Lafora Disease Cure, Now a Matter of Technical Hurdles

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Lafora disease (LD) is caused by mutations in two genes (EPM2A and EPM2B) that in yet incompletely understood ways regulate glycogen metabolism. The AAV9 virus is a natural dweller of the human brain, has no difficulty crossing the blood-brain barrier, is non-immunogenic, and has a plasmid which neither integrates the human genome, nor is silenced. We are presently testing the replacement of Epm2a and Epm2b in LD mice to rescue their phenotypes. At the same time, it is now clear that Lafora bodies are the pathogenic insult to the brain, that preventing Lafora body formation prevents the disease, and that Lafora body formation can be prevented by reducing glycogen synthesis. We are using AAV9 to introduce CRISPR/Cas9 nucleases targeted against glycogen synthase, PTG, and glycogenin, to stop brain glycogen and thus Lafora body formation. Towards the same goal, we are testing antisense oligonucleotides and triple helix forming oligonucleotides against the same three targets. Again towards the same end, we are screening focused libraries of potential glycogen synthesis inhibiting small molecules, as well as large unselected libraries, in order to identify glycogen synthesis inhibitor compounds. Finally, we are working towards introducing amylase, the only known enzyme that can digest Lafora bodies, into the murine brain through inactivated diphtheria toxin in one set of experiments, and carried by AAV9 in another. We hope that one, likely several, of the above approaches will progress towards a therapy for LD.

Novel approaches to Lafora disease: lessons learned from human stem cell analyses of Niemann-Pick, type C"

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Niemann Pick type C1 (NPC1) is a lysosomal storage disease that may share important characteristics with Lafora disease. I'll discuss our work, which has led to several surprising conclusions about NPC1 that might be relevant to the mechanism of disease action and potential therapy. We studied purified human neurons derived from induced pluripotent stem cells carrying NPC1 mutations. We found that loss of NPC1 protein in human neurons leads to trapped cholesterol in a late endosomal compartment and persistent activation of autophagy. This persistent activation of autophagy may be a response to the trapped cholesterol or to a cholesterol starvation response and appears to also result in large numbers of fragmented, depolarized mitochondria that generate elevated levels of reactive oxygen. Our hypothesis is that this mitochondrial fragmentation may be more toxic to neurons then trapping of cholesterol, especially in light of evidence that cholesterol can be trafficked by alternative pathways in NPCI neurons. We suggest that autophagy activation in neurodegenerative diseases characterized by accumulations of different materials may all activate autophagy persistently, which could lead to a common phenotype of toxic fragmented depolarized mitochondria.

Mechanisms of Lafora Disease

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Lafora disease (LD) is an autosomal recessive, teenage-onset fatal form of progressive myoclonus epilepsy characterized by accumulation of poorly branched, insoluble glycogen into structures termed Lafora bodies (LB). The disease results from mutations in the *EPM2A* gene, which encodes laforin, a dual specificity phosphatase, or in the *EPM2B* gene, which codes for malin, a putative E3 ubiquitin ligase. Studies from human patients and from mouse models of the disease support the involvement of a glycogen-associated phosphate in the formation of LB. A number of mechanisms, mostly based on *in vitro* or on overexpressing cultured cell lines, have been proposed. Some studies have implicated protein targeting to glycogen (PTG), a protein phosphatase 1 glycogen targeting subunit that dephosphorylates and activates glycogen synthase (GS) in the disease. It has been suggested that malin, recruited to glycogen by binding to laforin, ubiquitylates PTG, GS and glycogen debranching enzyme (AGL) so that these glycogen metabolizing enzymes are ubiquitylated and consequently degraded. Malin has also been suggested to degrade laforin. Other mechanisms proposed to be involved or even causative of the disease include autophagy, proteosomal activity and ER stress. An important question is whether Lafora bodies are causative of the Lafora disease. Different proposed mechanisms for the pathogenesis of the disease will be discussed as well as potential approaches for treatment. *[Supported by NIH grants DK27221 and NS056454]*

Increased oxidative stress and impaired antioxidant response in Lafora disease

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Lafora Disease (LD, OMIM 254780, ORPHA501) is a fatal neurodegenerative disorder characterized by the presence of glycogen-like intracellular inclusions called Lafora bodies and caused, in the vast majority of cases, by mutations in either *EPM2A* or *EPM2B* genes, encoding respectively laforin and malin. In the last years, several reports have revealed molecular details of these two proteins and have identified several processes affected in LD, but the pathophysiology of the disease still remains largely unknown. Since autophagy impairment has been reported as a characteristic treat in both Lafora disease cell and animal models, and since there is a link between autophagy and mitochondrial performance, we sought to determine if mitochondrial function could be also altered in those models. Using fibroblasts from LD patients, deficient in laforin or malin, we found mitochondrial alterations, oxidative stress and a deficiency in antioxidant enzymes involved in the detoxification of reactive oxygen species (ROS). Similar results were obtained in brain tissue samples from transgenic mice deficient in either the *EPM2A* or *EPM2B* genes. Furthermore, in a proteomic analysis of brain tissue obtained from *Epm2b-/-* mice, we observed an increase in a modified form of peroxirredoxin-6, an antioxidant enzyme involved in other neurological pathologies, thus corroborating an alteration of the redox condition. These data support that oxidative stress produced by an increase in ROS production and an impairment of the antioxidant enzyme response to this stress play an important role in the development of LD.

Ontogeny of Lafora bodies and Neurodegenerative Changes in a Laforin-deficient mice model

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Lafora disease (LD) is an autosomal recessive, always fatal progressive myoclonus epilepsy with rapid cognitive and neurologic deterioration. One of the pathological hallmarks of LD is the presence of cytoplasmic PAS+ polyglucosan inclusions called Lafora bodies (LBs). Current clinical and neuropathological views consider LBs to be the cause of neurological derangement of patients. A systematic study of the ontogeny and structural features of the LBs has not been done in the past. Therefore, we undertook a detailed microscopic analysis of the neuropile of a Laforin-deficient (epm2a-/-) mice model. Wild type and epm2a-/- mice were sacrificed at different ages and their encephalon processed for light microscopy. Luxol-fast-blue, PAS, Bielschowski techniques, as well as immunocytochemistry (TUNEL, Caspase-3, Apaf-1, Cytochrome-C and Neurofilament L antibodies) were used. Young null mice (11 days old) showed necrotic neuronal death in the absence of LBs. Both cell death and LBs showed a progressive increment in size and number with age. LBs type I emerged at two weeks of age and were distributed in somata and neurites. LBs type II appeared around the second month of age and always showed a complex architecture and always restricted to neuronal somata. Their number was considerable less than LBs type I. Bielschowski method showed neurofibrillary degeneration and senile-like plaques. These changes were more prominent in the hippocampus and ventral pons. Neurofibrillary tangles were already present in 11 days-old experimental animals, whereas senile-like plaques appeared around the third to fourth month of life. The null mice encephalons of null mice were not uniformly affected: Diencephalic structures were spared whereas cerebral cortex, basal ganglia, pons, hippocampus and cerebellum were notoriously affected. This uneven distribution was present even within the same structure, i.e.,: hippocampal sectors. Of special relevance was the observation of the presence of immunoreactivity to neurofilament L on the external rim of LBs type II. Perhaps, LBs type II is not the cul-de-sac of a metabolic abnormality. Instead, we suggest that LB type II is a highly specialized structural and functional entity that emerges as a neuronal response to major carbohydrate metabolism impairment. Early necrotic cell death, neurocytoskeleton derangement, different structural and probably functional profiles for both forms of LBs, a potential relationship between the external rim of the LB type II and the cytoskeleton and an uneven distribution of these abnormalities indicate that LD is both a complex neurodegenerative, and glycogen metabolism disorder.

Susceptibility to pentylenetetrazole of Laforin and malin deficient mice

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Objective: Genetically engineered mice lacking expression of either laforin ($Epm2a^{-/-}$) or malin ($Epm2b^{-/-}$) display a number of neurological and behavioral abnormalities similar to those found in patients suffering from Lafora disease. Both $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice show altered motor activity, impaired motor coordination, and episodic memory deficits. They also present different degrees of spontaneous epileptic activity. $Epm2a^{-/-}$ mice present tonic-clonic seizures, and both $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice show spontaneous single spikes, spike-wave, polyspikes, and polyspike-wave complexes with correlated myoclonic jerks. Intracellular inclusions immunostained for ubiquitin were abundant in the same regions as PAS-positive inclusions. Number and size of PAS-positive and ubiquitin immunostained Lafora aggregates increased with age in both mutants. The objective of this study is to analyzed the sensitivity of $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice to the administration of the convulsant drug pentylenetetrazol (PTZ), an antagonist of the γ-aminobutiric acid type A (GABA$_A$) receptor, commonly used to induce epileptic tonic-clonic seizures in laboratory animals.

Methods: PTZ-induced epileptic activity, including myoclonic jerks and tonic-clonic seizures, was analyzed in 2 age groups of mice comprising representative samples of young adult and aged mice, after administration of PTZ at sub-convulsive and convulsive doses.

Results: $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice showed a lower convulsive threshold after PTZ injections at sub-convulsive doses. A lower convulsive threshold and shorter latencies to develop epileptic seizures were observed after PTZ injections at convulsive doses. Different patterns of generalized seizures and of discharges were observed in $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice.

Significance: $Epm2a^{-/-}$ and $Epm2b^{-/-}$ mice present an increased sensitivity to the convulsant agent PTZ, reflecting different degrees of increased GABA$_A$ receptor-mediated hyperexcitability.

Glucan phosphatases: the intersection of neurodegeneration and starch metabolism.

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Research into Lafora disease (LD) and starch metabolism are surprisingly linked by a family of enzymes that we recently discovered called glucan phosphatases. Glucans are the most abundant polymer in plants, with cellulose serving as the structural component and starch as the energy reserve. Instead of starch, humans utilize glycogen as their primary carbohydrate energy storage molecule. Recent discoveries show that the metabolism of both starch and glycogen is dependent on the action of glucan phosphatases.

Plants release energy from starch via a recently identified three-step process: dikinases phosphorylate starch, amylases degrade it until they reach a phosphate moiety, and glucan phosphatases dephosphorylate starch to reset the cycle. In the absence of glucan phosphatases, starch becomes hyperphosphorylated and starch granules grow in size while plants are unable to access the energy stored in the starch and plant growth is stunted.

The *EPM2A* gene encodes laforin and recessive mutations in *EPM2A* result in LD. We demonstrated that laforin is a human glucan phosphatase that removes phosphate from phosphorylated glucans, e.g. glycogen. In the absence of laforin activity, glycogen transforms into a hyper-phosphorylated, water-insoluble, starch-like Lafora body (LB). LBs are the suspected cause of neuronal apoptosis, neurodegeneration, and eventual death of LD patients.

We have determined the first structures of glucan phosphatases. Using these structures, we gained new insights into the molecular basis of this medically relevant enzyme family. Additionally, we have defined their unique mechanisms of catalysis, substrate specificity, and interaction with glucans. We identified glucan-interacting platforms necessary for substrate engagement and dephosphorylation. Some LD patient mutations are within these newly identified regions. Cumulatively, we define the role of laforin in glycogen metabolism, establish how laforin mutants contribute to the fundamental biology of LB formation, and determine laforin's molecular role in LD. This structure/function approach allows us to obtain atomic level insights that can be translated into diagnoses, bioassays, and putative treatments.

Lafora body proteomics

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The presence of Lafora bodies (LBs) in various tissues of Lafora disease (LD) patients has first been reported over 100 years ago. LBs are PAS-positive deposits consisting in large part of polyglucosan – a carbohydrate that is usually referred to as abnormally structured, highly phosphorylated, insoluble glycogen. It is generally assumed that LB accumulation in neurons triggers LD symptoms, but the mechanisms leading to LB formation are still uncertain. Since LBs also contain a significant amount of protein, we reasoned that among these we could possibly find enzymes and/or other protein factors involved in LB metabolism. Therefore, we developed a workflow for the isolation of native LBs from different tissues of 9-months-old laforin-deficient ($Epm2a^{-/-}$) mice and subsequently identified associated proteins using high-sensitivity mass spectrometry. In total, we identified and semi-quantitatively analyzed 143 proteins. 51 of these were found in LBs from at least two of the three tissues analyzed (brain, heart, and skeletal muscle). Many of these generally highly abundant candidates represent proteins known to relate to glycogen metabolism underlining the feasibility and significance of our study. In addition, we identified a number of proteins potentially or de facto involved in disease-related processes, including autophagy, apoptosis, inflammatory response, ubiquitin proteasome system and multidrug resistance. Verification of expression levels of these candidates in different tissues of $Epm2a^{-/-}$ and wild-type mice is still ongoing, but we are positive that our data confirm the involvement in LD establishment of different pathways making LD a complex neurodegenerative disease.

BRAIN GLYCOGEN METABOLISM AND NEURODEGENERATION

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The metabolic activity of the brain is largely supported by externally provided glucose. However, some glucose is stored locally in the form of glycogen, mainly in astrocytes, which can degrade this polymer to provide neurons with energy-rich substrates. Strikingly, neurons accumulate large glycogen-like deposits under a variety of disease conditions, i.e. Lafora disease (LD) and Adult polyglucosan body disease (APBD). Working with primary cultured neurons, as well as with genetically modified mice and flies, we have found that—against general belief—neurons contain low but measurable amounts of glycogen and that, in addition to glycogen synthase, these cells express the brain isoform of glycogen phosphorylase, thus allowing glycogen to be fully metabolized. Most importantly, neuronal glycogen metabolism protects cultured neurons from hypoxia-induced death and flies from hypoxia-induced stupor.

To study whether glycogen is primarily responsible for the neurodegeneration in Lafora disease, we generated malin knockout mice with impaired (totally or partially) glycogen synthesis. These animals did not show the increase in markers of neurodegeneration, the impairments in electrophysiological properties of hippocampal synapses, or the susceptibility to kainate-induced epilepsy seen in the malin knockout model. Interestingly, the autophagy impairment that has been described in malin knockout animals was also rescued in this double knockout model. Conversely, two other mouse models in which glycogen is over-accumulated in the brain, independently of the lack of malin, showed altered autophagy.

Our findings change the current view of the role of glycogen in the brain and reveal that neuronal glycogen metabolism participates in tolerance to hypoxia. They also reveal that glycogen accumulation accounts for the neurodegeneration, as well as the impaired autophagy, observed in the malin knockout model.

Hyperphosphorylation of Glucosyl C6 Carbons and Altered Structure of Glycogen in the Neurodegenerative Epilepsy Lafora Disease

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Functional metabolism of storage carbohydrates is vital to plants and animals. Besides chemical similarities of animal glycogen and plant amylopectin both types of polyglucans contain low amounts of phosphate esters whose abundance varies. In the model plant Arabidopsis insufficiency in starch phosphorylation or dephosphorylation results in largely impaired starch turnover and starch accumulation. In humans deficiency of the glycogen phosphatase laforin leads to the progressive neurodegenerative epilepsy, Lafora disease. Patients lacking laforin progressively accumulate unphysiologically structured insoluble glycogen-derived particles (Lafora bodies) in many tissues including brain.

We could show that glucosyl C6 phosphate, which was believed absent in glycogen but has a crucial role in plant starch metabolism, is present in many glycogen preparations examined. Several NMR techniques independently proved the existence of 6-phosphoglucosyl residues in glycogen and confirmed the recently described phosphorylation sites C2 and C3. Additionally, carbon C6 is severely hyperphosphorylated in glycogen of Lafora disease mice, and laforin is capable of removing C6 phosphate from glycogen. Gradual glycogen degradation experiments revealed that C6 phosphate is more abundant in central parts of the glycogen molecules and in regions possessing longer glucan chains. Likewise, glycogen of Lafora disease mice consistently contains a higher proportion of longer chains while most short chains were reduced as compared to wild type.The results imply that, as in starch metabolism, 6-phosphoglucosyl residues in glycogen metabolism have an important function that is related to branching and therefore to glycogen structure which is impaired in Lafora disease. Better understanding of the enzymology underlying glycogen phosphorylation will lead to new possibilities of Lafora disease treatment.

Glycogen Phosphorylation and Lafora Disease

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Lafora bodies, which contain abnormally branched, hyper-phosphorylated glycogen, are a consistent feature of Lafora disease and suppression of their formation by genetically limiting glycogen accumulation alleviates symptoms in mouse models of the disease. Mammalian glycogen normally contains small amounts of covalent phosphate (perhaps one phosphate per 500-1500 glucoses). Current evidence suggests that the phosphate exists as monoesters at C2, C3 and C6 of constituent glucose residues. Laforin, the phosphatase encoded by EPM2A, one of the two most common Lafora disease genes, catalyzes the hydrolysis of all three linkages in vitro. Furthermore, in mice lacking laforin, the phosphorylation state of glycogen is elevated suggesting that laforin functions as a glycogen phosphatase in vivo. The origin of the phosphate in glycogen is still under investigation. Whelan had originally postulated that phosphate, as a bridging C1-C6 diester, was introduced by a glucose-1-P transferase that utilized UDP-glucose as donor, subsequent hydrolysis possibly generating the C6 monester. This activity has not been further characterized and transfer of the beta-phosphate of UDP-glucose to glycogen by muscle extracts was eliminated by disruption of the glycogen synthase gene. This result led to the observation that purified glycogen synthase was able to introduce the beta-phosphate of UDP-glucose into glycogen at a low rate, consistent with a catalytic side reaction or error. We postulated that such a reaction could account for C2, and perhaps C3, phosphorylation via the formation of cyclic glucose phosphodiester intermediates during catalysis. This hypothesis is supported by a crystal structure of glycogen synthase obtained with glucose-1,2-cyclic phosphate (GCP) bound at the catalytic site of glycogen synthase and consistent with the proposed mechanism. Furthermore, incubation of glycogen synthase with UDP-glucose in the absence of acceptor resulted in the formation of GCP. These results provide a plausible explanation for the occurrence of C2 phosphomonoesters in glycogen. *[Supported by NIH grants DK27221 and NS056454]*

Setting up for a CURE: Which disease mechanism(s) should we target for a CURE in Lafora PME?

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In 2013-2014 (25 years after CFTR was discovered) the Cystic Fibrosis Foundation together with Vertex Pharmaceuticals looked to complete the cure for Cystic Fibrosis (CF) and started phase III trials on the corrector drug VX-809 (moves the CFTR or cystic fibrosis transmembrane regulator gene to the cell surface) in combination with the doorman, Kalydeco (opens channels to let chloride flow in and out) to correct the Delta F 508 mutation. In April 2014, at the AAN annual meeting, Massimo Pandolfo revealed results of phase I trials of antioxidant therapy in Friedreich's Ataxia designed to correct the disrupted iron-sulfur biosynthesis produced by reduced expression of the mitochondrial protein FRATAXIN and resulting oxidative stress.

Here, we in the research community for Lafora PME, marvel at, admire and envy these advances. For many years, many of us have talked about finding a cure. Eain Cornford and his team, part of our epilepsy program at UCLA/VA in West LA, worked for over 10 years delivering laforin contained in immunoliposomes as the "Trojan horse" and obtained best results when delivered in utero in pregnant mice with Lafora disease. Translating this technique to humans would be an enormous challenge and Dr. Cornford has since retired and so our work on the blood-brain-barrier and immunoliposome delivery of laforin is at a standstill.

Today, most of the research community in Lafora PME know what has to be done -- in vitro curative drug trials in Lafora PME using induced pluripotent stem cells that contain EPM2A or EPM2B mutations and in vitro curative drug trials using neuronal cells in culture that contain the EPM2A or EPM2B mutations, introduced by CRISPR technology. From here, the path would be drug trials in transgenic mice and then drug trials in humans with LD. The community knows what has to be done, what enterprise it should be. How do you get the funds to support such an enterprise?

On May 12, 2014, NINDS released a Notice of Intent to publicly announce a Funding Opportunity for the NINDS Epilepsy Centers without Walls Program on Disease Modification or Prevention (U54). The estimated publication date is June 2014 with first estimated application date being November 2014. See more at: http://grants.nih.gov/grants/guide/notice-files/NOT-NS-14-028.html.

So, can we unite the Lafora disease research community in a common goal and find a cure for LD? Here is our "once in a generation" opportunity. NINDS has budgeted 20 million for worthy projects.

To get you thinking about a center without walls for Lafora disease, here are some questions I pose to you today:

1) How do you want to organize? The initial spark and PI and leadership should come from the basic science labs. I envision a central core clinical database with data on all LD patients, worldwide, that would be open to all collaborating site PIs. There would also be a central core of skin fibroblasts and even IPSCs that would be open to collaborating site PIs and to several collaborating research units, worldwide, who are working on correcting disease mechanisms. To help the hunt for curative drugs, this core should help and acquire such drugs for testing from pharma, NIH, private university labs depending on disease mechanisms to be tested and assays developed (122,000 chemicals were tested in CF).
2) Which disease mechanism(s) should be corrected? I list several possibilities below and invite you to suggest other disease mechanisms that you view as being the crucial defects that should be corrected.
3) Should disease mechanisms be tested first in Knockin transgenic models of more common mutations? Examples: p.R241X in exon4 of EPM2A; p.G2795S in DSPD of EPM2A; missense mutation p.P69A in RING domain of NHLRC1.
4) In addition to in vitro drug trials, accelerated approval and compassionate use of a drug that corrects the crucial disease mechanism would need to be approved by IRBs and FDA.

So, for the minutes allotted for my talk let's instead discuss the disease mechanisms you consider crucial for correction if we are to cure Lafora PME. Can we come to agreements and chart a course – realizing that not one academic team can take on this challenge, and no pharma company would embark on such an expensive drug search in a rare disease and hope to recoup investments. This is why such an opportunity to create a center without walls for Lafora disease is timely, ripe and we can unite as a group and compete for the NIH funds.

Clearly the world community wants to see CURES in the epilepsies. This is the second $20 million investment by NIH for the epilepsies. Which epilepsy should take a higher priority than Lafora disease—the most rapidly progressive fatal epilepsy?

Suggested disease mechanisms that should be corrected

Which would you target to develop a CURE in Lafora Disease?

I. Decrease glycogen synthesis

Why? Because of 2 hypothesis in LD --
1) increased glycogen phosphorylation
2) increased glycogen synthase
    A. Decrease Phosphate Incorporation from UDP-glucose during glycogen synthesis
    OR
    B. Decrease Hyperphosphorylation of glucose/C6 carbons in glycogen
    OR
    C. Increase GSK3 ser9 phosphatase to inactivate glycogen synthase

II. Resist Endoplasmic Reticulum Stress and Apoptosis

III. Reverse proteasomal dysfunctions and ER-stress
Stress increases glucose 6 phosphate allosterically hyperactivating glycogen synthase 1 whose dephosphorylation is increased by Laforin

IV. Decrease mRNA decapping enzyme Dcp1a and facilitate micro RNA gene silencing

V. **Enhance Autophagosome Biogenesis** inhibited by Laforin Mutations

VI. Serum glucocorticoid-induced Kinase1 (SGK1) with Laforin mutations which impair glucose transporter and decrease glucose update and lead to glycogen accumulations….**therefore, SGK1 should be inhibited**