpα* and WT flies (Sun et al., 2001).

Nematode (*Caenorhabditis elegans*)

The sodium/potassium-transporting ATPase subunit alpha gene, *eat-6*, in *C. elegans* roundworms is the one-to-one orthologue of the *ATP1A3* gene in *Homo sapiens*, with a protein identity of 73.5% and a DNA identity of 67.0% (NCBI HomoloGene 113729) (Table 2). Moreover, almost all the residues known to be mutated in *ATP1A3*-related disorders are conserved in the *C. elegans* EAT-6 protein (Tables S1 and S2). To model the *ATP1A3* G316S mutation found in ARA, the glycine at position 304 of EAT-6, corresponding to NKA α_3 G316, was mutated to serine in two independent *eat-6* alleles, *eat-6*^{rt251} and *eat-6*^{rt252}, using CRISPR/Cas9-mediated gene editing (Sorkaç et al., 2016). Worms homozygous for the *eat-6*^{rt251} and *eat-6*^{rt252} alleles were viable (Sorkaç et al., 2016), indicating that the G304S mutation does not cause a complete loss of function. This is consistent with *in vitro* studies by Sweadner et al. (2016), suggesting that G316S is a hypomorphic allele of NKA α_3 resulting in decreased function.

As young adults (3 days old), heterozygous *eat-6*^{rt252} (*eat-6*$^{rt252/+}$), but not heterozygous *eat-6*^{rt251} (*eat-6*$^{rt251/+}$), worms exhibit signs of bradykinesia, characterised by a decreased rate of rhythmic contractions of their neuromuscular feeding organ (pharynx) relative to control worms (Sorkaç et al., 2016). However, *eat-6*$^{rt251/+}$ and *eat-6*$^{rt252/+}$ mutants both showed a reduced rate of pharyngeal pumping when aged to 8 days, indicating that the phenotypic deficit has a progressive nature (Sorkaç et al., 2016). Rhythmic contractions of the pharyngeal muscle pump liquid and suspended particles (bacterial food) in through the mouth, concentrate the bacteria and expel the excess liquid back out of the mouth. Feeding behaviour is relevant to AHC because patients can have difficulties in feeding and swallowing, whereas missed meals can trigger paroxysmal episodes (Neville and Ninan, 2007). By comparison, knockdown of *Atp1a3* expression in hypothalamic neurones was found to reduce food intake following fasting in rats (Kurita et al., 2015).

In the presence of 1 mM aldicarb, an acetylcholinesterase inhibitor that leads to acetylcholine accumulation at the neuromuscular junction and paralysis of *C. elegans* over time, *eat-6*$^{rt251/+}$ and *eat-6*$^{rt252/+}$ worms show paralysis at a significantly faster rate than WT worms (Sorkaç et al., 2016). This hypersensitivity to aldicarb is indicative of defects in neuromuscular junction function. The ataxia-relevant phenotypes of the *eat-6* mutants modelling the ARA-related G316S mutation suggest that *C. elegans* may be a useful model organism for the *in vivo* study of other mutations causing *ATP1A3*-related disorders.

Concluding remarks and future perspectives

As behavioural abnormalities are the core symptoms of *ATP1A3*-related disorders, studies including behavioural measures in intact living animals are a powerful way to help resolve the pathophysiology of *ATP1A3*-related disorders. Genetically altered NKA α_3 mouse models have enhanced our understanding of the phenotypic effects of mutations causing *ATP1A3*-related disorders. The replication of AHC features in *Myshkin*, Mashlool, Matoub and *Atp1a3*^{D801Y} mice (Table 3), and the access provided to their neurophysiology, highlight these models as valuable tools for the exploration of pathophysiological mechanisms and potential treatments. Nonetheless, decades of research on other neurological disorders, such as Alzheimer's disease, have shown that therapeutics that are disease modifying in mice are not necessarily disease modifying in human patients (Breijyeh and Karaman, 2020), perhaps reflecting some intrinsic physiological differences between humans and mice.

By comparison, the non-mammalian model systems are disadvantaged by their greater genetic and physiological distance from humans, although this is mitigated by the high conservation of NKA α_3 relative to other proteins (Table 2). The neurological phenotypes exhibited by *Drosophila*, *C. elegans* and zebrafish with interference of *ATP1A3* orthologues demonstrate their capacity as useful tools to quickly and inexpensively assess the physiological and behavioural consequences of putative causative mutations in *ATP1A3*-related disorders. Moreover, their small size and short generation interval make them more suitable than mice for high-throughput screens of drugs and genetic modifiers. As there are welfare concerns surrounding the use of *Atp1a3* mutant mice with severe phenotypes (e.g. fatal seizures), the alternative models offer potential replacements to study aspects of *ATP1A3*-related disorders, under the principles of replacement, refinement and reduction (3Rs) in animal research.

Although the translational potential of invertebrate models is less obvious compared with mice, *Drosophila* models, in particular, have demonstrated their utility in drug discovery programmes for fragile X syndrome (Monyak et al., 2017) and *KRAS*-mutant metastatic colorectal cancer (Bangi et al., 2019). However, a consequence of the advent of CRISPR/Cas9 gene editing (Komor et al., 2017) is that genetic alteration of NKA α_3 is no longer restricted to conventional 'genetically tractable' model organisms. There is now scope to generate NKA α_3-mutated models from a wider range of animal species, including larger mammals, such as rabbits (Sui et al., 2018), and non-human primates, such as marmosets (Kumita et al., 2019). Given the current lack of published animal models for CAPOS, RECA and other *ATP1A3*-related disorders (Table 1), and the paucity of effective therapies, it is likely that NKA α_3 animal models will continue to play an important role in *ATP1A3*-related disorder research in the years ahead.

Competing interests

S.J.C. is a Handling Editor for Disease Models & Mechanisms.

Funding

H.W.Y.N. was supported by the British Association for Psychopharmacology and an MSc Studentship from the Ethel and Gwynne Morgan Trust. J.A.O. was supported by a National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) training grant (NC/S001719/1). S.J.C. was supported by Biotechnology and Biological Sciences Research Council (BBSRC) grant BB/R019401/1.

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