A Murine Central Nervous System-Derived Cell Line Carrying a NPC1 Gene Mutation and Expressing Multipotent Neural Stem Cell-Like Properties

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Understanding the subcellular and functional deficits and developing effective therapeutic strategies for the central nervous system (CNS) are principal issues in NPC research. Suitable mutant CNS cell lines could greatly expedite progress in these areas, but have not been available. We have now obtained and characterized a cell line originally arising as a highly proliferative growth within primary cultures of neocortical cells from the BALB/c npc1^{nih} mouse. The line is maintained under simpler culture conditions than required for "neurospheres" and continues to show rapid expansion after over 20 passages. In addition to dividing progenitor-like cells, identifiable neurons, astrocytes and oligodendrocytes but not microglia are present under maintenance conditions as evaluated morphologically and by cell type specific antigenic markers (β-tubulin isotype III and MAP2; GFAP and S100β; O4, O1 and galactocerebroside; MAC1, respectively). This argues that the line is of neuroectodermal lineage. Culture medium formulations using combinations of cytokines and growth factors have been explored to selectively stimulate development of individual cell types. For example, neuronal development is promoted by serum withdrawal and by addition of leukemia inhibitory factor or bone morphogenetic protein (BMP) 2, with cells showing neuritic processes and neuronal markers. Astrocytes are favored in the presence of BMPs 2 or 4 in medium also supplemented with epidermal growth factor and basic fibroblast growth factor (bFGF). Oligodendrocytes are favored in serum-free medium containing N-terminal sonic hedgehog and the majority develop more mature galactocerebroside-positive phenotypes by extended culture periods in non-supplemented serum-free medium. All cells display filipin-detected unesterified cholesterol in subcellular patterns consistent with the NPC1 defect, and this staining is particularly prominent in cells identified as neurons by virtue of β-tubulin isotype III expression. Confocal microscopy also shows coincidence of filipin-positive staining with LAMP2-positive vesicles, compatible with lysosomal storage. The extent of glycolipid storage is currently under investigation. This cell line should prove useful for detailed cell and molecular studies on NPC1 disease, as well as for screening assays directed at testing pharmacologic and macromolecular agents for CNS therapy.

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NPC1 I1061T mutant protein is functional but unstable due to targeting for proteasomal degradation by the ER quality control machinery

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Over 200 disease-causing mutations have been identified in the NPC1 gene. The most prevalent mutation, NPC1 I1061T, is predicted to lie within the cysteine-rich luminal domain and is associated with the classic juvenile-onset phenotype. Previous studies have shown that NPC1 I1061T is expressed at lower levels and exhibits altered banding patterns on Western blotting as compared to WT. We hypothesize that NPC1 I1061T protein is unstable due to altered processing in the secretory pathway. Our studies using human fibroblasts homozygous for the I1061T mutation (n=3 cell lines) find NPC1 I1061T and WT mRNA levels are similar, but NPC1 I1061T protein levels are decreased to 10-20% of WT. Metabolic labeling studies demonstrate that during 0-4 hours of *de novo* synthesis, WT protein undergoes a glycosylation pattern shift from primarily endoH-sensitive to endoH-resistant species, whereas NPC1 I1061T is nearly exclusively endoH-sensitive. While the $t_{1/2}$ of WT or NPC1I1061T endoH-sensitive species is ≤7 hours, the $t_{1/2}$ of WT endoH-resistant species is ≥18 hours. NPC1 I1061T protein expression is increased using treatments (glycerol and reduced temperature) that relax ER quality control. Mutant protein expression is also increased by treatment with MG132, but not chloroquine, indicating that degradation is via the proteasomal pathway. Unexpectedly, overexpression of NPC1 I1061T in NPC1I1061T mutant fibroblasts resulted in late endosomal localization of the mutant protein and complementation of the NPC mutant phenotype, likely due to saturation of the ER quality control checkpoint. Our findings shed light on the mechanism by which the I1061T mutation causes disease, and provides support for chemical chaperone therapy as an approach to treat NPC disease caused by the NPC1 I1061T mutation.

Using Molecular Evolution to Dissect the Function of NPC2

(Niemann-Pick Type C2)

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Mutations in two genes (NPC1 and NPC2) cause defects in transport and processing of cholesterol and other lipids, resulting in the neurodegenerative disease Niemann-Pick Type C. Thus far, little is known about the physiologically relevant activities of these proteins or the precise mechanisms by which they cause disease. As a means to gain insight into these issues, we are studying the molecular evolution of the NPC1 and NPC2 proteins, and report here on our analysis of NPC2. We have identified the set of NPC2 proteins in vertebrate organisms, performed multiple sequence alignments and phylogenetic analysis, and used this information to calculate position-specific conservation scores. By mapping these scores onto the NPC2 crystal structure, we have identified evolutionarily constrained surfaces of the human NPC2 protein. This analysis has allowed us to identify three regions that are likely involved in functionally significant protein-protein interactions. Using similar approaches to study the multi-gene insect NPC2 family, we have identified several regions that are conserved within subfamilies but divergent between them, suggesting that these regions are involved in functional differentiation between the insect NPC2 subfamilies. Finally, we have identified in NPC2 a hydrophobic "knob" that is well-conserved at the level of composition, but poorly conserved in primary sequence, suggesting that it may be involved in binding membranes. This analysis should provide the basis for directed experimental dissection of NPC2 function, which in the long term will hopefully lead to more effective NPC therapy.
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Lysosomal accumulation of neurotrophin receptor Trk and cholesterol in GM1-gangliosidosis mouse brain

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GM1-gangliosidosis is one of the most common neurodegenerative glycosphingolipid storage disorder, caused by genetic deficiency of lysosomal β-galactosidase (β-gal) with consequent disruption of the degradative pathway of GM1-ganglioside (GM1). To understand events underlying progressive neurodegeneration of this disease, we examined Trk neurotrophin receptor signals in β-gal-/- mouse brains. The level of TrkA associated with GM1 was increased in detergent-resistant membrane fraction of β-gal-/- mouse brains. Brain concentration of phosphorylated TrkA was significantly elevated in β-gal-/- mice relative to wild type control mice, following the downstream signal of PLCγ was also elevated. Immunocytochemical studies showed that phosphorylated Trk in β-gal-/- mouse brains was concentrated in cytoplasmic granules, which was co-localized with GM1 and cholesterol. These results indicate that accumulation of GM1 and cholesterol substantially impaired endosomal/lysosomal function, resulting in increased aberrant neurotrophin signaling in GM1-gangliosidosis mouse brains.

The role of NPC1 in synaptic and neuronal function

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Several symptoms of NPC disease, including seizures, cataplexy and disturbances of the sleep wake cycle, suggest that loss of NPC1 leads to alterations in synaptic transmission. Even relatively minor changes in neurotransmitter release from individual neurons can cause imbalances between excitatory and inhibitory input and a compensatory remodeling of the synaptic network. To be able to devise treatment strategies targeting imbalances in neurotransmission, it is important to identify the steps of neurotransmission that are impaired by loss of NPC1 and to elucidate the underlying mechanisms that cause changes in neurotransmitter release in NPC disease. We have previously shown that NPC1 is present in axons and presynaptic terminals and that loss of NPC1 leads to some biochemical and morphological alterations of the synapse. Here we have investigated whether synaptic vesicle release is altered in NPC1-deficient neurons. Using low density cultures of hippocampal neurons isolated from wildtype and *Npc1*$^{-/-}$ mice, we have monitored spontaneous and evoked synaptic vesicle release with a fluorescent dye, FM1-43. Preliminary experiments indicate an overall increased synaptic vesicle pool in *Npc1*$^{-/-}$ neurons, and an increase in spontaneous synaptic vesicle release, but no changes in vesicle release following a high frequency train of action potentials. The lack of alterations in evoked release suggests that exocytosis is not affected by loss of NPC1 but could also be due to altered short term plasticity. We are currently investigating the kinetics of exo- and endocytosis of synaptic vesicles in wildtype and *Npc1*$^{-/-}$ neurons in more detail. Earlier studies have shown a relative depletion of cholesterol in axons of *Npc1*$^{-/-}$ compared to wildtype neurons. To test whether cholesterol distribution is indeed altered in NPC1-deficient synapses, we have prepared synaptic plasma membrane fractions and detergent-resistant domains from isolated nerve endings from wildtype and *Npc1*$^{-/-}$ mice. Surprisingly, preliminary experiments showed no significant differences in synaptic plasma membrane cholesterol between wildtype and *Npc1*$^{-/-}$ mice. Analysis of detergent-resistant membranes indicates an increased formation and altered protein composition of cholesterol-rich membrane domains in *Npc1*$^{-/-}$ compared to wildtype synapses. Further studies are currently underway to verify the purity of these fractions and to characterize the nature of the observed alterations in *Npc1*$^{-/-}$ synapses in more detail.

Altered cyclic AMP signaling in Niemann-Pick type C fibroblasts

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Relatively little is known about the exact function of NPC1, particularly how losing its function results in cholesterol accumulation. NPC1 expression is driven by cAMP pathways and NPC1 mRNA is known to be increased in NPC cells suggesting a chronic increase in the cAMP pathway. We propose that NPC cells respond to the loss of NPC1-mediated trafficking of cholesterol to the plasma membrane by increasing the cAMP pathway in order to increase NPC1 expression. Our preliminary data support this hypothesis in that NPC fibroblasts demonstrate reduced protein expression of the cAMP-specific phosphodiesterase PDE4D compared to wt fibroblasts. Consistent with the finding that PDE4D protein expression is decreased in NPC fibroblasts is an increase in cAMP production in response to the beta-adrenergic receptor agonist isoproterenol (2.0 fold stimulation over unstimulated in wt versus 26.1 fold stimulation in NPC fibroblasts). The proposed consequence of a chronic increase in cAMP signaling is altered cholesterol transport regulation. To address the potential role of PDE4D in cholesterol trafficking, free cholesterol was visualized using filipin staining in wt fibroblasts that were treated with rolipram, a PDE4D specific inhibitor. Inhibition of PDE4 function with rolipram leads to peri-nuclear free cholesterol accumulation similar to what is observed in NPC cells, strongly implicating a cAMP-dependent pathway in the regulation of cholesterol trafficking. Conversely, treatment of NPC fibroblasts with the PKA inhibitor Rp-cAMPs significantly improves cholesterol processing further pointing to the involvement of the cAMP pathway. We propose that the cAMP pathway influences cholesterol processing through the regulation of β-arrestin-2 (βarr2). Chronic increase in the cAMP pathway would initiate an elevation in βarr2 expression, and this predicted increase in βarr2 protein expression was observed in NPC fibroblasts compared to wt controls. Over expression of βarr2 in epithelial cells results in a NPC-like peri-nuclear cholesterol accumulation supporting a causative role for βarr2 in this process. Because βarr2 is important in regulating endocytic recycling pathways it could impact cholesterol processing. Altered cholesterol trafficking in NPC would lead one to expect different βarr2 localization throughout the cell. Immunostaining for βarr2 in both NPC and wt fibroblasts did in fact show not only a more robust staining in NPC cells when compared to wt controls, but also an increase in the staining of the peri-nuclear area of NPC fibroblasts consistent with the region where cholesterol accumulates. Further understanding the implications of altered cAMP signaling and its relationship to aberrant cholesterol accumulation would potentially lead to therapeutic advancements in NPC.

Evaluating NPC-1's functional role in both sensing and facilitating the intracellular trafficking of lysosomal stores of amine-containing molecules

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ABSTRACT

The true substrate of NPC1 is controversial and not fully understood at the current time. In this presentation, we provide experimental evidence to support a novel functional role for NPC1, which involves the intracellular trafficking of amine-containing molecules. Specifically, we propose that NPC1 has the ability to sense when lysosomes become markedly concentrated with these molecules, which leads to the formation of a hybrid organelle capable of facilitating their cellular release. We demonstrate that the hybrid organelle originates from a heterotypic fusion event involving lysosomes with late endosomes. The hybrid organelle is unique in that is capable of significant expansion, referred to as vacuolization, the function of which is presumed to quickly dissipate osmotic pressure associated with the highly concentrated amine. The hybrid organelle subsequently facilitates the release of the lysosomal cargo to the plasma membrane. To better understand this specific trafficking event we employed a pulse-chase technique to localize ^{3}[H]-dextran to lysosomes and monitored secretion to the extracellular space as a function of time. It is well known that NPC1 is required for the efficient release of multiple lysosomal cargo relative to cells with mutated NPC1[1]. Conversely, agents have been identified that can cause a NP-C disease phenotype by apparently inhibiting these secretion events[2]; however, to our knowledge, no one has previously shown that NPC1 function in this secretion event could be stimulated, which, in turn, may provide insight into the true substrate of the protein. Using this secretion assay we revealed that certain amines were able to significantly increase the secretion of lysosomal dextran above untreated normal cells; whereas the same amines had no influence on cells without functional NPC1, ruling out a nonspecific influence. Furthermore, the relatively short exposure time required for the aforementioned stimulation rules out the possibility of NPC1 up-regulation as a cause. The stimulation appeared to be concentration dependent and was limited to those amines that were subject to extensive accumulation into lysosomes. These results provide an important foundation for future studies aimed at the identification of the true functional role and subsrates for this intriguing protein.

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Ncr1p, the yeast ortholog of the human Niemann Pick C1 protein, regulates the exit of sterols from the yeast vacuole

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The Niemann Pick Disease type C (NP-C) is characterized by accumulation of free cholesterol and glycosphingolipids within the endosomal/lysosomal system in various cell types including the neurons. Over 95% of NP-C cases are caused by mutations in the NPC1 gene, which encodes an integral membrane protein containing a "sterol sensing domain". Recent studies have identified Ncr1p, a vacuolar membrane protein, as an ortholog of NPC1 in the budding yeast *Saccharomyces cerevisiae*. However, no changes in cellular sterol homeostasis including uptake, synthesis and esterification have been detected when the *NCR1* gene is deleted. In this study, we identified two conditions that can enrich vacuolar membrane with sterols: acute glucose starvation and overexpression of the *ERG6* gene, which encodes a methyltransferase for ergosterol biosynthesis. Under these conditions, significantly more sterols accumulated in the vacuole of the *ncr1Δ* strain than that of the wild type strain. We also found that deletion of *NCR1* protected against sterol induced cytotoxicity. We propose that the primordial function of Ncr1p is to help recycle sterols from the yeast vacuole during prolonged starvation when membrane lipids or lipid droplets are ingested by the yeast vacuole for reuse. Lastly, the functional relationship between Ncr1p (a membrane sterol sensor), oxysterol binding proteins (cytosolic sterol carriers) and AAA ATPases is discussed.

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Molecular Characterization of Niemann-Pick C2 Disease

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ABSTRACT

We will describe different NPC mutant mouse strains that are available to the community and our research using these models. Central to NPC disease are defects that result in the storage of cholesterol and other lipids within the lysosomes of affected individuals. However, the exact nature of the storage material and the molecular events that underlie its accumulation remain unclear. As a step towards understanding the pathophysiological processes accompanying NPC disease, we are conducting subcellular fractionation studies using brain and liver from mice with mutations in either NPC1 or NPC2. Both presymptomatic and symptomatic NPC1 and NPC2-deficient mice exhibit a marked decrease in the buoyant density of lysosomes but not of other organelles based on marker enzyme analysis. We will discuss ongoing studies in characterizing alterations in the lysosomal system in NPC-mutant mice and describe the use of these animals in the study of lysosomal proteins and associated diseases.

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Characterization and Development of a New Mouse Model of Niemann Pick Type C (NPC) Disease

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We have identified and characterized a novel subline of mice (npc1NMF164) containing a point mutation in the npc1 gene. There are a number of potential experimental advantages of these mice compared to the currently available models of this disorder, and our analyses indicate that they exhibit many of the hallmark abnormalities associated with NPC disease in mice. The mutation corresponds to a single amino acid change (D1005G) within the large cysteine-rich luminal loop of the NPC1 protein, which contains a disproportionately high percentage of the identified mutations in the human NPC1 gene (>35% between residues 855-1098). This mutation is very close to 3 of the most frequently observed NPC1 mutations in humans: I1061T, the most common human mutation, S1004L, a milder mutation, and P1007A, a mutation found in 5-20% of patients and associated with a milder, more adult-like disease onset. The mutation in the npc1NMF164 mice is a partial loss of function, making it distinct from the mutations in other existing mouse models (npcnih, npc1spm), which are null alleles. as confirmed by the failure of the npc1NMF164 mutant to complement the npcnih and npc1spm alleles of NPC disease, the effect of the npc1NMF164 allele as a compound heterozygote with npcnih-null alleles, and the contrast between the npc1NMF164 mutation and the mutation in the npc1spm allele (with the latter determined by DNA sequencing). In addition, the progression of the disease is slower in the npc1NMF164 mice, although like npcnih mice there is a lower than expected frequency of homozygotes produced from heterozygote pairs. Western blot analysis of NPC1 protein in liver (antibody kindly provided by Dr. W. Garver) revealed npc1 mRNA levels in mutant npc1NMF164 mice that were10-20% of wild type levels. Analysis of tissue samples collected from the liver and brain (cortex, cerebellum) of 15 to 120 day-old mice revealed abnormally high levels of unesterified cholesterol and sphingomeylin expression, respectively. Histological analyses of cerebellum, hippocampus, and cortex also showed abnormal cholesterol accumulation (using BC-theta toxin staining), as well as astrocyte and microglial activation, and the loss of cerebellar Purkinje cells at older ages than in npcnih mice. Phenotypically, the npc1NMF164 mice exhibit the same characteristics as the original npcnih strain, though at older ages. Gait analysis at 60-65 days of age, when there is a noticeable effect of NPC on npcnih mice, failed to reveal ataxia or gait abnormalities in npc1NMF164 mice. However, the characteristic tremor and gait abnormalities associated with this disorder are noticeable in the npc1NMF164 mice by 80-90 days. There is a later onset and slower rate of weight loss in the npc1NMF164 mice compared to npcnih mice, consistent with the less severe, more slowly progressing phenotype. The npc1NMF164 mice also have an extended lifespan, and often live 90-110 days, notably longer than the 65-75 day lifespan of npcnih mice. There are a number of potential advantages of npc1NMF164 mice as a model of NPC disease: 1) unlike npcnih mice, npc1NMF164 mice have a C57BL/6 inbred genetic background, which is often used in generating transgenic and “knockout” lines of mice. This makes npc1NMF164 mice potentially more useful than npcnih mice for crossbreeding with other sublines of genetically engineered mice; 2) the difference in genetic background is advantageous for array-based comparisons of gene expression, and provides a potential avenue for confirming experimental results obtained from genetic analyses or treatment studies of npcnih mice; 3) because of the slower disease onset and progression, homozygous mutants are fertile, thereby reducing the need for heterozygous breeding pairs and larger breeding colonies, and 4) while the widely used npcnih mouse model most closely represents the rapid, severe infantile onset form of NPC disease that accounts for ~ 20% of the total cases, the mutation in the npc1NMF164 mouse is in a region where a high proportion of milder human mutations are found, making this a potentially valuable model for understanding and developing treatments for the more common, later onset forms of human NPC disease. Support provided by the Ara Parseghian Medical Research Foundation to R.A.M., K.L.S., and R.W.B., and NIH UO1-NS41215 to support the Neuroscience Mutagenesis Program at Jackson Laboratory.

Chemical compounds that reduce cholesterol accumulation in NPC cells

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We carried out a high content screen of a primary library of 15,000 compounds on CHO cell lines with a Niemann-Pick C phenotype, and found 14 compounds that significantly reduced cellular cholesterol levels. We then obtained a library of 4000 related compounds and screened these, yielding 6 compounds which were very effective in reducing cholesterol in CT-60 cells and non-toxic. We have carried out biochemical analysis of the mechanisms of action of these 20 compounds from the two screens. Several of the chemicals were found to inhibit hydrolysis of cholesteryl esters as compared to control, solvent-treated cells. This suggests that lysosomal acid lipase (LAL), the enzyme responsible for hydrolysis of cholesteryl esters is being inhibited by these compounds. One chemical (1A13) increases esterification by acyl-coenzyme A:cholesterol acyl transferase and also increases extracellular cholesterol efflux. We are currently screening a library of 40,000 compounds on a human NPC1-mutant cell line, and we have found many compounds that are effective in reducing cholesterol accumulation when used at 10 μM. We are rescreening those compounds at lower concentrations.

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Niemann-Pick type C disease involves infiltration of brain by activated T cells

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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. Since neuroinflammation appears to play an important role in the neurodegenerative process in other lysosomal storage disorders, we studied this process in NP-C mice. Our results show that neuroinflammation is also involved in mouse NP-C. Adult brains of NP-C mice have not only reactive macrophages, but also T cells also infiltrate specific brain regions. Brains from control and untreated NP-C mice were immunostained with antibodies to markers of T cells: CD4, CD8 and CD25. In addition, brains were analyzed by flow cytometry to calculate the numbers and kinds of T cells found in 50 day old NP-C mouse brains. Our results show that while wild type mice have no T cells in their brains, untreated NP-C mice have significantly increased numbers of CD4+, CD8+, CD4+/CD25+ and CD8+/CD25+ T cells. Treatment of NP-C mice with the neurosteroid allopregnanolone, which we have shown is effective in increasing lifespan and ameliorating some of the neuronal pathology in NP-C mice, substantially reduced the numbers of CD4 and CD8 immunopositive T cells. Concomitant with increased numbers of T cells in NP-C mouse brains, we found increased concentrations of several cytokines associated with those T cells. Allopregnanolone treatment also reduced the concentrations of some of those cytokines. In vitro, allopregnanolone reduced T cell proliferation, indicating that allopregnanolone could affect T cell infiltration and accumulation by several different mechanisms. These results indicate that infiltration of naïve and activated helper CD4+ and cytotoxic CD8+ T cells, and release of cytokines, contributes to the neuropathology of NP-C. Our results further demonstrate additional ways that allopregnanolone treatment may alter the course of neuronal pathology.

Proteasomal degradation of NPC1

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We and others reported reduced protein levels of NPC1 in fibroblasts from patients with NPC1 mutations. Importantly, cells from patients with juvenile/adult forms retained relatively high levels of the protein (1), suggesting that the NPC1 protein level may be one of the factors that determine the disease severity. It was unlikely that this decrease was secondary to any cellular phenotype of NPC cells, because NPC2-deficient cells contained increased levels of NPC1 (1). It was neither due to an impairment of transcription, because these cells contained rather increased levels of NPC1 mRNA (2). We report that a proteasome inhibitor MG132 caused an accumulation of ubiquitinated NPC1, suggesting proteasomal degradation of NPC1 and accelerated degradation of mutant proteins. Pulse-chase analysis in COS cells revealed that an I1061T mutation decreased the half-life time of expressed NPC1 protein. Wild-type as well as loss-of-function mutant NPC1 proteins associated with molecular chaperones HSP70, HSP90 and calnexin. Accordingly, overexpression of HSP70 in human fibroblasts with I1061T homozygous mutations by adeno-HSP70 or treatment with geranylgeranylacetone increased the level of the mutant protein. In COS cells, co-expression of an E3 ligase CHIP (carboxyl terminus of HSP70-interacting protein) enhanced MG132-induced accumulation of ubiquitinated NPC1. MALDI-TOF mass spectrometry has revealed three lysine residues on the cytosolic side, K318, K792 and K1180, as potential acceptors of ubiquitin. Substitution of the three lysine residues with alanine yielded a mutant protein with a steady-state level approximately 10 times higher than the wild-type protein. These findings indicate that NPC1 undergoes proteasomal degradation and that its biosynthesis is an inefficient process: 90% of the wild-type protein is degraded away. These findings also suggest the possibility to restore endosomal cholesterol flow by stabilization of the mutant NPC1 proteins.

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Title: The Role of Cholesterol in the Processing of the Amyloid Precursor Protein in Lipid Rafts

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Background: The amyloid peptide Aβ that accumulates in the Alzheimer's disease (AD) and Niemann-Pick type C (NPC) brains is generated by the sequential cleavage of the amyloid precursor protein (APP) by the enzymatic activities β- and γ-secretase. Aβ generation is thought to occur in lipid rafts (LRs), which are membrane microdomains localized at the cell surface and the endocytic pathway and are mainly constituted by cholesterol and sphingolipids. Cholesterol homeostasis is impaired in the NPC brain, leading to gross enlargement of LRs. Because amyloidogenesis is known to occur in these structures, it is thought that Aβ accumulation in NPC may result from abnormal LR function **Aim:** Our long-term interest is to elucidate the molecular mechanisms by which LR enlargement in the NPC brain may contribute to enhanced Aβ generation. **Methods**: Isolation of LRs from cerebella of NPC1 knock-out (BALB/cNctr-*Npc1*^{m1N}/J) and APP/NPC double knock out mice. Characterization of the amyloidogenic machinery components in LRs by Western blotting and immunofluorescence. **Results:** We have identified significant differences in the localization of components of the amyloidogenic machinery in the LRs of different APP and NPC1 genotypes. **Conclusion:** We will discuss the implications of these results for our understanding of how LR enlargement in NPC may contribute to enhanced generation and accumulation of Aβ in the NPC brain.

Mechanism of Neurosteroid Protection in a Mouse Model of Niemann-Pick C Disease

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Previous studies have shown that neonatal treatment with the neurosteroid allopregnanolone delays onset of neurological symptoms and prolong the lifespan of *npc1*$^{-/-}$ mice. To establish whether the mechanism of neuroprotection in *npc1*$^{-/-}$ mice involves $GABA_A$ receptor activation, we compared treatment of natural allopregnanolone and *ent*-allopregnanolone, a stereoisomer that has identical physical properties of natural allopregnanolone but is not a $GABA_A$ receptor agonist. *Ent*-allopregnanolone provided identical functional and survival benefits as the natural compound in the *npc1*$^{-/-}$ mice, strongly supporting a $GABA_A$ receptor-independent mechanism for allopregnanolone action. To further define this mechanism, p7 *npc1-/-* mice were treated with one of three neurosteroids (allopregnanolone, *ent*-allopregnanolone, and ganaxolone) or with vehicle alone during a time course study (n=4 per group). Since each neurosteroid was in solution with cyclodextrin, an additional control group treated with cyclodextrin alone was added at 8hr and 24hr time-points. Cerebellar tissue was harvested and total RNA was isolated for each mouse in each group. Samples were measured for genome-wide RNA expression levels using GeneChip® Mouse Expression 430_2.0 arrays. Resulting data were analyzed for significant expression differences between each neurosteroid and the control groups. Specific analysis was conducted to isolate differential gene expression due to neurosteroid treatment. Genes showing large fold differences and a statistical significance by a 2-way ANOVA were investigated using pathway analysis software. Results of analyses and their relevance with respect to NPC therapeutics will be presented.

Functional analysis of NPC1 protein

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The NPC1 protein is comprised of 1,278 amino acids with an apparent mass of 170–190 kD. Published topological analysis is consistent with the proposal that the protein has 13 transmembrane domains, three large lumenal domains, and a cytoplasmic tail. Yet the data verifying this topology and transmembrane domain number are less extensive than has been widely assumed. Five transmembrane domain-prediction programs yield different results for the NPC1 sequence (and for NPC1L1), and the proposed topology (1) is based on the assumed orientation of the sterol sensing domain and the accessibility of 5 FLAG tags introduced within recombinant NPC1 (1). Placement of the highly charged FLAG tag adjacent to a transmembrane domain may yield misleading results given its intrinsic hydrophobicity. Thus, while the location of potential asparagine-linked glycosylation sites and numerous cysteine residues make the lumenal orientation of the three large lumenal domains highly likely, the precise number of transmembrane domains is not yet firmly established. NPC1 is related to the prokaryotic RND (resistance-nodulation-division) superfamily of proton gradient-driven, multi-drug transporters. The related regions comprise only a small proportion of NPC1, highlighting the importance of studying the ~80,000 daltons of NPC1 protein mass that resides within the lumen of late endosomes. To avoid the challenges of working with an extremely hydrophobic protein, we are attempting to study NPC1 lumenal domains by expressing them individually in bacteria and in mammalian cells. We have replaced the lumenal loop-generating transmembrane domains with anti-parallel coiled coil sequences that should recapitulate the loop orientation of these domains. The crystal structure of the bacterial RND protein, AcrB, suggests this approach should be successful. We have successfully expressed lumenal domains 2 and 3 in bacteria in a soluble form. Domain 2 has been further purified by glutathione agarose chromatography. Our plan is to use these constructs to identify NPC1 intra- and inter-molecular binding partners in endosomes. By identifying partners, we hope to gain insight into the function of NPC1 protein in human cells. In addition, these purified subdomains may be valuable reagents for future structural analyses.

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Liver X Receptor agonists increase cholesterol loss from the CNS and attenuate microglia inflammatory responses to enhance the survival of *Npc1*-null mice.

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The liver X receptors (LXRα and LXRβ) are ligand-activated transcription factors of the nuclear hormone receptor superfamily. Cholesterol-derived oxysterols bind these receptors and promote LXR-dependent transcription of genes promoting cholesterol efflux and catabolism. We hypothesized that LXR agonists may have a positive effect on Niemann-Pick type C1 disease, a neurodegenerative disorder resulting from the mutation of a protein (NPC1) implicated in the intracellular trafficking of cholesterol. Upon administration of the synthetic LXR agonist T0901317 (T1317), *Npc1*-null mice demonstrated improved performance on behavioral tests, increased longevity, increased cholesterol loss from the cerebellum and increased Purkinje cell survival. This drug crossed the blood-brain barrier (BBB), as the mRNA levels of known LXR target genes were increased in the cerebellum of these mice. LG268, a ligand for the retinoid X receptor, which serves as the obligate heterodimer partner of LXR also appears to cross the BBB to increase the expression of RXR/LXR target genes. A role for LXR is also suggested by the finding that mice with genetic deletion of both LXRβ and NPC1 have shorter lifespans than the *Npc1*-null mice. Triple-knockout mice (LXRα/LXRβ/NPC1) have also now been generated and are currently being evaluated. Among the effects of T1317 on brain histology in the *Npc1*-null mice, we observed that the morphology of resident microglia was altered suggesting a role of LXRs in reducing neuroinflammation. The number of microglia in the cerebellum of T1317-treated *Npc1*-null mice was still increased, but these cells now displayed a stellate, resting morphology in contrast to the activated, amoeboid state of microglia in untreated *Npc1-/-* cerebellum. Isolated primary microglia express both LXR subtypes. Utilizing the BV-2 murine microglia cell line, we evaluated the effect of T1317 on gene expression and cellular response to proinflammatory agents such as lipopolysaccharide (LPS) and oxidized LDL (oxLDL). In BV-2 cells, both TNFα and IL-1β mRNA levels peaked 4 hours following treatment with LPS. After a 4 hr exposure to 10 ng/ml LPS, IL-1β and TNFα mRNA levels were decreased by 43% and 10% respectively, in BV-2 cells pretreated for 16 hours with T1317. In BV-2 cells exposed to 50 μg/ml oxLDL for 8 hr, administration of T1317 decreased the gene expression of IL-1β by 42% and TNFα by 64%. Similar changes in inflammatory cytokine expression were seen with other synthetic LXR ligands. This repression of LPS- and oxLDL-mediated upregulation of proinflammatory cytokines was even more pronounced when the agonists for both LXR (1μM T1317) and RXR (50 nM LG268) were applied to microglia. These anti-inflammatory effects are also observed in primary microglia from both wildtype and *Npc1*-knockout mice. To further study the role of inflammation in the progression of Niemann-Pick disease, *Npc1*-null mice are being crossed to a mouse strain with genetic deletion of TNF*a.* These results suggest that one possible mechanism through which LXR agonists positively affect the progression of NPC1 disease is by suppressing neuroinflammation. *This work was supported by a grant from the Ara Parseghian Medical Research Foundation (JJR).*

Title: NPC-db, a novel Niemann-Pick Type C Disease Variation Database

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Abstract

Niemann-Pick type C (NPC) disease is a rare autosomal-recessive lysosomal storage disease typically accompanied by progressive impairment of nervous system and liver function. Biochemically, the disease presents with an inhibited egress of cholesterol and glycosphingolipids from endosomal and lysosomal compartments in neuronal and non-neuronal cells. In the majority of NPC patients, mutations in the NPC1 gene can be identified. About 5% of patients show mutations in the NPC2 gene. Many different mutations can caus e NPC disease and multiple variants not associated with the disease are known in both genes. A continuously updated collection of gene variants is lacking to date and only limited information is available on genotype-phenotype correlation.

We have created the novel Niemann-Pick Type C disease variation database (NPC-db) [http://npc.fzk.de]. This database aims to provide a comprehensive overview of the sequence variants in NPC1 and NPC2 including information on their functional consequences and associated haplotypes. Moreover, genotype data and clinical information from individual NPC patients provide informat ion on the impact of functional variants.

NPC-db addresses professionals and non-professionals dealing with NPC disea se on a clinical, diagnostic, research or personal basis. The user is encouraged to search contents and submit novel information, thereby contributing to gener ate a valuable open-access tool which will allow a better understanding of the molecular and clinical details of NPC disease.

PXR Expression in the CNS

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The 5'-regulatory sequence of the PXR gene varies extensively between mammals. While the 5'-region of mouse and rat PXR is 2.9 kb, the 5'-region of human PXR is 16 kb. We created luciferase reporter plasmids containing the -2.9kb and the -16 kb human PXR promoter regions. We stably introduced the -2.9 kb and -16 kb PXR –luciferase reporters into the mouse genome to create PXR humanized mice. These mice were used to determine if the evolutionarily conserved 2.9 kb region of the promoter is sufficient for PXR expression in a variety of tissues, including brain, or whether the additional 13 kb of regulatory sequence in the human PXR gene leads to tissue specific differences in gene expression. The human PXR transgenic mice allow us to non-invasively monitor in real-time transcriptional regulation of human PXR. In addition, we are collaborating with Drs. Frank Gonzalez and Ting Wang (NIH) who generated a mouse lacking mouse PXR but humanized with the same human PXR BAC we used for the 16 kb reporter mice. This mouse expresses human PXR driven by the human PXR promoter. Studies are ongoing to examine human PXR expression in various mouse tissues, including neurons and purkinje cells of the cerebral cortex of the brain, using quantitative real-time PCR and immunohistochemistry. In addition, since the ligand binding domains vary extensively between mouse and human PXR, the humanized mouse will identify whether neurosteroids such as allopregnanolone, or other human specific ligands, can activate human PXR in the brain in vivo. In total, these mice allow for investigation of PXR control and gene expression in many tissues, including brain, and may advance our understanding of how this gene is regulated and activated in various tissues in humans.

In vivo studies of Niemann-Pick Type C Disease: Why do we need new mice?

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Niemann-Pick Type C Disease is a neurovisceral disorder caused by mutations in the *NPC1* gene. Although the protein NPC1 is ubiquitously expressed, only a specific subset of cells and tissues seem to require its function. In the mouse model of the disease, one hallmark is patterned Purkinje neuronal cell loss. Purkinje neurons are considered the main producers of neurosteroids and provide the only neuronal output from the cerebellum. However, the consequence of their perturbed function and death in Niemann-Pick Type C Disease has never been assessed. We do know from past work using chimeric mice that Npc1 acts cell autonomously within these neurons allowing us to perform a more sophisticated mosaic analysis. In order to study the effect of rescue or loss of Npc1 specifically in Purkinje neurons, we have chosen to employ a genetic Tet-off expression system.

The Tet-off expression system is a versatile binary transgenic system. This binary design allows the use of tissue-specific promoters to drive tTA expression. Transgenic mice that produce tTA in cerebellar Purkinje neurons, liver, and other cell-types are available commercially or from academic labs. tTA regulates expression of a target gene under control of a tetracycline-response promoter element (TRE) and its activity can be reversibly and quantitatively controlled by exposure to doxycycline. This allows for inducible tissue-specific expression of a TRE-regulated transgene. Two transgenes have been designed whose target genes are driven by TRE. The first produces functional mouse Npc1 tagged at C-terminus with fluorescent EYFP. This transgene will be used to rescue Npc1 in *Npc1* mutant mice with cell type and temporal specificity, for example from only Purkinje neurons or only at a certain time during a mouse's life. The protein can be detected and followed in living cells by way of the fluorescent marker EYFP. The second transgene produces fluorescent EGFP with a short hairpin microRNA sequence against mouse *npc1* mRNA. The EGFP serves as a marker for RNA interference activity. This transgene will be used to eliminate Npc1 selectively from wild type mice, the inverse of the rescue experiment, in order to learn what aspects of pathology occur under those conditions. An update of the status and initial testing of the founder mice for these these two transgenes will be reported.

Although we have chosen to prioritize our effort to the study of Npc1 in Purkinje neurons, once working transgenic mice are established, the effect of rescue or loss of Npc1 can be studied in a myriad of tissues. The goal is to answer when and where Npc1 is needed to ameliorate disease progression.

Purification of human NPC1 and characterization of its binding to sterols and lipids

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NPC1 contains a sterol-sensing domain and is clearly involved, either directly or indirectly, in intracellular cholesterol trafficking. NPC1 is likely to function in the export of sterols or other amphipathic molecules from the late endosomes, powered by a H^+ gradient. Our goals are to determine the molecular functions of NPC1 with respect to binding of substrates, transport and flipping of drugs and lipids, and sterol transfer from late endosomes. We are using simple *in vitro* systems; purified NPC1 in detergent solution or reconstituted proteoliposomes.

We have developed a greatly improved method for purification of NPC1 from CHO cells expressing FLAG-tagged protein in an NPC1 knockout background. A purified membrane fraction was isolated from intact cells, using sucrose density gradient centrifugation, and extracted with CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate). Affinity purification using anti-FLAG agarose resulted in highly purified NPC1, which was then concentrated >20-fold. The FLAG peptide used for elution was subsequently removed by gel filtration chromatography. We routinely obtain ~0.6 mg of NPC1 (>90% purity) at a concentration of ~0.1 mg/mL in HEPES buffer with 2 mM CHAPS.

In buffers containing 15 mM CHAPS/0.2 mM Fos-choline-13 (FC13) (above the detergent critical micelle concentration), FPLC analysis showed that purified NPC1 exists as a mixture of monomers, dimers and trimers, whereas in 2 mM CHAPS/0.2 mM FC13 (below the critical micelle concentration), the protein forms larger aggregates. Both purified NPC1 and NPC1 in the membrane fraction bound cholesterol, as shown by photo-crosslinking to ^{3}H-azicholesterol (7-azi-5α-cholestan-3β-ol, [3,5,6-^{3}H]), followed by SDS-PAGE and autoradiography.

Several fluorescent cholesterol derivatives were used in binding studies with purified NPC1. To help overcome the very low aqueous solubility of many of the sterols, 0.2 mM FC13 was included in the buffer. Initial experiments with BODIPY-labelled sterol showed that even under these conditions, binding to purified NPC1 (observed by enhanced BODIPY fluorescence) was a slow process, with equilibrium binding achieved after 3 hours. Slow kinetics of binding were also observed for SCAP, and likely arise because even "solubilized" sterol is present as large aggregates that exchange into NPC1 micelles very slowly. Four different fluorescent cholesterol derivatives were shown to bind to purified NPC1; dehydroergosterol, cholestatrienol, NBD-cholesterol and BODIPY-cholesterol ester. Binding was indicated by enhanced fluorescence of the labelled cholesterol, and substantial quenching (24-35%) of NPC1 Trp fluorescence. Binding affinity was high, with K_d values in the range 2-4 μM. Interestingly, very low Trp quenching was observed with native cholesterol and non-fluorescent sterol-mimicking drugs, including GW707, U18666A and ezetimibe. It seems likely that the NPC1 sterol binding site is not located close to Trp residues, so that direct quenching as a result of binding is very low. Fluorescent sterols, on the other hand, can efficiently quench NPC1 Trp fluorescence by FRET (fluorescence resonance energy transfer). Other potential substrates were also tested for Trp quenching of purified NPC1. Sphingosine showed high Trp quenching, with a K_d of 37 μM, suggesting that it is a low affinity NPC1 substrate. Oleic acid, sphingomyelin and lactosylceramide showed no quenching, suggesting that either they are not substrates, or delivery to NPC1 is a problem.

These experiments clearly show that purified NPC1 binds cholesterol and several fluorescent cholesterol analogues with high affinity, and the lipid sphingosine with lower affinity.

Subcellular localization of glycosphingolipids and signaling in NPC disease

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Niemann-Pick C (NPC) is an autosomal recessive neurodegenerative lipid storage disease, characterised by a cell autonomous loss of cerebellar Purkinje neurons. Most cases of NPC disease (>90%) result from mutations in the NPC1 protein, which localizes to late endosomes and lysosomes. NPC is characterised by the storage of a complex and unique pattern of lipids including sphingomyelin, sphingosine, glycosphingolipids (GlcCer, LacCer, GM2, GM3, GM1) and cholesterol. The disease is neurodegenerative and the most abundant storage lipids in the brain are glycosphingolipids (GSLs). Increasing evidence suggests that cell signaling processes are disrupted in NPC. NPC cells have been shown to accumulate GSL rafts and both endosomal trafficking and signaling are disrupted. However, the identification of the subcellular locations of GSLs within NPC cells is an important first step toward elucidating how storage affects NPC cells. Previously pharmacological depletion of GSLs or increasing intracellular calcium has been shown to reverse altered annexin 2 distribution and function. More recent data suggests that toll-like receptors show altered subcellular location and signaling. Are other lipid raft signaling processes affected? Are they normalized by GSL depletion or increased intracellular cations?
In summary, the accumulation of GSL rafts in late endocytic / lysosomal compartments may lead to defective raft function including disrupted signaling. **Can the mislocalisation of endosomal lipid rafts in NPC cells be reversed? Can normalization of lipid raft location in NPC cells also lead to the reversal of altered signaling?**

Specific Aims: Previous studies have indicated that both lipid and protein trafficking are disrupted in Niemann-Pick C disease. The first aim of this proposal is to identify the altered **sub-cellular** location of lipid-raft in Niemann-Pick C. The second aim is to build upon pilot studies investigating the effect of altered lipid rafts and cation (pH) gradients on cell signaling in NPC cells.

Aim 1) Intracellular distribution

1) What are the sub-cellular compartments where GSLs, sphingosine and cholesterol accumulate in NPC cell culture models using sub-cellular fractionation and confocal microscopy?

Aim 2) Is cell signalling altered in NPC cells?

2a) How does the intracellular accumulation of different GSL rafts effect signal transduction in NPC cells?
2b) What effects do GSL depletion and increases in intracellular cations have on NPC TLR4 and EGF cell signaling processes?

Structural and Functional Characterization of NPC2

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We will discuss research conducted jointly in the Lobel and Stock laboratories focused on structural and biochemical characterization of ligand binding to NPC2. We will also discuss ongoing investigations of intestinal cholesterol absorption that are being pursued as part of our efforts to characterize the pathway(s) in which NPC1 and NPC2 function.

Structural and biochemical characterization of ligand binding to NPC2. The crystal structure of bovine NPC2 bound to cholesterol-3-O-sulfate has defined the sterol-binding cavity, which is formed only in the presence of the ligand. The hydrophobic portion of the sterol is inserted between the two sheets of NPC2 and the protein molds itself around the ligand, creating a perfectly fitting tunnel that penetrates deep into the interior of the protein. This malleable binding mode can accommodate variations in both protein and ligand composition. Mutagenesis and ligand-binding studies have begun to define the range of allowable substitutions. The results suggest that the physiologically relevant ligands bound by NPC2 are likely to be dictated by the specific subcellular repertoire of sterols available for binding rather than by stringent selectivity of the binding cavity.

Role of NPC1 and NPC2 in intestinal cholesterol absorption: Similarity between NPC1 and NPC1L1 prompted us to investigate potential roles for NPC1 and NPC2 in intestinal cholesterol absorption. The efficiency of cholesterol uptake and sensitivity to ezetimibe (an inhibitor of cholesterol uptake via NPC1L1) was studied by the fecal dual isotope technique. Cholesterol uptake was found to be similar in wild-type and NPC1- and NPC2-deficient mice. Ongoing studies are in progress to probe the potential involvement of NPC1 and NPC2 in subsequent steps required for trafficking of cholesterol out of enterocytes.

The role of NPC2 in lysosomal cholesterol transport.

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Mutations in two genes, npc1 and npc2, have been identified as the causes of Niemann-Pick type C disease. NPC2 is a soluble intralysosomal protein that binds cholesterol in vitro. Our recent studies of NPC2 suggested that it could play a crucial role in facilitating cholesterol egress from the late endosome/lysosome compartment. Based on these studies, was predicted that electrostatic interactions are important for NPC2-membrane interactions that are involved in cholesterol transport. Thus, we are undertaking structure-function studies using NPC2 variants with single amino acid substitutions in charged surface residues. Remarkably, despite normal cholesterol binding properties, several NPC2 point mutants demonstrate dramatic reductions in their ability to transfer cholesterol between membranes. Further studies have addressed the potential role of glycosphingolipids in NPC. While gangliosides have not been shown to bind to NPC2, it is well appreciated that GM2 and GM3 accumulate in NPC disease, and there is some controversy as to whether ganglioside accumulation may represent the primary defect in the disease. We therefore examined whether the rate of cholesterol transfer from NPC2 protein to membranes was modulated by the incorporation of lactosyl ceramide or several gangliosides into model membranes. The results demonstrate an overall minor impact of these glycosphingolipids on cholesterol transport rates. Ligand binding studies using a DHE competition assay further support a sterol-specific role for NPC2 in cholesterol trafficking.

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Intervention Points in Niemann Pick type C Disease; Insight from NPC-Protein Interactions

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We now know the genetic cause of Niemann-Pick type C (NP-C) disease; variation in two genes, NPC1 or NPC2 [1-3]. However, surprisingly little is known about the mechanisms of action or substrates of the NPC pathways or what impacts the course of the disease. Our underlying hypothesis is that NPC proteins do not work alone. Further, we speculate that background genetic segregations in such interactions may explain the marked divergence in age of disease onset that arises even within families [4]. We view NPC1 proteins as "rheostats" i.e. membrane proteins that must sense, signal and transport. This places the protein at a critical nexus of interactions where it contacts both carrier proteins that shuttle membrane components through the cell and also interacts with "conventional" signal transduction pathways. We have exploited the 2 billion year conservation of NPC1 family members (i.e. yeast to humans) to define the interactions of the yeast NPC1 ortholog (a.k.a. NCR1) with the remainder of the genome, as a model of NP-C disease. We have performed genetic, pharmacogenetic and physical interaction-screens that detail interaction nodes for this protein family. To pursue this "translational" approach, we have developed a strategy that permits us to test hypotheses in yeast, cultured mammalian cells, animal models (primarily murine) and ultimately human subjects

NCR1, encoding the yeast ortholog of human NPC1 exhibits physical and genetic interactions with several components of subcellular transport, including oxysterol binding proteins and actin related proteins that we presume are the cytoplasmic components of lipid transport. Moreover, NPC1 pathways intersect with several signal transduction cascades. Aberrant activation of these pathways due to loss of NPC1 significantly influences cell metabolism and promotes purkinge cell death. These pathways represent clear points of therapeutic intervention that fortuitously have been targeted in other diseases (including Alzheimers). Two "test" cases of this approach are presented whereby NPC1 deficient mice will be treated with dietary Coenzyme Q10 or Glycogen Synthase Kinase inhibitors.

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Inflammation in the Niemann-Pick type-C brain – Characterization of Microglia

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Niemann-Pick Type-C (NPC) disease is a fatal neurodegenerative disorder characterized by cholesterol accumulation in late endosomes/lysosomes. Microglia are resident immune cells of the central nervous system, which upon activation secrete potentially neurotoxic molecules such as tumor necrosis factor-alpha (TNF-α). Inappropriate activation of microglia has been implicated in several neurodegenerative disorders including NPC disease. We hypothesize that microglial dysfunction plays an important role in neurodegeneration in NPC disease. To test this hypothesis, primary microglia cultures were prepared from *Npc1-/-* mice. Filipin staining of unesterified cholesterol shows that NPC1-deficient microglia have an altered cholesterol distribution. Additionally, treatment of wild-type microglia with U18666A, a compound which mimics the cholesterol accumulation in NPC disease, causes microglia to assume an activated morphology. Moreover, immunohistochemical analysis shows an accumulation of active microglia in *Npc1-/-* cerebellar brain slices. Taken together, these results suggest that cholesterol accumulation activates microglia, which in turn could lead to increased secretion of potentially toxic molecules such as TNF-α. In support of this idea, immunocytochemical staining of *Npc1-/-* microglia cultures reveals elevated levels of TNF-α and TNF-Receptor-1. Furthermore, the TNF-α content of conditioned media from lipopolysaccharide-stimulated *Npc1-/-* microglia is higher than that of *Npc1+/+* microglia. We also found that elimination of the Fc-receptor common gamma chain, required for antibody-based autoimmunity, in *Npc1-/-* mice does not improve the clinical outcome of the disease. This observation is consistent with the idea that cholesterol accumulation causes microglial dysfunction, rather than the production of autoantibodies leading to an autoimmune response. Further studies will aim to determine whether these microglial changes translate to neurotoxicity, and will evaluate the role of microglia in NPC neurodegeneration.

The Role of Dynein-Driven Motility in the Trafficking of NPC1 Membranes.

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NPC1 encodes a multipass transmembrane protein with sequences suggesting a role in cholesterol transport, however the specific functions of NPC1 remain unknown. In this study, we built on previous work suggesting that NPC1 containing membranes were dependent on microtubules (MTs) and MT motors to examine the motility of NPC1 using live-cell imaging. Utilizing a GFP-NPC1 expression construct, we observed that NPC1 membranes display two models of motility. The most obvious was large spherical membranes (LSM) which displayed bidirectional MT-based motility typical of late endosomes and lysosomes. This motility could be blocked by ablating MTs with nocodazole or by disrupting dynein-driven transport with p50(dynamitin) expression or expression of dominant-negative dynein phosphorylation site mutants. The latter induced accumulation of engorged LSMs at the cell periphery. The second type of motility resembled tubulo-vesicular (TV) extensions which emanated from the LSMs and probed other membrane compartments. Although both MT ablation and dynein disruption also affected the TV membranes, live cell imaging suggested that these membranes moved along nonradial vectors. To test if NPC1 mutations disrupt normal NPC1 function by perturbing motility, we generated a GFP-NPC1 construct harboring the I1061T mutation and compared wild-type and mutant NPC1 in living cells. In contrast to the wild-type protein, the mutant protein displayed defects in the motility of both LSM and TV membranes. In particular, mutant NPC1 membranes displayed the LSM but not TV morphology suggesting an inability to interact with MTs or MT motors. To test if this was common theme among NPC1 mutations, we analyzed additional mutants (C98S, P692S, and Y635C) and confirmed the loss of LSM and TV membrane motility in all cases. Because the TV membranes appeared to interact with both MTs and actin filaments, we disrupted the actin cytoskeleton and measured effects on TV motility. Latrunculin A treatment led to an increase in the formation of TV membranes for both wild-type and mutant NPC1 membranes. This suggests that NPC1 membranes interact with both actin filaments and MTs and that the balance of these interactions dictates the structure of NPC1 membranes. Because latrunculin A treatment drove mutant membranes towards a more normal phenotype, we tested the ability of other small molecule inhibitors to influence NPC1 function. Rapamycin (inhibitor of mTOR) has been shown to induce inhibitory phosphorylation of cytoplasmic dynein and H-89 (inhibitor of PKA) has been shown to reduce phosphorylation of dynactin. Both treatments reduced formation of TV membranes and the overall motility of wild-type membranes. Together these results suggest that NPC1 containing membranes are dependent on cytoplasmic dynein and that modulators of dynein influence the normal motility of NPC1 membranes. Supported by the Ara Parseghian Medical Research Foundation.

Effect of allopregnanolone administration on disease markers in NP-C cats

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The only large animal model of Niemann-Pick type C (NP-C) disease is a colony of cats housed at the School of Veterinary Medicine of the University of Pennsylvania. The disease in cats, due to a point mutation in the NPC1 gene, is a homologue of the disease in children with similar clinical, biochemical, and neuropathologic abnormalities. In the past two years, we have extended the natural history study of feline NP-C disease progression and successfully used allopregnanolone to partially treat neurologic and hepatic components of the disease. Statistically significant differences between age-matched NP-C and normal cats were identified in measures of weight gain, onset of neurological dysfunction, post-rotatory nystagmus, nerve conduction velocity, brain stem auditory evoked response testing, liver enzyme concentrations, chitotriosidase activity, nuclear magnetic resonance measures (T2 values of the white matter, apparent diffusion coefficient (ADCav) of the gray and white matter, and proton spectroscopy of the gray and white matter of the brain), and brain and liver histology. Preliminary proteomic analysis of cerebrospinal fluid (CSF), which can be readily collected in cats, for biomarkers of NP-C disease suggests significant differences in protein composition exist between NP-C and normal cats. All these differences serve as markers of disease and demonstrate that the feline model has been developed to a point where it is a significant resource for evaluating experimental therapies of NP-C disease.

We have thus far treated seven NP-C cats with allopregnanolone and have shown significant improvements in many of our measures of NP-C disease. Cats that began treatment with allopregnanolone after 3 weeks of age (AlloLate) showed significant improvements in tibial nerve conduction velocity, liver enzyme concentrations, chitotriosidase activity, and brain and liver pathology. Improvements in liver pathology and chitotriosidase activity were an unexpected finding. However, there was no improvement in onset and severity of signs of neurologic dysfunction or in lifespan. A cohort of cats treated with allopregnanolone at 1, 3, 7, 14, and 21 days (AlloEarly) are currently being assessed to determine whether the earlier administration of allopregnanolone improves neurologic disease in an animal model other than mice. Preliminary data suggests that the early administration of allopregnanolone may delay the onset of neurological dysfunction in cats.

Our continuing goals are to use this well-characterized large animal model to assess the efficacy of experimental therapies of NP-C disease and to better understand NP-C disease pathophysiology.

Combinatorial Approaches to Therapy for Niemann-Pick C Disease

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Niemann-Pick type C (NPC) disease is a complex lysosomal disorder characterized by slowly progressive CNS dysfunction. Abnormalities in brain include massive storage of glycosphingolipids and cholesterol, microglial activation, and selective neuronal cell death in regions like cerebellum (Purkinje cell loss). Other features of neurodegeneration in NPC disease include neurofibrillary tangles (NFTs) identical to Alzheimer's and frontotemporal dementias, and Lewy bodies (LBs) as seen in Parkinson's disease and Lewy body dementia. Treatment strategies that successfully limit CNS impact of NPC disease unfortunately remain limited. The only existing therapy is miglustat (NB-DNJ), a small molecule inhibitor of glycosphingolipid synthesis which has shown promise in animal studies and human trials. In addition to this drug, several emerging therapies include allopregnanolone and other documented activators of Pregnane X Receptors (PXR) which significantly increase longevity in animal studies. Other candidate compounds that may provide clinical benefit include anti-inflammatory, anti-oxidant and anti-apoptotic agents. While these drugs are not viewed as being capable of correcting the *primary* defect in NPC disease, they may have the ability to slow certain aspects of disease progression. This could be particularly true if they exhibited synergistic action when used in combination with other therapies. As a means to begin assessing the therapeutic benefit of combinatorial therapy for NPC disease, we have treated NPC1-/- mice with both miglustat and allopregnanolone. Allopregnanolone was administered by subcutaneous injection (25 mg/kg) at postnatal day 7 (P7) and at weekly intervals thereafter. Miglustat was administered once daily by intraperitoneal injections (300 mg/kg) beginning at P10 and converted to oral therapy (1200/mg/kg/day) at weaning (3 wks) using published procedures. Consistent with earlier studies, NPC1-/- mice receiving no treatment lived ~11 wks, while those on miglustat lived 16-17 wks (~45% increase) and those on allopregnanolone 21 wks (~90% increase). Longevity of animals on both treatments, while variable, in some cases was as a long as 28 wks (~155% increase). The latter finding shows significant synergy between miglustat and allopregnanolone and is consistent with the likelihood that the two drugs are working through independent mechanisms to lessen disease progression. Combinatorial therapies using these agents in conjunction with other drugs (e.g., anti-inflammatory agents) may delay disease and improve survival to an even greater degree. Furthermore, even though the relationship between the NPC1 defect and the presence of NFTs and LBs remains to be clarified, if therapeutic approaches outlined above, e.g., PXR activation, are successful in reducing this pathology in NPC disease, such therapies may also provide similar benefit to Alzheimer's, Parkinson's and related dementias as well.

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Signaling pathways leading to neuronal death in NPC: Finding new therapeutic targets.

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Niemann Pick type C (NPC) disease is a fatal autosomal recessive disorder characterized by the accumulation of free cholesterol and glycosphingolipids in the endosomal-lysosomal system. NPC patients suffer a markedly progressive neuronal loss, mainly of cerebellar Purkinje neurons. There is strong evidence indicating that cholesterol accumulation and trafficking defects activate apoptosis in NPC brains. Here,
we show that the pro-apoptotic c-Abl/p73 system, the phosphorylated p73 form and the p73 target genes are expressed in the cerebellum of NPC mice. Furthermore, inhibition of the c-Abl /p73 system at P28 decreases neurological symptoms, increases Purkinje cell survival and reduces apoptosis in the cerebellum of NPC mice. Moreover, this pro-survival effect was correlated with reduced mRNA levels of p73 pro-apoptotic target genes. In addition, we found that earlier inhibition of the c-Abl/p73 system at p7 increase the lifespan in a 10% in NPC mice. Finally, our preliminary results show that local CNS inhibition of the c-Abl/p73 pathway greatly increases the number of Purkinje neurons in NPC mice.
Our results show that the c-Abl/p73 pathway is involved in NPC neurodegeneration and suggest that treatment with c-Abl/p73 inhibitors may be useful in delaying progressive neuron death of NPC patients.

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Cell cycle activation synchronizes with onset of cytoskeletal pathology in Niemann-Pick Disease Type C Mice

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Although many studies support an involvement of cyclin-dependent kinases (cdks) in the neurodegenerative cascades of Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Niemann-Pick Type C (NPC), the question of which cdk is essential, is yet unanswered. To address this question, we investigated the earliest changes in cdks and phosphorylation of the cytoskeletal proteins in the murine model harboring a spontaneous *npc1* gene mutation ($npc^{-/-}$ mouse). This model mimics human NPC in displaying activation of cdk5, cdc2, and cdk4, with concomitant cytoskeletal pathology and neurodegeneration. Axonal spheroids, the characteristic lesion composed of hyperphosphorylated neurofilament and tau proteins were first detected in the brains of 3-week-old $npc^{-/-}$ mice. However, the phosphorylation activities of cdk5/p25 and cdk4/cyclin D were not increased until 4 weeks. In contrast, activation of the mitotic cdc2/cyclin B kinase coincided with the earliest detection of spheroids in 3 week-old

$npc^{-/-}$ mice. Our studies illustrates that key cell cycle regulators are upregulated in $npc^{-/-}$ mice, and that the timing of this upregulation is concomitant with appearance of cytoskeletal pathology. Of the various cdks, ie, cdc2, cdk4 and cdk5, the cdc2 kinase may be most crucial for initiating NPC neuro-cytoskeletal pathology. Targeted inhibition of cdc2 may offer a novel means for controlling onslaught of NPC neurodegeneration.

ERK1/2 is not essential for neuropathology in murine model of Niemann-Pick Disease Type C

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Different reports indicate that MEK/ERK1/2 signaling, in addition to its classical involvement in proliferation, differentiation and survival, can also promote neuronal cell death and can be involved in the cytoskeletal impairment and neurodegeneration of Alzheimer's disease, Parkinson's Disease and Niemann- Pick Disease Type C (NPC). In order to elucidate whether MEK/ERK1/2 pathway is crucial for NPC neuropathology, we continuously infused PD98059, a specific pharmacological inhibitor of MEK1, with the concentration of 5μmoles/day, into the lateral ventricles of *npc* $^{-/-}$ mouse model for a 2-week period, initiated at a pathologically incipient stage. Despite the obvious reduction of ERK1/2 activity, no significant attenuation of motor defects, number of spheroids, Purkinje neuron death and cytoskeletal hyperphosphorylation was observed in PD98059 treated *npc*$^{-/-}$ mice compared to vehicle control. In addition, unexpected increase of tau phosphorylation was observed and remarkably, hyperphosphorylated tau seemed to accumulate in oligodendrocytes rather than in neurons. Our data demonstrate that MEK/ERK1/2 is not essential for the pathogensis of NPC, and that inhibitors of this pathway would not be beneficial for treating NPC.

Astrocyte-directed expression of wild type *NPC1* attenuates neuronal cholesterol storage and extends survival in npc1$^{-/-}$ mice

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Although early death of Purkingje cells is a hallmark of Niemann-Pick Type C (NPC), the contribution of different neural cells in the neuropathology of the disease remains root. The important of glial cells for neuronal survival is undoubted, but it is not known whether astrocytes play a significant role in NPC. To address the role of glial cells in the neuropathology of NPC, we used the glial fibrillary acidic protein (GFAP) promoter to target expression of wild type *NPC1* gene in astrocytes of *npc1*$^{-/-}$ mice. We found that the GFAP^{E}, *NPC1*$^{-/-}$ line (GFAP Tg) harboring 24 copies of the transgene has a survival of 172±23.8 days (N=10), p≤0.0001- ie, 2.5 times that of the original *npc1*$^{-/-}$ line. The GFAP Tg mice maintain normal body weights up to the time of death. More strikingly, they are fertile; their double transgenic offsprings with 56 copies of *NPC1* survived over 230 days (3 times the usual survival). Histopathological studies demonstrated reduced numbers of axonal spheroids, decreased numbers of reactive astrocytes, and restoration of myelin tracts. Fluoro-jade staining revealed a significant attenuation of neurodegeneration in the cortex of these mice. Using SMI32 neurofilament antibody to identify neurons, and filipin dye for cholesterol, we found a marked reduction of cholesterol accumulation in brainstem neurons of the GFAP Tg mice relative to *npc1* $^{-/-}$ mice. The results show that GFAP-driven expression of wild type NPC1 protein in astrocytes greatly enhanced survival of *npc 1*$^{-/-}$ mice, and ameliorated degeneration, cytoskeletal abnormalities, and lipid storage in neurons. Our results suggest that NPC1 serves an important function in astrocytes, and that attempting correction of this function in *npc1*$^{-/-}$ mice may have tremendous benefit for neuronal survival and hence the treatment of NPC in humans.