



Third symposium ATP1A3 in Disease:
Genotype/phenotype correlations, modelling and
identification of potential targets for treatment

August 29-31, 2014

Lunteren
The Netherlands

Venue:

Hotel de Lunterse Boer, Boslaan 87, 6741 KD Lunteren

and

Conference Center De Werelt,
Westhofflaan 2
6741 KH Lunteren



Program Committee

David Goldstein
Thomas Friedrich
Jan Koenderink
Tsveta Schyns
Arn van den Maagdenberg

Organizers

Jan Koenderink
Tsveta Schyns
Thomas Friedrich

The “Third Symposium: ATP1A3 in Disease” is kindly supported by:

	ENRAH European Network for Research on Alternating Hemiplegia of Childhood www.enrah.net
	AHC Association of Iceland http://www.ahc.is/
	CureAHC Cure Alternating Hemiplegia of Childhood www.cureahc.org
	AESHA Spanish Association for Alternating Hemiplegia of Childhood www.aesha.org
	A.I.S.EA Onlus Italian Association for Alternating Hemiplegia www.aisea.org
	AFHA French Association for Alternating Hemiplegia www.afha.org
	AHCF US Foundation for Alternating Hemiplegia of Childhood www.ahckids.org
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	AHCUK Alternating Hemiplegia of Childhood UK Support Group http://ahcuk.co.uk/
	AHC-Deutschland e.V. German Association for Alternating Hemiplegia of Childhood www.ahckids.org
	AHC CANADA Canadian Association for Alternating Hemiplegia of Childhood www.ahc-canada.ca

Street map of Lunteren

De Lunterse Boer
Huisje van Elvi
De Lunterse Boer

De Werelt
Congrescentrum
De Werelt

Hugo de Vries
Parkhotel Hugo de Vries

De Wormshoef
Hotel Esthuys
de Wormshoef

Train Station
Lunteren

200 m



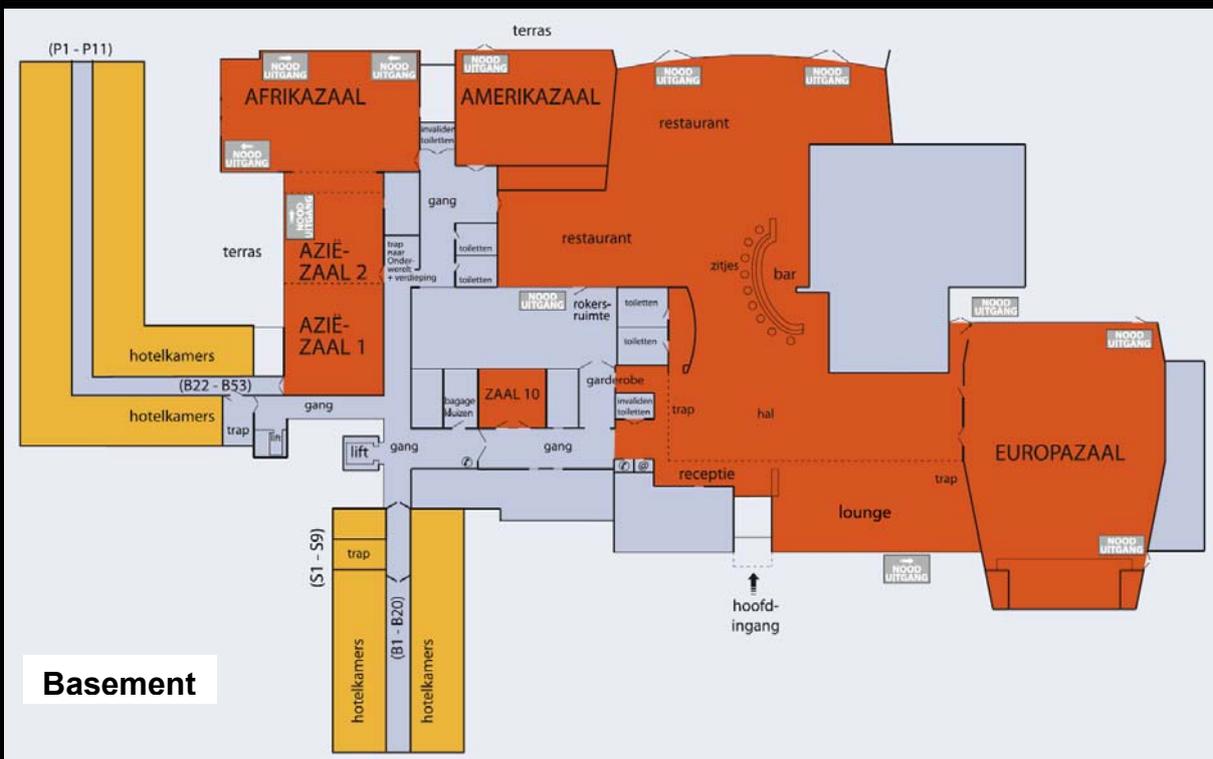
Orientation map of The Netherlands with Railways



Site map: De Werelt Conference Center



Floor Plan



Welcome to the

Third Symposium ATP1A3 in Disease: Genotype/phenotype correlations, modelling and identification of potential targets for treatment

As a follow-up of the two previous symposiums on the same topic, which have taken place 2012 in Bruxelles and 2013 in Rome, this meeting aims at presenting the most recent progress in research on Alternating Hemiplegia of Childhood (AHC) and other ATP1A3-related disorders. It is our wish that it may promote, concentrate and unify the international efforts on diagnosis, medical aspects and - eventually - clinical treatment and cure, which so many affected patients deserve by heart.

Since we all are devoted to the patients and their families, the meeting will start with an interactive program for the AHC families. The first session of the scientific program addresses results from genetics research and the correlations between genotype and phenotype from different research groups all over the world, and several poster presentations also address this topic. The second session is dedicated to the clinical spectrum of ATP1A3-related disorders, which also span Rapid Dystonia Parkinsonism (RDP) and CAPOS syndrome (Cerebellar ataxia, Areflexia, Pes cavus, Optic atrophy, and Sensorineural hearing loss). This will be followed by a session on the molecular aspects of ATP1A3 diseases in different model systems.

As a new format, we joined the scientific program of the symposium with the first day of a large conference, the ASBMB Special Symposium "14th International Conference on Na⁺,K⁺-ATPase and Related Transport ATPases". The idea behind it is to increase the awareness of the great P-ATPase community for ATP1A3-related diseases, stimulate discussions between clinicians, geneticists and researchers concentrating on molecular, cell biological and medical aspects - hopefully leading to novel cooperations to forward diagnosis and cure of AHC and other P-type-ATPase-related diseases. This part of the program covers a broad range of presentations by outstanding researchers working on physiology, medicine and therapy.

Finally we would like to express our thanks for the great contribution of the large number family support groups, for their presence during the meeting, and to the generous donations by the sponsors of this symposium.

We hope you will find a pleasant and stimulating atmosphere here in The Netherlands to share knowledge, exchange ideas, discuss topics - and meet colleagues and friends.

Your Organizing Committee:

*Jan Koenderink
Tsveta Schyngs
Thomas Friedrich*

...and Welcome to The Netherlands!*

Despite its size of 41,500 square km, which sets the country somewhere between Tennessee and Kentucky, The Netherlands is a country of several superlatives: It has the largest port in Europe (Rotterdam), the most gale-proof shore protection system (the Delta works blocking the delta of Rhine, Maas and Scheldt against the North Sea), and with a population of about 16.8 millions, it is one of the most populated areas worldwide. Its population density of 406 per square km is only outnumbered by Bangladesh, Taiwan and South Korea. Nobody entering The Netherlands can fail to recognize that the country is the most bicycle-friendly place on the globe. According to statistics, every inhabitant possesses a bike and people make extensive use of their *fiets*. The three largest cities share their responsibilities for the nation, with Amsterdam being the capital, The Hague as the seat of the government and Rotterdam as its umbilical cord for economy and trade.



The name *nederland* means the „low land“, which literally expresses that only about 50 % of its area are more than one meter above sea level, and a considerable portion of 26 % is even below the waterline. The fact that 18 % of the land is man-made well explains the Dutch's special preference for wind mills, which were less frequently used to grind grain but essentially to pump water for keeping the „low lands“ dry. Perplexing to outsiders, the name Holland appears to be used as a synonym and seems to harbor the root *hoch* (= high). Both conceptions are incorrect in a strict

sense, since *Holland* sets the two most populated provinces (North and South Holland) on its western shore as *pars pro toto* for the whole country (but its nevertheless appropriate for naming the national football team), whereas *Holland* historically stems from the word *holtland* (*holt* = wood), which was first used in 866 to name the forest-rich region around Utrecht.

The Netherlands have left a significant impact during the naval discovery of the world, in which the Dutch distributed place names all over the globe (New York was founded as *Nieuw Amsterdam*, Australia was first named *Nieuw Holland*). But also sea trade, the system of mercantile production, as well as science (Huygens, Snellius, Leeuwenhoek), fine arts (Rembrand, van Dijk, van Gogh), religion and philosophy (Erasmus von Rotterdam, Spinoza, Descartes) and print publishing (Elsevier) were greatly influenced by Dutch people. As a country whose shore line matches its overland frontiers, the Dutch have developed a strong sense for the benefits of interacting with overseas. Likewise, the Dutch are keen of water sports, and in winter, when the waterways are frozen, they enjoy ice skating over endless miles as a



* ...a view on The Netherlands from the perspective of a German, hope you like it (Thomas :-)

national pleasure. Although the Dutch make jokes about themselves that it is impossible to argue with them (especially if the subject is on football or the Dutch Royals), they are nevertheless open-minded and warm-heartedly welcome visitors. People are known as honestly steady-minded, with an outstanding resilience against external hardships that resides on a strong sense for the virtues of social coherence. Thus, it is no wonder that the United Nations' World Happiness Report of 2013 ranks the Netherlands in the fourth place.



...more and less famous *Oranje* fans

The fertile soil and the Polder grasslands have made the Netherlands one of the world's largest producers of dairy products (with uncountable flavors of cheese), vegetables, fruits and flowers. Agriculture accounts for 21 % of the nation's export value and the country is the second largest exporter of agricultural products, only topped by the United States. Nevertheless, the brand names of Dutch industry (Philips, DAF trucks, Fokker aircraft, Royal Dutch Shell, Heineken) and finance (ABN-AMRO, ING, Randstad) also resound worldwide.

Less known to outsiders are the more secluded areas of the countryside, with 20 National Parks that preserve lakes, heathland, forests, dunes, tidal flats and other habitats. Many of its national forests are located in the central province of Gelderland. Its capital is Arnhem, and the conference site **Lunteren** is only 25 km away from it. Not far from Lunteren are the National Parks *De Hoge Veluwe* and *Veluwezoom*, which are parts of the largest contiguous forest in the country. The major cities in the vicinity are Utrecht, Arnhem and Nijmegen.



The old church and the seal of Lunteren

The small size and the excellent public transportation system makes The Netherlands easy to explore. Sites that should not be missed include the Rijksmuseum with the city and canals of Amsterdam, the cities of Utrecht, Harlem and Delft, the historical windmills in Kinderdijk, the Keukenhof Gardens, the Delta Project, to name just a few. Lovers of historical architecture will find countless spots to admire, friends of the fine arts may also want to visit the van Gogh Museum and the Hermitage Museum in Amsterdam, the Mauritshuis Art Gallery in The Hague, and many more. And those who desire a rest far off the busy metropolises may find peace in the Veluwe forests or on the sandy beaches along the shore or on the Friesian islands.



The Dutch people highly appreciate use of their language, so if you remember *alstublieft* for 'please', *dank je wel* for 'thank you', *graag gedaan* for 'you are welcome', *goede dag* for 'good day', *mijnheer* and *mevrouw* for 'Madam' and 'Sir', you will get along with pleasure.

Dutch scientists have left some trace in P-type ATPase research. Thus, hosting the „Third Symposium ATP1A3 in Disease“ here is a late tribute by the organizers to Prof. Jan Joep H. H. M. de Pont, who unfortunately is no longer among the P-ATPase community today.



Third symposium ATP1A3 in Disease:
Genotype/phenotype correlations, modelling and
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August 29-31, 2014

Lunteren
The Netherlands

Program

Friday

August 29

Venue:

de Lunterse Boer, Boslaan 87, 6741 KD Lunteren, The Netherlands

<i>ATP1A3 Meeting registration & hotel check-in</i>		18.00-22.00
Meeting registration continues next morning at 8 a.m.		
<i>Opening and buffet dinner</i>		19.30-21.30
<i>AHC Parents interactive program</i>		
Opening address	<i>Jan Koenderink / Thomas Friedrich</i>	19.30
AHC Parents Welcome address	<i>Willem Zorge</i>	19.35
AHC parents interactive program		20.30
<i>How to focus and speed up research on AHC? Our children can't wait!</i>		
Moderated discussion and video trailer	<i>Jeff Wuchich</i> <i>Sigurður Jóhannesson</i>	20:30-21.30

***Third Symposium ATP1A3 in Disease:
Genotype/phenotype correlations, modelling and
identification of potential targets for treatment***

<i>Scientific Program</i>

Saturday	August 30
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<i>ATP1A3 Meeting registration (continued)</i>	08.00-08.30
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<i>Session 1: Genetics and phenotype /genotype correlation in AHC/RDP</i>	08.30-10.05
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<u>Chair:</u> Allison Brashear	08.30-08.40
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Erin Heinzen ATP1A3 in neuropsychiatric disease: where we came from and where we are going	08.40-09.00
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Masayuki Sasaki Genotype–Phenotype Correlations in Japanese Patients with AHC (2nd report)	09.00-09.20
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Soňa Nevšimalová Alternating hemiplegia of childhood – phenotype and genotype correlations: Czech and Slovak database	09.20-09.35
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Arn van den Maagdenberg The hunt for AHC2: does it exist?	09.35-10.05
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<i>Break</i>	10.05-10.20
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<i>Session 2: Clinical spectrum of ATP1A3 diseases</i>	10.20-12.10
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<u>Chairs:</u> Mohamad Mikati	10.20-10.30
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Allison Brashear ATP1A3 Mutations: An Expanding Phenotype	10.30-10.50
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Alan Fryer A novel recurrent mutation in ATP1A3 causes CAPOS syndrome	10.50-11.10
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Sanjay Sisodiya Asystole in alternating hemiplegia of childhood	11.10-11.30
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Hendrik Rosewich Clinical spectrum AHC/RDP/CAPOS	11.30-11.50
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General Discussion Morning Session	11.50-12.10
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<i>Lunch</i>	12.10-13.20
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Session 3: Molecular aspects of ATP1A3 diseases	13.20-16.00
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Chair: Thomas Friedrich

Fiorella Gurrieri 13.20-13.40
Development and characterization of human cellular models of AHC

David Goldstein 13.40-14.00
Modeling ATP1A3 mutations using multi electrode arrays

Mohamad Mikati 14.00-14.20
The D801N knock in mouse model of AHC

Nastacia Vedovato 14.20-14.40
Mechanisms of Na/K-ATPase pump dysfunction caused by mutations underlying the neurological disorders AHC and RDP

Break 14.40-15.00

Session 3 (continued):

Chair: David Goldstein

Karl Weigand 15.00-15.20
Functional consequences of mutations in ATP1A3 causing alternating hemiplegia of childhood

Steven Petrou 15.20-15.40
Proton transport function of ATP1A3 correlates with clinical severity in AHC

Alfred George 15.40-16.00
Impaired Cell Surface Expression of ATP1A3 Mutations Associated with Alternating Hemiplegia of Childhood

Session 4: Perspectives AHC / RDP	16.00-17.00
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Chair: Tsveta Schyns 16.00-16.10

David Goldstein / Jan Koenderink / Allison Brashear 16.10-17.00
Genetic, medical and molecular achievements needed to improve patients treatment

Posters should be placed at Conference Center De Werelt before 18:00

Join ATPase Conference 18.00-23.00
see following page

Joint meeting



Venue:
Conference Center De Werelt
Westhofflaan 2, 6741 KH Lunteren, The Netherlands

Saturday

August 30

Opening ceremony Thomas Friedrich / Jan Koenderink 18.00-18.15

Opening keynote lecture

Jerry Lingrel 18.30-19.30
Role of the $\alpha 2$ isoform of the Na^+, K^+ -ATPase in blood pressure regulation and
heart function

Diner and welcome reception 20.00-23.00

Poster session "ATP1A3 in Disease" Symposium 20.00-23.00

Session 1:

Physiology, Medicine and Therapy

08.30-12.00

Chair: Kathleen Sweadner

08.30-08.40

Karin Lykke-Hartmann

08.40-09.10

Co-morbid Psychiatric Manifestations Couple to Glutamate Defects in a Familial Hemiplegic Migraine type 2-mutation Mouse Model

Vivien Schack

09.10-09.30

Somatic mutations in Na⁺,K⁺-ATPase and plasma membrane Ca²⁺-ATPase lead to aldosterone-producing adenomas (Conn's syndrome)

Bente Vilsen

09.30-10.00

New aspects of Na⁺,K⁺-ATPase structure and mechanism in health and neurological disease

Break

Vladimir Matchkov

10.20-10.40

Mutation in the α_2 isoform of Na⁺,K⁺-ATPase associated with Familial Hemiplegic Migraine type 2 (FHM2) leads to elevated contractility and vasodilatation of cerebral arteries in mice

Rajini Rao

10.40-11.10

Isoform-Specific Role of Secretory Pathway Ca²⁺-ATPase 2 (SPCA2) in mammary physiology and breast cancer

Sigrid Langhans

11.10-11.30

A sodium-mediated feedback loop that regulates EGFR trafficking

Anita Aperia

11.30-12.00

The role of NKA in health and disease

Lunch

12.00-13.30

Session 2:

Physiology, Medicine and Therapy

14.00-16.00

Chair: Gustavo Blanco

14.00-14.10

Kathleen Sweadner

14.10-14.40

Mutations of ATP1A3: consequences in the realms of atoms, cells, mice, and humans

Ling Yi

14.40-15.00

Adaptor protein complexes 1 and 2 (AP-1, AP-2) mediate anterograde and retrograde neuronal trafficking of the copper transporter ATP7A

Judth Heiny

15.00-15.30

Isoform-specific role of the NKA alpha subunits in skeletal muscle

Hanne Poulsen

15.30-15.50

Characterization of the ATP1A3 mutation causing CAPOS

End of Joint Special Topic Meeting: ATP1A3 in Disease

Remove ATP1A3 symposium posters before 17.30

List of Posters (corresponding authors underlined)

- #1 (sub 1) Behavioral and electrophysiological analyses of Atp1a3 knockout mice and implication for pathophysiology of dystonia
Keiko Ikeda, Satomi Chiken, Atsushi Nambu, Hiroki Sugimoto, Kiyoshi Kawakami
- #2 (sub 4) A road-map from genetic studies to RDP- and AHC- understanding and treatment
Martin Kubala
- #3 (sub 5) Probing structural consequences of ATP1A3 mutations with molecular simulations
Wojciech Koplec, Bastien Loubet, Hanne Poulsen, Himanshu Khandelia
- #4 (sub 6) A novel mutation in ATP1A3 gene in a patient with RDP and some similarities with AHC
Alessandro Capuano, Barbara Garavaglia, Federico Vigevano, Nardo Nardocci
- #5 (sub13) AHC Federation of Europe
Sigurður Jóhannesson
- #6 (sub17) Functional consequences of Rapid onset Dystonia Parkinsonism-associated NKAalpha3 mutations for membrane expression and sodium homeostasis in primary hippocampal neurons
Nicolas Fritz, Evgeny Akkuratov, Thomas Liebmann, Hjalmar Brismar, Anita Aperia
- #7 (sub 20) Widening the phenotypic spectrum of ATP1A3 mutations: R756H mutation in a family with developmental delay, and fluctuating ataxia
Fatima Jaffer, Helen Kingston, Henry Houlden, Michael G. Hanna, Sanjay M. Sisodiya
- #8 (sub 21) The International IAHCRC Consortium for the research on AHC: a successful model for the progress of the research on this rare disease
Rosaria Vavassori, Alexis Arzimanoglou
- #9 (sub 22) Altered motor memory in behaviour and electrophysiological analyses in Atp1a3 heterozygous knockout mice
Kiyoshi Kawakami, Shinichiro Satake, Hiroki Sugimoto, Keiko Ikeda
- #10 (sub 23) Assessing motor and cerebellar function in Na⁺/K⁺-ATPase $\alpha 3$ +/D801Y mice
Toke J. Isaksen, Thomas H. Holm, Karin Lykke-Hartmann
- #11 (sub 24) Modeling neurological deficits of Alternating Hemiplegia of Childhood in mice
Thomas Holm, Toke J. Isaksen, Simon Glerup, Ernst-Martin Füchtbauer, Anders Heuck, Poul Nissen, Karin Lykke-Hartmann

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- #12 (sub 25) AHC French National Network
Dominique Poncelin
- #13 (sub 26) Characterization of human neuroblastoma cell lines expressing different levels of E815K mutation
Martina Rinelli, Sara Cocco*, Roberto Piacentini, Lorena Di Pietro, Cristian Ripoli, Giovanni Neri, Francesco Danilo Tiziano, Claudio Grassi, Fiorella Gurrieri*
- #14 (sub 27) The Italian Association A.I.S.E.A. and the support to the national and international research on AHC
Rosaria Vavassori, Paola Bona
- #15 (sub 28) Alternating Hemiplegia of Childhood: update on pharmacological treatment of an Italian cohort of 29 patients - preliminary results.
Livia Pisciotta, Marcella Gherzi, Michela Stagnaro, Rosaria Vavassori, Melania Giannotta, Edvige Veneselli, Elisa De Grandis
- #16 (sub 30) Transgenic Rescue of Alternating Hemiplegia-Related Behavioural Phenotypes in Atp1a3 Mutant Mice
Greer Kirshenbaum, John Roder, Steven Clapcote
- #17 (sub 31) ATP1A3 mutations and genotype-phenotype correlation of alternating hemiplegia of childhood in Chinese patients
Xiao-Ling Yang, Hua Gao, Jie Zhang, Xiao-Jing Xu, Xiao-Yan Liu, Xi-Ru Wu, Liping Wei, Yue-Hua Zhang
- #18 (sub 32) AHC Multidisciplinary Clinic
Mohamad Mikati, Jeff Wuchich

ATP1A3 in neuropsychiatric disease: where we came from and where we are going***Erin Heinzen***

Duke University, Durham, NC, USA

Mutations in ATP1A3 were first discovered to cause rapid-onset dystonia parkinsonism (RDP) in 1999. The phenotypic spectrum of ATP1A3 was dramatically expanded when multiple collaborative groups identified ATP1A3 mutations as the cause for alternating hemiplegia of childhood (AHC) in 2012. There are now 67 mutations in ATP1A3 reported to cause a wide range of neuropsychiatric diseases, including mutations shown to cause novel syndromes as well as derivatives of the disease-specific phenotypes. Building from the initial successes of collaborative research, the ATP1A3 working group is making great strides towards predicting the prognosis of patients with particular ATP1A3 mutations, and in deciphering the molecular consequences of these mutations and how they lead to disease. Since last year's meeting, the largest genotype-phenotype study was completed in 155 patients, a mouse model of the most commonly seen ATP1A3 mutation found in AHC patients was completed, analyses of a large group AHC patients to assess the risk of cardiac arrhythmias associated with ATP1A3 mutations was performed, and studies to look for additional AHC genes are underway. This exceptional progress underscores the need for collaborative work going forward.

Genotype–Phenotype Correlations in Japanese Patients with AHC (2nd report)

Masayuki Sasaki, Atsushi Ishii, Kenji Yokoshi, Hideaki Shiraishi, Yoshiaki Saito, Shinichi Hirose

National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan

keywords: Genotype-Phenotype Correlations, Japanese AHC

Objective:

Clinical severity of alternating hemiplegia of childhood (AHC) is variable among patients. To investigate genotype–phenotype correlations in Japanese patients with AHC, we analyzed the clinical information and ATP1A3 mutations in patients with AHC. Methods: Forty seven Japanese patients who were clinically diagnosed with AHC participated in this study. ATP1A3 mutations were analyzed using Sanger sequencing. Detailed clinical information was collected from family members of AHC patients and clinicians responsible for their care.

Results:

Gene analysis revealed 43 patients with de novo heterozygous missense mutations of ATP1A3: Glu815Lys in 13 cases (30%), Asp801Asn in 14 cases (33%), and other missense mutations in 16 cases. Ten cases confirmed by mutation analysis were added to the results of the last year. Only 1 of 10 cases was a newly diagnosed infant. Clinical information was compared among the Glu815Lys, Asp801Asn, and other mutation groups. Patients with Glu815Lys showed neonatal onset, severely delayed motor development, especially they did not acquire walking except 1 case. Statistical analysis revealed significant differences in the history of neonatal onset, gross motor level, status epilepticus, and respiratory paralysis in the Glu815Lys group compared with the other groups. In addition, 9 patients who did not receive flunarizine had severe motor deteriorations. Conclusions: The Glu815Lys genotype appears to be associated with the most severe AHC phenotype. Although AHC is not generally seen as a progressive disorder, it should be considered a disorder that deteriorates abruptly or in a stepwise fashion, particularly in patients with the Glu815Lys mutation.

**Alternating hemiplegia of childhood – phenotype and genotype correlations:
Czech and Slovak database**

Soňa Nevšimalová, D. Kemlik, M. Kolníková, P. Sykora, B. Petrak, A. Krepelova

Charles University Prague, The Czech Republic

keywords: Alternating hemiplegia in childhood, phenotype genotype correlation

Introduction:

Alternating hemiplegia in childhood (AHC) is a rare but severe disorder, in some cases with increased mortality and shortened life expectancy. Since different mutations in ATP1A3 gene causing the disease were detected, the phenotype - genotype correlations are very important.

Patients and methods:

Ten patients (7 males, 3 female, aged 18 months - 38 years) were followed-up for several weeks up to 30 years. The diagnosis was based on their clinical history, neurological findings, and supplemented by neurophysiological (EEG, EMG, EP, PSG) and neuroimaging (SPECT, PET, MRI) methods. For Sanger sequencing of the ATP1A3 gene, DNA from peripheral blood samples was extracted and primers for DNA amplification and sequencing were used to cover all coding regions of the gene.

Results:

In 4 patients Asp801Asn mutations, in 2 cases Gly947Arg mutation, and in the most severely affected patient Glu815Lys mutation were revealed. In one case with atypical late onset of the clinical symptoms genetic evaluation found no mutation; 2 cases are still under genetic investigation. Both Asp801Asn and Gly947Arg mutations can be seen as relatively benign with a stationary course of the disease in adults, while the Glu815Lys mutation has a very severe outcome. Video-presentations of all three types of mutation include clinical picture of affected patients (at different age), and illustrations of ictal and interictal neurophysiological and neuroimaging examinations.

Conclusion:

As the genotype-phenotype correlations are very important for the disease prognosis and outcome, the Czech-Slovak AHC Consortium was established.

The hunt for AHC2: does it exist?***Arn van den Maagdenberg***

Leiden University Medical Centre, Leiden, The Netherlands

Gene hunting in AHC led to the identification of ATP1A3 as the major gene in alternating hemiplegia of childhood (AHC). Several studies have shown that not all patients had a mutation in ATP1A3, which suggests that additional AHC genes may exist. An update will be provided of the hunt for the AHC2 gene. The choice of gene identification strategy, the accuracy of patient diagnosis, and the possibilities for follow-up research that is needed to find sufficient proof for the AHC2 gene, including some of the major challenges that are encountered in the gene hunt, will be presented. Identification of AHC2, if it exists, is important as it may give additional opportunities to develop therapies for AHC.

ATP1A3 Mutations: An Expanding Phenotype

How Do Mutations in ATP1A3 Impact the Brain?

Allison Brashear, Jared Cook, Kathleen Sweadner, Laurie Orzelius, Christopher Whitlow, Adrian Oblak, Bernadino Ghetti

Wake Forest University School of Medicine, Winston-Salem, NC, USA

keywords: ATP1A3 phenotypes, MRI, Pathology, RDP, Rapid Onset Dystonia Parkinsonism

RDP and AHC appear to be different diseases, though both are caused by mutations in ATP1A3 (Reference). Rapid-onset dystonia-parkinsonism (RDP) may have onset throughout the lifespan while alternating hemiplegia of childhood (AHC) occurs in children younger than 18 months. Both diseases report triggers. Both RDP and AHC sufferers exhibit motor symptoms (particularly dystonia), but in RDP symptoms are usually abrupt in onset and remain predominantly fixed. AHC is characterized by an episodic and fluctuating course. Mutations causing RDP or AHC may cause the following symptoms: dystonia, Parkinsonism, epilepsy (including status epilepticus), hemiplegic episodes, abnormal ocular movements, and developmental delay, psychosis, depression, anxiety, and gait disorders in ages ranging from newborns to 87 years. Our recently reported new findings of neuronal dropout in the globus pallidus, subthalamic nucleus, red nucleus, inferior olivary nucleus, cerebellar Purkinje and granule cell layers, and dentate nucleus in the brain of 4 siblings with ATP1A3 mutations and MRI changes in other patients with different ATP1A3 mutations suggest that ATP1A3 mutations cause structural changes in human brain.

A novel recurrent mutation in ATP1A3 causes CAPOS syndrome*Alan Fryer*

Department of Clinical Genetics, Liverpool Women's and Alder Hey Children's Hospital, Liverpool, United Kingdom

In 1996 Nicolaidis et al (J. Med. Genet. 33:419-21) described a UK family where a mother and her two children presented with a combination of cerebellar ataxia, areflexia, pes cavus, optic atrophy and sensorineural hearing loss (with the acronym "CAPOS" syndrome). In all three cases, there was an acute neurological presentation with hypotonia and cerebellar ataxia in early childhood following a febrile illness. Recovery was variable in its completeness and episodes recurred with subsequent febrile illnesses. All three developed a persistent cerebellar ataxia with areflexia, visual impairment due to optic atrophy and sensorineural deafness. In the mother the neurological symptoms have been very slowly progressive over the years. In 2009 a Canadian family with four affected members (father and three children) presented a similar phenotype. Exome sequencing was undertaken in probands from the two families and an identical mutation c.2452G>A (p.Glu818Lys) in ATP1A3 gene was identified in both and this was subsequently shown to segregate with the syndrome in both families and to have arisen de novo in the mother of the UK family. A second UK family with three affected members was identified and the disorder was again shown to be due to the c.2452G>A mutation. The clinical features of the 10 patients will be presented. A third UK family has now been identified with the identical mutation – arising de novo in this case. Thus CAPOS syndrome expands the spectrum of phenotypes associated with mutations in ATP1A3 – novel features include sensorineural hearing loss (shown to be an acoustic neuropathy in two cases), optic atrophy and the presence of areflexia and pes cavus.

Asystole in alternating hemiplegia of childhood***Sanjay Sisodiya***

UCL Institute of Neurology, London, United Kingdom

Clinical evaluation in a single person with AHC, before the discovery of its genetic cause, raised the possibility of cardiac disorder. Detailed cardiological evaluation with an implanted loop recorder captured brief periods of asystole. In the context of a known risk of sudden unexplained death in the condition, the patient had a permanent pacemaker implanted, with subsequent cessation of the events which had led to cardiological investigation. This single case prompted evaluation of cardiac function in other people with the condition. Initially, we have undertaken a study of baseline 12-lead ECGs through the Consortium. Fifty individuals have now been studied, using either existing ECG or newly-acquired records. About a half of the individuals show changes in the ECG, most of which are subtle but definite. These results will be presented and the implications considered.

Clinical spectrum of ATP1A3 related disorders: AHC/RDP/CAPOS***Hendrik Rosewich***

Georg August University Göttingen, Göttingen, Germany

Over the last four decades three different rare diseases, alternating hemiplegia of childhood (AHC), rapid-onset dystonia parkinsonism (RDP) and CAPOS syndrome (cerebellar ataxia, pes cavus, optic atrophy and sensorineural hearing loss) have been described and defined clinically. Subsequently the primary genetic cause has been identified using classical genetic techniques like linkage analysis and candidate gene approaches as well as next generation sequencing methods. Interestingly, all three diseases are caused by heterozygous mutations in the *ATP1A3*-gene, coding for the alpha3-subunit of a P-type Na⁺/K⁺-ATPase. Even though the classical presentation of each disease is quite unique, the thorough analyses of the individual phenotypes revealed a substantial overlap of the main clinical characteristics and impressively prove that AHC/RDP and CAPOS syndrome are prototypic disorders in a clinical continuum of ATP1A3 related disorders. Clinical symptoms shared by AHC/RDP and CAPOS syndrome include dystonia, bulbar symptoms like dysarthria and dysphagia as well as abnormal ocular movements. Moreover the symptoms of all three diseases can be caused by trigger mechanisms like febrile illness.

In the next years more patients with mutations in the *ATP1A3* gene will be identified. These patients will even extend the phenotypic spectrum of ATP1A3 related disorders. The identification and exact description of the entire phenotypic spectrum is the prerequisite for genotype-phenotype correlation studies and for assessing measurable outcomes for clinical trials. Achieving consensus on definitions and treatment strategies is therefore a challenging task for the whole ATP1A3 community. Furthermore, the expanding phenotypic spectrum will contribute to the understanding of protein expression and its cellular function. The association of three different diseases to alterations in one gene raises the interest of basic researchers, neurologists as well as the pharmaceutical industry for the development of potential treatments.

Development and characterization of human cellular models of AHC

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Introduction:

Alternating Hemiplegia of the Childhood (AHC) is a rare neurodevelopmental disorder characterized by recurrent episodes of paroxysmal hemiplegia associated with epilepsy, dystonia, intellectual disability and dysarthria. It occurs mostly in sporadic form, although families with an autosomal dominant pattern of inheritance have been reported. We have recently collaborated to identify *ATP1A3* as the main gene responsible of AHC (Heinzen et al., 2012). *ATP1A3* encodes for the alpha3 subunit of the neuronal Na⁺/K⁺-ATPase pump; mutations in this gene are also responsible for another condition, the dystonia-parkinsonism syndrome (DYT12) with early onset. We are currently developing and characterizing two different cellular models of AHC: a neuronal model, exploiting a human neuroblastoma cell line (SH-SY5Y), and the other based on HeLa cells. Both cell lines have been stably transfected with constructs (kindly provided by Dr. Erin Heinzen) expressing the most frequent *ATP1A3* mutations found in patients with AHC, namely the D801N, E815K, G987R. The rationale behind the choice of the double model is related to the fact that while SH-SY5H cells display a neuronal-like phenotype but express the endogenous *ATP1A3* gene, HeLa cells are not excitable but do not express the endogenous *ATP1A3*.

Materials and methods:

ATP1A3 cDNA with the 3 most common mutations observed in patients with AHC (E815K, D801N and G947R) and one of DYT12 (D801Y) were cloned into the pIRES-eGFP eukaryotic expression vector. After linearization, the two cell lines were permanently transfected with the different constructs. Transfected cells were selected by antibiotics resistance to G418; the expression of the transgene has been confirmed by the expression of the GFP. Resting membrane potential has been evaluated by patch-clamp recordings, performed in current-clamp mode. Intracellular ionic concentration of Na⁺ and Ca²⁺ has been determined by confocal microscopy, using the CoroNa-AM and the Fluo-4-AM fluorescent probes, respectively. For electrophysiological studies, SH-SY5H cells were differentiated into neuronal-like cells for one week, according to standard protocols.

Results:

We have at first demonstrated that the mutant constructs are expressed in transfected cells. Subsequently, we have characterized so far the phenotypic effect of the E815K mutation in the presence and absence of the endogenous *ATP1A3*. HeLa cells expressing the mutation showed a partial depolarization of the plasma membrane, compared to the cells transfected with the wt construct (-60.9±1.6 mV [n=21] vs. -70.2±1.5 [n=22], respectively, P<0.01). The SH-SY5Y cells, in addition to a partial depolarization of the resting potential (-43.7±1.1 mV [n=20] vs. -50.4±1.4 [n=21], P<0.01), also exhibited cytoplasmic accumulation of Na⁺ (13.4±3.0 mM

[n=73] in ATP1A3-E815K cells vs. 4.8 ± 0.9 mM [n=72] in ATP1A3-wt cells, $P < 0.01$) and Ca^{2+} (206.8 ± 20.0 nM [n=95] vs. 95.4 ± 21.7 nM [n=141], $P < 0.001$). Further phenotypic analyses are currently ongoing.

Conclusions:

Our data indicate that mutations in ATP1A3 responsible for AHC have a dominant negative effect. The availability of a cell model with phenotypic abnormalities specifically related to the mutation may allow to obtain information on the pathogenesis of AHC and may provide an experimental platform for the identification of candidate compounds suitable for the treatment of the condition.

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

Modeling ATP1A3 mutations using multi electrode arrays

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Mutations in ATP1A3 have been associated with distinct neurological diseases, but the underlying functional differences of the mutations associated with different diseases remains to be elucidated. Here we explore the use of multi electrode arrays to assess the effects of ATP1A3 mutations on *in vitro* neuronal networks. We first show that neurons from an D801N mutant mouse model, a model of the most severe disease associated with ATP1A3 mutations, Alternating Hemiplegia of Childhood (AHC), form neuronal networks that differ in a number of key features from wildtype networks. AHC-causing mutations result in decreased ATPase activity; therefore we next attempt to recapitulate the mutant features by selective pharmacological inhibition of ATP1A3 with ouabain at low doses. Wild-type neurons treated with low doses of ouabain show overall decreased neuronal activity but fail to replicate the mutant neuronal features associated with the D801N mutation, probably due to a limited range of doses. We also show that Flunarizine, a non-selective calcium channel blocker that has had beneficial effects in a fraction of AHC patients, has the effect of exacerbating mutant neuronal features. Given Flunarizine's mechanism of action, these results are unsurprising, but do not explain the drugs beneficial effect in some patients. Future studies will include the use of other drugs and compounds to increase ATPase activity in an attempt to correct mutant features.

The D801N knock in mouse model of AHC***Mohamad Mikati***

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We generated the D801N mutant mouse and compared mutant and wild-type (WT) littermates. Behavioral tests, amygdala kindling, flurothyl-induced seizure threshold, spontaneous recurrent seizures (SRS), and other paroxysmal activities were compared between groups. In-vitro electrophysiological slice experiments on the hippocampus were performed to assess for predisposition to hyperexcitability. Mutant mice manifested a distinctive phenotype similar to that of humans with Alternating Hemiplegia of Childhood (AHC). They had abnormal impulsivity, memory, gait, motor coordination, tremor, motor control, endogenous nociceptive response, paroxysmal hemiplegias, diplegias, dystonias, SRS, and predisposition to kindling, to flurothyl induced seizures, and to sudden unexpected death. Hippocampal slices of mutants, in contrast to WT animals, showed hyperexcitable responses to pulse-trains of electrical stimuli delivered to the Schaffer collaterals. Our model, thus, reproduces the major characteristics of human AHC, and indicates that ATP1 α 3 dysfunction results in abnormal short-term plasticity with increased excitability. This model should help in understanding the underlying molecular pathways of AHC and may also help in identification of novel therapeutic strategies and agents.

Mechanisms of Na⁺/K⁺-ATPase pump dysfunction caused by mutations underlying the neurological disorders AHC and RDP*Natascia Vedovato and David C. Gadsby*

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Distinct mutations of neuron-specific $\alpha 3$ Na⁺/K⁺-ATPase, most arising de novo, cause Alternating Hemiplegia of Childhood (AHC) and Rapid-onset Dystonia-Parkinsonism (RDP, or DYT12). AHC has more severe symptoms that extend to developmental and cognitive defects. To examine whether the two sets of mutations alter Na⁺/K⁺-pump function differently, we studied, one at a time, the six most prevalent AHC mutations and six RDP mutations in relatively ouabain resistant *Xenopus* $\alpha 1/\beta 3$ Na⁺/K⁺-pumps expressed in *Xenopus* oocytes. We selected AHC mutants: D813N (*Xenopus* $\alpha 1$ numbering; corresponding to human $\alpha 3$ D801N), E827K (E815K), G959R (G947R), S823P (S811P), as well as I286N (I274N) and D935Y (D923Y) which, like D813N, involve an amino acid also mutated in RDP. Selected RDP mutations were: I286T (I274T), D813Y (D813Y), and D935N (D923N), plus T625M (T613M), I770S (I758S), F792L (F780L). We tested their influence on outward pump current during 3Na⁺/2K⁺ exchange, as well as on inward proton current generated by the same pump population. As the mutated amino acids are conserved in all α isoforms of all species, disease-linked dysfunction is expected to be evident in these $\alpha 1$ isoform mutants. We found a clear distinction between effects of the most prevalent AHC mutation, D813N, and those of a severe RDP mutation, D935N: D813N abolished Na⁺/K⁺ exchange while sparing proton import, whereas D935N somewhat diminished Na⁺/K⁺ exchange but essentially abolished proton import. Ongoing analyses of the two selected mutation sets partly concur with this pattern, but with notable exceptions. Thus, in AHC mutants I286N, G959R, and S823P, as in D813N, Na⁺/K⁺ exchange is practically abolished, but proton import appears almost unchanged. Also, in RDP mutant I286T, as in D935N, Na⁺/K⁺ exchange is somewhat impaired (though to a smaller extent than in AHC mutants), and proton current diminished. Diverging results from those of D813N include apparent unaltered Na⁺/K⁺-pump function in E827K (though expression levels seemed reduced), and diminished, but not abolished, Na⁺/K⁺ exchange and proton import for D935Y. Similarly, RDP mutant effects unlike those of D935N include absence of Na⁺/K⁺ exchange, without diminution of proton current, for T625M, D813Y, and possibly I770S (which, like F792L, requires further analysis).

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Functional consequences of mutations in ATP1A3 causing alternating hemiplegia of childhood

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keywords: characterization ATPase-activity, expression, mutants, phenotype, biochemistry

Since 2012, de novo mutations in ATP1A3, the gene encoding the $\alpha 3$ -subunit of Na^+, K^+ -ATPase, are associated with the neurodevelopmental disorder Alternating Hemiplegia of Childhood (AHC). Although knowledge about the affected gene is paramount, the next step in understanding the mechanism-of-disease of these mutations requires biochemical characterization of Na^+, K^+ -ATPase containing these mutations. In this study, we have studied the functional consequences of 14 AHC-associated mutations: S137Y, D220N, I274N, S772R, G775C, D801N, I810S, S811P, E815K, C927Y, D923N, G947R, A955D, and D992Y. We have looked at ATPase activity, phosphorylation, ouabain binding, and ouabain- K^+ antagonism parameters using purified membrane fractions from baculovirus-infected insect cells expressing the above-mentioned mutant enzymes. In short, our results indicate that the tested mutants can be divided into four different groups: mutants that (I) possess characteristics similar to wild type enzyme, (II) show a decrease in ATPase activity, (III) do not possess ATPase activity but are still able to bind ouabain, and (IV) show neither ATPase activity nor ouabain binding. Together, these findings might help understand how the variation in symptoms observed within patients is explained at the molecular level.

Proton transport function of ATP1A3 correlates with clinical severity in AHC*Steven Petrou, Melody Li*

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keywords: ATP1A3, AHC, biophysics, sodium pump, oocytes

Variation in AHC clinical presentation is common, including duration and frequency of hemiplegia, extent of intellectual disability and presence of co-morbidities such as seizure and respiratory paralysis. In 2012, mutations in the ATP1A3 gene were identified as the primary cause of AHC and recently a clear genotype-phenotype relationship has emerged. The mutation D801N was found in mild-moderate AHC cases, whereas the mutation E815K was associated with severe AHC cases. In this study we hypothesised that biophysical changes caused by individual mutations underlie the clinical variation observed in AHC. ATP1A3 encodes for the human sodium pump that was studied in the *Xenopus* oocyte expression system. We examined three biophysical properties of three disease spectrum-spanning mutations. The first biophysical property was forward cycling, which describes the exchange of potassium and sodium, the major role of the pump in neurons and cells. The second was the dominant negativity of mutations, where mutant sodium pump subunits may interfere with wild type sodium pump function. The third was that mutations may alter the proton transport function of the sodium pump. These experiments aim to not only explore pathological mechanisms in AHC but also to find a biophysical correlate of severity of clinical presentation in AHC.

Our analysis revealed that AHC mutations all result in a profound loss of forward cycling function consistent with earlier reports. Co-expression of AHC mutant alpha subunits decreased wild type sodium pump function raising the possibility that a novel dominant negative mechanism contributes to pathology. Proton transport function was assessed and a correlation was found with disease severity. The lowest amount of proton transport was observed in the most severe AHC mutant. Our working hypothesis is that mutations that cause AHC are clearly linked to major loss of pump function together with a dominant negative effect producing an effect beyond that of haploinsufficiency. Within AHC there is a clear association with severity and degree of proton pump function and further investigation is needed to understand how this may contribute to clinical severity and whether this has implications for therapy.

Impaired Cell Surface Expression of ATP1A3 Mutations Associated with Alternating Hemiplegia of Childhood

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keywords: ATP1A3 mutation trafficking, high throughput screening

Mutations in ATP1A3 have been identified as the genetic cause of alternating hemiplegia of childhood (AHC). Loss of Na⁺/K⁺-ATPase activity has been proposed as the likely functional consequence of mutations, but specific mechanisms have not been demonstrated. We investigated the hypothesis that impaired plasma membrane expression may contribute to loss-of-function. We performed experiments designed to ascertain the functional and biochemical consequences of the three most common ATP1A3 mutations (D801N, E815K, G947R) associated with AHC. For functional studies, we adapted a fluorescence-based thallium uptake assay system to monitor time-dependent uptake into HEK-293 cells stably expressing either wildtype (WT) or AHC-associated mutant alleles of ATP1A3. A trivalent FLAG epitope was added to the distal carboxyl terminus to enable biochemical studies. Thallium uptake assays demonstrated greatly reduced function for the three AHC-associated mutations compared with WT-ATP1A3. To determine plasma membrane expression levels, we employed cell surface biotinylation coupled with immunoblotting. We found heterogeneity among the four ATP1A3 alleles with WT and D801N exhibiting similar levels of cell surface expression, and both E815K and G947R exhibiting greatly reduced plasma membrane expression without greatly reduced total cellular protein levels. These findings suggested that E815K and G947R may have impaired protein trafficking to surface membranes. We hypothesized that small molecules might exist, which could correct impaired trafficking and restore cell surface expression of mutant pumps. As a pilot study, we performed an initial screen of 2,729 drugs and drug-like compounds to find agents capable of restoring transporter activity (assayed by thallium uptake) and plasma membrane expression for E815K and G947R. From this screen, we identified four compounds with activity in a fluorescence-based thallium uptake assay system. In subsequent validation experiments using biochemical techniques (e.g., cell surface biotinylation coupled with western blot analysis), at least two of the compounds show some level of rescue of plasma membrane expression. Additional experiments are underway to further characterize these effects. We conclude that reduced cell surface expression may contribute to ATP1A3 loss-of-function in AHC.

Role of the $\alpha 2$ isoform of the Na^+/K^+ -ATPase in Blood Pressure Regulation and Heart Function

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The Na^+/K^+ -ATPase is present in almost all cells and plays an important role in transporting Na^+ and K^+ across the cell membrane and as well as playing a signaling role. There are four isoforms of the catalytic α subunit; $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 4$, as well as two β subunits, $\beta 1$ and $\beta 2$. The Na^+/K^+ -ATPase is also associated with FYFD proteins, which regulate its activity.

There has been a major interest in understanding the specific role of each of the α isoforms as their tissue distribution and expression during development differ significantly. For example, the major isoform in the heart is $\alpha 1$ while $\alpha 2$ is a minor isoform. Nevertheless, this isoform appears to play a role in this organ. In the vascular system, $\alpha 1$ is also the major isoform with $\alpha 2$ and $\alpha 3$ being relatively minor isoforms.

Our laboratory has studied the role of the various isoforms in a number of tissues, but has recently been concentrating on the $\alpha 2$ isoform in the heart and the vascular system. These studies raise issues relating to the tissue/cells involved in blood pressure regulation and the role that $\alpha 2$ plays in cardiac function. While it appears that the $\alpha 2$ isoform plays a role in both tissues, the data are somewhat mixed and not completely understood. Experiments addressing these issues will be discussed.

Co-morbid Psychiatric Manifestations Couple to Glutamate Defects in a Familial Hemiplegic Migraine type 2-mutation Mouse Model***Karin Lykke-Hartmann***

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Migraine headache represents one of the most frequent pain disorders, affecting millions of people worldwide. Familial Hemiplegic Migraine type 2 (FHM2) is a severe subtype of migraine with aura and co-morbidities like epilepsy/seizures, cognitive impairments and psychiatric manifestations, which also shares similarities with common migraine. FHM2 disease-mutations locate to the *ATP1A2* gene encoding the astrocyte-located α_2 -isoform of the sodium-potassium pump ($\alpha_2\text{Na}^+/\text{K}^+$ -ATPase). To functionally assess the FHM2-causing G301R mutation, we created and analyzed an *Atp1a2* G301R knock-in mouse. Here we show that mice heterozygous for the FHM2-associated G301R-mutation ($\alpha_2^{+/G301R}$) phenocopy FHM2 by mimicking migraine, social phobia and depression. Female-specific $\alpha_2^{+/G301R}$ phenotypes coupled to the female sex hormone cycle and were rescued by a progestin-only contraceptive treatment. *in vitro* assays showed that impaired glutamate uptake function coupled to the G301R-mutation. Induction of cortical spreading depression resulted in reduced K^+ -clearance in $\alpha_2^{+/G301R}$ mice, revealing that astrocytic $\alpha_2\text{Na}^+/\text{K}^+$ -ATPase actively contributes to this process. Our data demonstrate that psychiatric manifestations are parts of the FHM2-pathology and link the $\alpha_2\text{Na}^+/\text{K}^+$ -ATPase to the glutamate system, with the female sex hormone cycle exerting aggravating effects hereon.

Somatic mutations in Na⁺,K⁺-ATPase and plasma membrane Ca²⁺-ATPase lead to aldosterone-producing adenomas (Conn's syndrome).***Vivien Schack***

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While mutations in the alpha2- and alpha3-isoforms of Na⁺,K⁺-ATPase have been known since 2004 to cause neurological disorders, no disease was associated to mutations in the ubiquitous alpha1-isoform of the pump, until our recent report¹. Conn's syndrome is characterized by hypertension and muscle weakness caused by adrenal hypersecretion of aldosterone leading to renal sodium retention and increased excretion of potassium. We identify somatic mutations in Na⁺,K⁺-ATPase alpha1 of adrenal glomerulosa cells as a cause of Conn's syndrome. In a collection of 308 aldosterone producing adenomas (APAs), we found 16 (5.2%) mutations in Na⁺,K⁺-ATPase and 5 (1.6%) in plasma membrane (PM) Ca²⁺-ATPase. In our subsequent analysis of 112 APAs, we identified additional 7 (6.3%) with mutations in Na⁺,K⁺-ATPase, including one novel mutation, Gly99Arg². Intriguingly, in both P-type ion pumps the APA mutations involve the ion binding region. Our analysis of cell culture expressed mutant Na⁺,K⁺-ATPase shows that the APA mutations reduce the affinity for K⁺ 50- to 500-fold. This is a consequence of replacing residues in the interaction sphere of the K⁺ binding glutamate in transmembrane segment TM4. A leucine in TM1 is found replaced with arginine in several of the APAs. This leucine is a gatekeeper that helps closing the gate to the K⁺ binding pocket in the K⁺ occluded state of the Na⁺,K⁺-pump³. The APA mutations in the PM Ca²⁺-ATPase affect the homologous Ca²⁺ occlusion mechanism. The recurrence of mutations affecting conserved regions involved in interaction with the transported cations in two paralogs is suggestive of a gain-of-function effect. The APA mutations of the PM Ca²⁺-ATPase lead to intracellular accumulation of Ca²⁺ in the glomerulosa cells, which is also the ultimate consequence of the APA mutations of the Na⁺,K⁺-pump, due to membrane depolarization and inhibition of the Na⁺/Ca²⁺ exchange protein, thus converging into a common mechanism explaining the aldosterone hypersecretion.

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New aspects of Na⁺,K⁺-ATPase structure and mechanism in health and neurological disease***Bente Vilsen***

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Mutations in the $\alpha 2$ - and $\alpha 3$ -isoforms of Na⁺/K⁺-ATPase have been found associated with the neurological disorders familial hemiplegic migraine type-2 (FHM2) and rapid-onset dystonia parkinsonism (RDP)/alternating hemiplegia of childhood (AHC), respectively. Several of the neurological disease mutations have been found to affect specifically the Na⁺ specific site III without disturbing K⁺ binding (1, 2). The recent high resolution crystal structure of the Na⁺/K⁺-ATPase in the E1 form with bound Na⁺ (3) provides a sound basis for interpreting mutagenesis studies directed at Na⁺ site III. This site consists of residues Tyr771, Thr772, Thr774, Ser775, Asp808, Gln923, and Asp926 (pig $\alpha 1$ numbering). The docking of the C-terminus into the transmembrane domain near site III works as a brake or damper that prevents excessive movements of the M5 helix, thereby stabilizing site III (3). Therefore, mutations affecting the disposition of the C-terminus reduce Na⁺ affinity. Replacement of Asp926 in site III with asparagine causes the most conspicuous reduction of Na⁺ affinity seen so far for a neurological disease mutation (1) and a 3-fold increase of intracellular Na⁺ in cell culture studies (2), resulting in RDP/AHC. We now report that the Na⁺ affinity of Asp926 mutants can be rescued by secondary mutation of a glutamate located far away from Na⁺ site III, thus implying long-range communication and demonstrating that Asp926 is not indispensable to Na⁺ binding. The rescuing gain-of-function mutation represents a new principle for improving Na⁺/K⁺-ATPase function in neurological disease states with reduced Na⁺ affinity.

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Mutation in the α_2 isoform of Na^+, K^+ -ATPase associated with Familial Hemiplegic Migraine type 2 (FHM2) leads to elevated contractility and vasodilatation of cerebral arteries in mice***Vladimir Matchkov***

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The vasogenic theory of migraine suggests that the aura (a phase preceding the headache and characterized by visual, sensory and/or motor disturbances) is associated with vasoconstriction-induced hypoxemia in the brain, while the subsequent headache is caused by a rebound vasodilation. FHM 2 is associated with few point mutations in the α_2 isoform Na^+, K^+ -ATPase. Mice bearing a mutation corresponding to the inherited mutation in FHM2 patients (G301R) were used in functional studies of middle cerebral arteries.

Middle cerebral arteries from heterozygote G301R mice were not different in total α_2 Na^+, K^+ -ATPase mRNA in comparison with WT, but $51 \pm 11\%$ of their mRNA contained G301R mutation. G301R mice had elevated blood pressure and unchanged heart rate. Inner diameter of cerebral arteries from G301R mice was significantly larger than in WT. G301R arteries were more sensitive and had higher maximal contraction to U46619, endothelin and K^+ -depolarization. This was associated with increased depolarization and sensitization to $[\text{Ca}^{2+}]_i$ (in spite of reduced Ca^{2+} influx) in G301R arteries. pNaKtide, a peptide inhibiting the Na^+, K^+ -ATPase-dependent Src activation, abolished differences between G301R and WT mice. In the presence of pNaKtide middle cerebral arteries from G301R and WT mice responded similarly by force, $[\text{Ca}^{2+}]_i$ and membrane potential to agonist stimulation.

G301R middle cerebral arteries have pronounced relaxation to elevated $[\text{K}^+]_{\text{out}}$ in comparison with WT. This was associated with increased hyperpolarization of smooth muscles which was blocked by $30 \mu\text{M}$ BaCl_2 . BaCl_2 abolished the difference in relaxations between the groups. G301R arteries had an elevated mRNA of inward rectifying K^+ channels, $190 \pm 32\%$ of the WT.

In accordance with the vasogenic theory of migraine FHM2 associated mutation of the α_2 Na^+, K^+ -ATPase leads to both elevated contractility and increased relaxation of cerebral arteries. These abnormalities are, at least in part, mediated via disturbances in Src-kinase/ Na^+, K^+ -ATPase complex.

Isoform-Specific Role of Secretory Pathway Ca^{2+} -ATPase 2 (SPCA2) in mammary physiology and breast cancer

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There are two isoforms of Secretory Pathway Ca^{2+} -ATPases in tetrapods, including mammals. SPCA1 is expressed ubiquitously and has an essential, housekeeping function in providing Ca^{2+} and Mn^{2+} to the Golgi/vesicular compartments for protein processing, sorting, modification and quality control. Although SPCA2 shares similar transport characteristics, it has a restricted tissue expression and is highly induced in the mammary gland upon lactation. We show that elevation of SPCA2 in mammary cancers is associated with decreased survival, increased cell proliferation and tumorigenesis. These properties are derived from the ability of SPCA2 to elicit store-independent Ca^{2+} entry (SICE) by interacting with cell membrane Ca^{2+} channels, including Orai1. In lactating mammary glands, SICE contributes to the transepithelial transport of Ca^{2+} from blood to milk, where it accumulates in high concentrations. We are also investigating a chaperone-like function for SPCA2 in trafficking of cell membrane proteins, including E-cadherin that may play a role in epithelial-mesenchymal transitions in cancer. Thus, although expressed redundantly with SPCA1, SPCA2 has unique, isoform-specific functions in mammary physiology.

A sodium-mediated feedback loop that regulates EGFR trafficking***Sigrid Langhans***

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EGF regulates cell proliferation and/or migration by triggering molecular cascades that regulate cytoskeletal reorganization, cell cycle progression and gene expression. Endocytosis of activated EGF receptor (EGFR) to specific endocytic compartments is required to terminate EGF signaling. Trafficking of EGFR relies on microtubule tracks that transport the cargo vesicle to their intermediate and final destinations and can be modulated through posttranslational modification of tubulin. Recent studies show that acetylation of alpha-tubulin regulates intracellular cargo transport including transport of EGFR-containing vesicles. Interestingly, Na^+, K^+ -ATPase binds to and is regulated by acetylated tubulin and may function as an anchorage site for microtubules. On the other hand, EGF can trigger sodium influx upon receptor binding. Thus, we sought to better understand the relationship between Na^+, K^+ -ATPase, acetylated tubulin and ligand-activated EGFR signaling. We now show that EGF-induced sodium influx regulates EGFR trafficking through increased microtubule acetylation to modulate the speed of EGF-containing vesicles, downregulate EGFR levels and terminate EGFR signaling. Increased sodium influx induced either by sodium ionophores or Na^+, K^+ -ATPase blockade by cardiac glycosides mimicked the EGF-induced effects on EGFR trafficking through histone deacetylase (HDAC) 6 inactivation and accumulation of acetylated tubulin. In turn, blocking sodium influx reduced tubulin acetylation and EGF-induced EGFR turnover. Knockdown of HDAC6 reversed the effect of sodium influx indicating that HDAC6 is necessary to modulate sodium-dependent tubulin acetylation. These data suggest a novel pathway in which EGF modulates EGFR trafficking through sodium influx, leading to HDAC6 inactivation and tubulin acetylation. We suggest that an increase in intracellular sodium is a component of an auto-regulatory loop that regulates termination of EGFR signaling through trafficking of the EGF-EGFR complex and includes HDAC6 and tubulin acetylation.

The role of NKA in health and disease, Na⁺,K⁺-ATPase, signalling function and specific role of neuronal ATP1A3**Anita Aperia**

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It is now well recognized that Na⁺,K⁺-ATPase has a signaling function, that is triggered by the binding of non-saturating concentrations of ouabain and other cardiotonic steroids (CTS) to the α subunit. Signaling pathways can involve intracellular calcium oscillations, Src phosphorylation and activation of PI3 kinase and Akt. Our group has focused on the calcium pathway. We have shown that ouabain bound Na⁺,K⁺-ATPase interacts with the inositol-1,4,5-phosphate receptor, which functions as a center for signaling cascades. Using COS cells and primary rat renal proximal tubule cells, we have demonstrated that non-saturating concentrations of ouabain and other CTS trigger highly regular intracellular calcium transients with a frequency of approximately 7 mHz. Each cytosolic calcium wave is followed by a delayed mitochondrial calcium transient, which is similar in amplitude, but slower in recovery than the cytosolic wave. Importantly, one of the read-out of this signal is protection from apoptosis via the mitochondrial pathway. In cells challenged with two apoptotic triggers, shigatoxin and excessive albumin uptake, we have demonstrated that nM concentrations of ouabain rescues from apoptosis by preventing accumulation of the apoptotic factor Bax on the mitochondrial membrane, by preventing mitochondrial membrane depolarization and by restoration of cellular levels of the anti-apoptotic factor Bcl-xL. Taken together, these results suggest that CTS triggered Na⁺,K⁺-ATPase signaling creates a feed-back loop to the mitochondria to preserve life.

For a long time Na⁺,K⁺-ATPase has been considered as an almost inert housekeeping enzyme in the brain. From what we know today, it seems likely that we are just in the beginning of an era when a role for Na⁺,K⁺-ATPase dysfunction in a variety of neurological and neurodegenerative diseases will be revealed. The severe consequences of ATP1A3 mutations have led to the conclusion that the α 3 isoform is required for normal neuronal function. Neurons must cope with transient increases in Na_i from approximately 10 to 40 mM during periods of high synaptic activity, and only α 3, which has an approximately 3-fold higher K_m than the α 1 subunit, will have the capacity to rapidly restore such an increase in Na_i. We have in ongoing studies identified an attenuated capacity to deal with large increases in Na_i in three of the RDP mutants. -The majority of neuronal ATP1A3 molecules are in lateral diffusion in the plasma membrane, but they tend to cluster in excitatory synapses. Mobility can be attenuated by extra-cellular interaction with other molecules, which may have both physiological and pathological consequences.

Mutations of ATP1A3: consequences in the realms of atoms, cells, mice, and humans***Kathleen J. Sweadner***

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Mutations of ATP1A3 in the neurological diseases AHC, RDP, and CAPOS are not randomly distributed in the $\alpha 3$ subunit protein. A preponderance of the most clinically severe mutations are buried in the membrane, in contrast to the distribution of mutations in ATP1A2 $\alpha 2$ subunit causing FHM and related syndromes. Molecular modeling of ATP1A3 mutations that are buried near the ion binding sites suggest a principal effect on the unwound α -helical kinks that accommodate occluded ions. An important question is whether destabilization of the transmembrane spans leads to distinct cellular and clinical outcomes. We have examined three novel de novo mutations in the P-domain, where few AHC or RDP mutations are found. They produced different human phenotypes: a fatal infantile epilepsy, a case with apnea and developmental delay, and an adult with cerebellar atrophy. The clinical severity was related to the activity of Na^+, K^+ -ATPase expressed in cells. The $\alpha 3$ subunit of Na^+, K^+ -ATPase is found in a majority of neurons, with or without co-expression of $\alpha 1$. Several lines of evidence point to inhibitory neurons for a particularly abundant level of $\alpha 3$. Neuropathology was detected in RDP and correlated with the expression of high $\alpha 3$ and low $\alpha 1$ in corresponding neurons in mice. The presence of $\alpha 1$ may be protective, partially offsetting reduced Na^+, K^+ -ATPase activity that affects electrophysiological parameters. Two lines of mutant mice were studied, one with knockout of one copy of *Atp1a3*, and the other with the D801Y mutation. The heterozygote knockout showed vulnerability and aberrant motor output during stress, with a relatively minor reduction in total Na^+, K^+ -ATPase activity, but an increase in $\alpha 1$. The heterozygote knock-in mutation had a larger reduction in activity, and showed a disparate combination of symptoms: both hypotonia and hyperactivity at baseline; Rotarod performance well above WT; hyperactive response to swim stress and to ketamine; and aberrant motor output after swim stress and ketamine. The functional phenotypes show a diversity that underscores the expanding phenotype spectrum in humans.

Adaptor protein complexes 1 and 2 (AP-1, AP-2) mediate anterograde and retrograde neuronal trafficking of the copper transporter ATP7A

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ATP7A is a P-type ATPase in which diverse mutations lead to X-linked recessive Menkes disease or occipital horn syndrome. Recently, two previously unknown ATP7A missense mutations, T994I and P1386S, were shown to cause an isolated adult-onset distal motor neuropathy without clinical or biochemical features of the other ATP7A disorders. These mutant alleles cause subtle defects in ATP7A intracellular trafficking, resulting in a larger pool at the plasma membrane compared to wild-type ATP7A, which localizes mostly in the trans-Golgi network. We reported earlier that ATP7A-T994I interacts abnormally with p97/VCP, a protein implicated in two other inherited motor neuropathies. We also demonstrated that ATP7A-P1386S causes unstable insertion of the 8th and final transmembrane segment, thus destabilizing the ATP7A C-terminal tail in at least a proportion of the mutant molecules. We exploited this mutant allele to evaluate the mechanism(s) of normal ATP7A trafficking and demonstrated that the adaptor protein complexes 1 and 2 (AP-1, AP-2) of the clathrin-coated-vesicle traffic machinery each physically interact with ATP7A. Mutagenesis experiments indicate that the ATP7A carboxyl-terminal dileucine motif (L1477L1478) is necessary for this interaction. The ATP7A-P1386S mutation disturbs interactions with the adaptor protein complexes and leads to abnormal axonal localization in NSC34 motor neurons, in distinction from the somatodendritic localization of wild type ATP7A at basal copper concentrations. Taken together, these results suggested that AP-1 normally tethers ATP7A at the trans-Golgi network in the somatodendritic segment of motor neurons. ATP7A-P1386S alters the stability of the carboxyl-terminal tail, leading to disruption of this interaction and release of ATP7A to the axons or axonal membranes. We hypothesize that these latter effects are intensified by loss of interaction with AP-2, impeding retrograde trafficking of ATP7A-P1386S to the somatodendritic region, and disturbing ATP7A-mediated copper transport in polarized motor neurons. We also documented altered calcium signaling upon glutamate stimulation in ATP7A-P1386S transfected NSC34 motor neurons. All these new findings further illuminate the normal molecular mechanisms of ATP7A trafficking, and suggest a possible pathophysiological basis for ATP7A-related distal motor neuropathy.

Isoform-specific roles of the Na⁺,K⁺-ATPase alpha subunits in skeletal muscle

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Adult skeletal muscles express two isoforms of the catalytic alpha subunit of Na⁺,K⁺-ATPase. α 1 is the minor isoform, while α 2 comprises up to 90% of total alpha subunit. Interestingly, despite a high density of mature α 2 isozyme in the membrane, the α 2 isozyme was found to be largely inactive in resting skeletal muscles of rats and mice. Basal α 2 activity, measured as its ouabain-sensitive electrogenic contribution to the resting potential, provides only about 25% of the transport activity needed to maintain resting ion homeostasis. Moreover, as shown by other laboratories, basal Na⁺,K⁺-ATPase activity represents less than 5% of the total capacity of skeletal muscles for active Na/K transport. To search for other possible roles of α 2, we developed a genetically altered mouse model having a skeletal muscle-targeted deletion of the α 2 subunit (*sk α 2*^{-/-}). We found that α 2 activity is rapidly recruited in contracting muscle and that its contraction-related transport is absolutely required to maintain membrane excitation and resistance to fatigue. Therefore, the skeletal muscles use these two isoforms, each with different capacities and modes of regulation, to handle their wide demand for Na⁺/K⁺ transport. α 1 activity provides most of the “housekeeping” transport to maintain basal ion homeostasis, while α 2 functions as a “turbocharger” that can be rapidly recruited to meet the greatly increased demand of contracting muscles for Na⁺/K⁺ transport. A key question is: what are the molecular mechanisms by which the activity of α 2 is inhibited in resting muscle, and recruited rapidly upon the onset of muscle activity. Because the basic enzymatic properties of the alpha isozymes do not show large differences when studied in cultured cells or expression systems, we focused on their physiological context in vivo. Ongoing studies examine their subcellular localization, interactions with FXD1 and beta subunits and other protein partners, regulation by FXD1 phosphorylation, and substrate affinities. These questions are being addressed using western blot, co-IP, immunohistochemistry, measurements of isozyme activity in vivo and in vitro using ATPase assay, ICP-MS, and recordings of pump currents from isolated single muscle fibers.

Supported by the National Institutes of Health, USA

Characterization of the ATP1A3 mutation causing CAPOS

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keywords: ATP1A3, Na K ATPase, CAPOS, electrophysiology

In addition to AHC and RDP, the extremely rare neurological syndrome CAPOS (Cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss) was recently added to the list of diseases caused by mutations in ATP1A3. All patients characterized share an E818K substitution. Using exogenous expression of the human alpha3 E818K in *Xenopus* oocytes, we have examined the functional consequences of the mutation. We observe electrophysiological differences between the CAPOS and AHC mutations, which are likely to be the underlying cause of the different pathophysiological consequences.

Poster abstracts

#1 (sub 1)

Behavioral and electrophysiological analyses of Atp1a3 knockout mice and implication for pathophysiology of dystonia

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keywords: knockout mice, dystonia, basal ganglia. behavior, electrophysiological analysis

ATP1A3 is the causative gene for rapid-onset dystonia parkinsonism (RDP) and alternating hemiplegia of childhood (AHC). Many of the mutations found in ATP1A3 of patients are substitution mutations and most of the positions of mutations are distinct between RDP and AHC. They are mostly loss of function mutations and lead to decreased activity of Na⁺,K⁺-ATPase. We previously established Atp1a3 gene deficient mice Atp1a3^{+/-} and found that they showed increased symptoms of dystonia when being administered kainate into cerebellum and that the inhibitory neurotransmission from molecular-layer interneuron to Purkinje cells in the developing cerebellum was enhanced (Ikeda et al., J. Physiol., 2013). To evaluate whether these mice are suitable model animal for RDP, we performed behavioural and electrophysiological analyses. We found that the Atp1a3^{+/-} mice exhibited shorter stride length at 4 weeks of age and shorter stride was persistently observed in chronically-stressed condition. Shorter hanging time in the hanging box test was also observed after chronic restraint stress in Atp1a3^{+/-}. To explore the cellular bases for dystonia symptoms, we examined neuronal activity of the basal ganglia, especially internal (GPi) and external (GPe) segments of the globus pallidus, under awake condition. The spontaneous discharge rates of GPi neurons were significantly lower in Atp1a3^{+/-} compared with wild type, while that of GPe was not significantly different. In the wild-type mice, cortical stimulation evoked triphasic response composed of early excitation, followed by inhibition and late excitation in the GPi and GPe. In the GPi of Atp1a3^{+/-}, pronounced early excitation with single or double peaks followed by strong inhibition was observed. In the GPe of Atp1a3^{+/-}, the excitation was followed by sustained excitation. Because these changes in the GPi are similar to those observed in the model mice of DYT1 dystonia (Chiken et al. J Neurosci, 2008), it is possible that electrophysiological changes in the basal ganglia and the cerebellum (Ikeda et al., J. Physiol., 2013) are the basis for the symptoms of dystonia in Atp1a3^{+/-}.

Poster abstracts

#2 (sub 4)

A road-map from genetic studies to RDP- and AHC- understanding and treatment

Martin Kubala

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keywords: rapid-onset dystonia parkinsonism, alternating hemiplegia of childhood, statistics

Abstract:

Rapid-onset dystonia parkinsonism (RDP) and alternating hemiplegia of childhood (AHC) are associated to mutations in the ATP1A3 gene. However, the mechanism of how ATP1A3 mutations result in clinical manifestation of RDP or AHC is unknown. Moreover, low number of identified patients presents a serious limitation to performance of clinical trials.

Deep understanding to the whole process should precede the clinical trials, because an unsuccessful clinical test would hinder further research in the field for a long period. In order to achieve it, transfer of knowledge among experts from genetics, structural biology, cell biology and physiology is needed, with high impact on transfer-reliability.

The contribution will present a road-map for a rational way from laboratory experiments to successful clinical trial with identification of convergent and divergent points as well as missing information.

Acknowledgement:

Support by the grant LO1204 from the National Program of Sustainability I, and grant OP VK CZ.1.07/2.3.00/20.0057 from Czech Ministry of Schools, Youth and Sports is acknowledged.

Poster abstracts

#3 (sub 5)

Probing structural consequences of ATP1A3 mutations with molecular simulations

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keywords: Molecular Dynamics Simulations, Na K-ATPase, AHC, Protein dynamics

Despite growing experimental data, the structural effects of ATP1A3 disease-causing mutations remain elusive and difficult to study. On the other hand, molecular simulations provide fast and reliable insight into the protein 3D structure and dynamics that eventually define their function, down to the single atom resolution, which is typically not accessible by the majority of experimental methods.

Here, we present molecular dynamics simulations of Na⁺,K⁺-ATPase as a tool for studying an impact of disease causing mutations on the behavior of the protein and its local environment. We illustrate the concept with recently found ATP1A1 mutations characteristic for adrenal hypertension, for which simulations are in excellent agreement with electrophysiology measurements and describe the observed protein malfunction on the molecular level. Furthermore, we present preliminary simulation data on three ATP1A3 mutations that cause AHC, namely D804N, E818K and G950R. We believe that knowing molecular mechanisms underlying the Na⁺,K⁺-ATPase impairment would ultimately lead to better treatments for ATP1A3 related diseases.

Poster abstracts

#4 (sub 6)

A novel mutation in ATP1A3 gene in a patient with RDP and some similarities with AHC

Alessandro Capuano, Barbara Garavaglia, Federico Vigevano, Nardo Nardocci

Bambino Gesù' Children Hospital, Rome, Italy

keywords: Rapid onset dystonia parkinsonism, Alternating hemiplegia of Childhood. new mutation ATP1A3 gene

Introduction:

Rapid-onset dystonia parkinsonism (RDP) is a rare condition of adolescence and young adulthood characterized by abrupt onset of dystonia within hours to weeks with associated parkinsonism features. None of known mutations described in AHC was reported in RDP suggesting that the two diseases are allelic variants; nevertheless patients presenting an overlapping of symptoms have been described.

Case report:

A 13 years old female was referred to our Center presenting progressive ideomotor slowdown, apathetic state, ideomotor slowdown, facial hypomimia with sialorrhoea, bradykinesia, rigidity and dystonic postures. Symptoms established in two hours, worsening in the first 24 hours, and then remained stable in the next days. The hallmarks of neurological exams were: gait abnormalities characterized by dystonic postures of the feet, reducing arm swing movements; normal oculomotor movement; facial hypomimia and minor difficulties in swallowing with subsequently hypersalivation; difficulties in tongue protrusion, slurred speech, dystonic postures of both hands, increased muscular tone, no pyramidal signs were detected. Routine blood tests including plasma lactate and ammonia, routine tests of blood coagulation, screening of metabolic defects as well as autoimmunity tests were in the normal range. Brain MRI was performed showing a mild enlargement of left temporal horn. EEG recording was normal. Family history was negative for major neurological diseases. The patient was born at term, from an uneventful pregnancy. When she was 3 months she presented focal clonic seizures involving the right side of the body. EEG confirmed epileptic activity in the left hemisphere, brain MRI was normal. Seizures were well controlled by phenobarbital and carbamazepine as add-on therapy. In the first year of life she also presented paroxysmal episodes characterized by generalized weakness, eye deviation, hyperextension of the neck (dystonic?), hypotonia in right hemisome. These episodes could last many hours. After first year of life these episodes disappeared. Genetic analysis of ATP1A3 gene revealed a sporadic mutation in a high pathogenic site of the protein structure c. 1747G>T p.Asp583Tyr. L-Dopa was ineffective, lorazepam 1 mg tid was effective.

Conclusions:

Our case with a novel mutation expands the phenotypical spectrum of the ATP1A3 gene.

Poster abstracts

#5 (sub 13)

AHC Federation of Europe

Sigurður Jóhannesson

AHCFE, Reykjavik, Iceland

keywords: AHC Federation of Europe, Alternating Hemiplegia of Childhood, AHC documentary

The poster explains the role of AHC Federation of Europe and current projects.

The poster will identify 4 items:

1. The purpose of AHC Federation of Europe
2. Members of the Federation
3. The ATP1A3 Symposium in Lunteren
4. AHC documentary

Poster abstracts

#6 (sub 17)

Functional consequences of Rapid onset Dystonia Parkinsonism-associated NKAalpha3 mutations for membrane expression and sodium homeostasis in primary hippocampal neurons

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keywords: Rapid Onset Dystonia Parkinsonism, Na⁺ K⁺ ATPase alpha3 mutations, Membrane expression, Intracellular Sodium concentration

Rapid-onset Dystonia-Parkinsonism (RDP) is a rare brain disorder arising during childhood, with extremely severe consequences for patients. Whole exome sequencing of patients diagnosed with RDP have revealed 11 single mutations in ATP1A3, a gene encoding the sodium-potassium pump Na⁺,K⁺-ATPase (NKA) alpha 3 subunit (NKAalpha3), which is specifically expressed in neurons in the central nervous system. Our group has previously shown that due to its lower affinity for sodium when compared to the ubiquitous NKAalpha1, NKAalpha3 is particularly well armed to restore intracellular sodium levels after high neuronal activity.

To study the functional impacts of NKAalpha3 mutations, we used primary cultures of hippocampal neurons transiently transfected to express a fluorescently tagged WT-NKAalpha3 or NKAalpha3 carrying one of four mutations associated with RDP (I274T, T613M, F780L, D801Y). To evaluate and compare membrane expression of each of the engineered NKAalpha3, we developed an imaging-based method using calibrated fluorescent beads. We found that all mutation-carrying NKAalpha3, except the ones carrying D801Y, were consistently expressed at the plasma membrane. Total levels of expression were varying and T613M, the most common mutation associated with RDP, displayed levels closest to WT. For these reasons, T613M was chosen for further analysis with sodium imaging experiments using the Na⁺-sensitive dye, Asante Natrium Green-2. Recordings were made of basal intracellular sodium and of rate of sodium recovery after pump activity was stimulated or after NMDA challenges to mimic intense neuronal activity. Sodium levels were analyzed in the soma and dendrites. To correct for inconsistencies in culture conditions, data are expressed as ratio between transfected and un-transfected neurons on the same sample. Expression of T613M led to dramatic changes in baseline Na⁺ levels and pump rate of recovery. Thus, our study shows that the mechanism of RDP development can involve an increase in intracellular sodium concentration and a decrease in the maximum rate of restoration of sodium concentration in neurons, which during severe stress that triggers RDP may alter the pathophysiological state of neurons.

Poster abstracts

#7 (sub 20)

Widening the phenotypic spectrum of ATP1A3 mutations: R756H mutation in a family with developmental delay, and fluctuating ataxia

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keywords: ATP1A3, Ataxia, Motor delay

Mutations in ATP1A3 result in alternating hemiplegia of childhood (AH) and rapid-onset dystonia-parkinsonism (RDP) and CAPOS. Intermediate forms between the two disorders are increasingly recognised and related to mutations in this gene.

We present a new familial intermediate phenotype associated with the R756H mutation, thereby widening the spectrum of ATP1A3-related disorders.

The proband, now in her forties, presented with developmental motor and speech delay: she walked aged 28 months with ataxia, and first spoke aged 24 months. Aged 5 years, after an acute viral illness, she was unable to mobilise and required the use of a wheelchair for 9 months, and developed persistent dysarthria, gait and limb ataxia. Episodes of worsening cerebellar dysfunction were noted, triggered by intercurrent illness, stress, and tiredness. She developed choreoathetoid movements, dystonia of the lower limbs and progressive deterioration of cerebellar dysfunction in her thirties. On examination, she has dysarthria, spasticity in her limbs, globally diminished reflexes with upgoing plantar responses, an ataxic gait and choreoathetoid movements. Eye movements are normal, and there are no features of parkinsonism. MRI shows mild cerebellar atrophy.

The proband's daughter, aged 20 years, presented with motor and speech delay. Aged 2.5 years, after a febrile illness, she was unable to mobilise for several months, and developed permanent limb ataxia. Aged 5 years she had a quadriplegic episode, with dysphagia and dysarthria lasting one month, and developed a persistent cerebellar syndrome worsening with intercurrent illness and stress. On examination, she has normal eye movements, mild dysmetria and dysdiadochokinesia, and a normal gait. She has spasticity in her limbs and had globally reduced reflexes with normal plantar responses. MRI is normal.

Whole-exome sequencing revealed a missense mutation, R756H in the ATP1A3 gene segregating with disease and absent in dbSNP, EVS, and control exome databases. This mutation has been previously reported in a case of infantile RDP with motor delay and ataxia. Our cases reveal an intermediate form between AH and RDP presenting in early childhood with developmental delay, and fluctuating episodes of ataxia, leading to a progressive cerebellar syndrome with age.

Poster abstracts

#8 (sub 21)

The International IAHCRC Consortium for the research on AHC: a successful model for the progress of the research on this rare disease

Rosaria Vavassori, Alexis Arzimanoglou

IAHCRC, Verderio (LC), Italy

keywords: Alternating Hemiplegia of Childhood, AHC, IAHCRC Research Consortium, Genotype-Phenotype Correlations ATP1A3 AHC

The International Consortium for the Research on AHC IAHCRC was formed in 2012 to carry out a collaborative research that led to the identification of the ATP1A3 gene as the main cause of AHC.

The Consortium involves clinicians, geneticists and researchers working at University centers in Europe, USA and Australia; it works in close collaboration with health professionals and patient organizations, most of whom were already implicated in the EU-funded projects "ENRAH for SMEs" (2005-2007) and nEUroped (2008-2011).

In 2013 the Consortium launched a new collaborative study aimed to identify possible correlations between the clinical phenotype associated with AHC and mutations in the ATP1A3 gene, with the goal to investigate whether different mutations could, in part, be responsible for the clinical heterogeneity observed in the disease. The data were collected from the largest international cohort of AHC patients to date (155 patients), and the results of their analysis are now under publication.

Further collaborative studies, clinical, genetic and molecular, are currently in execution by the centers of the Consortium. Each center has the liberty to initiate and/or pilot a study with all or part of the centers members of the Consortium and to develop external collaborations.

The Consortium is a network based on an organizational and IT model developed in compliance to the ENRAH and nEUroped models; its key features are a set of clear rules, included in a charter, for the sharing of the patient data and of the working information regarding the studies carried out by the centers, and a set of common formats and procedures for the data assessment, collection and keeping at the centers, to be usable for all the collaborative studies of the Consortium, current and future.

This model can easily include any new centers in the network, thus allowing a fast and ethic involvement of a larger and larger number of patients, and a fast and efficient sharing of their data for both retrospective and prospective studies; in the future, these could also be used for therapeutic trials, the final aim of all these studies being the development of specific treatments for the AHC patients.

Close collaboration with national patient associations and mixed networks (such as ENRAH) represents a guarantee for the development of a better health and social care for all the affected patients.

Poster abstracts

#9 (sub 22)

Altered motor memory in behaviour and electrophysiological analyses in Atp1a3 heterozygous knockout mice

Kiyoshi Kawakami, Shinichiro Satake, Hiroki Sugimoto, Keiko Ikeda

Jichi Medical University, Yakushiji, Shimotsuke, Japan

keywords: Atp1a3 knock-out mice, rotarod, cerebellum, LTD

The Na⁺-pump alpha3 subunit gene is responsible for rapid-onset dystonia parkinsonism (RDP) and alternating hemiplegia of childhood (AHC). To get insight into the pathophysiology of the diseases, we generated Atp1a3 deficient heterozygous mice (heterozygotes) and analyzed their motor function. When the mice were trained for between P14-P16, the performance of rotarod of wild-type mice (WT) was better compared with that of heterozygotes at P26. The rotarod performance without training at P26 was similar between WT and heterozygotes. The results suggest the existence of different motor memory between WT and the heterozygotes. It is commonly accepted that a persistent change in the efficacy of the synaptic transmission is the basic mechanism underlying learning and memory. The cerebellum is the key structure of the motor function and exhibits a synaptic plasticity called cerebellar long-term depression (LTD). We therefore performed electrophysiological analyses to examine the induction of LTD at the parallel fiber (PF)-PC synapse using the slice preparation of the cerebellum. The induction of LTD was significantly impaired in the heterozygotes. The results suggest the existence of differences of motor memory and function of cerebellar neural circuit in the heterozygotes. We will discuss possible mechanism for the impairment of LTD and implication for RDP/AHC pathophysiology in patients.

Poster abstracts

#10 (sub 23)

Assessing motor and cerebellar function in Na⁺/K⁺-ATPase α 3+/D801Y mice

Toke J. Isaksen, Thomas H. Holm, Karin Lykke-Hartmann

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keywords: ATP1A3, Cerebellum Motor-related behavioural tests

The distinct neurological disorders Dystonia parkinsonism (RDP) and Alternating hemiplegia of childhood (AHC) is caused by mutations in the ATP1A3 gene encoding the α 3 subunit of the Na⁺/K⁺ pump.

Among common symptoms observed are persistent motor and movement deficits, such as ataxia and dystonia. In line with this it has been reported that ouabain-mediated inhibition of the Na⁺/K⁺ pump in the cerebellum of mice resulted in ataxia and dystonia.

This study will functionally assess possible contributions of cerebellum in the pathology of RDP/AHC, using the heterozygous knock-in mouse line α 3+/D801Y harbouring the D801Y in vivo-relevant RDP disease-mutation. Noteworthy, AHC causing disease-mutations includes a mutation in the same residue that is the D801N mutation.

Motor-related behavioural tests, such as balance beam, gait analysis and rotarod is used to assess motor or movement deficits. Additionally, quantitative and qualitative molecular studies of ATP1A3 in cerebellum are being assessed by western blotting and IHC.

Preliminary motor-related behavioural tests show that the α 3+/D801Y mice have substantial motor and movement difficulties. On the balance beam, α 3+/D801Y mice have significant more slips and takes longer time to cross compared to wild type littermates. Altered posture is evident in the α 3+/D801Y mice, and gait analysis indicates that this might include uncoordination of hind limbs.

This study will improve the understanding of RDP/AHC, in particular with regards to cerebellum function. This could lead to better drug treatment of patients suffering from the severe neurological disorders.

Poster abstracts

#11 (sub 24)

Modeling neurological deficits of Alternating Hemiplegia of Childhood in mice

Thomas Holm, Toke J. Isaksen, Simon Glerup, Ernst-Martin Füchtbauer, Anders Heuck, Poul Nissen, Karin Lykke-Hartmann

Aarhus University, Aarhus, Denmark

keywords: Alternating Hemiplegia of Childhood, Animal Disease Model, Behavior, Central Nervous System

Introduction:

Alternating hemiplegia of childhood (AHC) is a neurological disease caused by mutations in the *Atp1a3* gene encoding the neuron-specific $\alpha 3$ isoform of the Na^+/K^+ -ATPase. AHC manifests within the first 18 months of birth. Children with AHC exhibit a wide range of neurological symptoms including hemiplegia, dystonia, ataxia, nystagmus, hyperactivity, seizures and developmental delays. Often, episodes are triggered by stressful events.

Aim:

We have created an AHC mouse model harboring the D801Y in vivo-diseases mutation ($\alpha 3^+/\text{D801Y}$) to analyze the pathology of AHC.

Methods:

The $\alpha 3^+/\text{D801Y}$ mice were subjected to behavioral tests assaying anxiety, locomotion, balance and capacity for learning and memory.

Results:

In the open field test the $\alpha 3^+/\text{D801Y}$ mice were hyperactive, traveling twice the distance of their wild type littermates. The $\alpha 3^+/\text{D801Y}$ also showed increased anxiety, as they spend more time in the periphery of the apparatus. The $\alpha 3^+/\text{D801Y}$ mice showed severe ataxia on the balance beam relative to their wild type littermates. Lastly, $\alpha 3^+/\text{D801Y}$ mice performed significantly worse in the passive avoidance and Barnes maze tests, indicating impaired learning and memory skills.

Perspectives:

The $\alpha 3^+/\text{D801Y}$ mice display several of the most prominent phenotypes reported for AHC patients including anxiety, hyperactivity, ataxia and reduced learning. Thus, the $\alpha 3^+/\text{D801Y}$ mice constitute a potentially very promising disease model for AHC. Future studies will focus on the biochemical mechanisms responsible for this disease. Using a combination of neurotransmitter assays and immunohistochemical characterization, we hope to help improve the treatments, currently available to AHC patients.

Poster abstracts

#12 (sub 25)

The AHC French National Network

Dominique Poncelin

President of AFHA (French AHC organization)

keywords: AHC, Alternating Hemiplegia of Childhood, research projects France

This poster presents how AHC (Alternating Hemiplegia of Childhood) research is organized in France, gathering both scientists (clinicians, geneticists) and AFHA (French AHC Family organization). Moreover this poster presents a short sum up of current research projects on AHC in France.

Poster abstracts

#13 (sub 26)

Characterization of human neuroblastoma cell lines expressing different levels of E815K mutation

*Martina Rinelli**, *Sara Cocco**, *Roberto Piacentini*, *Lorena Di Pietro*, *Cristian Ripoli*, *Giovanni Neri*, *Francesco Danilo Tiziano*, *Claudio Grassi*, *Fiorella Gurrieri*

Catholic University of Rome, Rome, Italy

keywords: AHC, SH-SY5Y, ATP1A3 mutations, E815K, pIRES-eGFP

We are currently developing and characterizing a cellular model of AHC, using a human neuroblastoma cell line (SH-SY5Y). SH-SY5Y have been stably transfected with constructs (kindly provided by Dr. Erin Heinzen) expressing the E815K, one of the most frequent ATP1A3 mutations found in patients with AHC. 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 cDNA bearing the E815K mutation has been cloned into the pIRES-eGFP eukaryotic expression vector. After linearization, cell line has been permanently transfected with the constructs. Transfected cells were selected by antibiotics resistance to G418; the expression of the transgene has been inferred from the expression of the GFP. Mixed cell population underwent to clonal selection, and single clones were plated separately. So far we have obtained 4 different clones expressing the E815K mutation. The levels of expression of endogenous and mutated ATP1A3 mRNA have been determined by absolute real time PCR. We observed, as expected, wide variability in the levels of expression of the transgene: in the mixed population we observed much higher (up to 6 folds) levels of the E815K allele, that were not found in single clones, suggesting that cells expressing high levels of the mutation may undergo to negative selection. So far, the E815K clones we have selected display different ratios of mutated/wt allele; among those, the most promising clones are those with a 1:1 ratio. Electrophysiological characterization is currently undergoing and data will be presented.

*These authors contributed equally to the work

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Poster abstracts

#14 (sub 27)

The Italian Association A.I.S.EA and the support to the national and international research on AHC

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keywords: A.I.S.EA Onlus, I.B.AHC Biobank Clinical Registry, Alternating Hemiplegia of Childhood, AHC

A.I.S.EA Onlus, the Italian Patient Association for Alternating Hemiplegia of Childhood (AHC), was created in 1999 with the main goals to support the Italian AHC families and promote a better health and social assistance for the AHC patients, to raise the awareness about the disease, to promote and support the research of an effective treatment.

In 2004, the service I.B.AHC - Biobank and Clinical Registry for AHC, was activated to effectively support the research on AHC, thanks to a project designed, funded and coordinated by A.I.S.EA, in close collaboration with its Scientific Committee.

The service is managed by the I.B.AHC Consortium, composed by the technical staff of A.I.S.EA, by the clinicians of the Reference Centers in charge of the main clinical studies on AHC, by the researchers of the main labs for the genetic and molecular research and the diagnostic testing, and by the treating physicians of the recruited patients.

The I.B.AHC service is composed by two main linked repositories, the Clinical Registry, containing the clinical data and the video-photographic documentation, and the Biobank containing the DNA samples of the recruited patients and of their parents. These two repositories are accessible by all the research groups, national and international, for their research projects related to AHC, provided that they are scientifically valuable and non-duplicated.

In 2012 the genetic cause of AHC was identified, mainly thanks to an international collaborative research (Heinzen et al., 2012) in which both the contribution of the I.B.AHC Consortium and the availability of the I.B.AHC service played an important role for the confirmation of the results, by providing the largest, non-duplicated and validated case-series.

The researchers and clinicians of the I.B.AHC Consortium are now working for several studies, national and international, to better understand the malfunctioning in the brain cells caused by the AHC-mutations in the ATP1A3 gene, and in perspective to develop an effective treatment to correct it.

A.I.S.EA is still providing its support to all these studies, both financial, technical and logistical.

Poster abstracts

#15 (sub 28)

Alternating Hemiplegia of Childhood: update on pharmacological treatment of an Italian cohort of 29 patients - preliminary results.

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keywords: Alternating Hemiplegia of Childhood, Treatment, Flunarizine

Background:

Alternating Hemiplegia of Childhood (AHC) is a very severe and intractable disorder. In particular, many drugs have been tried as a prophylaxis for the paroxysmal movement disorders, but no therapy is currently completely effective in controlling these most disturbing symptoms. Given the disease rarity, prospective, randomized and controlled studies are lacking. Aim of the study is to review the pharmacological data about prophylactic and acute treatment of an Italian cohort of patients and correlate treatment response to clinical, demographic and genetic data.

Methods:

Data on 29 patients (15 M, 14 F, age range 5-42 years) have been collected through the Italian Biobank and Clinical Registry for AHC (www.ibahc.org) and verified and completed by families and patients interviews. Results: our study confirm that flunarizine is actually the most used long-term treatment in AHC to prevent the non-epileptic paroxysmal attacks: reduction of frequency and severity has been reported in 16/28 (57%), while less duration has been observed in 14/28 (50%). Flunarizine has been used for a mean period of 11.9 years, ranging from 27 years to 12 months. Side effects caused tapering in 32% (9/28) and interruption in 11% (3/28) of cases. Moreover, three out of 28 (11%) failed to respond. Of the many other prophylactic drugs tried, only few have been partially efficacious: acetazolamide (3/6), clonazepam (2/4), 5-hydroxytryptophan (1/2), amitriptyline (1/1) and ketogenic diet (1/1). As far as acute treatment of attacks is concerned, benzodiazepines helped in 4/25, melatonin in 2/4 and niaprazine in 1/13.

Conclusions:

The study of the type and the intensity of treatment response in a cohort of genotypically characterized AHC patients, together with the recent discovery of the biological basis of the disorder, will contribute to clarify the pathophysiology and will give the rationale for the use of newer and more efficacious molecules.

Poster abstracts

#16 (sub 30)

Transgenic Rescue of Alternating Hemiplegia-Related Behavioural Phenotypes in ATP1A3 Mutant Mice

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keywords: alternating hemiplegia, transgenic rescue, Na⁺/K⁺-ATPase α 3, Atp1a3, mice

Missense mutations in the ATP1A3 gene encoding Na⁺,K⁺-ATPase α 3 have been identified as the major cause of alternating hemiplegia of childhood, the most common permanent symptoms of which are ataxia and cognitive impairment. Heterozygous Myshkin mice with a missense mutation in Na⁺,K⁺-ATPase α 3 have phenotypic abnormalities that model some symptoms of alternating hemiplegia, including low body weight, motor dysfunction, cognitive impairment, and increased susceptibility to seizures. We previously reported that inheritance of a wild-type Atp1a3 transgene increased brain Na⁺,K⁺-ATPase activity by 16% and suppressed the enhanced epileptic propensity of Myshkin mice.

The aim of the present study was to determine whether this transgenic intervention had therapeutic effects on the alternating hemiplegia-related motor and cognitive deficits of Myshkin mice. Myshkin mice carrying the wild-type Atp1a3 transgene were subject to a range of behavioural tests, in comparison with wild-type and Myshkin littermates without the transgene.

Myshkin mice with the wild-type Atp1a3 transgene showed significant improvement compared to non-transgenic Myshkin mice in motor and cognitive tests.

Inheritance of the wild-type Atp1a3 transgene suppressed phenotypic abnormalities that model the most common permanent symptoms of alternating hemiplegia. This finding thus provides a solid rationale for testing the efficacy of in vivo virus vector-mediated Atp1a3 transfer in reversing the alternating hemiplegia-related phenotypes of Myshkin mice, as a prelude to possible future clinical translation to alternating hemiplegia patients with ATP1A3 mutations.

Poster abstracts

#17 (sub 31)

ATP1A3 mutations and genotype-phenotype correlation of alternating hemiplegia of childhood in Chinese patients

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Alternating hemiplegia of childhood (AHC) is a rare and severe neurological disorder. ATP1A3 was recently identified as the causative gene. Here we report the first genetic study in Chinese AHC cohort. We performed whole-exome sequencing on three trios and three unrelated patients, and screened additional 41 cases and 100 controls by PCR-Sanger sequencing. ATP1A3 mutations were detected in 95.7% of AHC patients. At least 93.3% were de novo. Four late onset, atypical AHC patients were also mutation positive, suggesting the need for testing ATP1A3 mutations in atypical cases. Totally, 13 novel missense mutations (T370N, G706R, L770R, T771N, T771I, S772R, L802P, D805H, M806K, P808L, I810N, L839P and G893R) were identified in our study. By homology modeling of the mutant protein structures and calculation of an extensive list of molecular features, we identified two statistically significant molecular features, solvent accessibility and distance to metal ion, that distinguished disease-associated mutations from neutral variants. A logistic regression classifier achieved 92.9% accuracy by the average of 100 times of five-fold cross validations. Genotype-phenotype correlation analysis showed that patients with epilepsy were more likely to carry E815K mutation. In summary, ATP1A3 is the major pathogenic gene of AHC in Chinese patients; mutations have distinctive molecular features that discriminate them from neutral variants and are correlated with phenotypes.

Poster abstracts

#18 (sub 32)

AHC Multidisciplinary Clinic

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Alternating Hemiplegia of Childhood is a complex disease involving multiple systems, including problems in the neurological, psychiatric, and psychological, motor control, educational and, to a lesser extent, cardiac and sleep, areas. The multi-disciplinary Alternating Hemiplegia of Childhood Clinic at Duke University is geared to address those needs and to foster data collection and research to ultimately benefit these patients. The clinic started in March 2013 and has directly served 29 patients since then. Of these, 13 have had comprehensive evaluations each spanning three days, three have had inpatient evaluations, 18 have been seen in the outpatient clinic, eight were phone consultations for patients that were not able to travel with their parents and/or their physicians. These patients came from various geographical areas including the following states in the US: California, Florida, Georgia, Kentucky, New York, North Carolina, Pennsylvania, Texas, Virginia, and Vermont; foreign patients have included patients from the British Virgin Islands, Chile, India, Lebanon, Saudi Arabia, and Venezuela. Coordinated care and social support for the families while undergoing evaluations by the various members of the team and Cure AHC made it possible to facilitate initial contact, travel and lodging when needed, and administrate subsequent therapy. Collaboration with the referring physician and the primary care physician with the Duke and Cure AHC team has also been key to the success of this program.

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