

5th Symposium
ATP1A3
in Disease

24-26th August 2016

Institute of Neurology
33 Queen Square
London

Organising Committee:

Helen Cross
Sanjay Sisodiya
Steve Clapcote
Tsveta Schyns
Jill Bailey

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AHCUK

Alternating Hemiplegia of Childhood

Welcome!

We would like to welcome the AHC community to London for the 5th Annual Symposium on ATP1A3 in Disease. This year's meeting is the latest in a series of successful events, bringing together people affected by ATP1A3-related conditions, their families and carers, clinicians and scientists, all working towards better understanding and treatment of these difficult diseases. We all look forward to hearing about progress in the field over the last year, and to setting the stage for the next year.

We hope you will find the meeting rewarding and enjoyable!

Sanjay Sisodiya – **Chair**

Organising Committee:

Jill Bailey

Steven Clapcote

Helen Cross

Jan Koenderink

Tsveta Schyns

London was the first great metropolis of the industrial age. In 1800 it was a major European city of one million inhabitants. By 1911 it was the greatest city in the world – the first city of the British Empire with over seven million people – greater than the combined populations of Paris, Berlin, St Petersburg and Moscow. This phenomenal expansion was unique in Europe – both for the speed of its growth and the way in which it happened. Even today, London remains unlike any other European City with its own distinctive form which is a direct result of its history.

Westminster Abbey

Kings, Queens, statesmen and soldiers; poets, heroes and villains – the Abbey is a must-see living pageant of British history. This building is 700 years old. It is in the heart of London – next to Big Ben and the Houses of Parliament.

St. Paul's Cathedral

St. Paul's, with its world-famous dome, is an iconic feature of the London skyline. Inside you can enjoy the Cathedral's awe-inspiring interior, you can walk in the footsteps of royalty and political leaders on the Cathedral's floor; climb the dome to try the unique acoustics of the Whispering Gallery; or go higher to enjoy some of the most spectacular views over London from the Stone and Golden Galleries; or head down to the crypt where our nation's heroes are buried.

The Tower of London

The Tower has an incredibly varied history, having served as a prison, a zoo, an arsenal, the Mint, and a royal residence since the first stones were laid for William the Conqueror. You can see Henry VIII's suits of armour and the crowns and garments of kings and royals from centuries past. Stand on the execution site of 3 different queens, or join one of the famous Beefeater tours to hear the Tower's fascinating tales of pain, passion, and punishment.

London Tower Bridge

Step inside the most famous bridge in the world to explore its iconic structure, spectacular views and glass floor, modern exhibitions and magnificent Victorian Engine Rooms.

Buckingham Palace

Buckingham Palace serves as both the office and London residence of Her Majesty The Queen. It is one of the few working palaces remaining in the world today. You can visit the nineteen magnificent State Rooms which provide the setting for ceremonial occasions and official entertaining. All rooms are furnished with many of the greatest treasures from the Royal Collection. At the moment the exhibition 'Fashioning a Reign: 90 Years of Style from The Queen's Wardrobe is on show.

The London Eye

The London Eye is next to Westminster Bridge gracefully rotating over the River Thames opposite the Houses of Parliament and Big Ben. At 135 metres, the Coca-Cola London Eye is the world's tallest cantilevered observation wheel; a feat of design and engineering. It has become the modern symbol representing the capital and a global icon. The gradual rotation in one of the 32 high-tech glass capsules takes approximately 30 minutes and gives you an ever-changing perspective of London.

Big Bus London

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**DIRECTIONS TO THE 5TH SYMPOSIUM ON ATP1A3 IN DISEASE,
QUEEN SQUARE**

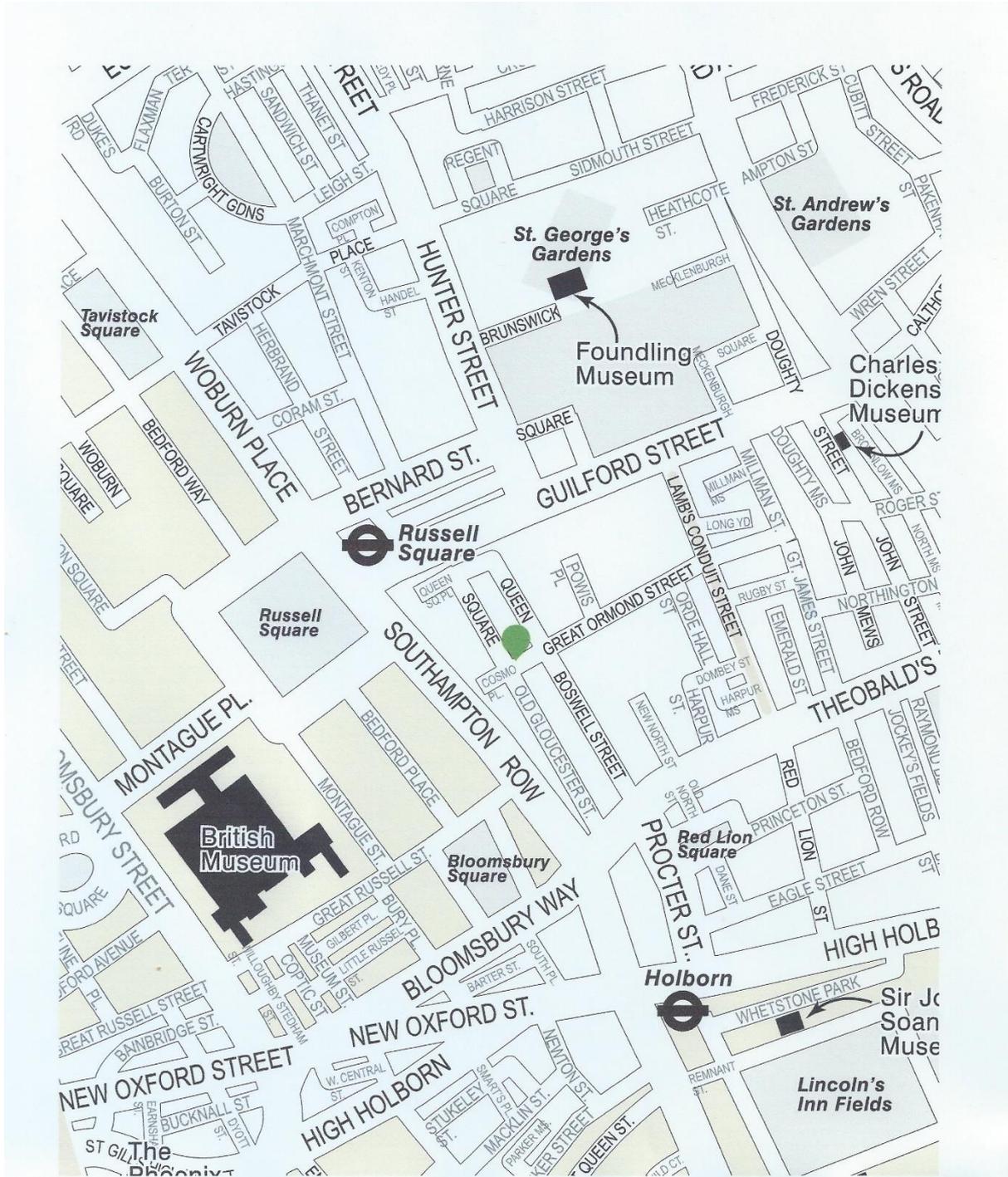
FROM THE HOLIDAY INN – BLOOMSBURY

Exit out of hotel and turn right
Turn left onto Marchmont Street
At the end of the road turn right onto Bernard Street
(Opposite Russell Square Station)
Turn left onto Herbrand Street
Turn left opposite President Hotel
Cross road at 'no-entry' sign to car park
Go down side street
Turn left at square
Follow past main hospital entrance
Number 33 is a few doors away

DIRECTIONS TO RETURN TO THE HOLIDAY INN – BLOOMSBURY

Exit right out of venue
Travel past sign of 'Institute of Neurology'
Turn left and cross the road
Turn right onto Herbrand Street
Turn right onto Bernard Street
Cross road
Turn left onto Marchmont Street
Turn left to entrance of The Holiday Inn

Map of surrounding area for Queen Square, London



SYMPOSIUM PROGRAMME

Wednesday 24th August, 2016

19.00 P.M. – 22.00 P.M. WELCOME TO LONDON

THE HOLIDAY INN – BLOOMSBURY, FOYER RECEPTION AREA

Registration 7.00 – 9.30 p.m.

Buffet 7.30 – 9.30 p.m.

Welcome Speech – AHCUK 7.45 p.m.

Opening Message – Professor Sanjay Sisodiya 7.50 p.m.

Victoria A. Platt – Secretary AHC Foundation, USA 8.00 p.m.

A presentation of the topics and discussions raised at the July 2016 AHCF Family Meeting in Indianapolis, Indiana, USA

Sigurdur Holmar Jóhannesson – President, AHC Federation of Europe. AHC today and in the future. 8.15 p.m.

Interaction and discussions with AHC Family Associations 8.30 – 10.00 p.m.

Thursday, 25 August 2016

Time	Agenda		Abstract, page
	Morning session: Clinical developments in ATP1A3-related disease		
	Chairs: Helen Cross, GOSH, London and Mohamad Mikati, Duke, USA		
09:00	Allison Brashear Wake Forest University School of MedicineUSA	Plenary Introduction: Where we are in ATP1A3-related disease today?	1
09:45	Diane Doummar Hospital Armand Trousseau, Paris, France	Encephalopathies associated with ATP1A3 mutation	2
10:00	Hendrik Rosewich Medical University Göttingen, Germany	CAPOS	3
10:15	Madeleine Scharf Inst Experimental Immunology, Lübeck, Germany	Neuronal Na ⁺ /K ⁺ ATPase as an autoantibody target in paraneoplastic neurologic syndrome	4
10.30	Coffee Break		
11:00	<p>What are the clinical events in ATP1A3-related disease? Chairs: Hendrik Rosewich, Medical University Göttingen, Germany and Sanjay Sisodiya, UCL, London</p> <p>A video and clinical panel session to discuss events that are seizures confirmed on EEG recording, those that are hemiplegic episodes and that are undiagnosed.</p> <ul style="list-style-type: none"> Facilitators: murine experts (Steve Clapcote, Leeds, UK; Karin Lykke-Hartmann, Aarhus University, Denmark, Mohamad Mikati, Duke, USA), human epilepsy (Mohamad Mikati, Duke, USA; Helen Cross, GOSH, London; Beate Diehl, UCLH, UK; Alexis Arzimanoglou, Lyon, France), human movement disorders (Kailash Bhatia, UCL and Lucinda Carr, GOSH, London). Intended outcome: consensus definitions of events in AHC, key for therapy trials. 		
12:30	Lunch in Foyer with poster session		

	Afternoon session Research developments in ATP1A3-related disease		
	Chairs: Erin Heinzen, Columbia University, USA and Steve Clapcote, Leeds University, UK		
14:30	Arn van den Maagdenberg, LUMC, the Netherlands	AHC Gene 2 – an update	5
14:50	Jennifer Kearney Northwestern University, USA	Genetic modifiers for ATP1A3	6
15:10	Karin Lykke-Hartmann, Aarhus University, Denmark	Further analysis of the Atp1a3 D801Y knock-in mouse model	7
15:30	Bente Vilsen Aarhus University, Denmark	ATP1A3 neurological disease mutations affecting Na ⁺ -site III: Structural and functional perspectives and rescue of compromised function	8
15:50	Discussion		
16.00	Coffee Break		
16.30	Hugh Piggins The University of Manchester, UK	Circadian Disruption in the Myshkin Mouse Model of Mania Independent of Deficits in Molecular Clock Function	9
16:50	Minako Hoshi, Kyoto University, Japan	ATP1A3 as target of beta-amyloid assembly	10
17.10	Ronald Melki, CNRS Paris, France	α -synuclein interaction with α 3-Na ⁺ /K ⁺ -ATPase and relation to decline?	11
17.30	Jan Koenderink Radboud UMC, The Netherlands	Biochemical and electrophysiological analysis of ATP1A3 mutations	12
17:50	Discussion		
18:00	End of Day 1 Program		
19.00	Conference Dinner, Holiday Inn London Bloomsbury		

Friday, 26 August 2016

Time	Preliminary draft agenda		Abstract, page
	Morning session: Translation to Treatments in ATP1A3-related disease Chairs Sanjay Sisodiya, UCL, UK and Tsveta Schyns, ENRAH, Belgium		
09:00	Francesco Muntoni GOSH, London, UK	Plenary Introduction: Gene therapy in a neurological disease, the journey: Duchenne Muscular Dystrophy	13
09:45	Natalya Fedosova Aarhus University, Denmark	On the way to isoform – specific drugs	14
10:00	Steve Clapcote, Leeds University, UK	Update on the Myshkin mouse model of AHC	15
10:15	Emmanuel Roze, Paris, France	Sharing the experience of a clinical trial in alternating hemiplegia	16
10:30	Coffee break		
11:00	Helen Cross, GOSH London, UK	The role of dietary therapy in AHC	17
11:15	Elisa de Grandis, University of Genoa, Italy	Flunarazine and AEDs – where are we now?	18
11:45	Juan Kaski, GOSH, London, UK	Cardiac involvement in AHC: treatment beyond the brain?	19
12:15	Poster Bursary Award Winner Christine Simmons Northwestern University, USA	AHC Patient-specific iPSC-derived Neurons Exhibit Depolarized Resting Membrane Potential and Altered Excitability	20
	CONCLUSIONS		
	LUNCH AND END OF MEETING		

ABSTRACTS

ORAL PRESENTATIONS

1. Where are we with ATP1A3-related disease today?

A. Brashear¹, MD, MBA and K. J. Sweadner, PhD²

¹*Professor & Chair, Department of Neurology, Walter C. Teagle Endowed Chair, Wake Forest School of Medicine, Winston Salem, NC USA*

²*Associate Neurobiologist, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA*

ATP1A3-related disorders include RDP, AHC and CAPOS. However, reports of intermediate cases suggest a broader phenotype. This presents challenges for the diagnosis and treatment of ATP1A3. The broad phenotype means missed or masquerading diagnoses and more reliance on genetic testing. The variability in phenotype and the existence of triggers complicate the design of clinical trials.

Moreover, the recognition of non-motor phenotypes underscores that ATP1A3 mutations produce disease of the nervous system rather than a pure motor phenomenon. There are syndromes include intermediate phenotypes to RDP and AHC, patients with primary psychiatric disease, with epilepsy, with loss of primary sensory system function, and with cerebellar atrophy. The neuropathological findings in the brain and MRI imaging in motor and non-motor areas also suggest that the location of damage in the brain affects the phenotypic presentation.

With the broadening phenotype, the focus will turn to the development of disease therapies: both presymptomatic and/or post symptomatic treatment. The pre-symptomatic will lead to a push for early identification of gene carriers with a focus on understanding and prevention of triggers, and the need for pre-symptomatic intervention with pharmacotherapy. Post-symptomatic therapy would include treatment acutely for those who develop symptoms as well as the potential intervention with pharmacotherapy and/or direct stimulation or surgery in the brain. Acute treatment would be limited to those with a family history or to those in which the diagnosis of ATP1A3 is suspected in the acute setting.

A pressing question is whether there is a genotype-phenotype relationship. We have analyzed the genetics and genomics of the gene family of the Na,K-ATPase catalytic subunits, as well as the distribution of their mutations in the protein structure. The results suggest a different perspective. The mutation patterns found so far for ATP1A1, ATP1A2, ATP1A3, and ATP1A4 are mostly distinct, despite the strong sequence homology of the four genes. What is most striking is that entire groups of mutations are “missing” from the known disease-associated mutations in each gene. This implies that when the missing mutations are eventually discovered, very different manifestations may be recognized clinically.

Overall heightened awareness of the broad spectrum of ATP1A3 diseases and an understanding of how the pump may be altered by specific mutations open doors for prevention and treatment. The overlap of clinical, imaging, genetic and bench work in studying ATP1A3 disease is a unique opportunity to develop a new line of therapies for neurologic diseases.

2. Encephalopathies associated with ATP1A3 mutation

Diane Doummar

Hospital Armand Trousseau, Paris, France

Early-onset epileptic encephalopathy without alternating hemiplegia. A third case with novel ATP1A3 mutation

Diane Doummar¹ Pauline Marzin², Nathalie Dorison¹, Anna Kaminska³, Caroline Nava¹, Thierry Billette de Villemeur¹, Delphine Héron², Cyril Mignot²

1. Neuropédiatre, hôpital Trousseau, Paris ; centre de référence mouvements anormaux de l'enfant

2. Assistance-Publique Hôpitaux de Paris, Département de Génétique and Centre de Référence Déficiences Intellectuelles de Causes Rares and GRC UPMC « Déficience Intellectuelle et Autisme », Groupe Hospitalier Pitié-Salpêtrière, Paris, France

3. Neurophysiologie, Hôpital Necker, Paris

We report a 16 year-old girl with an early onset encephalopathy carrying a novel ATP1A3 mutation. Her epilepsy started at six weeks of age, and manifested as frequent convulsive and non convulsive seizures associated with episodes of non epileptic abnormal movements without alternating hemiplegia.

She had severe intellectual disability. At 15 years, we analyzed the DNA of the patient and her parents using the TruSight One sequencing panel (Illumina), which led to the identification of the g.42479820C>A de novo heterozygous sequence variant in ATP1A3 resulting in the unreported p.Asp742Tyr amino acid substitution.

Clinical manifestations were close to those reported by Paciorkowski et al, 2015, in two patients. These three cases confirm the existence of a rare phenotype due to ATP1A3 mutations which features are similar to those of the most severe forms of alternating hemiplegia of childhood without hemiplegic attacks.

3. CAPOS

Hendrik Rosewich

Medical University Göttingen, Germany

4. Neuronal Na¹/K¹ ATPase is an autoantibody target in paraneoplastic neurologic syndrome

Madeleine Scharf

Inst Experimental Immunology, EUROIMMUN AG, Lübeck, Germany

Objectives: To identify an autoreactivity in a 66-year-old woman who presented with combined brainstem and cerebellar syndrome including vertical gaze palsy, severe progressive ataxia, and spastic tetraparesis, an acute deterioration of vision, dysarthria, and dysphagia with concurrent diagnosis of a colon adenocarcinoma.

Methods: Patient's serum and CSF underwent comprehensive autoantibody screening by indirect immunofluorescence assay and immunoblot. For autoantigen purification, a histo-immunoprecipitation technique was developed followed by mass spectrometrical analysis. Recombinant candidate antigens were expressed in HEK293 and used to verify the identification.

Results: Indirect immunofluorescence assay screening revealed strong immunoglobulin G reactivity with neuronal tissues in serum and CSF, but not with a panel of 28 recombinantly expressed established neural autoantigens. The hitherto unknown target antigen was identified as the neuronal Na¹/K¹ ATPase. Epitope mapping and competitive inhibition experiments showed that the autoantibodies were directed against the membrane-spanning alpha 3 subunit (ATP1A3) of the enzyme but did not bind to extracellular epitopes. Immunohistochemical analysis revealed overexpression of this subunit in the patient's tumor.

Conclusions: We describe a case of an anti-ATP1A3-associated neurologic disorder. Mutations in the gene encoding this neuronal surface protein have already been recognized as the cause of infantile alternating hemiplegia, rapid-onset dystonia parkinsonism, and CAPOS syndrome. Although the autoantibodies are unlikely to be pathogenic, they are likely to be rare biomarkers for the apparently paraneoplastic neurologic syndrome or for the tumor itself.

Neurology® 2015;84:1–7

GLOSSARY

ATPase = adenosine triphosphatase; ATP1A3 = alpha 3 subunit of neuronal Na¹/K¹ ATPase;

CAPOS = cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss.

5. AHC Gene 2 – an update

Searching for AHC gene mutations and developing approaches to analyse them

Arn M.J.M. van den Maagdenberg, PhD; Leiden University Medical Center, Leiden The Netherlands

As not all patients with alternating hemiplegia of childhood (AHC) carry a mutation in the coding regions of the ATP1A3 gene, a gene hunt was initiated aimed at identifying a second gene for this disabling disorder. The search for additional AHC genes turned out more challenging than imagined, not in the least because of clinical heterogeneity among ATP1A3-negative cases, many being classified as atypical. Despite considerable efforts e.g. exome sequencing genomic DNA of the patient and parents (known as trio design) to search for de novo mutations this research has not yielded clear cut candidate genes. The status will be discussed on behalf of the gene hunters in the consortium. Also efforts to approach screening of the ATP1A3 gene in a more targeted manner will be discussed.

While the AHC2 gene remains hidden – if it exists – scientific efforts are directed towards developing approaches to analyse gene mutations of genes affecting brain function. The use of transgenic animals that express gene mutations, previously identified in patients, seems a fruitful approach. Given the hemiplegic phenotype and neuronal dysfunction leading to epilepsy, lessons may be learnt from investigating related disorders, such as familial hemiplegic migraine. Approaches to investigate brain dysfunction, including cortical spreading depression, epileptic seizures, as well as optogenetic approaches to modulate brain activity will be discussed, as they may also be useful for unravelling the pathophysiology of AHC.

6. Genetic modifiers for ATP1A3

Jennifer Kearney

Associate Professor, Northwestern University, Chicago, USA

Genetic modifiers can profoundly influence neurological disease phenotypes. ATP1A3-related diseases includes clinical phenotypes with varying degrees of severity, suggesting a potential contribution of genetic modifiers. Significant progress can be made by studying genetic modifiers in model systems. I will present evidence for genetic modifiers in model organisms with ATP1A3 mutations. Additionally, I will discuss our recent work on genetic modifiers in mouse models of epileptic encephalopathies to illustrate the paradigm for modifier gene identification.

7. Multi-electrode array work update

David Goldstein

Columbia University, USA

8. ATP1A3 neurological disease mutations affecting Na⁺-site III: Structural and functional perspectives and rescue of compromised function

Bente Vilsen, Department of Biomedicine, Aarhus University, 8000 Aarhus C, Denmark

Several neurological phenotypes derive from ATP1A3 mutations. The effects of some of these mutations on Na⁺,K⁺-ATPase function have been studied in vitro. I will discuss the ATP1A3 disease mutations as well as information derived from studies of corresponding mutations of ATP1A1 in the light of the high-resolution crystal structures of the Na⁺,K⁺-ATPase. Na⁺ binds at three sites, I, II, and III, of which I and II overlap with the K⁺ binding sites, whereas site III is unique and Na⁺ specific. In several ATP1A3 disease mutants the compromised function can be traced to disturbance of the Na⁺ specific binding site III. Hence, it has been known for some time that RDP and AHC mutations F780L, D923N, and Y1013dup disturb Na⁺ binding selectively without effect on K⁺ binding. E815K (the second most common AHC mutation), E818K (CAPOS mutation), E277K (AHC mutation), and E951K (overlapping AHC-RDP phenotype mutation), reverting the charge, are all likely to disturb Na⁺ site III indirectly by perturbation of a crucial hydrogen bonding network apparent in the crystal structure. AHC mutations Y768H/C will affect Na⁺ site III directly by alteration to the Na⁺ binding aromatic function. The effect of the third most common AHC mutation G947R may also be attributed to perturbation of Na⁺ site III – via destabilization of Y768. Moreover, the most common RDP mutation T613M may disturb Na⁺ binding indirectly by a shift of the E1-E2 conformational equilibrium in favor of E2. Recently, a secondary mutation was found to rescue the defective Na⁺ binding at site III caused by RDP/AHC mutation D923N. A possible mechanism of the rescue will be discussed. A perspective is that it may be feasible to develop an efficient pharmaceutical mimicking the rescuing effect, which optimally would rescue the compromised function of a variety of ATP1A3 mutants with reduced affinity of Na⁺ site III.

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5. Holm, R., Einholm, A.P., Andersen, J.P., and Vilsen, B. (2015). Rescue of Na⁺ affinity in aspartate 928 mutants of Na⁺,K⁺-ATPase by secondary mutation of glutamate 314. *J. Biol. Chem.* 290, 9801–9811.

9. Circadian Disruption in the Myshkin Mouse Model of Mania Independent of Deficits in Molecular Clock Function

Hugh D. Piggins

Faculty of Life Sciences, University of Manchester, Manchester, UK M13 9PT

Alterations in environmental light and the body's intrinsic circadian timing system have strong clinical and pre-clinical associations with depression and bipolar disorders. The neural origins underpinning these behavioural changes remain unclear, although genetic deficits in the molecular circadian clock can result in altered mood-associated phenotypes. In this study, we performed a detailed circadian and light-associated behavioural characterisation of the Na⁺/K⁺-ATPase (NKA) α 3 heterozygous mutant, known as the Myshkin (Myk/+) mouse. The Myk/+ mouse is model of mania with face, construct and predictive validity, but unlike other rodent models, the NKA α 3 mutation does not reside within core circadian molecular clockwork. The effect of this mutation on the brain's neural circadian system was then investigated through molecular (PER2::LUC) and electrophysiological investigation of the master circadian pacemaker, the suprachiasmatic nuclei (SCN) of the hypothalamus. Light input and glutamatergic signalling to the SCN were concomitantly assessed through behavioural assays and Fura-2 calcium imaging. In vivo wheel-running, metabolic and behavioural assays revealed a plethora of abnormalities including lengthened period, elevated daily metabolic rate, and instability in wheel-running activity. Gross aberrant responses to light were widely evident including accentuated re-setting, accelerated re-entrainment to simulated jet-lag and an absence of locomotor suppression. Strikingly, bioluminescent recording of circadian clock protein (PER2) output from SCN explants ex vivo revealed no overt deficits in the Myk/+ molecular clock function at single-cell or SCN network-level. However, electrophysiological recordings from individual SCN neurons demonstrated reduced day-night variation in excitability, while calcium imaging revealed elevated responses to glutamate, the main neurotransmitter of the retinal light input pathway to the SCN. Therefore, the Myk/+ model demonstrates profound circadian and light-responsive behavioural alterations with an aetiological basis independent of molecular circadian clock disruption. Instead, these findings implicate alterations in the light input pathway and its processing as key contributors to these behavioural disruptions and suggest new mechanistic implications for sleep and circadian disruption in human affective disorders.

Supported by project grants from the BBSRC and HFSP.

10. ATP1A3 As A Target of Alzheimer Amyloid- β Assembly

Minako Hoshi, Ph.D.

Associate Professor

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Alzheimer's disease (AD) involves neuron dysfunction and loss. This brain damage is thought to be caused by a small protein, the amyloid β -protein ($A\beta$), which forms aggregates that are neurotoxic. This neurotoxicity has been explained by multiple mechanisms. We have previously isolated in vivo $A\beta$ aggregates termed amylospheroids from AD patients' brains. Recently we have found a new neurotoxic mechanism that involves the interaction between the patient-derived amylospheroids and the neuron-specific Na^+/K^+ -ATPase $\alpha 3$ subunit (Ohnishi et al. PNAS 2015). This interaction causes neurodegeneration through pre-synaptic calcium overload, which explains earlier observations that such neuronal hyperactivation is an early indicator of AD-related neurodegeneration. Importantly, amylospheroid concentrations correlate with disease severity and progression in AD patients. Further, we found that the 4th extracellular loop (Ex4) region of NAK $\alpha 3$ encompassing Asn879 and Trp880 is essential for ASPD-NAK $\alpha 3$ interaction, because tetrapeptides mimicking this Ex4 region bound to the ASPD surface and blocked ASPD neurotoxicity. This ASPD-binding tetrapeptide is of sufficiently small molecular weight (602.6) that it could be a lead compound for the design of peptidomimetic drugs. Our findings open up new possibilities for knowledge-based design of peptidomimetics that inhibit neurodegeneration in AD by blocking aberrant ASPD-NAK $\alpha 3$ interaction.

11. α -synuclein assemblies sequester neuronal $\alpha 3$ -Na⁺/K⁺-ATPase and impair Na⁺ gradient

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The deposition of fibrillar alpha-synuclein (α -syn) within inclusions (Lewy bodies and Lewy neurites) in neurons and glial cells is the hallmark of synucleinopathies such as Parkinson's disease, dementia with Lewy bodies and multiple system atrophy (Mc Cann et al, 2014). α -syn fibrillar aggregates have the ability to spread from one cell to another in a prion-like manner (Goedert, 2015) and trigger synucleinopathies when injected to model animals (Peelaerts et al., 2015). The underlying molecular mechanisms, in particular the binding mode of α -syn fibrillar assemblies to cell membranes and the consequences of binding, are poorly understood. We assessed the binding of well-defined α -syn mega-dalton assemblies (Bousset et al., 2013; Pieri et al., 2016) to the membrane of neuronal cells. We demonstrated that α -syn assemblies form clusters within the plasma membrane of neurons in a manner dependent on the nature of the α -syn high-molecular weight species (Shrivastava et al., 2015). Using a proteomic-based approach, we identified the $\alpha 3$ -subunit of Na⁺/K⁺-ATPase (NKA) as a cell surface partner of α -syn assemblies (Shrivastava et al., 2015). The interaction strength depended on the state of α -syn, fibrils being the strongest, oligomers weak, and monomers none. Mutations within the neuron-specific $\alpha 3$ -subunit are linked to rapid-onset dystonia Parkinsonism (RDP) and alternating hemiplegia of childhood (AHC). We showed that freely diffusing $\alpha 3$ -NKA is trapped within α -syn clusters resulting in $\alpha 3$ -NKA redistribution and formation of larger nanoclusters (Shrivastava et al., 2015). This creates regions within the plasma membrane with reduced local densities of $\alpha 3$ -NKA, thereby decreasing the efficiency of Na⁺ extrusion following stimulus. Thus, the interaction of $\alpha 3$ -NKA with extracellular α -syn assemblies reduces its pumping activity as its mutations in RDP/AHC. These results and the role of NKA misdistribution in the early stages of synucleinopathies will be discussed in a context where other misfolded protein aggregates have been shown to bind to cell membranes and/or interact with NKA, thus, perturbing normal NKA distribution either by sequestration or exclusion.

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12. Biochemical and electrophysiological analysis of ATP1A3 mutation

Jan B. Koenderink, Charlotte A. Hoogstraten, Muriël Messchaert, Karl M. Weigand, Herman G.P. Swarts, Thomas Friedrich

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Alternating Hemiplegia of Childhood (AHC) is a neurodevelopmental disorder caused by de novo mutations in ATP1A3, the gene encoding the $\alpha 3$ subunit of Na,K-ATPase. Na,K-ATPase regulates electrogenic transport by exporting three Na⁺ ions in exchange for two K⁺ ions across the cellular membrane by hydrolysis of one adenosine triphosphate molecule. Maintaining these ionic gradients has been shown to be of great importance in a variety of cellular functions, including neuronal activity. Recently, it has been reported that functional impairments of the $\alpha 3$ pump isoform (ATP1A3) can be found in AHC. In addition, a loss of proton inflow has been correlated with the severity of the disease, with E815K as most severe and D801N in milder cases. The functional consequences of these mutations for the Na,K-ATPase have been investigated very limited.

We aimed to further characterize these common AHC mutations and included several other mutations related to AHC or ATP1A3-related disorders, including RDP and CAPOS. Human ATP1A3 was expressed in Sf9 insect cells and *Xenopus laevis* oocytes. Na,K-ATPase functionality were measured using biochemical ATPase activity assays, electrophysiological approach two-electrode voltage clamp and transport assay in which rubidium (K⁺ analogue) uptake is measured.

13. Gene therapy in a neurological disease, the journey: Duchenne Muscular Dystrophy

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Antisense oligonucleotide (AON) induced exon skipping, also known as splice switching AON, are increasingly being used in Duchenne muscular dystrophy (DMD), where several hundreds of children with deletions eligible to skipping of exons 44; or 45; or 51 or 53 have been studied in phase I, II and III studies.

Two main chemistries are in use at the moment, the 2' O Methyl (2'OMe) AON backbone; and the phosphorodiamidate morpholino (PMO) AON backbone.

Both backbones have been quite extensively studied before in relevant animal models such as the mdx mouse and, the PMO, also in the more severe dystrophic dog models with encouraging results.

These preclinical promising results have been paralleled by similarly encouraging results of the phase I and II studies of both chemistries. More recently however the failure of the only phase III study (in which a 2'OMe AON targeting exon 51 was studied) raised concerns regarding both the rationale for exon skipping induced restoration of the reading frame in DMD, and the possibility that current chemistries may achieve sufficient correction to induce a clinical benefit to DMD patients.

In my presentation I will review both the data from the preclinical model; the efficiency of the current AON to induce dystrophin expression; the biological significance of different levels of dystrophin and the available data from the clinical trials available to date. I will discuss why on the whole the positive data from the current approaches are encouraging although they raise issues related to the efficacy and also long term safety of some of the products under development. I will also discuss the potential of using AONs to target the central nervous system, and progress in the preclinical development of next generation AONs.

Disclosure. The Author is involved in clinical trials on antisense oligonucleotides in DMD with Prosensa (2' O methyl antisense for skipping exon 45 and exon 53); and as the Chief Investigator, in collaboration with Sarepta Therapeutics, in a European Commission funded study on a morpholino antisense to skip exon 53 (EU grant SKIP-NMD).

14. On the way to isoform-specific drugs

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Isoform-specific inhibitors of the enzymes are tools to clarify mechanisms of the isoform-related disorders and in certain cases have a potential of becoming drugs.

In brain the catalytic subunit of the Na,K-ATPase is represented by three isoforms, mutations in one of them ($\alpha 3$) have drastic physiological consequences. In the cell the direct outcome of these mutations would be either an alteration in the intracellular ion composition or an activation/inhibition of different signaling cascades or networks associated with the enzyme.

In this context, application of the isoform-specific drugs is a way of interference with the homeostasis in order to elucidate the underlying mechanisms of the disorder and to analyze the role of individual isoform in the pathogenesis. Their design, however, demands detailed information on the structure of the binding site and type of its interactions with the inhibitor.

The solution of the crystal structure of the $\alpha 1$ isoform in complex with very different cardiotonic steroids (CTS) ouabain, digoxin and bufalin was the first step in the process. Mapping of the CTS-binding cavity and marking out its boundaries under physiological conditions can be achieved by the EPR technique and will add to the above description. In addition, it will define the spacial position of the sugar units (known to improve affinity and contribute to isoform selectivity). The presented results will be used to expose structural determinants of the CTS specificity towards Na,K-ATPase and to provide the guidelines for the improvement of their isoform selectivity.

15. Update on the Myshkin mouse model of AHC

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Heterozygous missense mutations of the ATP1A3 gene, encoding the Na⁺,K⁺-ATPase α 3 subunit, have been identified as the primary cause of alternating hemiplegia of childhood (AHC), a rare neurodevelopmental disorder that manifests as episodic hemiplegia starting in the first 18 months of life. Na⁺,K⁺-ATPases are membrane-bound transporters that harness the energy of ATP hydrolysis to pump three Na⁺ ions out of the cell in exchange for two K⁺ ions moving inwards. Most ATP1A3 mutations in AHC patients are clustered in or near transmembrane α -helix TM6, including mutation I810N that has been identified in three cases to date. All of the AHC mutations studied result in a catalytically inactive Na⁺,K⁺-ATPase α 3.

Heterozygous Myshkin (Atp1a3Myk/+; Myk/+) mutant mice carry an I810N mutation that reduces total Na⁺,K⁺-ATPase activity (α 1 + α 2 + α 3) in the whole brain by 42%. Myk/+ mice move with a paretic, tremulous gait that becomes transiently more severe after stress. Other phenotypic abnormalities include neuronal hyperexcitability, cognitive impairments¹, social behavioural deficits², and increased susceptibility to epileptic seizures.

We have used the Myshkin model as a tool to advance understanding of the underlying neural mechanisms of AHC and develop novel therapeutic strategies. We found that a wild-type Atp1a3 transgene that increases brain-specific total Na⁺,K⁺-ATPase activity by a modest 16% was sufficient to rescue the phenotypic deficiencies of Myk/+ mice in body weight, motor coordination and contextual fear conditioning. This transgenic intervention did not, however, rescue the deficient performance of Myk/+ mice in the visible platform version of the water maze³. In light of these findings, other therapeutic interventions are currently being tested.

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16. Sharing the experience of a clinical trial in alternating hemiplegia

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Drugs are usually disappointing in alternating hemiplegia of childhood due to limited efficacy and/or poor tolerability. Although a broad range of medications has been tried their use is not evidence-based. One reason for this is that implementation of good quality controlled trial is challenging in rare disorders, such as alternating hemiplegia of childhood.

To address this issue we set up a trial with the following characteristics : i) cross over design that reduce the number of required participants ii) inclusion of patients from age 15, which may increase the number of potential participants iii) possibility of keeping the current treatment at stable dose during the trial to avoid deterring potential participants. Iv) trial supported by patient's association (AFHA) to favor good interaction with families and recruitment

We conducted a randomized, double-blind, placebo-controlled, crossover pilot study to assess the efficacy and safety of triheptanoin on the paroxysmal events in patients with alternating hemiplegia of childhood. All the ten included patients completed the study. The results will be available at the end of the year.

We will present the general design of the study as well as the difficulties we have encountered. More generally, we will interactively discuss the issue of how to do clinical trial in alternating hemiplegia of childhood.

17. The role of dietary therapy in Alternating Hemiplegia in Childhood

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UCL Institute of Child Health & Great Ormond Street Hospital for Children

The ketogenic diet has long been used in the management of children with drug resistant epilepsy. Although the exact mechanism of action is unknown, it is accepted that there are many metabolic pathways on which the diet may have an effect, and consequently different mechanisms may be responsible in any one type of epilepsy. This aside a diet high in fat will have an effect on pathways involving energy metabolism. The ketogenic diet has been reported to be beneficial in isolated cases of AHC the result of ATP1A3 mutations. However the picture is muddled with coexistence in one case of a change in the SLC2A gene coding for Glut1 deficiency – a condition where the ketogenic diet remains the treatment of choice. Work suggesting a role for medium chain fatty acids as an active component of the diet provides promise for the future.

18. Flunarizine and AEDs – where are we now?

Elisa de Grandis, MD, PhD and IB.AHC Italian Consortium

Alternating Hemiplegia of Childhood (AHC) is a severe and intractable disorder. Since the description of the disorder, many antiepileptic (AEDs) and antimigraine agents have been used, mostly to control the paroxysmal attacks that represent the most disturbing symptoms of the disease. However, because of the rarity of the disorder, prospective, randomized, controlled trials are lacking.

A part from occasional efficacy of benzodiazepines and recently of topiramate (a blocker of voltage-dependent sodium channels), there is general agreement that AEDs have no effect on the episodes of AHC. At the same time, no significant efficacy has been found with migraneous drugs, except for flunarizine, that is actually the most used long-term treatment to prevent the non-epileptic paroxysmal attacks. Although its efficacy is variable and many patients fail to respond, several case reports and case reviews reported flunarizine efficacy in reducing the number of attacks and their duration. Flunarizine is an unselective blocker of voltage-dependent calcium channels and it has recently been demonstrated that it also blocks sodium currents; thus preventing high frequency firing of cortical neurons and decreasing cortical hyperexcitability.

We reviewed the pharmacological data about prophylactic and acute treatment of an Italian cohort of 30 patients (16 M, 14 F, age range 5-42 years). Pharmacological, clinical and genetic data have been collected through the Italian Biobank and Clinical Registry for AHC and completed by family interviews. Our study confirms that flunarizine is the most used and effective drug in preventing paroxysmal attacks in AHC, with reduction of duration and frequency of attacks in 50 % of the patients and lower intensity in 32.1 %. Genotype does not seem to influence flunarizine response. Moreover, we did not find any correlation between flunarizine's effectiveness and developmental outcome and duration of treatment.

Eighty % of our patients received AEDs during their life, often in polytherapy. None of these AEDs have been reported efficacious on non-epileptic paroxysmal attacks.

Among the many other prophylactic therapies tried, few have been partially efficacious (benzodiazepines, niaprazine, acetazolamide, melatonine, olanzapine, ketogenic diet). No clear rationale exists towards their use, but it is possible that these therapies may have secondary effects on attacks, reducing provoking factors. Larger studies are warranted in a cohort of genotypically characterized AHC patients to clarify the pathophysiology of the disease and give the rational for the use of more efficacious molecules.

19. Cardiac involvement in AHC: treatment beyond the brain?

Juan Kaski, GOSH, London, UK

Cardiac channelopathies, such as long QT syndrome, Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia, are associated with an increased risk of malignant arrhythmias and sudden cardiac death. Increasingly, a link between cardiac and neurological channelopathies is being recognized, with several cardiocerebral conditions identified. Alternating hemiplegia of childhood is a rare disorder caused by de novo mutations in the ATP1A3 gene, expressed in neurons and cardiomyocytes. Premature mortality can occur in this patient group and is not fully explained. We have recently reported a high prevalence of electrocardiographic abnormalities in an international collaborative cohort of patients with alternating hemiplegia, suggesting that cardiac dysfunction may account for some of the unexplained premature mortality in this condition. A follow up study of further cardiovascular evaluation in this cohort is ongoing.

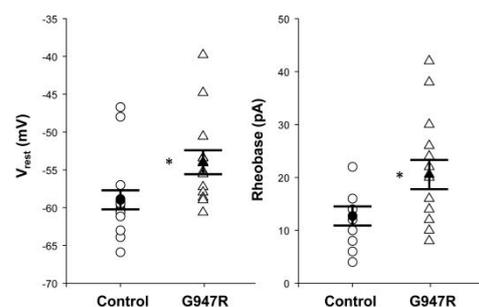
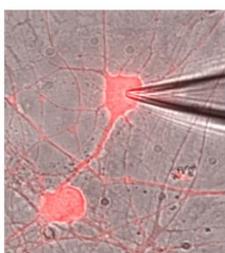
20 AHC Patient-specific iPSC-derived Neurons Exhibit Depolarized Resting Membrane Potential and Altered Excitability

Christine Q. Simmons,¹ Christopher H. Thompson,¹ Bryan E. Cawthon,² Grant Westlake,² Kathryn J. Swoboda,³ Evangelos Kiskinis,⁴ Kevin C. Ess² and Alfred L. George, Jr.¹ Departments of ¹Pharmacology and ⁴Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA; ²Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN, USA; ³Massachusetts General Hospital, Boston, MA, USA

Background – Mutations in *ATP1A3* encoding the catalytic subunit of the sodium/potassium ATPase expressed in mammalian neurons cause a spectrum of neurodevelopmental disorders including alternating hemiplegia of childhood (AHC). In this study, we investigated the electrophysiological properties of human neurons generated from AHC patient-specific induced pluripotent stem cells (iPSCs) to ascertain cell-autonomous functional disturbances underlying this neurological disease.

Methods – Dermal fibroblasts were reprogrammed to iPSCs with transcription factors c-Myc, Klf4, Oct4, and Sox2 using non-integrating plasmid transfection. Control iPSC lines from unrelated, unaffected subjects were also generated. Neuronal differentiation was initiated by neurogenin-2 (NGN2) induction and converted to mature cortical excitatory neurons by co-culturing with mouse glial cells. Excitatory neurons differentiated for 21-24 days were identified by neuronal morphology and co-expression of red fluorescent protein (coupled to NGN2 transgene expression) and used for electrophysiology studies. Whole-cell current clamp recordings were performed on neurons to measure resting membrane potential, rheobase current, spontaneous and evoked action potentials.

Results – Fibroblasts from two unrelated AHC subjects with the *ATP1A3* mutation G947R and two unrelated unaffected subjects (controls) were reprogrammed to iPSCs. Clonal iPSC lines were demonstrated to exhibit markers of pluripotency, have normal karyotypes, have no evidence of plasmid integration, were heterozygous for the *ATP1A3* mutation and were capable of differentiating into ectoderm, mesoderm and endoderm lineages. Cells differentiated by the NGN2 method exhibited neuron-like morphologies and were capable of firing action potentials. Whole-cell current clamp recordings demonstrated a significantly depolarized resting membrane potential in AHC-derived neurons ($V_{rest} = -53.9 \pm 1.6$ mV, n=16) as compared to control neurons ($V_{rest} = -58.9 \pm 1.2$ mV, n=16; $p=0.002$). We also observed a greater proportion and higher frequency of spontaneous action potential firing in



AHC-derived neurons (7 of 16 cells firing at a rate of 0.7 ± 0.3 Hz) compared to control cells (3 of 16 cells firing at a rate of 0.1 ± 0.02 Hz). Action potentials evoked while clamping the membrane potential to -80 mV exhibited a significantly greater

rheobase current, the minimum amount current require to elicit a single action potential, in

AHC-derived neurons compared to control neurons (AHC: 20.5 ± 2.8 pA, n=15; vs control: 12.7 ± 1.8 pA, n=16; p=0.02). However, over a wide range of stimulating current injections, no differences in the number of action potential fired were observed between control and AHC neurons. A depolarized resting membrane potential was also observed in neurons differentiated from iPSCs derived from an unrelated AHC subject with the same *ATP1A3* mutation.

Conclusions – Neurons differentiated from AHC patient-specific iPSCs exhibit a depolarized resting membrane potential and altered action potential firing behaviors. These findings are consistent with impaired function of neuronal sodium/potassium ATPase and may represent a primary cell-autonomous defect underlying AHC.

Funding – This work was supported by grants from the Alternating Hemiplegia of Childhood Foundation.

POSTER PRESENTATIONS

ABSTRACTS

P1 The RDP T613M mutation, from Protein to Patient

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RDP was reported first time in 1993 and is characterized by abrupt onset of disabling symptoms, including generalized dystonia, severe bradykinesia and gait instability. This dramatic presentation takes less than a month to develop, is triggered by stress and/or fever and is treatment refractory to both drugs and deep brain stimulation. RDP is caused by mutations in the ATP1A3 gene which encodes alpha3 subunit of Na,K-ATPase (NKA). The most common mutation observed in RDP patients, T613M, is located close to the nucleotide-binding domain. Recently we diagnosed a 21 year old Uzbek female with severe and disabling generalized dystonia and bradykinesia developed after a stressful experience at age 11. She harbors a de novo T613M mutation in the ATP1A3 gene. Her treatment refractory presentation prompted us to study the molecular and neuronal origin of the disease in primary rat hippocampal neurons and by creating a knock-in mice model of T613M ATP1A3 using CRISPR/Cas technology. We first performed an in silico study, which predicts that the T613M mutant is structurally unstable around the ATP binding site. This should affect forward cycling of the protein and ion transport. The cellular study has been performed on hippocampal neurons derived from embryonic day 18 rat, and co-cultured with astrocytes for 2 weeks, at which time the neurons have developed a well branched dendritic tree with high spine density, and abundant expression of both the ubiquitous NKA alpha1 and the neuronal NKA alpha3 catalytic subunit. The ratio between alpha1 and alpha3 varies somewhat, depending on type of neuron. Wild type and mutant T613M alpha3, both tagged with pHlorin in the extra-cellular domain, were transiently expressed in the neurons and studies were performed after 2-3 days. We demonstrated that the T613M mutant is equally well expressed in the plasma membrane as wild type alpha3. We showed in live imaging experiments, using the Na⁺-sensitive dye ANG2, that the T613M mutation affects sodium homeostasis by increasing basal Na concentration and by retarding the Na⁺_i recovery after a brief exposure to NMDA, used to mimic intense neuronal activity. The resting membrane potential and spontaneous spiking activity were studied during current clamp recordings, and show that T613M-expressing neurons have a higher resting membrane potential and are more prone to respond to depolarization with high frequency action potential firing than neurons expressing wild type alpha3. We will start studying the T613M knock in mouse in July and hope to be able to report preliminary observations from behaviour and electrophysiological studies at this meeting. Currently we know that homozygotes are not viable.

P2 The functions of $\alpha 3$ subunit of Na,K-ATPase during early development

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The major function of Na,K-ATPase is maintaining sodium and potassium gradient but there are indications that minor alpha subunits of Na,K-ATPase could play some additional functions as well. While it is commonly accepted that $\alpha 3$ subunit of Na,K-ATPase expressed only in neurons, during embryonic development $\alpha 3$ subunit may have different expression pattern and therefore could have other functions. The main aim of our study is to identify the functional role of $\alpha 3$ subunit of Na,K-ATPase on the model of individual vertebrate development.

In the ongoing study by employing using immunohistochemistry and in situ hybridization on embryonic mouse slices we documented unexpected patterns of expression of $\alpha 3$ subunit. We found that on E12 $\alpha 3$ subunit was expressed only in the heart while on E15 it was found in the heart, neurons and muscles.

To analyze which organ is the most affected by the lack of $\alpha 3$ subunit function during development we performed a phenotypical analysis using immunohistochemistry of D801N homo- and heterozygous mouse embryos at embryonic stage E15. We found significant differences between homo- and heterozygous embryos in size of head and body, changes in the liver, pancreatic and thorax structure, reduction in the number of cells which express $\alpha 3$ in the cortex and in the number of glutamatergic neurons in lateral cortex near the pallium-subpallium boundary.

In follow-up functional study we plan to evaluate expression of $\alpha 3$ subunit in chicken embryos in order to evaluate how the nervous system is developing in healthy organisms and how AHC mutations might affect it.

P3 Phospholipid flippase ATP8A2: The isoleucine found mutated in neurological disease (CAMRQ) patients is an essential part of a hydrophobic gate of the lipid transport machinery

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The asymmetric distribution of phospholipids between the two leaflets of cell membranes depends on translocation of specific phospholipids across the bilayer by flippases (P4-ATPases), a subfamily of P-type ATPases. The phosphatidylserine flippase ATP8A2 is expressed in the central nervous system, and mutation of ATP8A2 has been found associated with the CAMRQ (cerebellar ataxia, mental retardation and dysequilibrium) syndrome. Analysis of the amino acid sequence suggests that the overall structure and domain topology of ATP8A2 is similar to that of the “classic” ion pumping P-type ATPases Ca²⁺-ATPase and Na⁺,K⁺-ATPase, whose structures are well known from crystallography, but the mechanism of phospholipid transport is poorly understood. The flipping of a phospholipid molecule, consisting of a polar/charged head group and two hydrocarbon chains (~20 Å long, half of the lipid bilayer thickness), appears much more complex than the transport of small inorganic ions such as Ca²⁺, Na⁺, and K⁺ (diameter only 2–3 Å), an enigma referred to as the “giant substrate problem”. We have investigated ATP8A2 by mutagenesis and specific functional assays in combination with computational modeling studies. A clue to identification of a unique, putative lipid transport pathway in the flippase protein and the mechanism of transport of the giant substrate was obtained by studying mutants with alteration to the isoleucine of transmembrane helix M4, which is found mutated in CAMRQ patients. The movement of this isoleucine, working as a hydrophobic gate, seems to propel the lipid head group along in a water-filled groove during the flipping process. The position of the isoleucine in the amino acid sequence is homologous to a glutamate crucial to the ion translocation by Ca²⁺-ATPase and Na⁺,K⁺-ATPase (and found mutated in alternating hemiplegia of childhood), thus illustrating the conservation of basic features of the transport mechanism between flippases and ion pumps.

P4 A new case of AHC due to ATP1A3 deletion responding to ketogenic diet

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Here, we describe a 2-year-old boy who was admitted in our clinic for focal clonic seizures in the right hemisphere at the age of 3 months. Neurological examination revealed marked axial and distal hypotonia. Brain MRI was normal; EEG showed spikes and slow waves in left fronto-temporal region. Carbamazepine was started. Twenty days later, episodes characterized by upgaze deviation and tonic-clonic movements of both arms followed by eyes and head right or left deviation appeared. These episodes occurred during sleep and lasted 5 to 10 minutes. Ictal EEG showed bilateral central spike-and-wave complexes with spreading. Full metabolic analysis on CSF and blood gave normal results. Other antiepileptic drugs were added with no efficacy. From the age of 5 months, developmental delay became more evident. An antiepileptic therapy based on more than 4 drugs did not control seizures. At the age of 12 months he started to present frequent episodes of apnea with sudden oxygen desaturation, cyanosis, gaze deviation and hypotonia occurring during sleep and lasting about 5 minutes. In the following months, episodes of hypotonia, gaze deviation followed by nystagmus, and hemiplegia arose; they typically occurred when awake, lasted about 30 minutes each and could also repeat in clusters for 3 to 4 days. Since the history and the characteristics of episodes resembled AHC, we performed ATP1A3 gene sequencing analysis and we found a novel three base-pair deletion (c.2227_2229delGAC; p.D743del). This mutation was not found in both parents and in 100 unrelated healthy controls. In 2015 he started ketogenic diet (KD). KD was well tolerated and no major side effects were reported. After one-year follow-up, KD resulted in a completely disappearance of paroxysmal episodes. He is still on two-based antiepileptic drugs therapy, his development is delayed but he is improving.

P5 Structure of Na⁺,K⁺-ATPase in the Na⁺ releasing E2P state

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The Na⁺, K⁺-ATPase maintains the electrochemical gradients for Na⁺ and K⁺ across the plasma membrane and potentiates for example ion channel receptors/action potentials and secondary active transport. Na⁺,K⁺-ATPase is also of key importance for the regulation of intra- and extracellular ion homeostasis such as in K⁺ clearance from the interstitial space and Na⁺ extrusion in firing neurons. We aim to solve the crystal structure of the Na⁺, K⁺-ATPase ternary complex in a BeF₃⁻ stabilized form that mimicks the E2P phosphoenzyme intermediate releasing Na⁺ to the extracellular environment. We present a low-resolution structure of this key intermediate and present progress towards a functional understanding of this intermediate state, which is of key importance for the mechanistic understanding of disease-causing mutations of Na⁺,K⁺-ATPase associated with AHC and RDP.

P6 Key words: phenotype, Alternating Hemiplegia of Childhood

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Alternating hemiplegia of childhood is an early onset neurodevelopmental disorder characterized by paroxysmic episodes of alternating hemiplegia, variable degrees of intellectual disability and dystonic movements. The main causative gene, ATP1A3, is also responsible for other neurodevelopmental disorders. While the neurological profile of this condition is well defined, the question whether a recognizable pattern of physical anomalies does exist in this condition is still open. We performed a morphological evaluation of 30 patients at different ages. All patients were evaluated independently by each author and evaluation sheets were compared, discussed and agreed afterwards. This study started before the identification of ATP1A3 as the causative gene, and the patients were selected upon their neurological picture. Four of these 30 patients tested negative for ATP1A3 mutations and were excluded from the present work. On physical ground, almost all patients shared a similar physical phenotype consisting of hypotonia, long face, thin eyebrows, strabismus, hypertelorism, long palpebral fissures, downturned mouth, slender habitus. Such phenotype is sufficiently typical to generate a recognizable gestalt. We also evaluated patient's photographs taken from the parents in early childhood (6-20 months) to delineate a clinical profile possibly recognizable before the neurological signs suggest the diagnosis. Our data suggest that the typical early gestalt is sufficient to advise the molecular analysis of ATP1A3, even in absence of the pathognomonic neurological signs. Finally, since a number of patients is now adult, some information can be drawn on the phenotypic evolution of the facial appearance of patients with alternating hemiplegia of childhood.

P7 Cognitive deficits are associated with GABAergic neurotransmission in a mouse model for Alternating Hemiplegia of Childhood

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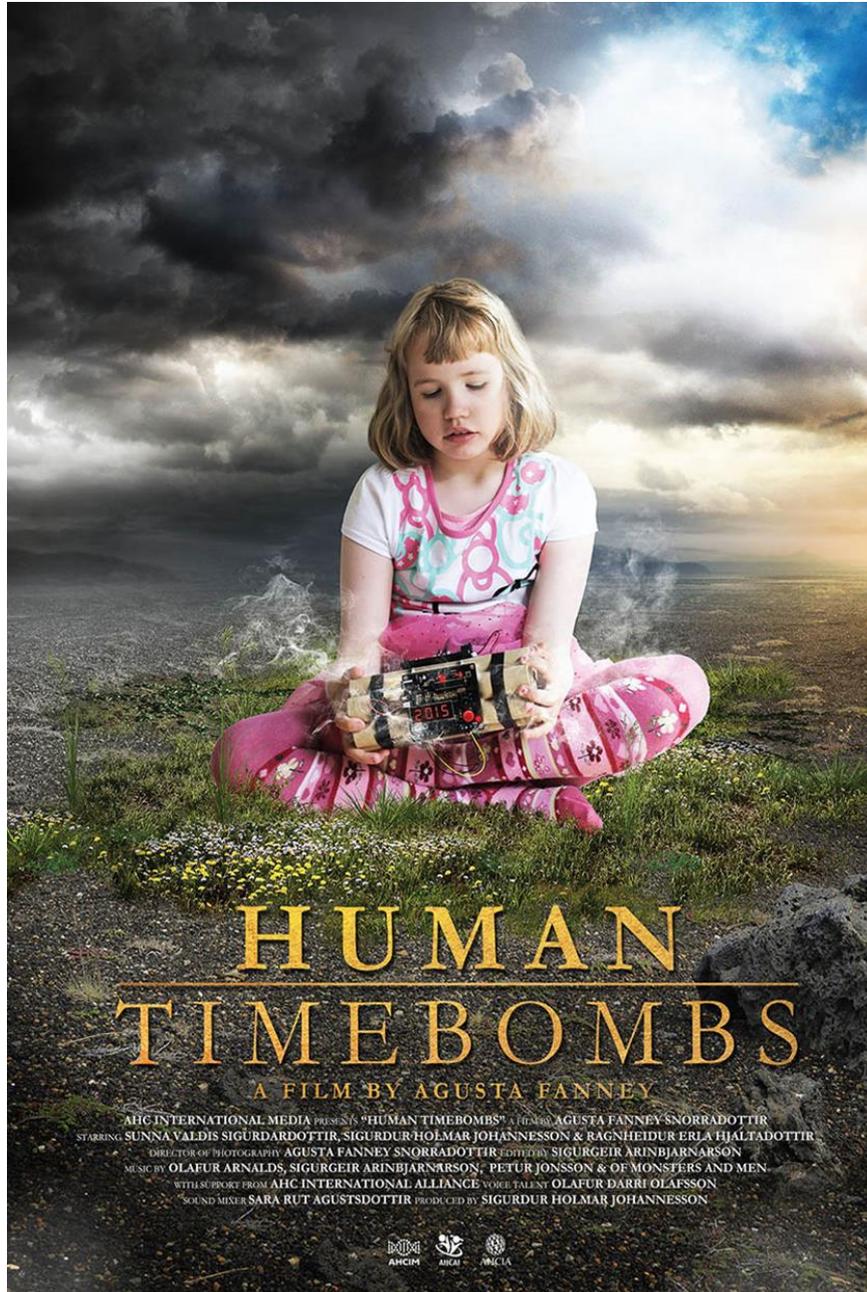
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Mutations in the ATP1A3 gene encoding the Na⁺, K⁺ ATPase α 3 isoform cause the neurological diseases, Rapid onset Dystonia-Parkinsonism (RDP) and Alternating Hemiplegia of Childhood (AHC). These diseases are associated with cognitive deficits, motor dysfunction, high risk of epilepsy and behavioural deficits. The α 3 isoform is highly expressed in the inhibitory GABAergic neuronal subtypes. Introduction of a mutation in the α 3 isoform is expected to cause imbalance of the inhibitory systems. In support, a mouse model for AHC has previously been shown to have compromised thalamocortical circuitry. Using a new Atp1a3 knock-in mouse model, the α 3^{+/D801Y} mice display hyperactivity, reduced seizure threshold and have memory deficits associated with hippocampal dysfunction. Histochemical examination of this brain region revealed pronounced degradation of dentate gyrus granule cells. Supporting a role for GABAergic neurotransmission in ATP1A3-related diseases, treating the mice with low concentrations of the benzodiazepine, clonazepam normalized the memory performance in the mice. Our findings reveal the functional significance of the α 3 isoform in the control of spatial learning and memory, and offers promise for exploration of disease mechanisms and future therapeutic interventions.

P8 Human Time Bombs

Siggi Johannesson

AHC ICELAND



P9 Unravelling the pathogenesis of CAPOS syndrome: novel insights from the beneficial effects of acetazolamide

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Background: Whilst the clinical features of CAPOS syndrome have long been recognized, its genetic background was only recently elucidated. Given the potential recurrence of attacks and gradual progression of the syndrome, an effective therapeutic intervention is needed.

Cases: Four patients from two unrelated families initially presented during childhood with fever-induced episodes of cerebellar ataxia, dysarthria, motor weakness, opthalmoparesis, hypotonia, and areflexia. Targeted sequencing of the ATP1A3 gene unveiled a c.2452 G>A mutation in all four, consistent with CAPOS syndrome and indicating genetic homogeneity. Two of them, both still in childhood, were administered acetazolamide 125 mg b.i.d. which has proven successful in preventing further bouts of neurologic symptoms.

Discussion: The underlying mutation in CAPOS syndrome most likely induces a gain-of-function of Na/K-ATPase, which will generate excessive ion leakage, a negative membrane potential, and a decrease in neuronal excitability. In animal experiments, the extent of ionic currents across plasma membranes has previously been found to increase at elevated temperatures and higher pH values of the extracellular fluid. The latter observation might provide the rationale for the beneficial effect of the carbonic anhydrase inhibitor acetazolamide in our patients.

Conclusion: The clinical benefits in our patients, combined with a plausible experimentally underpinned pathologic correlate, might lend support to the introduction of an “acetazolamide trial” early in the course of CAPOS syndrome to prevent bouts of cerebellar ataxia and other neurologic symptoms, presumably by reducing the excessive pH-sensitive leakage of sodium and potassium ions across the neuronal plasma membrane.

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P10 Alternating hemiplegia of childhood associated with mutation of the ATP1A3 gene: neuropathological study.

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Introduction: Alternating hemiplegia of childhood (AHC) is a rare, severe neurodevelopmental syndrome associated with ATP1A3 mutations, characterized by hemiplegic episodes and progressive neurological dysfunctions including epilepsy. The neuropathology of AHC is unreported, though cases with the ATP1A3 I758S mutation presenting with rapid-onset dystonia-parkinsonism show pathology in neuronal populations, part of complex motor and sensory loops. We present the neuropathology in a 21 year old woman with AHC due to an ATP1A3 E815K mutation.

Methods: Using an immunohistochemistry panel (against neurofilament, GFAP, β A4, AT8, α -synuclein, calbindin, ZnT3 and ATP1A3) in 15 brain regions including hippocampus and cerebellum, we compare the pathology in AHC case to four epilepsy and three non-epilepsy control brains. Western blot analysis was used to quantify ATP1A3 protein expression.

Results: The AHC case showed hippocampal sclerosis without mossy fiber sprouting, and laminar neuronal loss and gliosis in the calcarine cortex. There was marked cerebellar atrophy and striking gliosis of the dentate nucleus and globus pallidus. No neuronal inclusions were identified. Greater immunoreactivity for ATP1A3 protein was observed in some regions than in others (for example the dentate nucleus, globus pallidus, and occipital cortex, hippocampus (CA1/subiculum), parahippocampal gyrus and cerebellar cortex) in all cases and controls.

Western blot analysis did not show regional differences in protein expression level in the AHC case or between controls.

Conclusion: In this case of AHC with an E815K mutation in ATP1A3, no differences in the distribution and cellular expression pattern of this protein were shown compared to controls. Prominent degeneration of the cerebellum could be a component of this condition or may be explained by seizures. Further analyses are underway.

P11 Search for New Candidate Genes for Alternating Hemiplegia of Childhood

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Background: Alternating Hemiplegia of Childhood (AHC) is a rare neurological condition affecting 1 in 100,000 to 1,000,000 children worldwide. The main cause of AHC has been traced to de novo mutations in the ATP1A3 (Na⁺/K⁺ transporting ATPase alpha 3) gene. However, approximately 20% of cases are ATP1A3 mutation negative and are of unknown genetic etiology.

Objectives: To pursue genetic studies of a cohort of ATP1A3 mutation negative and unknown cases to identify novel or rare variants that might cause AHC.

Methods: Whole-exome sequencing (WES) analysis was performed for 11 individuals with AHC whose ATP1A3 mutation status was negative or unknown. Data were filtered for novel or rare (found at most once across databases) protein-changing variants. Variants were confirmed by Sanger sequencing. Segregation analysis was confirmed by sequencing all available family members to determine inheritance: de novo, recessive, hemizygous, and compound heterozygous.

Results: WES analysis identified ATP1A3 mutations in four individuals who were previously ATP1A3 mutation status unknown: two carried a de novo D801N mutation and one carried a de novo V322D mutation, which have been previously associated with AHC. One individual carried a de novo V589F mutation, which is novel and not previously associated with AHC but predicted to be deleterious. For the remaining seven individuals who are ATP1A3 mutation negative, a de novo or inherited, novel or rare mutation(s) was confirmed in a single gene, different in each individual. These are: VCAN (Versican), TUBB4A (Tubulin beta-4a), ATP13A2 (ATPase type 13A2), SCN2A (Sodium channel, voltage-gated, type II, alpha), GRIN2C (Glutamate receptor, ionotropic NMDA 2C), SPG11 (Spastic paraplegia 11), and PCDHB3 (Protocadherin beta 3). All of these genes are expressed in the brain, and some have been previously associated with other disorders. For example, mutations in TUBB4A cause hypomyelinating leukodystrophy-6 and torsion dystonia-4. ATP13A2 mutations are associated with Kufor-Rakeb syndrome, or juvenile parkinsonism. SCN2A has been implicated in several seizure disorders and autism spectrum disorder.

Conclusions: Genetic analysis of ATP1A3 mutation negative and unknown cases of AHC has identified a novel ATP1A3 mutation and novel or rare mutations in new potential candidate genes. Expression in the brain and previous implication in other motor disorders provides some evidence toward a possible association between mutations in these genes to AHC. WES of additional cases may uncover recurrent hits in one or more of the genes, and functional analysis as to how the mutations may affect cellular processes will strengthen the evidence for these new candidate genes.

P12 Characterization of a human neuroblastoma model of AHC: towards cell lines expressing different levels of E815K mutation

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We are currently refining the characterization of a cellular model of AHC, based on a human neuroblastoma cell line (SH-SY5Y). SH-SY5Y have been stably transfected with constructs expressing the wild type form or four different ATP1A3 variants: E815K, D801N, D801Y, G947R. The rationale behind the choice of this model is related to the fact that SH-SY5H cells display a neuronal-like phenotype and express the endogenous ATP1A3 gene.

ATP1A3 cDNAs have been cloned into the pIRES-eGFP eukaryotic expression vector. After linearization, cell lines have been permanently transfected with the constructs. Transfected cells were selected by antibiotics resistance to G418. Mixed cell population underwent to clonal selection, and single clones were plated separately. The levels of expression of endogenous and mutated ATP1A3 mRNA have been determined by absolute real time PCR. The electrophysiological characterization has shown in E815K cells an accumulation of Na⁺ and Ca²⁺, as well as the reduction of the membrane resting potential.

The differentiation protocol of naïve SH-SY5Y cells with retinoic acid followed by neurobasal medium /B27 supplement led to the development of neuron-like cells showing Ca²⁺ transients, trains of action potentials, and the expression of MAP2 and NeuN neuronal markers. On the opposite, cell lines bearing ATP1A3 mutations started dying two days after the medium switch from retinoic acid to neurobasal medium /B27 supplement. We are currently characterizing the death processes activated in mutated cell lines.

Once the characterization of the models will be refined, our cell lines will be used to identify candidate compounds to the treatment of AHC. We will use a computational design for the identification of candidate compounds, that will be tested in cells for their effect in: 1) promoting Na⁺ and Ca²⁺ scavenging and 2) revert the death phenotype upon differentiation.

This work was supported by the Italian (AISEA), the French Association for AHC and the Spanish associations for Alternating Hemiplegia of the Childhood

P13 The IAHCRC-Cloud Platform: an effective tool to support the international studies of the IAHCRC Consortium, for the development of the collaborative research on the ATP1A3 rare diseases

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Alternating Hemiplegia of Childhood (AHC), Rapid Onset Dystonia and Parkinsonism (RD) and the CAPOS Syndrome are very rare neurological diseases caused by specific groups of mutations in the same ATP1A3 gene. The IAHCRC International Consortium, for the research on AHC and other rare diseases related to the ATP1A3 gene, was formed in 2012 to carry out its first international collaborative study that led to the identification of the ATP1A3 gene as the main cause of AHC. In 2013, the Consortium launched further collaborative studies, including the largest international cohort of AHC patients to date, whose results have already been published: the study of the genotype-phenotype correlations and the study on some cardiac conduction abnormalities of AHC. The project for the identification of the secondary gene(s) for AHC (Project GEN2-AHC), the second study of the heart disturbances in the ATP1A3 diseases (Project ECG2_ATP1A3) and the expansion of the common data elements used by the Consortium are all in progress; the launch of further IAHCRC Studies, clinical, genetic and molecular, is under evaluation. The Consortium involves clinicians and basic science researchers in Europe, USA and Australia; it works in close collaboration with health professionals and patient organizations worldwide.

The IAHCRC organizational and IT infrastructure is based on a network whose nodes consist of Clinical Databases and linked DNA Biobanks at the national level. The Nodes collect, keep and share the patient data and samples according to common data elements and common methods defined by the IAHCRC Workgroups in compliance to the international standards. This infrastructure has been very well established through the Charter and bylaws of the Consortium and has been evolving from the centralized proprietary model for the biobanking and the research data storage towards a distributed collaborative model. It can easily include new centers and nodes in the network, thus allowing a fast and ethical involvement of an increasing number of patients, and an efficient sharing of their data and samples for both retrospective and prospective large-scale studies.

The IAHCRC-Cloud Platform, developed using the RedCap© software tool and hosted by the IEMEST, a IAHCRC member, implements the IT infrastructure of the Consortium. Thanks to this on-line Platform, the IAHCRC Centers can collect their patient data in their Node Databases and share them securely and efficiently, through their direct inclusion in the Databases of the IAHCRC Studies to which the Centers are participating.

The data collected and shared in the IAHCRC-Cloud Platform are managed in full observance of the confidentiality and ethics rules set forth in the IAHCRC Charter, for the development of a fair and effective collaboration among all the researchers involved in the study of the ATP1A3 diseases.

P14 Differential distribution of Na⁺/K⁺-ATPase α 1 and α 3 isoforms in excitatory and inhibitory neurons in adult rodent brain

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Neurons are generally classified as excitatory neurons, releasing glutamate, and inhibitory neurons, releasing GABA. These two classes can be divided into several subclasses that express different subsets of ion transporters. All neurons express Na⁺/K⁺-ATPase (NKA), but little is known about the relative proportions of the expression of α 1 and α 3 isoforms in the different subtypes of neurons. Given the distinct symptomatology in rapid-onset dystonia parkinsonism, it seems likely that the expression and relative importance of the NKA α 1 and α 3 isoform may differ between different subtypes of neurons.

To address this question, we are performing an immunohistochemistry study on rat primary hippocampal neurons and slices from adult rat brain. The primary neurons are derived from E18 rats and cultured for 3 weeks, at which time they have a mature level of spines and synapses and display spontaneous activity. NKA α 3 was found to be highly expressed in inhibitory cells labelled for the GABA synthesizing enzyme, glutamate decarboxylase (GAD65/67). NKA α 3 was particularly high in axons and presynaptic boutons of inhibitory cells. NKA α 1 was found to be predominantly enriched in excitatory glutamatergic cells labelled for the vesicular glutamate transporter 1 (VGLUT1). NKA α 1 expression was relatively weak in inhibitory neurons. Immunohistochemistry in slices from adult rat brain confirmed that NKA α 1 and NKA α 3 have differential expression patterns in hippocampus. NKA α 1 was highly expressed in VGLUT1 positive glutamatergic granule cells and their projecting axons, while NKA α 3 appeared to predominantly localize to GAD65/67 positive inhibitory synaptic input residing on the granule cells. Ongoing immunohistochemistry experiments in a mouse line expressing EGFP in the majority of inhibitory neurons (LHX6) will further elucidate the extent of differential distribution patterns of NKA α 1 and NKA α 3 in whole rat and mouse brain.

Significance: The identification of the relative distribution of NKA α 3 in different brain regions and subclasses of neurons will be an important asset in the search for novel therapeutic strategies in diseases associated with ATP1A3 mutations.

P15 Associations between brain structure and executive function in patients with rapid-onset dystonia-parkinsonism

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Rapid-onset dystonia-parkinsonism (RDP) is caused by ATP1A3 gene mutations and characterized by bradykinesia, postural instability and dystonic movements. Recently, RDP has also been shown to affect several cognitive domains, including executive function¹. This study aims to investigate the neuroanatomical substrates underlying RDP-associated effects on executive function. We hypothesized that RDP may affect components of the cerebello-thalamo-cortical (CbTC) pathway thought to be affected in other movement disorders and known to play a role in executive function.

In this preliminary investigation, standard brain MRI voxel-based morphometry was used to measure gray matter (GM) volume in prefrontal cortex, thalamus and cerebellar structures among 6 RDP patients (age: mean±SD=37±20 years; 50% female) in a larger ongoing study of brain structure and cognitive function. Wisconsin Card Sort Task (WCST) total and perseverative errors were measured; age- and education-specific percentile scores were used for analyses.

Pearson correlation demonstrated statistically significant ($p \leq 0.05$) correlations (range: $r=0.80-0.86$) between WCST measures and GM volume in thalamus and cerebellar structures, but not prefrontal regions.

These data suggest that RDP-associated abnormalities of executive function may be mediated by thalamic and cerebellar portions of CbTC pathway rather than prefrontal regions that are more commonly ascribed to executive dysfunction in other central nervous system diseases.

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P16 ATP1A3 mutation without clinical symptoms of alternating hemiplegia in childhood

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The authors undertook a genotype-phenotype research of 10 cases with alternating hemiplegia in childhood (AHC) - 8 typical, 2 atypical. They found incidentally another unusual case of ATP1A3 mutation without clinical symptoms of alternating hemiplegia.

A 21-year-old girl had her first (and only) attack of mild hemiparesis with paresis of the facial nerve (associated with ataxia and hypotonia) during febrile illness at the age of 2.5 years. Neurological symptoms, particularly cerebellar ataxia, dysarthria, dystonic features, mild intellectual disability (IQ=62), and epilepsy had a slowly progression in the following years.

Exome sequencing of the trio (proband plus both unaffected parents) revealed novel heterozygous de novo mutation c.2266C>T in *ATP1A3* gene. Localized in the large cytoplasmic loop between transmembrane domains M4 and M5, the mutation changes converted Arg756 into Cys (p.Arg756Cys).

In spite of the absence of acute AHC symptoms – determining usually the diagnosis – the chronic course of clinical features may correspond with common outcome of AHC patients. De novo ATP1A3 mutation (c.2266C>T) enlarges a spectrum of unusual phenotypes of mutations associated with this gene.