Research Presented

2008 Lysosomal Diseases and the Brain Conference

Organized by the Children's Gaucher Research Fund

--- Speaker Abstracts ---

Molecular Mechanisms of Lysosome Biogenesis

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Lysosomes are membrane-bound organelles that serve as a major site for the degradation of biomacromolecules within animal cells.
Lysosomal hydrolases. The degradative function of lysosomes is carried out by over 50 luminal acid hydrolases, most of which are biosynthetically targeted to the lysosomal lumen by virtue of a specific post-translational modification: the acquisition of mannose 6-phosphate (M6P) groups on their N-linked oligosaccharide chains. Modification with M6P in the Golgi complex allows binding of the acid hydrolases to transmembrane M6P receptors (MPRs), which carry the enzymes from the *trans*-Golgi network (TGN) to endosomes. The acidic pH of the endosomes induces dissociation of the hydrolase-MPR complexes, after which the enzymes are delivered to lysosomes with the fluid phase while the MPRs return to the TGN to mediate further rounds of hydrolase sorting. A few acid hydrolases, including glucocerebrosidase, are transported to lysosomes independently of M6P and MPRs, by a process that may involve the lysosomal membrane protein, LIMP-2.

Lysosomal membrane proteins. The limiting membrane of the lysosome contains another specific set of proteins. These include additional hydrolytic enzymes, a proton pump that acidifies the lysosomal lumen, solute transporters for export of the products of lysosomal degradation, membrane fusion and fission factors that allow interactions of lysosomes with other organelles, and a group of highly

glycosylated “lysosome-associated membrane proteins” (LAMPs) or “lysosomal integral membrane proteins” (LIMPs) that protect the lysosomal membrane from degradation by the acid hydrolases and also participate in more specific processes such as autophagy. Most lysosomal membrane proteins are not modified with M6P and are therefore not transported to lysosomes by the MPRs. Instead, they have sorting signals within their cytosolic domains that direct them to lysosomes.

Lysosomal targeting machinery. Accurate biosynthetic targeting of both luminal and membrane proteins to lysosomes depends on the function of a complex molecular machinery that comprises over 100 different gene products. These machinery components function at different stages of lysosomal targeting pathways. Coat proteins including the GGAs, the AP-1 complex and clathrin act at the TGN to sort MPRs and their cargo acid hydrolases to endosomes. Sorting depends on recognition of signals in the cytosolic domains of the MPRs by the GGAs and AP-1. After release of the acid hydrolases in endosomes, another type of coat protein, retromer, mediates retrieval of the unoccupied MPRs from endosomes to the TGN. Yet another protein complex named GARP functions to receive at the TGN the incoming carriers containing free MPRs. Lysosomal membrane proteins can be transported to lysosomes by two pathways, a direct pathway going from the TGN to endosomes and lysosomes, and an indirect pathway involving transport from the TGN to the plasma membrane, and only then to endosomes and lysosomes. Work from our laboratory has shown that the indirect pathway is responsible for the transport of the bulk of the LAMPs to lysosomes, and depends on recognition of sorting signals in the LAMPs by the plasma membrane-localized AP-2 complex and clathrin. Another complex named AP-3 plays a role in targeting LAMPs from endosomes to lysosomes.

Lysosomal diseases: Genetic defects in single acid hydrolases or lysosomal membrane proteins are the cause of many lysosomal diseases, including most lysosomal storage disorders. Combined deficiencies in various acid hydrolases can result from defects in components of the targeting machinery, as is the case for I-cell disease or mucolipidosis type II, in which an inability to add M6P to the acid hydrolases leads to the secretion of most acid hydrolases into the extracellular space. Finally, mutations in other components of the targeting machinery impair the sorting of lysosomal membrane proteins, leading to defects in not only lysosomes but also a family of organelles named lysosome-related organelles (LROs) that includes melanosomes and platelet dense bodies. An example of the latter is the Hermansky-Pudlak syndrome type 2 caused by mutations in the AP-3 complex. The emerging understanding of the lysosomal targeting machinery may shed light into the pathogenesis of lysosomal storage disorders and may lead to novel means of therapeutic intervention.

Prospects for Treating the CNS Disease in Lysosomal Storage Disorders.

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The majority of lysosomal storage diseases exhibit both CNS and visceral pathology. While systemic administration of recombinant enzymes can be effective at addressing the visceral disease, this approach does not adequately address the CNS component. This is because lysosomal enzymes in systemic circulation only have a limited ability to traverse the blood brain barrier. To overcome this limitation, we have investigated alternate therapeutic strategies such as the direct administration of enzyme into the CNS, and evaluated other technology platforms like gene and small molecule therapies. Our experience with intracerebroventricular administration of recombinant acid sphingomyelinase (ASM) into the CNS of Niemann-Pick A mice showed that this route of delivery could result in broad dispersion of the therapeutic throughout the CNS. Near-global correction of the storage pathology was noted in the CNS of the treated mice. Amounts of the enzyme were also detected in the serum with resultant partial correction of the visceral disease suggesting that direct and periodic delivery of enzyme into the brain may be an approach to treat this disease. We also investigated gene therapy as another strategy to address the CNS manifestations and showed that the use, in particular, of recombinant adeno-associated viral (AAV) vectors would appear to hold promise. Intraparenchymal injections of AAV vectors encoding ASM into Niemann-Pick A mice resulted in correction of broad areas on the brain. This was likely facilitated by the ability of (i) the AAV vectors to undergo axonal transport from the site of injection to distal sites, and (ii) the enzyme to be secreted from the transduced cells, diffuse and cross-correct adjacent cells. However, combination systemic and brain injections of AAV-ASM vectors provided maximal benefit in terms of reversal of pathology, improvement in cognitive and motor functions and survival benefit. Results of substrate reduction therapy using small molecule inhibitors of glucosylceramide synthase have also shown promise for treating the glycosphingolipidosis. Oral administration of inhibitors that are able to cross the blood brain barrier into Sandhoff mice were effective in delaying the progression of the disease and extending their longevity.

Together, these data are supportive of the contention that the CNS manifestations associated with several lysosomal storage disorders may be addressable.

Common themes in Batten disease pathogenesis

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A great advantage of mouse models of NCL is the ability to study pathogenesis at multiple stages of disease progression, which is not currently feasible in the human disorder. Through systematic analysis of the CNS of these mice, it is not only possible to obtain landmarks of disease progression, but also to identify markers that may be ultimately studied non-invasively in affected individuals. Stereological methods provide the means to generate these data rapidly and efficiently, with no assumptions made about the size of structures sampled or the objects counted. Using this approach we have characterized changes in regional volume, neuronal number and glial activation and have revealed the highly localized nature of these events. The initial focus upon GABAergic interneurons has now moved to a more complex picture of neuronal vulnerability, with a combination of location and cellular phenotype as determinants of neuronal survival. Although cortical atrophy is a feature of each form of NCL, we have also revealed pronounced effects upon subcortical structures. Indeed, the thalamus appears a particular focus for both neurodegeneration and glial activation early in pathogenesis. Glial activation precede widespread neuronal loss and disease related symptoms by several months. Moreover, glia also appear to involved in the process of synaptic stripping which occurs before neuron loss and also shows marked regional specificity. However, the precise nature and timing of reactive and degenerative changes differs markedly between forms of NCL, challenging the long accepted classification of these disorders as a closely related group of disease. Armed with this detailed quantitative data we are now in a position to begin answering fundamental questions about the underlying neurodegenerative mechanisms and begin testing the efficacy of a variety of therapeutic approaches.

Autophagy in the pathophysiology of the brain

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Damaged and abnormal proteins accumulate in most cells and tissues with age, and these protein deposits are deleterious to cellular function. Protein accumulation results in part from the failure of the systems that normally take care of their removal. Our studies have focused primarily in one of these removal systems, autophagy, which mediates delivery of cytosolic components to lysosomes for their degradation. Alterations in autophagy have been recently identified to underlie the pathogenesis of different neurodegenerative disorders, highlighting the essential role of this process in neuronal homeostasis. We have been characterizing the contribution of alterations in a selective form of autophagy, known as chaperone-mediated autophagy to Parkinson's disease. We have found that a fraction of intracellular a-synuclein, one of the pathogenic proteins in this disorder, is degraded in lysosomes via CMA. Wild type a-synuclein binds to the different chaperones and to the lysosomal receptor involved in this process, and it is rapidly degraded after its translocation into the lysosomal lumen. In contrast, although mutant forms of a-synuclein also interact with the different components of this pathway, they fail to be translocated across the lysosomal membrane. The persistence of the mutant proteins bound to the translocation units at the lysosomal membrane results in blockage of CMA. Recently, we have found that a similar defect in CMA translocation and blockage of this pathway can be found in dopamine-modified forms of a-synuclein. We are currently investigating the mechanism that lead to CMA blockage, but have already shown experimentally that because of the important role of CMA in the cellular response to stress, impairment in CMA activity renders cells susceptible to stressors and often lead to cellular death. In this context, the decrease with age in CMA activity can become an important aggravating factor in the course of this disease. ur group is also developing different approaches aimed to restore normal CMA activity. These models would help us evaluate the importance of maintaining proper autophagic activity at different stages of Parkinson's disease and, by extension, in other neurodegenerative disorders.

GM1-ganglioside as apoptotic signal in ER- and mitochondria-mediated cell death in neurodegenerative GM1 gangliosidosis

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Gangliosides are emerging as important determinants of apoptosis in physiological and pathological conditions. In the lysosomal storage disease GM1-gangliosidosis, progressive accumulation of GM1-ganglioside (GM1), due to deficiency of acid β-galactosidase (β-gal), is associated with neurodegeneration. We have previously demonstrated that in the mouse model of this disease, abnormal buildup of GM1 at the ER membrane induces depletion of ER Ca^{2+} stores and activation of the unfolded protein response (UPR), resulting in neuronal apoptosis (Tessitore et al, 2004). Given the cross-talk between ER and mitochondria, we now asked whether GM1-mediated disturbance of Ca^{2+} homeostasis could directly or indirectly influence mitochondrial function, and involve this organelle in the apoptotic process. We found that GM1 progressively accumulates at the mitochondrial outer membrane and likely influences Ca2+ exchange between the ER and the mitochondria. A GM1-dependent mitochondrial Ca2+ overload induces mitochondrial membrane permeability (MMP), dissipation of the membrane potential, opening of the permeability transition pore and release of apoptogenic factors, which ultimately activate an apoptotic caspase-cascade (d'Azzo et al 2006). Based on our data, we propose a 'two-hit' model in which abnormal levels of GM1 at the ER membrane indirectly induces MMP via Ca2+-mediated signal released from the ER. Deciphering the precise mechanisms by which GM1 orchestrates neuronal cell death after it reaches a critical intracellular concentration will help to understand the pathophysiology of this disease and to design more efficacious therapies.

This research was supported in part by the NIH grant R01 DK052025, the Cancer Core Grant P30 CA21765, the Assisi Foundation, and the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude.

Therapeutic trials and pathogenetic studies in an animal model of metachromatic leukodystrophy

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Metachromatic leukodystrophy (MLD) is a lysosomal storagae disorder caused by the deficiency of arylsulfatase A (ASA). Patients are unable to degrade sulfatide which is one of the major myelin lipids. This leads to progressive demyelination which results in various final lethal neurologic symptoms. Enzyme replacement therapy (ERT) has evolved as a treatment option for several lysosomal storage diseases mainly for those without central nervous system (CNS) involvement. We have recently treated arylsulfatase A deficient mice which are a model for metachromatic leukodystrophy with 4 repetitive high doses of recompbinant human arylsulfatase A. Unexpectedly this caused also a partial reduction of sulfatide storage in the brain of treated mice. The effects of long term treatment could not be assessed since severe immune reactions did not allow for further injections. Therefore we have generated ASA knock out mice immunotolerant against human ASA. Immunotolerance to human ASA was achieved by constitutive transgenic low-level expression of an inactive human ASA mutant. These mice were injected with 52 doses of either 4 or 50 mg recombinant human ASA per kg body weight. ERT was tolerated without detectable side effects and treatment improved disease manifestations in a dose-dependent manner. Thus, low-dose treatment diminished sulfatide storage in kidney and peripheral nervous system (PNS) but not the CNS, whereas high-dose treatment was also effective in reducing storage in brain and spinal cord by 34% and 45%, respectively. Histological analyses confirmed partial decline of storage in spinal cord, but did not reveal corrective effects in brain stem and inner ear. Low- and high-dose treatment normalized the ataxic gait and impaired swimming in ASA knockout mice. Consequently, low enzyme doses are effective in ameliorating PNS pathology and function. High-doses are, however, required to additionally target the CNS disease. Neither dose can prevent storage progression in peripheral tissues and CNS completely.

Novel metabolic pathway of glucosylceramide involving Klotho-related protein: Insights into Gaucher disease

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Catabolism of glucosylceramide (GlcCer) primarily takes place in the lysosomes where acid b-glucosidase (glucocerebrosidase, GBA1) cleaves the b-glucosyl linkage between ceramide (Cer) and glucose with the assistance of a non-catalytic protein, saposin C and negatively-charged lipids. The enzyme is specifically and irreversibly inhibited by conduritol B epoxide (CBE). An inherited deficiency of the enzyme causes Gaucher disease, the most common lysosomal storage disease, in which GlcCer is accumulated in lysosomes of laden tissue macrophages. However, the accumulation of GlcCer in other cell types is not obvious in patients with Gaucher disease despite the significant decrease of GBA1 activity and thus the existence of an alternative catabolic pathway for GlcCer was speculated. We have developed a sensitive and reproducible fluorescent-based HPLC assay to measure the activity of b-glucocerebrosidases. Using this method, we detected the activity of a CBE-insensitive neutral glycosylceramidase in cytosolic fractions of zebrafish embryos, mouse and rat brains and human. The candidates for the enzyme were assigned to the Klotho (KL) whose family members share a b-glucosidase-like domain but whose natural substrates unknown. Among this family, only the KL-related protein (KLrP) is capable of degrading C6-NBD-GlcCer when expressed in CHOP cells, in which *Myc*-tagged KLrP was exclusively distributed in the cytosol. In addition, knockdown of the endogenous KLrP by siRNA increased the cellular level of GlcCer. The X-ray structure of KLrP at 1.6 Å resolution revealed that KLrP is a $(b/a)_8$ TIM barrel, in which E165 and E373 at the carboxyl termini of b-strands 4 and 7 could function as an acid/base catalyst and nucleophile, respectively. The substrate-binding cleft of the enzyme was occupied with palmitic acid and oleic acid when the recombinant protein was crystallized in a complex with glucose. GlcCer was found to well fit the cleft of the crystal structure of KLrP. These results clearly indicate that KLrP is a novel GlcCer-degrading enzyme in cytosol and suggest that the enzyme is involved in the novel catabolic pathway of GlcCer on the cytosolic faces of Golgi apparatus or ER. In this presentation, possible participation of KLrP in Gaucher disease will be discussed. Furthermore, a sensitive and specific method to determine GlcCer using sphingolipid ceramide *N*-deacylase (SCDase) will be reported.

Ameliorating Lysosomal Storage Diseases by Restoring Proteostasis

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A lysosomal storage disease can result from the deficient activity of one of greater than 50 lysosomal enzymes, leading to accumulation of corresponding substrate(s). Enzyme replacement therapy is not useful for neuropathic diseases because recombinant enzymes do no enter the brain. We have shown that diltiazem and verapamil, potent FDA approved L-type Ca^{2+} channel blocker drugs, increased the endoplasmic reticulum (ER) folding capacity, trafficking and activity of mutant lysosomal enzymes associated with three distinct lysosomal storage diseases. These compounds appear to function through a Ca^{2+} ion mediated upregulation of the proteostasis network. We have shown that increasing ER calcium levels appears to be a relatively selective strategy to partially restore partial mutant lysosomal enzyme homeostasis in diseases caused by the misfolding and degradation of non-homologous mutant enzymes. Because diltiazem crosses the blood-brain barrier, it may be useful for the treatment of neuropathic lysosomal storage diseases, and possibly other loss-of-function diseases, although efficacy needs to be demonstrated in a human. In a second study, two proteostasis regulators, not functioning through calcium signaling, have been discovered that alter the composition of the proteostasis network in the endoplasmic reticulum through the unfolded protein response. We demonstrate that these can be used to fold mutated enzymes that would otherwise misfold and be degraded by ERAD. These proteostasis regulators partially restore folding, trafficking and function to two non-homologous mutant enzymes, each associated with a distinct lysosomal storage disease. A further synergistic restoration of enzyme function was observed when an enzyme-specific pharmacologic chaperone was co-administered with a proteostasis regulator, owing to their distinct mechanisms of action. We propose that it may be possible to ameliorate loss-of-function diseases by using proteostasis regulators, either alone or in combination with a pharmacologic chaperone.

Genetic engineering of a lysosomal enzyme fusion protein for targeted delivery across the human blood-brain barrier

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Mucopolysaccharidosis Type I, Hurler's Syndrome, is a lysosomal storage disorder that affects the brain. The missing enzyme, a-L-iduronidase (IDUA), does not cross the blood-brain barrier (BBB). To enable BBB transport of the enzyme, human IDUA was fused to the carboxyl terminus of the heavy chain of a chimeric monoclonal antibody (MAb) to the human insulin receptor (HIR). The HIRMAb crosses the BBB on the endogenous insulin receptor, and acts as a molecular Trojan horse to ferry into brain the IDUA. Transfection of COS cells resulted in high levels of IDUA enzyme activity both in the medium and in the intracellular space. The size of the fusion heavy chain, as measured with Western blotting and antibodies to either human IDUA or human IgG, was increased about 80 kDa, relative to the size of the heavy chain of the parent HIRMAb. The IDUA enzyme specific activity of the affinity purified HIRMAb-IDUA fusion protein was 363 ± 37 units per ug protein, which is comparable to specific activity of recombinant IDUA. The accumulation of glycosoaminoglycans in Hurler fibroblasts was decreased 70% by treatment with the HIRMAb-IDUA fusion protein. Confocal microscopy showed targeting of the fusion protein to the lysosome. The HIRMAb-IDUA fusion protein bound with high affinity to the HIR, and was rapidly transported into the brain of the adult Rhesus monkey following intravenous administration. The HIRMAb-IDUA fusion protein is a new treatment for Hurler's syndrome, which has been specifically engineered to cross the human BBB.

New therapeutic approaches for lysosomal disorders of the brain

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Lysosomal storage diseases of the brain remain a major challenge and currently lack effective therapies. Three broad approaches can be considered for their management that involve either 1) targeting the primary cause, 2) targeting the secondary consequences of storage or 3) a combination of these approaches. The biggest obstacle to all of these approaches is identifying agents that can be effectively and safely delivered to the brain, or can access the brain from the periphery.

We have explored one approach for the treatment of the glycosphingolipidoses that aims to target the primary cause. This involves using a small molecule inhibitor

(miglustat) of glycosphingolipid(GSL) biosynthesis to reduce the flux of GSLs entering the lysosome. The aim is to balance the rate of GSL biosynthesis with the impaired rate of catabolism preventing/reducing storage and is termed substrate reduction therapy (SRT). To date the drug miglustat has been approved for use in type 1 Gaucher disease. Significantly, miglustat crosses the blood brain barrier and therefore also has the potential to reduce storage in the brain. Data will be presented that shown efficacy in mouse models of the GM1 and GM2 gangliosidoses.

We have also investigated targeting the secondary consequences of storage in mouse models and have found that CNS inflammation is a hallmark of GSL storage disease with CNS involvement. Non-steroidal anti-inflammatory drugs show efficacy and also synergise with SRT, providing proof of principle that combination therapy may hold considerable promise if translated into the clinic.

The first report of clinical efficacy of miglustat in a CNS storage disease has recently been reported. Patients with Niemann-Pick disease type C (NPC) were studied for 12 months on miglustat. Clinical stabilisation or improvement was reported in the treatment arm. This was in agreement with functional and improved survival previously described in a mouse model of NPC1.
NPC is not a primary glycosphingolipidosis but has secondary storage of GSLs. These GSLs may contribute to pathogenesis and is the rational for testing SRT in this disorder. We have investigated the potential mechanisms of pathogenesis in NPC and have unmasked a novel acidic store calcium defect in NPC1. Possible explanations for the efficacy of SRT in NPC arise from these studies and will be discussed. These mechanistic studies have also revealed a potential new approach to NPC therapy. Specifically, a widely available and well-tolerated natural product has shown efficacy in the NPC1 mouse, as it compensates for the low calcium status of acidic stores.

Taken together, these studies suggest that combination therapy for lysosomal disorders of the brain provides greatest benefit. In addition, they also demonstrate that unravelling pathogenic mechanisms can lead to novel strategies for intervention, using existing compounds/drugs. This has the potential to lead to more rapid translational studies relative to more conventional drug discovery approaches.

The Association between Mutant Glucocerebrosidase and the Synucleinopathies

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Several different studies implicate an association between mutations in glucocerebrosidase (*GBA*), the lysosomal enzyme deficient in Gaucher disease and the development of parkinsonism. Parkinsonian manifestations are described in some patients with Gaucher disease and in heterozygote relatives. Moreover, *GBA* mutations are encountered with increased frequency in different populations of subjects with parkinsonism with dfiifering ethnicities, suggesting their role as a risk factor. Genotyping of autopsy samples demonstrated that GBA mutations are associated with a spectrum of synucleinopathies, including individuals with diffuse Lewy body dementia (DLB) and Parkinson disease (PD). Immunofluorescence studies and confocal microscopy of brain samples from subjects with synucleinopathies who carried GBA mutations showed that mutant glucocerebrosidase was present in a-synuclein-positive inclusions in both GBA heterozygotes and homozygotes with parkinsonism. In control samples from parkinsonian subjects without GBA mutations, synuclein-positive aggregates did not show immunoreactivity to glucocerebrosidase. We suggest that GBA mutations may enhance synuclein aggregation by a toxic gain-of-function mechanism or may interfere with the lysosomal clearance of toxic a-synuclein oligomers.

We have screened a large library of small molecule, and have identified several classes of glucocerebrosidase inhibitors which have the potential to chaperone the mutant enzyme, enabling it to get to the lysosome. Optimized versions of these chemicals could potentially prove useful for the treatment of patients with Gaucher disease as well as individuals with parkinsonism carrying *GBA* mutations. Unraveling the relationship between Gaucher disease and the synucleinopathies will advance our understanding of the etiology, genetics, and pathogenesis of the both disorders, and also lead to the development of new therapeutic strategies.

Fate and function of glucosylceramide in mammalian cells

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Cells have thousands of different lipid molecules that serve both structural and signaling functions. One class of lipids that discriminates eukaryotes from prokaryotes is that of the sphingolipids. While in animals the phosphosphingolipid sphingomyelin is the most abundant component of lipid rafts, the complex glycosphingolipids in addition to being raft components provide specificity by their interactions with receptors in the same or opposed membranes. The basic membrane sphingolipids sphingomyelin and glucosylceramide are interesting for a different reason as well: they are synthesized from the proapoptotic second messenger ceramide and produce ceramide when they are degraded, often during signaling (1).

Until a decade ago, the biosynthesis of the sphingolipids seemed simple: sphingosine, newly synthesized or recycled from the lysosomes, is converted to ceramide in the ER, where after vesicular transport it receives a phosphocholine or glucose headgroup in the cis-Golgi lumen. Now we know that there are at least 6 ceramide synthases, that sphingomyelin is synthesized in the trans-Golgi, that the ceramide is supplied via the ceramide transfer protein CERT, and that CERT is regulated via phosphorylation. Unexpectedly, glucosylceramide is synthesized on the cytosolic surface of the Golgi. Consequently, it must cross the membrane to reach the site of complex glycolipid synthesis in the Golgi lumen.

We found that natural glucosylceramide does not cross the Golgi membrane and is not a substrate for the multidrug transporters ABCB1 and C1 (recent evidence suggests that ABCA12 is a glucosylceramide translocator specific for the wall of keratinocyte lamellar bodies). Instead, glucosylceramide is transported back to the ER by a protein-mediated mechanism, where it crosses towards the lumen and reaches the Golgi lumen by vesicular transport (2). In the meantime, glucosylceramide is required for protein sorting in the secretory pathway by affecting conditions in the lumen (3). As a possible explanation, we found that glucosylceramide affects the lumenal pH. These and other novel findings will be discussed.

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The involvement of astroglia in the neuropathology of Gaucher disease

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Gaucher disease is the most common lysosomal storage disorder (LSD). This metabolic disorder is caused by mutations in the gene encoding glucocerebrosidase (GlcCerase), and as a result, glucosylceramide (GlcCer) accumulates within the cell. Type 2 and 3, the most severe forms of the disease, are characterized by neurological impairment and neuronal cell death. Little is known about the molecular mechanisms leading from GlcCer accumulation to neurodegeneration and/or neuronal cell death. To date, the neuropathology was attributed mainly to neurons and the role of astrocytes in neuropathology has been largely ignored. Glial cells are the most abundant cell type in the brain and are an important source of neuro-active substances such as growth factors, and neurosteroids, which influence neuronal development, survival, and neuro-secretion. Over the past few years, glia were found to contribute to neuropathology in various neurodegenerative diseases including amyotrophic lateral sclerosis, Parkinson's disease and Huntington's disease.

Thus, we are performing gene arrays studies of cultured astrocytes after treatment with conduritol-B-epoxide (CBE), an active site-directed inhibitor of GlcCerase.

Our data suggest significant alterations in gene expression in these cells. Among the genes that were transcriptionally regulated are genes related to cell death, genes involved in the immune response and the gene encoding alpha-synuclein which is known to be involved in the pathology of Parkinsons disease.

We suggest that GlcCer accumulation in astrocytes is involved in the neuropathology of Gaucher disease and that the cross-talk between astroglia and neurons is a major contributor to disease progression. These results will hopefully shed light on the pathways activated in astrocytes upon GlcCer accumulation, and will thus lead to a clearer picture of the cellular mechanisms involved in neuronal cell dysfunction and death.

Pharmacological chaperones for the treatment of CNS disorders

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Background**:** Mutations in *GBA,* the gene that encodes β-glucocerebrosidase (GCase), lead to Gaucher disease due to reduced enzyme activity and accumulation of the sphingolipid, glucosylceramide (GlcCer). Pharmacological chaperones are orally available, small molecules which increase protein levels by selectively binding and stabilizing proteins in the endoplasmic reticulum (ER), preventing premature ER associated degradation, and promoting proper protein trafficking and processing (Steet et al. *PNAS* 109:13813; Lieberman et al. *Nat. Chem. Biol.* 3:101). The pharmacological chaperone AT2101 (isofagomine tartrate) is highly selective for β-glucocerebrosidase (GCase; Steet et al. *Biochem. Pharmacol.* 73:1376) and is currently under development for the treatment of Gaucher disease. In preclinical studies, AT2101 has been shown to cross the blood brain barrier and increase mutant and WT GCase levels in the brains of mice (2-fold). Clinical studies have demonstrated that AT2101 is generally safe and well tolerated and significantly increases wild-type and mutant GCase levels. These results suggest that pharmacological chaperone therapy may be useful in treating the CNS manifestations of types II and III Gaucher disease. Additionally, multiple studies have identified mutations in *GBA* as a potential risk factor for Parkinson's disease (PD) and other synucleinopathies. Interestingly, we have observed elevated levels of α-synuclein in plasma of some Gaucher patients. We hypothesized that mutated forms of GCase and/or the accumulation of GlcCer (or a related lipid) may

interfere with cellular processing of α-synuclein, predisposing the brain to the accumulation of α-synuclein. To test whether deficient GCase activity would lead to α-synuclein accumulation, we examined Gaucher mouse models that exhibit age-dependent accumulation of GlcCer in the brain. These mice combine a GCase mutation [D409H or V394L] with reduced expression of the GCase-activating protein saposin C. To test whether the converse (increasing wild-type GCase levels) could provide protection against α-synuclein accumulation, we administered AT2101 to a mouse model of PD ([*PDGF*β]*pr-hSNCA*; Masliah et al., *Science* 287:1265) in which wild-type human α-synuclein is moderately overproduced in the hippocampus, cortex, and olfactory bulb. Unlike the Gaucher mouse model, these mice express endogenous, wild-type GCase. **Results:** Mouse models for Gaucher disease demonstrated an accumulation of synuclein in the brain that was sensitive to the activity of GCase and/or the level of its substrate GlcCer (or a related lipid). Furthermore, the amounts of both aggregated (Campbell-Switzer-positive) synuclein and soluble synuclein in the brains of the PD mouse model were reduced by treatment with the GCase-specific pharmacological chaperone, AT2101. **Conclusions**: Enhancement of GCase activity in the brain may be beneficial in treating various synucleinopathies. These studies demonstrate that studying the underlying pathology of many lysosomal storage disorders can provide valuable insight into the pathogenic mechanisms of age-related neurodegenerative diseases. Such research may lead to the development of disease modifying therapies which could benefit both the rare-disease (lysosomal storage disorders) communities as well the populations with more common idiopathic neurodegenerative diseases.

Glucosylcerebrosid accumulation in gba2 knockout mouse

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Summary GBA2

Bile acid beta-glucosidase (GBA2) is a highly conserved, membrane-bound enzyme of the endoplasmic reticulum in many organisms. Although the ubiquitous presence of GBA2, no studies have been published about its functionality so far. To gain insight into the biological function of this enzyme, we generated mice

deficient in GBA2. First characterisations of these animals exhibited impaired male fertility with globozoospermia, and accumulation of glucosylceramides in liver, brain, testes, and other tissues, indicating the importance of this enzyme (Yildiz et al JCI 2006).

The most common inherited defect in glycosphingolipid breakdown is Gaucher's disease, an autosomal-recessive disorder arising from mutation in the gene encoding the lysosomal acid ß-glucosidase acid 1 (GBA1). GBA1 is abundant in lysosoms, which normally degrade large amounts of cellular membrane lipids. When GBA1 is absent or impaired, glucosylceramides accumulate within the macrophage lysosoms, and the engorged cells leads to hepatospleno-megalie, bone lesions, and in severe cases, impairment of central nervous systems function.
Both, GBA1 and GBA2 are glycolipid hydrolases and catalyse the same reaction: The hydrolysis of glucosylceramide to glucose and ceramide. Despite of this common function, they do not share any sequence identity and are expressed in different subcellular compartments. Patients with Gaucher disease show no clear correlation between phenotype and genotype, the striking biochemical similarities between GBA1 and GBA2 might indicate an important role of GBA2 in the pathogenesis of Gaucher disease.

Research Presented

2008 Lysosomal Diseases and the Brain Conference

Organized by the Children's Gaucher Research Fund

--- Poster Session Abstracts ---

Murine models of acute neuonopathic Gaucher disease

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Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder; caused by mutations in the glucosidase, beta, acid (GBA) gene that encodes the

lysosomal enzyme glucosylceramidase (GCase). GCase deficiency leads to characteristic visceral pathology and in some patients, lethal neurological manifestations. We have generated mouse models of the severe neuronopathic form of Gaucher disease. To circumvent the lethal skin phenotype observed in several of the previous GCase-deficient animals, we genetically engineered a mouse model with strong reduction in GCase activity in all tissues except the skin. These mice exhibit rapid motor dysfunction associated with severe neurodegeneration and apoptotic cell death within the brain, reminiscent of neuronopathic GD. In addition, we have created a second mouse model, in which GCase deficiency is restricted to neural- and glial cell progenitors and progeny. These mice develop similar pathology as the aforementioned mouse model, but with a delayed onset and slower disease progression, which indicates that GCase deficiency within microglial cells which are of hematopoietic origin is not the primary determinant of the CNS pathology. These findings also demonstrate that normal microglial cells cannot rescue this neurodegenerative disease. These mouse models have significant implications for development of therapy for patients with neuronopathic GD.

Expression of PTD (protein transduction domain)-glucocerebrosidase in *Pichia pastoris* for potential therapeutic treatment of Types 2 and 3 Gaucher disease.

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Although enzyme replacement therapy is effective for treatment of Type 1 Gaucher disease, its effectiveness for treating the Types 2 and 3 forms is limited because of the blood brain barrier that is impermeable to macromolecules. We have attempted to express glucocerebrosidase (GBA) in the methyltropic yeast *Pichia pastoris* as a human recombinant enzyme as well as chimeric enzyme fused with the eleven amino acid HIV Tat-protein transduction domain (PTD). The PTD has been documented to permeate all known animal cell membranes and also the blood brain barrier. Initial expression of GBA in wild type *P. pastoris* was unsatisfactory as the recombinant enzyme was hyperglycosylated to a 83 KDa form that was inactive. However, when GBA was expressed in the Glycoswitch5 strain of *P.*

pastoris in which the yeast *OCH1* gene responsible for hyperglycosylation was deleted, we succeeded in expressing GBA as an active enzyme of 63 KDa similar to the native GBA in human. In order to facilitate purification, we incorporated a hexa-histidine tag to GBA that permits affinity purification of the enzyme to homogeneity by using a Nickel-NTA column. However, it was noted that a pH above 7.5 is needed to protonate the histidine tag for GBA binding and this resulted in pH inactivation of GBA. To circumvent this problem, we replaced the histidine tag by the cellulose binding domain (CBD) that has specific affinity for cellulose between pH 4 to 9. GBA-CBD was expressed and secreted into the culture medium of *P. pastoris* Glycoswitch5, adsorbed onto cellulose at pH 6.0, and recovered as an active and stable enzyme upon proteolytic cleavage of a site between GBA and CBD. We are currently attempting to express PTD-GBA-CBD in *P. pastoris* for purification and uptake studies in cultured Gaucher fibroblasts, and eventually in a Gaucher mouse model for potential enzyme delivery to the CNS.

NPC1 deficiency leads to oxidative stress: protective effect of allopregnanolone

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Niemann Pick C disease (NPC) is an autosomal recessive neurodegenerative disorder caused by the abnormal function of NPC1 or NPC2 proteins, leading to an accumulation of unesterified cholesterol and glycosphingolipids (GSLs) in the lysosomes. The mechanisms underlying the pathophysiology in NPC disease are not clear. Oxidative damage is implicated in the pathophysiology of different neurological disorders and the effect of GSL accumulation on the intracellular redox state has been documented. Therefore, we determined whether the intracellular redox state might contribute to the NPC disease pathophysiology. Since the treatment of NPC mice with allopregnanolone (ALLO) increases their lifespan and delays the onset of neurological impairment, we analyzed the effect of ALLO on the oxidative damage in human NPC fibroblasts.

We first analyzed the intracellular concentrations of reactive oxygen species (ROS), lipid peroxidation, reduced (GSH) and oxidized (GSSG) glutathione, and the activity of glutathione reductase and catalase, in fibroblasts from NPC patients (FNPC) and in fibroblasts from normal controls (FNC). The levels of ROS and lipid peroxidation were higher in FNPC than in FNC. While no differences were found in the activity of glutathione reductase and in the levels of total GSH and GSSG , catalase activity was significantly reduced in FNPC with respect to FNC. In addition, FNPC were more susceptible than FNC to cell death through apoptosis after an acute oxidative insult. This process was mediated by activation of the NF-κB signaling pathway. To determine whether the NPC deficiency caused the oxidative stress condition observed in FNPC, we selectively knocked down the expression of NPC1 in primary cultures of fibroblasts and in human SHSY5Y neuroblastoma cells, using small interference RNA (siRNA). The down regulation of NPC1 protein caused a massive accumulation of intracellular unesterified cholesterol, mimicking the NPC phenotype, and increased ROS significantly in both fibroblasts and neuroblastoma cells. These results suggest a causal involvement of NPC1 deficiency in the oxidative stress condition observed in FNPC. Furthermore, the association between the down regulation of NPC1 protein expression and the increase in ROS levels in human SHSY5Y neuroblastoma cells, suggests that NPC1 deficiency would lead to a condition of oxidative stress not only in fibroblasts but also in neuronal cells.

Treatment of FNPC or NPC1 knockdown cells with ALLO or its enantiomer ent-ALLO significantly reduced ROS levels after 15-30 min. These data suggest that ALLO exerts an effect on the intracellular redox state that might be mediated through a non genomic mechanism. In addition, ALLO (50 nM) increased the catalase activity, reduced lipid peroxidation, and prevented peroxide-induced apoptosis and NF-kB activation in FNPC.
Thus, oxidative stress plays a role in the pathophysiology of NPC disease and ALLO might be beneficial in the treatment of the disease, at least in part, due to its ability to restore the intracellular redox state.

Is there activation of the unfolded protein response in neuronopathic Gaucher disease?

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Gaucher disease is the most common lysosomal storage disorder (LSD). This metabolic disorder is caused by mutations in the gene encoding glucocerebrosidase (GlcCerase), and as a result, glucosylceramide (GlcCer) accumulates within the cell. Type 2 and 3, the most severe forms of the disease, are characterized by neurological impairment and neuronal cell death. Over the past few years, our laboratory has shown in neuronal models of Gaucher disease that GlcCer enhances agonist-induced Ca^{2+}-release from the endoplasmic reticulum via the ryanodine receptor (RyR), and that the enhanced Ca^{2+}-release is directly responsible for neuronal cell dysfunction and death. Moreover, in *in vitro* studies, we have shown that GlcCer directly interacts with and modulates the activity of the RyaR, the major Ca^{2+}-release channel in the ER, and that this also occurs in human brain tissue obtained post-mortem from Gaucher disease patients. These findings suggest that defective Ca^{2+}-homeostasis may be a mechanism responsible for neuropathophysiology in acute neuronopathic Gaucher disease. However, it is still not clear how accumulated GlcCer leads to neuronal cell death.

Altered Ca^{2+}-homeostasis may lead to impaired ER activity and to accumulation of mis-folded proteins in the ER lumen. In order to restore normal ER function, the cell can activate the unfolded protein response (UPR). However, if the adaptive response fails, UPR can lead to cell death. The UPR was indicated in other LSDs such as GM1 gangliosidosis, and infantile neuronal ceroid lipofuscinoses (INCL). Therefore, we are examining the involvement of the UPR in neuronopathic Gaucher disease. Despite all of the above, to date, we have not found any indications for activation of the UPR in various models of the disease, among them cultured hippocampal neurons as well as cultured astroglia treated with an inhibitor of GlcCerase (conduritol B-epoxide) and the Gba and L444P mouse models.

Anti-inflammatory agents as a therapeutic potential in Niemann Pick type C disease

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Niemann Pick Type-C (NP-C) is an autosomal recessive neurodegenerative disease caused by mutations in NPC1 (95%) or NPC2 (5%), resulting in lysosomal accumulation of unesterified cholesterol and glycolipids. How lysosomal storage and trafficking defects lead to neurodegeneration is unknown. The NIH mouse model of NP-C has a mutation in the NPC1 gene, and exhibits several pathological features of the most severe NP-C patients. Our recent studies show an early neuroinflammatory process in the pathology of NP-C mice. Inhibition of neuroinflammation may therefore present a new therapeutic opportunity for this neurological disorder. FK506 (Tacrolimus), an immunosuppressant, and atorvastatin, a 3ß-hydroxy-ß-methylglutaryl coenzyme A reductase inhibitor, have immunomodulatory properties that show benefit in the treatment of neurodegenerative diseases that have a neuroinflammatory component, such as multiple sclerosis and Alzheimer's disease. To determine if suppression of the neuroinflammatory response is beneficial in treating NP-C, we treated NP-C mice with FK506 (5mg/kg/day, SC 3 times/week), or atorvastatin (10mg/kg/day, orally daily), starting at postnatal day 7. Both FK506-treated and atorvastatin-treated mice but not PBS-treated control mice had similar increases in lifespan, from 67 days (untreated NP-C mice) to 78 days. Both treatments also delayed the start of weight loss and loss of motor coordination by 2 weeks. We also treated NP-C mice with a combination of FK506 plus allopregnanolone, a neurosteroid that we showed was effective in increasing lifespan and delaying loss of locomotion and coordination. Treatment with allopregnanolone plus FK506 did not increase longevity further than treatment with allopregnanolone alone. However, this combination treatment rescued fertility in previously infertile male and female NP-C mice, indicating a new beneficial effect of dual therapy. Our results therefore indicate that FK506 and atorvastatin are effective treatments in NP-C mice. These drugs may be new and promising neuroprotective drugs for NP-C disease.

Presentation and natural history of Niemann-Oick disease Type C in the UK

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Aim- To audit all known patients with Niemann-Pick disease Type C in the UK up to February 2008 in respect of age at diagnosis, presenting symptoms, disease progression and distribution.
Method- A retrospective review of all data held on national database for 67 live patients and 48 deceased comparing data in neonatal, childhood, adolescent and adult presentations.

Results- the 67 live patients ranged in age from 1 yr 4 months to 53 years 7 months, with a mean of 17 years 2 months. All patients suffered at some time from most of the symptoms associated with NPD including ataxia, swallowing problems, neurodegeneration, dementia and seizures.
Age of onset went from stillbirths to age 49, age at diagnosis from 2 months to 49 years. The rate of neurodegeneration is variable but there is a similar pattern in each of the presentations.

Length of time between onset of symptoms and diagnosis can be very different and often correlates with proximity to a diagnostic centre.

Conclusion- having a clearer picture of the natural history of NPD will be an important factor when assessing the future care needs of these families.

The Pharmacological Chaperone AT2101 Increases Cellular and Tissue Levels of L444P -Glucosidase

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Gaucher disease is caused by mutations in the gene for the lysosomal enzyme acid β-glucosidase, also known as glucocerebrosidase (GCase). The two most common missense mutations lead to the expression of N370S and L444P mutant forms of the enzyme. Patients homozygous or heterozygous for the N370S mutation typically present with a non-neuronopathic form of Gaucher disease, whereas those

homozygous for the L444P mutation present with a neuronopathic form. We have shown previously that the small molecule pharmacological chaperone AT2101 (isofagomine tartrate) binds and stabilizes N370S GCase, resulting in more efficient trafficking of the enzyme to lysosomes and an increase in cellular GCase levels (Steet et al; PNAS 2006, 103: 13813-13818). In this study, we investigated the effect of AT2101 on L444P GCase levels *in vitro* and *in vivo*. Incubation with AT2101 reproducibly caused a significant and concentration-dependent increase in GCase levels (up to 235% above baseline) in fibroblast and lymphoblastoid cell lines derived from Gaucher patients homozygous for the L444P mutation. In mice that express murine L444P GCase and show an attenuated Gaucher disease phenotype (Mizukami et al; J. Clin. Invest. 2002, 109: 1215-1221), oral administration of AT2101 resulted in a significant and dose-dependent increase (2- to 5-fold) in GCase levels in all disease-relevant tissues that were tested (liver, spleen, lung, and brain). Increased GCase levels were sustained for up to 4 days after withdrawal of AT2101. The effect of AT2101 on L444P GCase was selective, as the levels of four other lysosomal hydrolases were not altered. In the same animals, 8-week AT2101 treatment resulted in statistically significant lowering of plasma chitin III and IgG levels. Similarly, 24-week AT2101 treatment caused a statistically significant reduction in spleen and liver weights. Importantly, oral administration of a single dose of AT2101 to rats (300 mg/kg) and monkeys (1000 mg/kg) showed detectable AT2101 levels in brain and cerebrospinal fluid, respectively, as well as in all other tissues examined, as measured by LC-MS/MS. Taken together, these data indicate that AT2101 can increase levels of L444P GCase in Gaucher patient-derived cell lines and in the tissues of mice that express murine L444P GCase. Moreover, the data indicate that *in vivo* AT2101 is orally available and distributes into multiple tissues, including the brain. As such, AT2101 may merit further evaluation for the treatment of patients with neuronopathic Gaucher disease.

Acid β-glucosidase induces negative-regulation of p38 proinflammatory signaling kinase

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Sphingolipids are important components of cells, many of which function as bioactive signaling molecules. Of these, ceramide is a central metabolite and plays key roles as a lipid second messenger in a variety of cellular responses. Ceramide controls cellular signaling by activating target molecules such as ceramide-activated protein kinases/phosphatases. Our recent studies demonstrated that activation of protein kinase C (PKC) by a phorbol ester (PMA) induces ceramide formation by stimulating the salvage pathway that recycles long chain sphingoid bases derived from complex sphingolipids. Functionally, the formation of ceramide derived from the salvage pathway was shown to accelerate dephosphorylation/inactivation of a proinflammatory signaling kinase p38 by activating ceramide-activated protein phosphatases. In this study, acid β-glucosidase (GBA1), which hydrolyzes glucosylceramide, was hypothesized to contribute to the elevation of ceramide with PKC activation and consequent dephosphorylation/inactivation of p38, and we characterized the ceramide signals dependent on GBA1.

To assess involvement of GBA1 in ceramide formation and signaling, genetic and pharmacological approaches were employed. Knock-down of GBA1 by small interference RNA (siRNA) significantly inhibited the generation of ceramide in response to PKC activation by PMA which promoted a decrease in glucosylceramide, suggesting GBA1-dependent formation of ceramide. Accompanied with inhibition of GBA1-dependent formation of ceramide by treatment with either GBA1 siRNA or an inhibitor of GBA1, p38 phosphorylation was dramatically increased following PMA treatment. These results suggest that the GBA1/ceramide pathway is involved in counteracting p38 activation. The proinflammatory signaling kinase, p38, is known to induce the generation of IL-6, but if and how GBA1/ceramide pathway controls the biosynthesis of IL-6 was poorly defined. Interestingly, knock-down of GBA1 significantly enhanced the generation of IL-6 induced by PMA or TNF-α. Inhibition of p38 by pharmacological and genetic approaches was revealed to abolish the induction of IL-6 enhanced by the defects in GBA1, suggesting p38-dependent induction of IL-6 in GBA1-defective cells.

Collectively, GBA1 plays a role in the salvage pathway of ceramide formation and could be important in the subsequent inactivation of p38, which is responsible for

IL-6 biosynthesis. Impairments of a GBA1-dependent ceramide signal toward p38 in cellular responses of patients with Gaucher disease may lead to pathogenic production of IL-6 which can propagate the inflammatory reactions by stimulating immune cells and even cause neurotoxicity.

Pharmacological chaperone development for Gaucher disease: pH dependence of enzyme structure and stability

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The human lysosomal enzyme acid-β-glucosidase (GCase) hydrolyzes the sphingolipid glucosylceramide, and mutations in GCase lead to Gaucher disease. Partially-active mutant GCase is retained in the endoplasmic reticulum (ER) and targeted for degradation, and as a consequence never reaches the lysosome. The details of structural and stability differences of GCase in the neutral-pH environment of the ER versus the acidic-pH environment of the lysosome are important for the development of pharmacological chaperone therapy for Gaucher disease, in which a small molecule stabilizes a mutant enzyme in the ER to enable lysosomal trafficking. Once in the lysosome, the partially active mutant enzyme can break down accumulated substrate and ameliorate clinical symptoms. In contrast to currently available therapies, pharmacological chaperones are capable of crossing the blood-brain barrier, and thus are likely to treat the severe form of Gaucher disease that affect the CNS. We have solved the crystal structure of apo GCase at various pHs and in complex with two inhibitors, one that acts as a

pharmacological chaperone, and a close analog compound that does not. In addition, we have measured the differences in stability at ER and lysosomal pH by differential scanning calorimetry. Our results provide detailed insight into how GCase is thermodynamically stabilized by competitive inhibitors, and suggest strategies for the development of new pharmacological chaperones for Gaucher disease and other lysosomal storage disorders.

The effect of Purkinje Neuronal Rescue on the severity of Niemann-Pick C disease

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Niemann-Pick Type C (NPC) Disease is an autosomal recessive disorder that affects lipid metabolism. Childhood onset predominates and symptoms include severe liver disease, breathing, feeding and eye movement difficulties, seizures, dystonia, and ataxia. Children can survive to adulthood but ultimately succumb to neurodegeneration if not liver failure. NPC is predominantly caused by the lack of function of NPC1, a protein involved in the trafficking of endosomal-lysosome cholesterol and possibly other sterols. Although, NPC1 is present in all tissues, only specific subsets of cells require its function for survival. We aim to uncover why.

In the mouse model of the disease, one hallmark is patterned cerebellar Purkinje cell loss. Purkinje neurons are considered the main producers of steroids in the brain and provide the only neuronal output from the cerebellum. However, their importance to the etiology of the NPC disease phenotype has never been assessed before. Here we rescue Purkinje neurons by expressing functional mouse Npc1 specifically in cerebellar Purkinje cells in an otherwise Npc1 null mouse. To do this, we utilize a tet-inducible system.

Tet-technology offers cell type specific and temporal control of disease genes. Gene expression can be adjusted on, off or to intermediate levels. Early events of disease onset can be assessed as well as the potential reversibility of pathology. Our initial findings using this tet-inducible approach include: outstanding Purkinje

neuron survival even during the morbidity stage of the disease, a slightly prolonged lifespan, improved nesting behavior, greater weight gain and healthier overall tissue morphology as compared to diseased mice. We suspect the effects we are seeing to be steroid related and are currently testing the steroidogenic capacity of Purkinje neurons with respect to Npc1 levels. Our data verifies that Npc1 acts cell autonomously in neurons and shows that rescued Purkinje cells can ameliorate the disease phenotype.

Exposure to a glucocerebrosidase inhibitor alters α-synuclein.

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The long-term goal of our research is to determine α-synuclein (α-syn)-related molecular pathways that contribute to pathogenesis in Gaucher disease and other human neurodegenerative diseases including Parkinson's disease (PD). Specifically, our objectives are to: (a) determine mechanisms of neuronal damage in the neuronopathic forms (i.e. types 2 and 3) of Gaucher disease, and (b) elucidate the biological, mechanistic link between non-neuronopathic Gaucher disease (type 1) and α-synucleinopathies, such as PD. A growing body of clinical literature indicates a relationship between Gaucher and parkinsonism; similarly, lysosomal storage abnormalities have been observed in experimental PD models. Together, these reports suggest that convergent mechanisms may contribute to neurodegeneration in these disorders. In this study, we test the hypothesis that the protein α-syn may represent a pathological substrate for neurotoxicity in Gaucher disease and other lysosomal storage diseases. We have generated data using a mouse model of glucocerebrosidase (GCase) inhibition by conduritol B epoxide (CBE) supporting this hypothesis. Our findings demonstrate, for the first time using a pharmacological approach, a biological connection between α-syn and GCase activity. After one injection of CBE into mice, enhanced α-syn expression is detected within neurons of the substantia nigra. Such alteration is important as increased α-syn levels promote the development of cell death and pathology in brain from humans with multiplication mutations of the α-syn gene. Astrocytic activation was also detected in the substantia nigra after a single administration of CBE, further suggesting that cellular injury and/or frank cell death are promoted by in vivo exposure to the inhibitor. Analyses by Western blot and confocal

microscopy revealed that the normal cellular distribution of α-syn is perturbed after GCase inhibition by CBE. After CBE exposure, we found that nigral levels of α-syn in the insoluble fraction are increased, suggesting an alteration in its solubility and/or intracellular localization. Consequently, accumulation of the protein occurs in the cell body of nigral neurons, indicating that normal intracellular α-syn trafficking is altered under conditions of reduced glucocerebrosidase activity. These studies indicate that α-syn could play a role in neuronal dysfunction in types 2 & 3 Gaucher disease, and further, provide a biological link between type 1, non-neuronopathic Gaucher disease and parkinsonism.

LC-MSMS LIPID ANALYSIS OF LUXOL FAST BLUE HIGHLIGHTED REGIONS IN X-LINKED ADRENOLEUKODYSTROPHY BRAIN TISSUE SAMPLES

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Background: X-linked adrenoleukodystrophy (ALD) is a neurogenerative disorder characterized by an increase of very long chain fatty acids (VLCFA) in plasma and other tissues. Luxol fast blue (LFB) was used as a white matter-selective magnetic resonance (MR) imaging contrast agent for human *ex vivo* brain tissue. 8 LFB stained blocks of postmortem ALD white matter were identified as (1) lesion, (2) inflammatory zone, (3) normal appearing white matter, and (4) cortex by MR imaging. 8 samples from non-ALD white matter and gray matter were prepared with LFB and analyzed. **Methods:** The samples were dried, weighed, and extracted by the method of Folch. The upper phases were assayed for VLCFA content by capillary gas chromatography. The lyso-phosphatidylcholines (LPC) and ceramides were analyzed by LC-MSMS. **Results:** On average the ALD upper phases had a 22 fold increase in C26:0 content. The non-hydroxy fatty acid ceramides were increased 5 fold in the ALD samples and there was a 1.8 fold increase in the C26:0 ceramide content. The analysis of the LPC species showed an average of a 3.3 fold increase in the total LPCs/mg dry wt. and the total C26:0 LPC had a 10.4 fold increase in the ALD samples. The 1-0-alkyl-lyso-PC (lyso-PAF) was increased in ALD samples 14 fold compared with control samples. **Conclusions:** The increased amount of ceramides in white matter diseases and the

increased C26:0 content of gangliosides and LPCs in ALD brain have been reported previously. The new finding of the increased lyso-PAF in ALD brain will determine a new approach in research of the demyelination process in ALD brain.

Identification of A-Galactosidase A Inhibitors Using High-Throughput Screening

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Fabry disease is an X-linked lysosomal storage disorder that is caused by a deficiency of the enzyme α-galactosidase A (α-gal). Many of the mutations in Fabry disease are missense alterations that may cause misfolding, decreased stability, and/or mistrafficking of the α-gal protein. Iminosugar analog enzyme inhibitors have demonstrated chemical chaperone activity that may correct enzyme folding and allow trafficking to the lysosome. In order to develop more broad chaperone therapy, new compounds need to be identified, especially enzyme activators. A fluorogenic α-gal enzyme assay has been optimized and miniaturized into a 1536-well plate format. The preparations used were purified green coffee bean enzyme, expressed and purified recombinant human enzyme, and enzyme from human spleen homogenate. This enzyme assay has been used in the high-throughput screening of a 170,000 compound library. Several novel structures of lead compounds have been identified.

Complexity of mutational analysis of genes located on 1q22; A region with a high risk of recombination

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The glucocerebrosidase (GBA) locus on chromosome 1q22 is very gene-rich, encompassing 15 genes and 2 pseudogenes within 200kb. The HapMap project demonstrates that this region has very low linkage disequilibrium, with recombination occurring frequently in different populations. The presence of contiguous, highly homologous pseudogenes for both GBA and metaxin 1 (MTX1) at this locus increases the likelihood of DNA rearrangements in this region. These recombinations and the presence of several hot spots for recombination around this locus can complicate genotyping of GBA in patients with Gaucher disease (GD) and may contribute to the difficulty in interpreting genotype-phenotype correlations in this disorder.

When screening patients for GBA mutations, complex alleles may be missed by simple mutational analysis, since PCR based genotyping may not always detect a DNA rearrangement. Mutation screening may also yield a false genotype, indicating homozygosity for a mutation when the other allele carries pseudogene sequence resulting from a recombination event. To identify all possible GBA recombinations and to determine the gene copy number, we combine several genotyping strategies: Sequencing of genomic DNAs is performed specifically examining all polymorphic sites in GBA, including two repeat sequences located upstream and between GBA and its pseudogene (GBAP) by short tandem repeat polymorphism (STRP)-based genotyping. Southern Blot analysis of genomic DNA using restriction enzymes SstII, SspI, EcoRI or HincII is performed to detect duplications or fusions in the GBA locus of less than 50 kb. Quantitative Real-Time PCR can also be used to detect DNA rearrangements within the GBA locus. Lastly, haplotype analysis using chromosome 1 markers can be used to identify a large deletion, recombination or uniparental disomy.

By applying these strategies, we identified 67 recombinant alleles among 550 alleles from patients with GD. These recombinant alleles included fusions between GBA and GBAP, duplications of GBA, GBAP or the metaxin pseudogene, and gene conversions between GBA and GBAP. Many of these alleles would have been missed by sequencing alone. Uniparental disomy of chromosome 1 was identified in one patient with GD who also had Charcot-Marie-Tooth. The child was homozygous for mutations in both GBA and MPZ as well as 23 chromosome 1 markers, confirming paternal isodisomy. Thus complementary methods are required to accurately perform genotyping at this locus.

A mouse model of the free sialic acid storage disorders shows dysmyelination of the central nervous system

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Lysosomal storage disorders (LSDs) are associated with the pathological accumulation of material in lysosomes, frequently leading to neurodevelopmental defects. The free sialic acid storage disorders, Salla disease and infantile sialic acid storage disease, constitute one group of LSDs. The causative mutation of these disorders is in the gene sialin, an anion/cation symporter that uses a hydrogen ion gradient to pump sialic acid out of lysosomes. These disorders are rare; consequently, the underlying pathophysiology during development remains unknown. A sialin knockout mouse recently became available through the NIH mouse knockout program. The mouse line was generated through targeted disruption of the first exon of the sialin gene. As a first step in validating this mouse as a model for the free sialic acid storage disorders, we confirmed the genetics and analyzed the behavior of these mice. Sialin knockout mice show decreased weight gain and uncoordinated gait compared to wild-type littermates. Pathological characterization of this mouse shows enlarged vacuolar structures, a hallmark of lysosomal storage disorders, in brain and spinal cord neurons. Importantly, using histology, electron microscopy, and Western blotting, we found decreased myelination of the central nervous system which is consistent with the human disease. However, how loss of sialin leads to dysmyelination of the central nervous system remains unclear. It is not known if loss of sialin function in neurons, oligodendroglia or both leads to this phenotype. Our preliminary studies indicate that oligodendrocyte precursor cells (OPCs) derived from sialin knockout and wild-type littermates show similar survival, proliferation and differentiation in vitro. This suggests that sialin function may be necessary for OPC migration or may be more important in neurons than in oligodendrocytes for proper myelin formation, possibly through regulation of sialylated glycoproteins and glycolipids.

The frequency of glucocerebrosidase mutations in cohorts with Parkinson Disease

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BACKGROUND: Recent studies have indicated a higher incidence of mutations in the glucocerebrosidase (GBA) gene in subjects exhibiting Parkinson disease (PD). Consequently, the aim of the current study was to screen for mutations in well characterized cohorts of patients with PD. DNA panels were obtained from the Coriell Institute's NINDS Human Genetics DNA and Cell Line Repository where extensive collections of DNA are maintained and cataloged. The panels screened in the study were from subjects with PD from various ethnic backgrounds, as well as panels of neurologically normal individuals used as controls.
METHODS: Analysis included 276 Caucasian and 184 Chinese PD subjects and 184 Caucasian, 92 Chinese control subjects. The GBA gene was amplified using PCR and all 11 exons were sequenced. Clinical information was compared in subjects with and without GBA mutations.

RESULTS: Among the Caucasian subjects sequenced, 12 (4.2%) carried mutations in GBA. These included 9 subjects with either L444P or N370S and 3 with rare alleles. Eighteen (18) were found with E326K or T369M, alterations believed to be polymorphisms. In the Chinese cohort, 9 subjects were found to carry mutations (4.2%) whereas only one control (1%) had a GBA mutation. The distribution among the entire series was 258 males and 199 females whereas, subjects with GBA mutations included 24 males and 16 females. Generally, the average age of disease onset for mutation carriers was 62 years, as compared to 63 years in the entire cohort. Twelve (12%) of the subjects with mutations had a family history of PD as compared to 15% of the entire cohort.

CONCLUSIONS: This study demonstrates that mutations are seen at a frequency of around 4% in PD subjects of different ancestries. Clinically the age of onset, and family history of patients did not differ significantly from the average for the entire cohort. The study demonstrates the utility of a well characterized DNA repository in determining mutation frequency in a given gene.

A viable mouse model of neuronopathic Gaucher disease: Acid β-glucosidase V394L/saposin C deficient mice have CNS glucosylsphingosine and glucosylceramide accumulation and progressive neurological deficits

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Gaucher disease is the most common lysosomal storage disease and is caused by defective acid b-glucosidase (GCase) function. Saposin C is a lysosomal protein needed for optimal GCase activity. To test the *in vivo* effect of saposin C on GCase, saposin C deficient mice (C-/-) were backcrossed to point mutated GCase (V394L/V394L) mice. The resultant mice (4L;C*) began to exhibit CNS abnormalities ~30 days: first as hindlimb paresis, and then progressive tremor, ataxia, incoordination, 25% weight loss and decreased ambulation. Death occurred ~48 days due to neurological deficits. Axonal degeneration was evident in brain stem, spinal cord and white matter of cerebellum accompanied by increasing infiltration of the brain stem, basal ganglion, thalamus and hindbrain regions by CD68 positive microglial cells, and activation of astrocytes. Electron microscopy showed inclusion bodies in neuronal processes, myelin layer separation and degenerating cells. Relative to V394L/V394L mice, 4L;C* mice had diminished GCase protein and activity. Marked increases (20-30 fold) of glucosylsphingosine (GS) and moderate elevation (1.5-3 fold) of glucosylceramide (GC) were detected in the brain. In contrast to the CNS, visceral tissues appeared normal and, in addition, no storage cells were found in liver or lungs of 4L;C* mice, but increases of GS and GC were. Neuronal cells in thick slices of the hippocampus from 4L;C* mice had significantly attenuated long-term potentiation than WT mice, suggesting synaptic impairment presumably resulted from substrate accumulation. The 4L;C* mouse mimics the CNS phenotype and biochemistry of some type 3, neuronopathic variants of Gaucher disease, and is a unique and viable model suitable for testing chaperone and substrate reduction therapies, and investigating the mechanisms for neuronopathic Gaucher disease.

The effects of the D409H and H255Q glucocerebrosidase mutations when present individually or in tandem.

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Gaucher disease is an autosomal recessive disorder that is caused by mutations in the gene encoding the enzyme glucocerebrosidase (*GBA*). Homozygosity for the common D409H mutation is associated with a unique type 3 phenotype manifesting with slowed horizontal eye movements, cardiac calcification and, at times, hydrocephalus and dysmorphic features. We evaluated an infant of Albanian ancestry with type 2 Gaucher disease who was reported to have a D409H/D409H genotype. Complete gene sequencing determined that the infant was also homozygote for the H255Q mutation. Previous reports have also shown that the D409H;H255Q tandem *GBA* mutation is associated with type 2 Gaucher disease. While there is one previous report of a Greek infant with type 2 Gaucher disease carrying only the H255Q mutation on one allele and a RecTL on the second allele, we recently confirmed that the H255Q allele also carried the D409H mutation, while the second allele was a RecNcil. Thus, there are no reports of the rare H255Q mutation occurring alone; it is always seen present in *cis* with the D409H mutation. The objective of this study was to identify the effect of each of these mutations on the glucocerebrosidase enzyme. The two mutations were introduced into a human *GBA* cDNA construct both independently and in tandem. Heterologous expression of either the single or double mutant *GBA* was studied in CHO cells to assess their impact on transcription, translation, and enzyme activity. The results showed that the D409H mutation caused an almost complete loss of enzyme activity as well as reduced protein levels, while the H255Q mutation did not confer a loss of enzyme activity, protein or transcription. This suggests that the H255Q mutation could be involved in protein trafficking or other post-transcriptional processing. While it is still possible that this mutation is actually a rare polymorphism since it has never been observed alone, the more severe clinical course associated with the double mutant allele suggests that the H255Q has some impact on the phenotype.