

Fourth International Symposium on Neuroacanthocytosis

July 1-2, 2008
London and Oxford

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1-1 Introduction: movement disorder phenomenology

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Chorea is a hyperkinetic movement disorder characterized by involuntary movements which flit and flow unpredictably from one body part to another. Dystonia is characterized by involuntary muscle spasms resulting in writhing movements and abnormal body postures. Chorea and dystonia are the main movement disorders exhibited by patients with neuroacanthocytosis, although tics and parkinsonism may also be present. The differential diagnosis in a patient with familial adult-onset chorea entails Huntington's disease (HD), which is caused by a triplet repeat expansion in the IT15 gene (also known as huntingtin) and which accounts for about 90% of cases of chorea of genetic etiology, as well as other distinct genetic disorders which can present with a clinical picture indistinguishable from HD. These disorders are termed HD-like (HDL) syndromes. So far, four such conditions have been recognized, including disorders attributable to mutations in the prion protein gene (HDL1), the junctophilin 3 gene (HDL2) and the gene encoding the TATA box-binding protein (HDL4), and a single family with a recessively inherited HD phenocopy, the genetic basis of which is currently unknown (HDL3). These disorders, however, account for only a small proportion of cases with the HD phenotype but a negative genetic test for HD, and the list of HDL genes and conditions is set to grow.

1-2 Follow-up of cases reported by Dr. R. Hardie in 1991

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In 1991, Dr. Hardie described the clinical and pathological features of 19 cases of neuroacanthocytosis, resulting in the largest series reported with this rare disorder. During the past 15 years, there have been many advances in our understanding of the neuroacanthocytosis syndrome, including the identification of several different molecular causes. We have revisited the original Queen Square series in an attempt to correlate the clinical picture and natural history of each case with the new genetic findings.

1-3 International efforts at case collection ("Virtual Institute")

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For more than fifteen years we have collected – with the generous support of physicians world-wide – chart data on individual patients (presently >150) affected by neuroacanthocytosis syndromes in order to delineate disease features and natural history. In addition to the data on McLeod syndrome (Danek et al. 2001) information on 106 cases with a diagnosis of chorea-acanthocytosis (ChAc) based on *VPSI3A* mutation analysis has been evaluated (Danek et al., 2009).

For a more systematic, prospective approach we now concentrate on ChAc after the Western blot for chorein became available as a diagnostic tool (Dobson-Stone et al. 2004). Since 2007 we have fully analyzed 110 blood samples from patients with suspected ChAc, sent in from all continents, and made a positive diagnosis in 42 new cases. This service is offered at no cost thanks to the support of Advocacy for Neuroacanthocytosis Patients (www.naadvocacy.org), but in return we ask for clinical data on the affected patients, collected according to a protocol newly set up with a group of neuroacanthocytosis specialists and to be entered locally by the physician in charge.

This patient registry forms a submodule within the European Huntington's disease network (www.euro-hd.net/html/na/submodule/registry). In addition to the data collection according to standardized questionnaires and scales, a systematic documentation of the patients' movement disorder, filmed according to a teaching video available for download, is encouraged. We also consider possibilities for uploading neuroimaging data.

The registry will be opened for data entry in July 2008 and eventually aims at allowing therapeutic studies for ChAc patients. Due to the rarity of the disease such studies critically depend on global collaboration. This can now be comfortably provided by our web-based platform, a "virtual institute" for neuroacanthocytosis information, research and treatment.

1-4 Epilepsy in chorea-acanthocytosis

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It is reported that 30-40% of chorea-acanthocytosis (ChAc) patients have seizures at some stage of their disease. Seizures can occur many years after the onset of a movement disorder, but they can also precede any other clinical manifestation by as much as 15 years. In such cases the diagnosis of ChAc may be established many years after the first seizure.

Unfortunately the majority of studies do not further classify the epilepsy, mostly 'generalised seizures' are described, however, some of them might well have had a focal onset and secondary generalisation.

We describe a patient who underwent evaluation for epilepsy surgery because of medically intractable temporal lobe epilepsy. Surgery was not an option as he had independent seizures arising from either temporal lobe. During this presurgical evaluation FDG-PET was performed and showed a markedly reduced metabolism of the caudate nucleus, MRI showed mild global atrophy. Later he developed orofacial tics with dyskinesia and the diagnosis of ChAc was made.

This patient was shown to have focal bilateral temporal lobe epilepsy, but in many ChAc patients a clear syndromic epilepsy classification may not be available. It might even remain unclear if they have generalised or focal epilepsy which could have an impact on the choice of anticonvulsant medication. Additionally further information about the epilepsy can improve our understanding of basic mechanisms of ChAc s: truly generalised epilepsies can be indicative of

molecular dysfunction, e.g. in ion-channels, whereas focal epilepsies suggest mechanisms of focal structural damage, e.g. on an embolic or inflammatory basis.

Therefore we recommend that epileptologists become involved, to achieve a distinct syndromic classification of epilepsy in ChAc patients with seizures.

1-5 McLeod syndrome: brain and neuromuscular pathology

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The McLeod neuroacanthocytosis syndrome (MLS) is an X-linked multisystem disorder with haematological, neuromuscular, and central nervous system (CNS) involvement. MLS is caused by mutations in the *XK* gene that encodes the XK protein, a putative membrane transport protein which is covalently linked to the Kell glycoprotein. The function of the XK/Kell-complex is not yet clarified. The Kell protein is an endothelin-3 converting enzyme generating the bioactive endothelin-3. The XK protein shares important homologies with the ced-8 protein of the nematode *C. elegans*, in which it acts as a cell death effector downstream of the caspase ced-3. CNS manifestations of the MLS comprise chorea, neuropsychiatric and cognitive abnormalities, and generalized seizures. Imaging studies in MLS patients revealed caudate nucleus and putamen atrophy as well as decreased glucose uptake in positron emission tomography studies without clear evidence for extrastriatal pathology. Neuropathological examination demonstrated unspecific neuronal loss and astrocytic gliosis in the caudate nucleus, putamen and, less pronounced, in the globus pallidum. Extended work-up did not demonstrate specific features of the pathological alterations. The severity of the striatal pathology varied considerably between the patients, also in those carrying an identical mutation. In contrast to choreoacanthocytosis (ChAc), there was no involvement of the substantia nigra and thalamus and only minor cortical gliosis was present in some patients.

Virtually all MLS patients had elevated serum creatine kinase levels, and about 50% develop weakness and muscular atrophy. In a series of 10 muscle biopsies of MLS patients, clear but unspecific myopathic changes were present only in four patients. All patients, however, had neurogenic changes of variable degree. Motor and sensory nerve examinations demonstrated axonal sensory-motor neuropathy. Cardiac manifestations of MLS developed in more than half of the patients and include congestive cardiomyopathy, dilated cardiomyopathy, atrial fibrillation and tachyarrhythmia. Cardiac histopathology is not specific and reveals focal myocyte hypertrophy, slight variation of myofiber size and interstitial fibrosis.

1-6 Chorein expression and neuropathology

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Background: Chorea-acanthocytosis (ChAc) is characterised by marked neurodegeneration within the caudate nucleus and presence of malformed erythrocytes. The connection between both phenomena and the underlying function of the mutated protein chorein remain still cryptic. For progress in understanding the disease, understanding the functional aspects is crucial.

Methods: Brain tissue of ChAc patients in comparison to Huntington's disease (HD) patients was investigated by histology, immunohistochemistry and stereology for 3D reconstruction as well as morphometric methods for cell differentiation and calculation. Western blot was used to

study chorein expression in various brain regions as well as peripheral tissues of healthy subjects. Further, blood samples of HD and pantothenate-kinase associated neurodegeneration (PKAN) have been examined for chorein presence in erythrocyte membranes.

Results: In ChAc a notable loss of neurons was found in neocortex and even more striking in striatum, exceeding the observations in late stage HD brains in these regions. Impressive neurodegeneration was confirmed by both, a remarkable astrogliosis and an increased number of activated microglial cells. This could also be documented by an increase in the astroglia/neuron-index in ChAc (47) and in HD (23) compared to healthy controls (3). In Western blot, chorein expression was throughout constant in different brain regions of healthy control subjects, but was absent in all corresponding tissues of ChAc patients. Two fragments present in healthy brain and missing in ChAc tissue are recognised at 160 kDa and 100 kDa.

Furthermore, we examined different peripheral tissues of healthy control subjects and found full length chorein synthesised in brain, blood, testis and muscle. Comparing brain and peripheral tissues, chorein expression pattern seems to be tissue specific. While full length chorein is missing in erythrocyte membranes of ChAc patients, it is present in all healthy controls examined so far ($n > 30$), as well as in five HD patients (PKAN results pending).

Conclusions: Neurodegeneration in ChAc is different than in HD regarding the degree of tissue loss and astroglia/neuron index but shows similarities regarding the affected brain regions, while chorein is expressed ubiquitously in non affected brains. Results also point out that different variants of chorein are synthesised in brain tissue. This can implicate a structural organisation of functional domains within the chorein protein. Chorein presence in erythrocytes of healthy controls and patients suffering of similar disorders supports the specificity of the diagnostic Western blot.

1-7 Caudate nucleus pathology and obsessive-compulsive disorder in Chorea-Acanthocytosis

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Chorea-acanthocytosis (ChAc) is an exceptionally rare autosomal recessive disorder (1 in 5 million) associated with seizures, chorea and peripheral blood acanthocytes. Patients often have dramatic caudate atrophy noted on magnetic resonance imaging. Notably, the disorder presents with adolescent or adult-onset obsessive-compulsive disorder (OCD) in up to 25% of individuals, implicating dysfunction of the caudate nucleus and its relay function in the lateral orbito-frontal loop (LOFL). MRI scans from a number of clinicians in Europe, Australia and the Americas were collected ($n=14$), and then age- and gender-matched against controls ($n=14$) and patients with Huntington's disease (HD, $n=14$). Caudate nuclei were traced using an established protocol; caudate volume corrected for intracranial volume was determined, and shape analysis was conducted on caudate shape using permutation analysis, following spherical harmonic modeling and parametric mesh generation. HD patients had a 10% reduction in caudate volume (not significant) and only modest shape changes compared to controls; ChAc patients in comparison showed a dramatic 80% reduction in size, a flattening of the caudate and the loss of most of the caudate head. This suggests that dramatic neuronal loss in the caudate results in the loss of its function in inhibiting motor acts, leading to OCD-like illness in a number of ChAc patients.

2-1 Drug therapies including botulinum toxin

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Neuroacanthocytosis is a neurodegenerative disease due to mutations of the *VPS13A* (Chorein) gene on chromosome 9q21. The condition is characterized by hyperkinetic movements of chorea and dystonia with prominent orofacial involvement and self mutilation, tics, parkinsonism, eye movement abnormalities suggestive of brain stem involvement, subcortical dementia and psychiatric features with impairment of frontal lobe function with age of onset in mid-life. Seizures, autonomic features, as well as myopathy and neuropathy may also be present. Treatment remains symptomatic. With respect to movements, chorea may respond to tetrabenazine or atypical neuroleptics, however, the former may worsen a co-existing depression. Dystonia may respond to anticholinergics. Focal dystonias, e.g. of the oromandibular region, can be treated with botulinum toxin injections. Parkinsonian features may respond to dopaminergic drugs and gait problems to amantadine. Deep brain stimulation has been explored in individual cases with mixed results. In later disease stages, patients may require a PEG, or suprapubic catheter. Speech therapy, physiotherapy and psychological therapy should also be offered to the patient when needed.

2-2 Rehabilitation - Qualitative Interviewing - A patient's perspective

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A person's identity is important in any treatment. It helps shape the way they cope. If you want to know how a person copes you have to ask the question.

The results of a qualitative interview with a patient will be presented. Qualitative interviewing, according to Kvale (1996), attempts to "unfold the meaning of peoples' experiences". This presentation then, gives a glimpse of the person, from Mozart to a desire for chocolate.

2-3 Biofeedback in dystonic syndromes

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The brain projects motor output on a specific group of muscles required for performing a specific task. During movement sensory feed-back afferents from the muscles provide input for motor control. Abnormal movement-related sensory input or abnormal sensorimotor processing in the brain results in abnormal postures or movements. We show the clinical proof of the impact of abnormal sensory feedback on movement in a patient with pseudoathetosis-dystonia caused by sensory polyneuropathy. Causalgiform dystonia-like cramps present probably the most extreme example of such abnormal peripherally induced sensorimotor disorder. Dystonia may also be caused by abnormal sensorimotor integration, resulting in a mismatch between sensory input versus motor output. In a number of patients with sensorimotor movement disorders the abnormal sensorimotor drive can be restored, overruled or evaded by behavioral manipulations of the sensorimotor loop. We show examples of the effect of sensory tricks in torticollis and oromandibular dystonia, the effect of blockade of a trigger muscle in midbrain tremor, and the

effect of evading the disordered sensorimotor loop in writers cramp. We have systematically treated patients with writers cramp with behavioral therapy. The patients are taught to recognize the relationship between the level of EMG activity as shown on a screen and the contraction of the muscle. Patients are trained to reduce the excessive EMG to the minimum possible level while they were writing. In a majority of patients this therapy results in long-lasting improvement of writing. In a pilot study we found that behavioral therapy restored pre-existing lowered levels of dopamine receptors, suggesting that dopaminergic abnormalities in the basal ganglia in these patients are probably a secondary adaptation rather than the cause of writers cramp. Most of these therapies can probably not be applied in patients with extensive brain pathology because they may require full co-operation of compensatory parts in the brain.

2-4 Deep brain stimulation for NA patients – forum

a) Deep Brain Stimulation in Pantothenate Kinase Associated Neurodegeneration

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Background: Pantothenate kinase-associated neurodegeneration (PKAN, OMIM 234200) is a progressive autosomal recessive disorder due to mutations in the pantothenate kinase 2 gene (PANK2). PKAN patients generally fall into 2 clinical categories: early onset, rapidly progressive (classic) disease or late onset, slowly progressive (atypical) disease. Motor involvement with gait disturbances due to dystonia, rigidity, and spasticity are present in both forms. Psychiatric symptoms (more prominent in atypical disease) include depression, emotional lability, and impulsivity. Repetitive actions, freezing, and palilalia are all common features of the syndrome. Pharmacologic and surgical interventions aim at improvement of psychiatric and motor symptoms.

Objective: Deep brain stimulation of the internal globus pallidus (GPi) has been proposed for treating progressive generalized dystonia and dyskinesia sometimes resulting in life threatening conditions in PKAN. We studied the results of GPi stimulation in PKAN.

Population and method: Eleven consecutive genetically proven PKAN patients (6 male) underwent surgery for DBS. Eight patients presented classic form of PKAN. Bilateral GPi implantation has been performed in all patients. The patients were assessed before and after surgery using the Burke-Fahn-Marsden's dystonia rating scale (BFMDRS) which includes a motor scale and a disability scale.

Results: At onset, the mean age of the patients was of 9.4 years (range, 1-17). At surgery, the mean age was 18.5 years (range, 8-39). Postoperatively, there was a sustained decrease of the dystonia in all but one patient with a mean global motor improvement of 60.5% (range, 6.5 to 91.5) and a mean global disability improvement of 42% (range, 4-82). Two patients presenting with classic forms of PKAN died after 56 and 14 months follow-up. The three atypical and four classic PKAN patients preserved autonomous gait at last assessment. Three patients experienced improvement of dysarthria. Hardware related complications occurred in one patient.

Discussion: Since our first publication reporting on the effect of GPi DBS in 6 PKAN patients, several case reports described sustained efficacy of GPi stimulation in PKAN. Two patients in life-threatening condition previous to DBS survived for more than five years with a satisfying quality of life. If the mean motor improvement is satisfying, the outcome for walking capacities

can be limited by freezing (common feature of the disease), sometimes increased by high frequency GPi stimulation. Speech involvement with severe dysarthria is rarely improved by DBS. Psychiatric symptoms were not modified by GPi DBS.

Conclusion: Since no curative therapy exists to date, we consider that DBS should be considered in the treatment of the severe generalized dystonia of PKAN. Pallidal stimulation reduces the dystonia along with the painful spasms and subsequently improves the functional autonomy of the patients in daily living.

b) Deep brain stimulation for neuroacanthocytosis. Lessons from three cases

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Neuroacanthocytosis (NA) is a group of neurodegenerative diseases characterized by various types of involuntary movements resistant to medical treatment. We report data concerning three NA patients who benefited from deep brain stimulation (DBS) with different targets: one in the posterior ventral oral nucleus of the thalamus (Vop) and the other two in the internal pallidum (GPi).

Patient 1 had a severe form of chorea-acanthocytosis, with violent truncal spasms, head banging, hypotonia and dysarthria. The frequency of trunk spasms and head banging dramatically decreased with Vop stimulation and the clinical benefit remained stable 1 year later but no clear effect was observed on dysarthria nor on hypotonia, which always impaired gait.

Patient 2 was a 32-year-old man with an 8-year history of choreatic-dystonic syndrome, dysarthria, recurrent distressing tasteless belching and dramatic tongue-biting. Walking was disturbed by intermittent dystonia of the left foot and bilateral choreatic movements causing a jerky gait. Trunk flexion movements and a moderate back-arching dystonia were occasionally observed. **Patient 3** exhibited a severe generalized chorea predominating on the left side with hypotonia, postural instability causing him to fall repeatedly and a moderate cognitive deterioration. In these two patients 40Hz stimulation applied to the GPi gave the best clinical benefit, improving chorea without effect upon hypotonia. Higher frequency stimulation (120Hz) was effective for dystonia but increased chorea, worsened dysarthria and induced drooling. Low frequency (10Hz) GPi stimulation was ineffective.

These preliminary data show that pallidal stimulation is effective on choreatic and dystonic symptoms in NA. However, it should be kept in mind that this evolutive disease has a wide spectrum of symptoms. The decision to perform surgery involves full assessment of the risk of side-effects and the clinical features of each patient. The latter must also guide the choice of target and stimulation parameters.

c) DBS frequency screening for programming optimization in a patient with chorea-acanthocytosis

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Objective: To explore the optimal frequency of therapeutic pallidal stimulation in a patient with genetically confirmed neuroacanthocytosis (NA).

Background: Pharmacological treatment of NA, including antipsychotics, tetrabenazine, tiapride and levitiracetam, has often been proven ineffective. The use of pallidal deep brain

stimulation (DBS) has been reported in five previous medication-resistant NA patients. A superior efficacy of lower stimulation frequencies (40Hz) has been reported, while classical high frequency stimulation (130 Hz) either failed to provide benefits or worsened NA symptoms.

Methods: Bilateral pallidal DBS was performed on a 49 year-old male with a two year history of medically resistant, progressive orobuccal dyskinesia with recurrent tongue biting associated with distressful, tasteless belching. Choreiform movements involving the lower extremities caused a mild gait disturbance. Diagnosis was confirmed by peripheral blood smear positive for acanthocytes, elevated CPK and genetic testing. Instead of applying a routine voltage screening during initial programming, we adopted a new paradigm maintaining constant voltage (3.0V) and pulse width (210 microsec) while performing a frequency screening with systematic changes every 24 hours. Tested frequencies ranged from 10 to 130 Hz. Results were evaluated using the Unified Huntington’s Disease Rating Scale (UHDRS).

Results: We observed best clinical results, as measured by the UHDRS, at 40 and 50 Hz stimulation using a single monopolar configuration and at 40 Hz using a double monopolar configuration. At 40Hz, maximum UHDRS score improvement from baseline of 64% (single monopolar) and 73% (double monopolar) was recorded. Stimulation at frequencies higher than 100 Hz were not effective and poorly tolerated.

Table 1: UHDRS scores following DBS programming screening at different stimulation frequencies

Frequency (Hz)	UHDRS Score	
	Single Monopolar (1-C+)	Double Monopolar (1-2-C+)
Baseline	33	33
20	15	12
30	15	11
40	13	9
50	12	17
>50	Not Tolerated	Not Tolerated

Conclusions: Pallidal DBS is a safe and effective treatment for patients with advanced NA resistant to currently available medications. Our data confirm that lower stimulation frequencies (40-50 Hz) are more effective to treat NA than high frequencies typically used for tremor and Parkinson’s disease treatment. New programming paradigms, highlighting the importance of frequency screening, should be further investigated to optimize DBS results in NA and other movement disorders.

d) Bilateral Deep Brain Stimulation of internal Globus pallidus in Chorea-acanthocytosis: New Neurological and Psychiatric Findings

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Neuroacanthocytosis syndromes are a group of rare neurodegenerative disorders with co-occurring neurological deficits and peripheral blood acanthocytosis. Although pharmacological treatment strategies have positive effects, in many cases there is loss of efficacy with longer disease duration. Deep brain stimulation, employed in other movement disorders, has been tested as a therapeutic option in neuroacanthocytosis syndromes. We describe the effects of bilateral internal globus pallidus deep brain stimulation on the neurological and psychiatric symptoms of two patients suffering from pharmaco-resistant chorea-acanthocytosis. The data presented here, including a detailed neurological and psychiatric evaluation of disease course under conditions of low and high frequency deep brain stimulation, are of considerable value in this regard. We claim that deep brain stimulation should be included as a potential therapeutic option in the treatment of neuroacanthocytosis syndromes, particularly in cases with drug-resistant motor disturbances.

3-1 Pantothenate kinase-associated neurodegeneration (PKAN): Hypotheses of pathogenesis and relevance to other neuroacanthocytoses

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Pantothenate kinase-associated neurodegeneration (PKAN) is characterized by dystonia, pigmentary retinopathy, basal ganglia iron accumulation and acanthocyte formation. The causative gene, *PANK2*, is one of four human genes to encode a pantothenate kinase, the key regulatory protein in coenzyme A biosynthesis. Pantothenate kinase 2 is unique among these homologs for being targeted to mitochondria.

Disease caused by mutations in *PANK2* is hypothesized to arise from a combination of stressors in the specialized cells and tissues affected in PKAN. These include energy demands, cellular and organellar membrane composition, differential activities of other pantothenate kinases, and compensatory mechanisms in the affected cells. Data will be presented to support a hypothesis of disease that centers on lipid dyshomeostasis. This mechanism is likely to underlie other disorders that share key features with PKAN.

3-2 Huntington's disease like-2

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Huntington's disease-like 2 (HDL2) is an autosomal dominantly inherited neurodegenerative disorder characterized by a progressive movement disorder and cognitive impairment. HDL2 is due to trinucleotide repeat expansions in the *JPH3* gene encoding for junctophilin 3, and is found almost exclusively in families of African ancestry, suggesting a founder effect. As with Huntington's disease (HD), age of onset is inversely related to the size of the repeat expansion, and there is likely to be anticipation with successive generations. Unlike HD, the expansion repeat size does not appear to determine whether the phenotype will be parkinsonian or choreiform. Acanthocytosis is found in approximately 10% of cases, for reasons which are unclear, and may result in diagnostic confusion. Neuropathological findings are very similar to those seen in HD, with striking atrophy of the caudate nucleus and putamen. As with HD, ubiquitin-immunoreactive and polyglutamine-immunoreactive intranuclear inclusion bodies are

found throughout the cortex, however, recent evidence suggests that cytotoxicity is more likely to be related to cytoplasmic RNA inclusions.

4-1 *Soi1/Vps13* in budding yeast

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We identified the yeast (*Saccharomyces cerevisiae*) *VPS13* gene through recessive suppressor mutations (*soi1* mutations) that affected localization of a *trans* Golgi Network (TGN) transmembrane (TM) protein, Kex2 protease (*Mol. Cell Biol.* 1996 16:6208-17; *J. Cell Biol.* 1997 139:23-36). Vps13p is required for retrograde vesicular transport from the late endosome/PVC (prevacuolar compartment) to the TGN of TM proteins (Kex2p, Ste13p & Vps10p – the lysosomal/vacuolar sorting receptor of yeast) and promotes forward transport of Kex2p and Ste13p from the TGN to the PVC by inhibiting a signal in the cytosolic tails of these proteins that favors retention in TGN, most likely by directing transport between the TGN and early endosome. Vps13p behaves as a peripheral membrane protein that is part of a high molecular mass complex. Three additional observations have been published on Vps13p in yeast that may be of significance for interpreting the function of the Chorea Acanthocytosis protein, Vps13A. First, John Kilmartin published striking evidence that Vps13p is a centrin/Cdc31p-binding protein, and we have obtained some evidence that corroborates this result (*J. Cell Biol.* 2003 162:1211-21). Centrin is a highly conserved EF-hand protein that binds to several proteins through interaction with a short α -helical motif. Calcium binding to the centrin-Sfi1p complex is thought to result in a contractile function at the centriole/spindle pole body. Second, we found that *soi1/vps13* null mutants were severely defective for sporulation in yeast. Aaron Nieman and coworkers subsequently found that *vps13* mutants are defective for initiation of prospore membrane formation, a process that begins by the recruitment of vesicles to the spindle pole body (*J. Cell Sci.* 2007 120:908-16). Finally, Michael Sherman and coworkers found *vps13* mutations among a collection of mutations that enhance the toxicity of polyQ₁₀₃ in yeast (*Mol. Cell Biol.* 2003 23:7554-65). Models that integrate this information will be discussed. We are currently attempting to verify possible Vps13p-interacting proteins identified through high-throughput proteomic screens and to identify additional interactors through mass spectrometric analysis of native and cross-linked complexes isolated from yeast.

4-2 The *Tetrahymena thermophila* Vacuolar Protein Sorting 13A protein (VPS13A) localizes to the membrane of phagosomes

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The ciliated protozoan *Tetrahymena thermophila* utilizes phagocytosis as a means of ingesting other microorganisms that serve as its food source. To learn more about the molecular mechanisms of phagocytosis, we recently carried out a mass spectrometry-based analysis of the *Tetrahymena* phagosome proteome, identifying 73 putative protein components (Jacobs *et al.*, 2006, *Euk. Cell*, **5**:1990). One of the identified proteins was VPS13A. Mutations in *VPS13* gene homologues have been implicated in the human genetic disorders Chorea-acanthocytosis and

Cohen syndrome, but the VPS13 protein has not been previously linked to phagocytosis. As a first step to study the function of this gene and document its role in phagocytosis, we characterized the *VPS13A* gene structure. RLM-RACE (RNA ligase mediated Rapid Amplification of cDNA Ends) and RT-PCR were performed to confirm/revise the *Tetrahymena* Genome Database *VPS13A* gene prediction. The results of these analyses eliminate 12 predicted exons and alter 3 predicted exon/intron boundaries, so that the revised gene structure consists of 17 exons that encode a protein of 3475 amino acids. In addition, there was no evidence of major mRNA splice variants, which has been observed in other systems. To localize the VPS13A protein, we constructed a strain of *Tetrahymena* in which the endogenous *VPS13A* genes have been replaced with copies bearing a green fluorescent protein fusion at their carboxy termini (*VPS13A-GFP*). Live-cell confocal microscopy indicates that the VPS13A-GFP protein localizes to the membrane of phagosomes within the cell. In time course analyses, VPS13A-GFP is present on the phagosome membrane at the earliest time points that we are able to analyze (1 min.), and remains associated with phagosomes throughout their passage through the cell. Phagosome association of VPS13A-GFP occurs well before acidification of the phagosome, which begins at ~20 min. These cytological analyses provide confirmation of the phagosome proteome analysis, and further implicate VPS13A in a phagocytosis related process. We are now pursuing genetic analyses to further study the role of VPS13A in phagocytosis. A *VPS13A* gene construct with a selectable drug resistance gene interrupting its coding region is being constructed and will be used to generate a *Tetrahymena VPS13A* knock-out strain to assess its function in phagocytosis. In addition, we are preparing a construct that will allow the inducible over-expression of the well-conserved amino terminal domain of VPS13A as a means of generating a potential dominant negative mutant. These, and other future studies in *Tetrahymena*, should provide insights into the function of the VPS13A protein, and may provide new insights into how mutations in similar genes result in human genetic diseases.

4-3 Chorein and other human VPS13 proteins

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The VPS13 protein family includes four members: VPS13A, VPS13B, VPS13C and VPS13D. Chorein, encoded by the *VPS13A* gene, is altered in Chorea-Acanthocytosis (ChAc) and the *VPS13B* gene, also known as *COH1*, is mutated in Cohen syndrome. Sequence analysis of these four large proteins did not reveal any domain that could provide information about the possible function of these proteins. However, their yeast homologue, Vps13p has been shown to be involved in the trafficking of several proteins between the trans-Golgi network and the prevacuolar compartment. We present here a review of the data obtained on VPS13 proteins. The investigation of their subcellular localisation shows that they are soluble cytoplasmic proteins that interact with membranes. A characteristic vesicular-like pattern is easily detected in cells expressing chorein; similar structures can also be detected for VPS13B, C or D but at a much lower occurrence. The characteristic vesicular-like pattern of chorein is altered in several of the mutated chorein proteins suggesting that this pattern reflects a functionally important localisation. We have used an immunoprecipitation approach to test whether the VPS13 proteins form homo- or heterodimers (or multimers). We also investigated the potential interaction between VPS13 proteins and candidate partners such as proprotein convertases or MADD.

4-4 Chorein (VPS13A) state and proteome analysis in chorea-acanthocytosis red blood cells

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Chorea-acanthocytosis (ChAc) is an autosomal recessive disorder characterized by striatal degeneration and erythrocyte acanthocytosis. Loss of function mutations in *VPS13A* gene, coding a large protein, chorein, cause ChAc. In the present study, we performed parallel proteomic analysis of protein expression in erythrocyte membrane of a ChAc patient. We found decreased or increased protein levels of several proteins, some of which are involved in the assembly of spectrin-actin network in erythrocytes. Recently, we produced a ChAc model mouse which shows the striatal neurodegeneration. Subsequently, we performed immunoblot and immunohistochemical analyses in the striatum of the ChAc model mouse. Consistent results were obtained from the experiments using ChAc patient's erythrocyte and brain striatum of model mice, suggesting the existence of a common pathway leading to acanthocytosis and neurodegeneration.

4-5 Genomic and transcriptomic analysis of the *VPS13A* gene in a Mexican American population sample

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We are using genome wide transcriptional profiles in lymphocytes from more than 1200 Mexican Americans in ~40 large families to assist us in a variety of projects aimed at identifying risk factors for common human disease. We have also recently demonstrated the utility of this dataset to study monogenic disease using cystinosis as an example. All known causal mutations for cystinosis have been found in the *CTNS* gene coding for the lysosomal cystine transporter cystinosin. We have used *CTNS* mRNA levels in lymphocytes as a quantitative trait and performed variance components based linkage analysis in our Mexican American families to identify potential regulatory loci. We found (and confirmed) strong evidence for *cis*-acting regulation and identified a putative *trans*-acting QTL on chromosome 9q. The *VPS13A* gene at this QTL is a strong candidate for the *trans*-acting factor as its mRNA expression in lymphocytes was negatively genetically correlated with *CTNS* expression levels. We are currently attempting siRNA knockdown in lymphoblastoid cell lines from our families to confirm a *trans*-acting role for *VPS13A* on *CTNS* mRNA expression.

Given the known involvement of *VPS13A* in chorea-acanthocytosis we have now used our datasets to look for potential *trans*-acting regulators of *VPS13A* mRNA expression. We have used a high density SNP dataset for 600 of our Mexican American individuals (genotyped for >500,000 SNP markers), in combination with our lymphocyte expression data for *VPS13A* in the same individuals, to perform a genome wide association scan. Our strongest associations are for SNPs within the *PVRL3* (rs873132; $p = 1.53 \times 10^{-6}$), *DRD5* (rs7685513; $p = 3.84 \times 10^{-6}$) and *PARD3* (rs7904348; $p = 4.37 \times 10^{-6}$) genes on chromosomes 3, 4, and 10 respectively. Interestingly, the *PVRL3* and *PARD3* genes code for known interacting proteins. It may also be

possible to confirm a *trans*-acting role for these genes using siRNA knockdown in our lymphoblastoid cell lines.

We have recently re-sequenced ~ 2kb of the proximal promoter of *VPS13A* in 189 founder individuals from our Mexican American families. We identified 16 SNPs (6 novel), that along with 262 known SNPs in this gene, have now been genotyped in our Mexican American families. Of the 278 SNPs that we successfully genotyped, 50 show strong association with *VPS13A* mRNA expression levels (i.e., *cis*-effects) and of the best 5 *cis*-acting SNPs, 3 SNPs showed significant association with *CTNS* mRNA expression levels (i.e., *trans* effects). We are currently analysing this genotype data in combination with our lymphocyte transcriptome data, to identify by association genes downstream of *VPS13A*. These genes may provide greater insight into the pathophysiology of chorea-acanthocytosis.

4-6 Pathogenesis of Huntington's disease-like 2 (HDL2)

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Huntington's disease-like 2 (HDL2) is an autosomal dominant, progressive, adult onset neurodegenerative disorder characterized clinically by chorea, dystonia, rigidity, bradykinesia, psychiatric syndromes, dementia, and an inevitable decline to death. Pathologically, HDL2 resembles Huntington's disease (HD), with cortical and basal ganglia degeneration and a loss of medium sized neurons in the striatum in a dorsal to ventral gradient. Staining with anti-ubiquitin and 1C2 reveals intranuclear protein inclusions in multiple brain regions. Some individuals with HDL2 appear to have acanthocytes, suggesting a potential relationship between HDL2 and neuroacanthocytosis syndromes. At least 25 HDL2 pedigrees have been identified; all pedigrees of known ethnicity are of definite or probable African origin. HDL2 is caused by a CAG/CTG expansion mutation on chromosome 16q24.3. We previously found that, in addition to protein aggregates, HDL2 brain contains RNA foci that are detectable with both a CAG riboprobe and with riboprobes specific to *JPH3* transcripts. Similar to foci detected in myotonic dystrophy 1 (DM1), the foci co-localize with muscleblind-like 1 protein (MBNL1), and nuclear MBNL1 in HDL2 cortical neurons is decreased relative to controls. In cell experiments, expression of a *JPH3* transcript with an expanded CUG repeat resulted in the formation of RNA foci that co-localized with MBNL1 and in cell toxicity. The toxicity was rescued by co-expression of MBNL1. These results imply that RNA toxicity may contribute to the pathogenesis of HDL2.

5-1 Production of antibodies against human VPS13 proteins

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The Advocacy for Neuroacanthocytosis Patients awarded us a grant in 2007 to develop new tools for the analysis of chorein and other VPS13 proteins. Two members of the *VPS13* gene family have an associated disorder: Chorea-Acanthocytosis (ChAc) for *VPS13A* and Cohen syndrome for *VPS13B*. One of the main problems in the research on the molecular biology aspects of ChAc is the difficulty in obtaining good antibodies able to detect chorein, the protein encoded by *VPS13A*. The same applies to the rest of the VPS13 proteins (B, C and D). These antibodies are needed for the detection of the endogenous proteins not only for basic research but also for diagnostic purposes. A western-blot-based diagnostic assay is already available for ChAc, where

an antibody against the N-terminal region of chorein is used. With the new antibodies we want to improve this assay and, hopefully, develop a similar one for Cohen syndrome. The approach we have followed in this project consists on over-expression of six or more different fragments for each VPS13 protein and purification of those protein fragments showing a good expression level. The purified proteins are then used as antigens to develop antibodies in different species, allowing the generation of a panel of antibodies that could be used in different combinations for a number of applications. Here we are presenting the results obtained in the cloning and over-expression of the VPS13 protein fragments as well as the initial characterisation of the first antisera obtained against six of these fragments. The next steps in the project will also be outlined.

5-2 Analysis of functional and post-translational modifications in red cells from neuroacanthocytosis

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Abnormally spurn-thorn shaped red cells, known as acanthocytes, characterise neuroacanthocytosis syndromes (NA). The enormous gap between genotype and phenotype clinical manifestations in NA suggests a possible important role of post-translation protein modifications in abnormal cell function. Here, we studied red cells from patients with chorea-acanthocytosis (ChAc) or McLeod syndrome (MLS). We evaluated hematological parameters, red cell index by ADVIA –Technicon (Bayer) and red cell cation content. In both ChAc (n=4) and MLS (n=5) patients we observed the presence of dense red cell fraction with marked significant reduction in red cell K⁺ content and a slight but significant increase in red cell Na⁺ content. We then fractioned red cells according to their density and we further analysed red cells membrane proteome and post-translational modifications focusing on red cell membrane phosphotyrosine (Tyr-) profile. We considered two fractions: F1 corresponding to red cells density < 1.074 and F2: corresponding to red cells density > 1.092, which contains denser red cells and acanthocytes. We generated bidimensional gels of membrane proteins from fractioned red cells of normal and ChAc patients. We analysed 3378 spots and a total of 91 spots significantly differently expressed were identified by MALDI-TOF MS/MS analysis. The identified proteins were divided into 6 major clusters according to their functions: (i) membrane-cytoskeleton proteins; (ii) metabolic enzymes; (iii) ubiquitin-proteasome system; (iv) membrane channel and transports; (v) phosphatase-kinases and (vi) chaperones. The differences in the spots of membrane-cytoskeleton proteins in red cell fractions (F1, F2) from ChAc subjects were mainly related to 2D mobility shift most likely due to post-translation modifications such as for ankyrin, band 4.1, protein p55 in F1 and F2 or for dematin in F2. We then moved to analysis of Tyr-phosphoproteome of fractioned red cells from both normal and ChAc patients. In both F1 and F2 fractions from ChAc red cells, we observed increase red cell Tyr-phosphorylation state of red cell membrane protein compared to normal controls. The perturbation of Tyr phosphorylation profile in dense ChAc red cells involved integral membrane protein, cytoskeleton proteins but also anchoring proteins. In addition, the increased membrane association of chaperone proteins such as heat shock proteins 70 and 27, support a perturbation of red cell membrane organisation participating in the generation of acanthocytic red cells in ChAc. Further studies need to be carried out to progress on the functional analysis of

mechanisms involved in red cell volume regulation processes and in post-translational events in red cells from NA patients.

5-3 Analysis of functional and post-translational modifications in red cells from neuroacanthocytosis

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The presence of abnormally shaped erythrocytes in the blood of patients with various forms of neuroacanthocytosis (NA) suggests that elucidation of the cause of the acanthocyte shape may yield clues to the mechanism underlying the neurodegeneration that is associated with mutations in chorein, XK protein, and junctophilin-3. Previous data suggested a central role in acanthocyte formation for alterations in erythrocyte band 3. This integral membrane protein occupies a central position in regulation of erythrocyte function, shape and removal. Recent data from the Verona and Nijmegen groups indicated the involvement of post-translational modifications of band 3, but also of other membrane and cytoskeleton proteins. Preliminary data showed proteomic approaches to be highly informative in these analyses. Therefore, in order to accelerate the discovery of novel clues on the pathophysiology of NA, the major aim of this project is to generate a comparative proteomic inventory of the membrane fractions of erythrocytes from NA patients with chorea-acanthocytosis (ChAc), McLeod syndrome, and Huntington disease-like 2 (HDL-2).

We performed proteomic analysis using the peptides obtained by tryptic digestion of gel slices after protein separation using one-dimensional SDS gel electrophoresis. Peptide sequences were determined using a nano-HPLC system connected to a LTQ-Fourier transform mass spectrometer (FTMS), and proteins were identified by searching an in-house data base, using Mascot 2.1. Semi-quantitative analysis was performed with a label-free, spectral counting exponentially modified protein abundance index (emPAI). The combination of these methods for identification and quantification was shown to be able to detect proteins in the picomolar range, and were validated by immunoblot analysis.

Since proteomic analyses are expensive and time-consuming, we first invested in the development of a method to generate reliable data with a minimal number of samples. In the resulting procedure, protein separation is restricted, which reduces the necessary number of FTMS runs sixfold. This yields results that are comparable to those obtained previously by much more elaborate methods, although there is some loss in the amount of – mainly qualitative - information.

Using this method, we then determined the protein composition of the erythrocyte membrane preparations obtained from four healthy control donors. These were identical to those obtained before by us and others. The inter-individual concentrations varied 5-10 percent, apparently dependent on the number of molecules per cell, the degree of hydrophobicity, and/or the degree and type of glycosylation.

A first analysis of the membrane protein composition of erythrocytes from patients with ChAc and HDL-2 showed considerable differences in the concentration of a number of proteins between ChAc and HDL-2 erythrocytes and control erythrocytes, but also between ChAc and HDL-2 erythrocytes. The proteins in question are mainly components of the cytoskeleton and the cytoskeleton-membrane interface (increased concentrations of ankyrin, spectrin, band 4.2 in

ChAc and HDL-2; increased p55 in ChAc but not in HDL-2). There were also considerable differences in the association of cytosolic proteins such as hemoglobin and glyceraldehyde 3-phosphate dehydrogenase, and in the amount of membrane-bound stomatin.

These analyses are presently being repeated and extended with samples from other patients. The first results of this project presented here confirm previous data from a pilot study reported in Kyoto, and complement and extend the data obtained by the Verona group. In combination with the latter, they strongly suggest that this approach is likely to inspire new theories and lead to more insight into the pathophysiology of neuroacanthocytosis.

6-1 The molecular basis of red cell membrane disorders

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Most genetic disorders of the red cell membrane are associated with a more or less pronounced hemolytic anemia and various shape abnormalities. Indeed, these disorders have been largely classified on the very change in erythrocyte morphology. The membrane is composed of a lipid bilayer and is studded with a variety of proteins spanning the bilayer and involved in transport functions. Specifically, there is, at the inner surface of the bilayer, a strong bidimensional skeleton tightly attached to the bilayer by anchoring proteins such as ankyrin-1, and playing a major role in red cell mechanical properties and morphology. **Hereditary elliptocytosis** (HE) and its aggravated form, **hereditary poikilocytosis** (HP), stem from mutations in the *SPTA1*, *SPTB* and the *EPB41* genes that encode spectrin α -chain, spectrin β -chain and protein 4.1R, respectively. Mutations of both spectrin chains lie at or next to the site where $\alpha\beta$ -spectrin dimers interact to form $\alpha_2\beta_2$ tetramers or higher order oligomers. They loosen this site and generate an ellipsoid. A very common allele of the *SPTA1* gene, allele α^{LELY} , leads to an aggravation of elliptocytosis when it happens to stand *in trans* to an elliptocytogenic allele of this gene. The loosening is accentuated (through a subtle mechanism), resulting in the fragmentation of the red cell, that is, poikilocytosis. The **Southeast Asian ovalocytosis** is a unique condition due to a 27 nucleotide-deletion in the *SLC4A1* gene that encodes the anion exchanger. The corresponding 9 amino acid loss lies at the very junction of the cytoplasmic and membrane domain of the anion exchanger. **Hereditary spherocytosis** (HS) is the most common genetic condition with an altered cell shape. Generally speaking, one of many possible proteins may be reduced or even absent (in the most severe cases): HS can be viewed as the result of quantitative defects. As a consequence, the lining of the inner surface of the membrane by the skeleton becomes more sparse. Microvesicles swarm out of the cell, diminishing its surface (normally in excess) and transforming the physiological biconcave disk into a spheroid. Mutations lie in at least five genes in HS: *SPTA1* and *SPTB* (however the mutations in these genes are different from the ones seen above, and primarily lead to an isolated defect of spectrin), *ANK1*, that encodes ankyrin-1, *SLC4A1* and *EPB42*, that encode the anion exchanger and protein 4.2, respectively. A huge collection of mutations have been gathered. Some of them, especially when they occur in the homozygous state, have proved very useful in the understanding of the supramolecular architecture and will be presented. **Hereditary stomatocytoses** (HSt) are now known to belong to the wider, ever diversifying group of genetic disorders of the **passive leak of monovalent cations across the membrane**. The shape abnormality (sometimes missing) relies on the conversion of the circular depression centering the normal red cells into a linear groove. The

generation of this shape is ill-understood. These conditions are rare. The most frequent is dehydrated hereditary stomatocytosis (DHSt) which, very interestingly, is part of a pleiotropic syndrome including, in addition to anemia, a pseudohyperkalemia and perinatal fluid effusions. The involved genes are still unknown for most of this set of conditions. However, a dramatic example is accounted for by cryohydrocytosis. This condition, which is characterized by a cation leak enhanced by the cold, is due to mutations lying in the anion exchanger! **Neuroacanthocytosis** (NA) is an association of neurological manifestations and acanthocytosis. NA has been described as inherited as an autosomal recessive disorder, as an autosomal dominant disorder, and as part of an X-linked disorder called McLeod syndrome (MLS) and related to the Kell blood group system. The links between acanthocytosis and the other symptoms constitute a fascinating problem yet to be solved.

6-2 Protein interactions in the erythrocyte membrane

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Erythrocyte membrane is a composite structure in which a plasma membrane envelope composed of cholesterol and phospholipids is anchored to a two dimensional elastic network of skeletal proteins through tethering sites on cytoplasmic domains of a number of transmembrane proteins embedded in the lipid bilayer. Direct interaction of several skeletal proteins with the anionic phospholipids provides additional tethering of the skeletal network with lipid bilayer. More than 50 transmembrane proteins of varying abundance ranging from a few hundred to approximately a million copies per red cell have been identified. A large fraction of these transmembrane proteins, approximately 25, specify the various blood groups. Transmembrane proteins exhibit diverse functional heterogeneity serving as cation, water and urea transporters, as adhesive proteins involved in interactions of red cells with other blood cells and endothelial cells, in cell signaling events and some with yet to be defined. Of direct relevance to structural integrity of the membrane are membrane proteins, band 3, glycophorin C and RhAG that link the bilayer to the spectrin based membrane skeleton. Band 3 and RhAG link the bilayer to the membrane skeleton through the interaction of their cytoplasmic domains with ankyrin while glycophorin C links through its interaction with protein 4.1R. We have recently documented that XK, the membrane protein deficient in acanthocytosis, binds to both 4.1R and spectrin. We speculate that this newly identified skeletal linkage of XK may play a role in shape abnormalities of acanthocytosis.

7-1 Recent progress on Kell and XK: Functional aspects of the two proteins learned from knockout mouse models

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Absence of XK protein, the McLeod phenotype, is responsible for red cell acanthocytosis and late onset forms of neuromuscular abnormalities. On red cells, XK, a putative membrane transporter, forms a heterodimer with Kell, an endothelin-converting enzyme-3 (ET-3) and Kell/XK is anchored, by binding with 4.1R, to the cytoskeleton forming a XK(Kell)/4.1R/actin/spectrin multi-protein complex. ET-3 exerts multiple functions primarily through activation of ET_B receptor which is coupled with various alpha subunits of heterotrimeric G

protein. Neither the functions of XK nor the role played by Kell in the XK/Kell complex have been defined. The expressions of Kell and XK on the red cell surface, are affected by absence of either partner protein; absence of XK (McLeod red cells) reduces Kell expression and absence of Kell (Kell Null red cells) reduces XK expression. In non-erythroid tissues, where XK expression exceeds Kell (or no Kell is expressed), XK may function differently from XK/Kell in red cells. In order to understand the function of XK alone (mainly non-erythroid) and of the erythroid XK/Kell/4.1R complex (also present in non-erythroid tissues), we studied knockout mouse models, lacking Xk, Kel or both Xk and Kel (Xk-KO, Kel-KO and Xk/Kel-double KO) and compared them to wild-type mice. Scanning skeletal muscles of the knockout mice, we found some abnormalities (internalized nuclei, angulated fibers and splitting fibers), in Xk-KO and Xk/Kel-KO mice and very mild changes in Kel-KO. Sciatic nerves, anterior horns of lumbar spinal cord and related nerve roots of all three knockout mice showed axonal neuropathy with variable degrees of secondary demyelination (Xk/Kel-double KO>Kel-KO>Xk-KO). Ion transport studies of the red cells of the knockout mice showed that their functions are affected in all three knockout mice lines. These results, taken together with other behavioral, motor function studies indicate that Kell and XK are functionally coupled and imply that Kell and XK may both participate in McLeod pathology and that absence of XK in the XK(Kell)/4.1R complex is a contributing factor to McLeod pathology.

7-2 Mitochondrial apoptosis cascade is involved in the brain pathology of ChAc-model mice

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Chorea-acanthocytosis (ChAc) is a hereditary neurodegenerative disorder caused by loss of function mutations in the *VPS13A* gene encoding chorein. Recently, we produced a ChAc model mouse using a gene targeting technique to delete exons 60-61 that corresponds to a human disease mutation. Neuronal degeneration was observed in the striatum of the ChAc-model mouse. We performed proteomic analysis of striatal protein in the ChAc model mouse that revealed an increased protein level of DARPP-32. DARPP-32, which regulates ion channels and receptors functions acting as an inhibitor of protein phosphatase-1 (PP1) at striatum, is a pivotal integrator of dopamine signals. PP1 and protein phosphatase 2A (PP2A) dephosphorylate Bcl-2 family proteins and regulate apoptosis. We then performed immunoblot and immunohistochemical analyses to detect alterations in protein expression levels of Bcl-2 family related with apoptosis in the striatum, the cerebral cortex, and the hippocampus for comparison between wild-type and ChAc-model mice. The expression levels of PP2A and Caspase-3 tend to be increased in the striatum and the hippocampus of the ChAc-model mouse. Our preliminary results suggest that Bcl-2 family proteins are involved in the ChAc brain pathology.

7-3 Molecular mediators and environmental modulators of corticostriatal neurodegeneration leading to motor, cognitive and psychiatric symptoms: Insights from Huntington's disease mice

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a CAG trinucleotide repeat expansion encoding a polyglutamine tract in the huntingtin protein and is the most common of at least nine polyglutamine brain diseases. HD involves selective degeneration of neurons in specific brain regions, particularly the striatum and cerebral cortex. HD patients exhibit motor abnormalities (including chorea), cognitive deficits (culminating in dementia) and psychiatric symptoms (the most common of which is depression). We have attempted to model motor, cognitive and psychiatric symptoms in the R6/1 transgenic mouse model of Huntington's disease (HD) and correlate the behavioural findings with changes in gene expression and cellular plasticity. Transgenic HD mice and wild-type littermates were compared across a range of cognitive, affective and motor tests, following housing under different environmental conditions. We have then investigated specific aspects: gene expression, neuronal morphology, synaptic plasticity and neurogenesis in selected brain regions of wild-type and HD mice, including the striatum, neocortex and hippocampus. Furthermore, our findings demonstrate that environmental factors, in particular environmental enrichment, can dramatically modify the disease process and delay the onset and progression of motor and cognitive symptoms. We have also been able to model both the cognitive deficits and affective abnormalities, and correlate them with deficits of adult neurogenesis and cortical plasticity. Cognitive and affective deficits were found to occur prior to onset of motor symptoms in HD mice and may be mediated by 'pathological plasticity' at the cellular level. We have been investigating the mechanisms mediating these experience-dependent effects, and have identified spatiotemporally regulated molecular and cellular changes in response to environmental stimulation. Our findings indicate that the modulatory effects of environmental enrichment are mediated by experience-dependent changes in transcription of specific genes, synaptogenesis and adult neurogenesis, some of which may be mimicked by a newly proposed class of therapeutics ('enviromimetics'). The relevance of these findings to chorea-acanthocytosis and related 'HD phenocopy syndromes' will be discussed.

8-1 Neuroacanthocytosis - new directions for collaboration and research

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The field may benefit from support from the National Institute for Neurological Diseases and Stroke together with the National Heart, Lung and Blood Institute (Bethesda, MD), as neuroacanthocytosis is a unique disease for research into neurodegeneration because of the relatively simple system of pathogenesis.

DNA from genetically-proven cases of NA diseases should be tested for SNPs to identify subtle variations in genes that may be modifiers. Rarer syndromes may be associated with genes that are yet to be found. New technologies which are suitable for these studies are now available at reasonable cost.

Genetic modifiers of the phenotypes should be explored by creating an international case database (as is currently underway).

Frontal temporal dementia linked to chromosome 3 should be added to the conditions being studied as a newly implicated gene (CHMP2B) involves the same pathway as VPS proteins.

The focus on acanthocytes might be a “red herring”, an artifact of the disease pathway rather than a crucial link or cause, thus research should be guided by the pathology of the diseases rather than the symptoms.

In discussing the use of descriptive clinical scales to evaluate patients with NA, different scales may be useful in diagnosis from those that may determine a treatment effect.

8-2 Neuroacanthocytosis – A structural point of view

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Neuroacanthocytosis (NA) symptoms can result from several gene defects that cause the loss or dysfunction of the respective proteins, namely chorein/VPS13A, Kx, junctophilin-3, PANK2, and additional new candidates like the glucose transporter GLUT1. These proteins have little in common. VPS13A is a homologue of yeast vacuolar protein sorting 13 protein, a large protein reminiscent of cytoskeletal or tethering proteins, Kx is a putative transporter of neutral amino acids, with 10 transmembrane domains, junctophilin-3 is a membrane protein of the endoplasmic/sarcoplasmic reticulum (ER/SR) and a component of junctional complexes with the plasma membrane mediating cross-talk between the cell surface and intracellular ion channels, and PANK2 is a pantothenate kinase, an enzyme involved in lipid metabolism.

Because of the lack of structural similarities, the question is whether these proteins share a common pathway, e.g. in metabolism, or if the mutated proteins are misfolded and prone to aggregation and thus create a proteinopathic phenotype, or both. Misfolded and aggregated proteins are recognized by chaperones and by the unfolded protein response (UPR)-proteins at the ER membrane and are subsequently ubiquitinated and degraded in the proteasomes. Larger aggregates cannot fit in the proteasome and are deposited in aggresomes, which are microtubule-associated inclusion bodies at the microtubule organizing centre (MTOC) near the centrosomes. The formation of the aggresome is largely believed to be a protective response, sequestering potentially cytotoxic aggregates and also acting as a staging centre for eventual autophagic clearance from the cell. Certain cellular inclusions seen in human disease are thought to represent an aggresomal response, for example the [Lewy body](#) seen in neurons in the brain in Parkinson's disease.

Apparently, many neurological diseases are caused by deposited material, mainly protein or lipid, which cannot be degraded in the normal way. Often, this is due to an enzymatic defect like in the sphingolipidoses, Niemann-Pick disease, Tay-Sachs disease, leukodystrophy, and other lysosomal storage diseases. The accumulation of undegraded material eventually leads to (neuronal) cell death by apoptosis and autophagy, processes that are highly regulated and connected. Autophagy is the major route for the clearance of mutated huntingtin in Huntington's

disease and α -synuclein in Parkinson's disease. In Alzheimer's disease, a key protein of autophagy, beclin1, was found to be reduced in affected brain regions and in beclin1 knock-out mice the lack of beclin1 led to intraneuronal amyloid β (A β) accumulation and neurodegeneration. There is also experimental evidence that the loss of autophagy in the central nervous system causes neurodegeneration and therefore restoring normal autophagy is thought to have therapeutic potential in neurodegenerative diseases.

Autophagy and the apoptotic pathway also play an essential role in erythrocyte maturation. When human erythroid precursor cells expel the nucleus, the resulting reticulocytes clear all remaining organelles by autophagy. Concomitantly, the large amounts of transferrin receptor (TfR) on the cell surface are internalised by endocytosis, Fe³⁺ is transported through the endosomal membrane and TfR is targeted to multivesicular bodies (MVBs) that bud off luminal vesicles from the limiting membrane, the so-called exosomes, which are expelled into the extracellular medium after fusion of MVBs with the plasma membrane (PM). MVBs and autophagosomes share signalling pathways, because in late stage erythropoiesis MVBs are fused to autophagosomes. The autophagosomes also fuse with lysosomes to generate autophagolysosomes, which digest all enclosed material and eventually fuse with the PM. This process is also essential for the restructuring of the PM from the bizarre shape of early reticulocytes to the smooth, discoid shape of mature erythrocytes and is tightly regulated. It is conceivable that a defect in this process may lead to a deviation of the discocyte shape, as in acanthocytosis.

Regarding the formation of the acanthocyte shape, there are two possible mechanisms according to the bilayer couple hypothesis (reviewed by Gordon Stewart in Neuroacanthocytosis Syndromes II): either the outer leaflet of the bilayer membrane expands or the inner leaflet contracts. Expansion of the outer leaflet could be caused by insertion of lipids that are not equally distributed between the outer and inner leaflet via the flip-flop mechanism like the sphingolipids or by insertion of (lipo)proteins like glycosylphosphatidylinositol (GPI)-linked proteins, which are known to be transferred from one cell to another. Contraction of the inner leaflet could be caused by loss of "inner" phospholipids like phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol phosphates (PIPs). Particularly the turnover of PIPs is tightly regulated and correlated with the attachment of cortical actin filaments. Aggregation of PI(4,5)P₂-containing lipid rafts could lead to unequal distribution of the actin cytoskeleton and thereby cause an acanthocytic shape. Moreover, attachment of cytosolic proteins to the inner leaflet like the annexin family- and BAR domain-proteins could cause an acanthocytic shape. Last, not least, a conformational change of a large transmembrane protein like band 3 could also create acanthocytes. It is not clear, which of these mechanism(s) might play a role in NA. If autophagy is defect in NA, certain proteins might survive, which normally would be destroyed. Since autophagy is essential for discocyte formation, that is creating the right balance between surface area and cytoskeleton attachment, it is feasible that a defect would create misshaped red cells.

The biogenesis of autophagosomes has been studied in yeast and mammalian cells. In yeast, 17 autophagy genes (Atg) have been identified. A key component that is regulating autophagy is the class III phosphatidylinositol 3-kinase, PI(3)KC3. In mammalian cells, the PI(3)KC3 complex contains the proteins UVRAG, Vps15, Vps34, and Beclin1. Recently, the protein Bif-1 was also

found to be associated with the PI(3)KC3 complex. Bif-1 contains an N-terminal BAR domain that is associated with lipids and due to its wedge-shaped structure induces curvature on membranes. Bif-1 also contains a coiled-coil domain for oligomerization and an SH3 domain for association with a variety of proteins like UVRAG, dynamin, amphiphysin, Bax, and huntingtin. One can envisage that Bif-1 forms oligomeric complexes with various proteins bound to the SH3 domains, possibly also VPS13A, which could then interact with each other.

Although many Vps proteins were found to play a role in the biogenesis of autophagosomes, VPS13A was not (yet) identified in the relevant complexes. However, it may play a role in endosome-vacuole/phagosome tethering rather than biogenesis. Several large tethering complexes are known that connect various endosomes with intracellular membranes, like the TRAPP complexes between ER and Golgi and within the Golgi apparatus, the CORVET complex tethering trans-Golgi network (TGN) vesicles with the TGN, the exocyst complex tethering TGN vesicles to the PM, and the HOPS complex tethering TGN vesicles to vacuoles. Vps proteins are tethered by the HOPS complex, however, Vps13 was not (yet) identified as part of this complex. However, Vps13A has been identified on phagosomes of *Tetrahymena* (L. Klobutcher) and human VPS13A was found to associate with large vesicles when overexpressed in mammalian cells (A. Velayos-Baeza). Moreover, VPS13A was identified in human erythrocytes suggesting that it plays a role not only in neurons but also in red cells and probably in the other cell types that express it. In summary, VPS13A may act as a tethering component targeting endosomes/MVBs to vacuoles/autophagosomes for the clearing of cell debris. Non-functional VPS13A may lead to accumulation of debris in the cell and eventually lead to cell death.

The Kell/Kx complex has no similarity to any tethering complex. The Kx protein that is mutated in McLeod Syndrome (MLS) has a similarity to transporters of neutral amino acids and oligopeptides. It is also similar to the *C. elegans* protein CED-8, which is involved in the apoptosis pathway. Mutations in CED-8 inhibit apoptosis and enhance cell survival but may lead to inefficient cell differentiation/maturation (of red cells and neurons), a process that relies on apoptotic steps and autophagy. There is a balance of anabolism and catabolism (apoptosis and autophagy) in the cell and this balance is dependent on the metabolic state. Neutral amino acids in the cytosol, particularly leucine, activate the mammalian target of rapamycin (mTOR), a Ser/Thr-kinase, which is a key regulator of cell metabolism stimulating protein synthesis and inhibiting autophagy. It is not known, if Kx actually transports amino acids, nor if the lack of Kx has an effect on mTOR signalling, however, the inhibition of apoptosis and autophagy may have long-term consequences on the autophagic removal of accumulated debris in the cell. The mTOR C2 complex is also an important regulator of the cytoskeleton and therefore could be involved in the formation of the red cell acanthocytic shape.

It is certainly desirable to get more insight into the function of the players that have been identified so far, i.e. VPS13A, Kx, JPH3, PANK2, and the newly discovered proteins. Insights might come from cell biological studies and biochemical analyses. Cell biological studies may start with the localization in the cell, overexpression of wild type and mutated proteins, and knock-down of the proteins, respectively. Live microscopy of the GFP-tagged proteins (wild type and mutants) will give insight into the dynamics of the membrane-bound proteins. The biochemical analyses will characterize the proteins and identify binding partners. When soluble

fragments can be produced, X-ray crystallography might be the ultimate goal for understanding the structural basis. Insights will also come from comparative proteomic analyses of red blood cells by identifying gained or lost proteins and changes in post-translational modifications in the acanthocytes. Because lipids play an essential role in membrane structure, it is also advisable to perform lipid analyses, preferably lipidomics in collaboration with a specialized laboratory.