Consequences of NPC1 and NPC2 loss of function in mammalian neurons

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Abstract

Genetic deficiency of NPC1 or NPC2 results in a devastating cholesterol-glycosphingolipidosis of brain and other organs known as Niemann–Pick type C (NPC) disease. While NPC1 is a transmembrane protein believed involved in retroendocytic shuttling of substrate(s) to the Golgi and possibly elsewhere in cells as part of an essential recycling/homeostatic control mechanism, NPC2 is a soluble lysosomal protein known to bind cholesterol. The precise role(s) of NPC1 and NPC2 in endosomal–lysosomal function remain unclear, nor is it known whether the two proteins directly interact as part of this function. The pathologic features of NPC disease, however, are well documented. Brain cells undergo massive intracellular accumulation of glycosphingolipids (lactosylceramide, glucosylceramide, GM2 and GM3 gangliosides) and cholesterol and concomitant distortion of neuron shape (meganeurite formation). In neurons from humans with NPC disease the metabolic defects and storage often lead to extensive growth of new, ectopic dendrites (possibly linked to ganglioside sequestration) as well as formation of neurofibrillary tangles (NFTs) (possibly linked to dysregulation of cholesterol metabolism). Other features of cellular pathology in NPC disease include fragmentation of the Golgi apparatus and neuroaxonal dystrophy, though reasons for these changes remain largely unknown. As the disease progresses, neurodegeneration is also apparent for neurons in some brain regions, particularly Purkinje cells of the cerebellum, but the basis of this selective neuronal vulnerability is unknown. The NPC1 protein is evolutionarily conserved with homologues reported in yeast to humans; NPC2 is reported in Caenorhabditis elegans to humans. While neurons in mammalian models of NPC1 and NPC2 diseases exhibit many changes that are remarkably similar to those in humans (e.g., endosomal/lysosomal storage, Golgi fragmentation, neuroaxonal dystrophy, neurodegeneration), a reduced degree of ectopic dendritogenesis and an absence of NFTs in these species suggest important differences in the way lower mammalian neurons respond to NPC1/NPC2 loss of function.

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1. Introduction

Niemann–Pick type C (NPC) disease is a neuro-visceral lipid storage disorder originally classified along with Niemann–Pick types A, B and D on the basis of clinical symptoms and biochemical features [1,2]. Niemann–Pick disorders were recognized as disease entities in the early part of the 20th century but shared numerous similarities with Gaucher disease and other so-called storage disorders and nosological confusion was common. The insightful definition of storage disorders as conditions caused by inherited deficiencies of specific lysosomal hydrolases by Hers in 1965 [3] led to the subsequent discovery of an acid sphingomyelinase defect in Niemann–Pick types A and B [4]. Types C and D, however, were left in limbo for an additional three decades as a result of the failure to find a similar lysosomal hydrolase deficiency that could account for storage. The discovery by Pentchev and colleagues in the early 1980s that cholesterol esterification was defective in cultured fibroblasts from NPC patients provided an important diagnostic tool for this disease. This discovery also led to the view that NPC disease
was primarily a cholesterol lipidosis [5], further splitting type C disease from types A and B in which sphingomyelin was the primary storage material. The enigma of NPC disease was resolved for the majority of cases in 1997 with the cloning of the defective gene and the discovery of the NPC1 protein [6]. NPC1 was found to be an integral membrane protein consisting of 1278 amino acids and 13–16 membrane-spanning domains. NPC1 showed high homology with Patched, a critical element in the sonic hedgehog signaling pathway, as well as with proteins involved in cholesterol homeostasis, including 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and with SCAP, the sterol regulatory element-binding protein (SREBP)-cleavage-activating protein (SCAP). The numerous links between NPC1 and possible functions involving cholesterol reinforced the view that the disorder was a primary cholesterol lipidosis. This idea, however, could not be substantiated in recent studies implicating NPC1 as a eukaryotic version of a prokaryotic “permease” as in this experimental system the NPC1 protein did not appear capable of transporting cholesterol [7]. Further, recent studies of the NPC1 homologue in yeast (NCR-1) have suggested that the primordial role of this protein is associated with subcellular sphingolipid distribution [8]. Interestingly, NPC disease has long been known to exhibit massive storage of gangliosides and other glycolipids in brain and, recently, it was shown that the accumulation of unesterified cholesterol in neurons appears largely dependent on the types of complex gangliosides expressed [9], once again raising significant questions as to the exact function of the NPC1 protein as well as the identity of the primary storage material in NPC1 disease.

While type D Niemann–Pick disease was found to be allelic with type C1 [6], a small percentage of cases (≈5%) with remarkable similarity to NPC1 disease was found to nonetheless have normal expression of NPC1 protein. This led to the discovery of NPC2, a second defective protein and one with identity to HE1, a cholesterol binding protein [10]. Interestingly, the suggested linkage between NPC2 and cholesterol (see also Ref. [11]) once again puts issues of cholesterol trafficking and metabolism at center stage in terms of understanding NPC disease. While it is not presently known whether NPC1 and NPC2 proteins directly interact in cells, recent studies on a murine NPC1–NPC2 double mutant are consistent with direct functional co-operativity between the two proteins [12].

In addition to human NPC1 disease, feline and murine models of NPC disease are also known, both of which were discovered after occurring spontaneously in normal cat and mouse populations [13–15]. Research colonies for both species have been established. Basic pathological features of these animal models closely resemble those observed in human cases of NPC disease and each model also exhibits clinically evident neurological dysfunction. Mutations of the NPC1 gene have also been produced in Drosophila [K. Bhat, personal communication] and in C. elegans [16], but detailed structural changes in neurons and the CNS of these simpler organisms are not yet well elucidated. This review therefore is focused on the consequences of NPC1 and NPC2 loss of function as they occur in human, cat and mouse neurons.

2. NPC1 and NPC2 loss of function in humans

NPC disease is clinically heterogeneous in humans, and various phenotypes have been documented in infants to adults [2,17,18]. Recent studies of the molecular genetics of NPC1 have revealed functional significance of the sterol-sensing domain in NPC1 protein and showed that the absence of NPC1 protein can be correlated with the most severe neurological form of the disease which shows infantile onset and demonstrable (severe) cholesterol metabolic defects [19]. Many of the NPC1 disease clinical variants, in contrast, were found associated with missense mutations in the cysteine-rich luminal loop. Not surprisingly, the heterogeneity of NPC1 disease in terms of clinical features and mutational characteristics is further reflected in CNS and visceral pathology. Hepatosplenomegaly with an accumulation of cholesterol, sphingomyelin and some glycolipids is prominent in infantile cases, but in the majority of juvenile or adult cases, hepatomegaly is not a usual pathological feature. In these later onset cases, neurodegeneration with formation of neurofibrillary tangles, in addition to lipid storage, is the usual pathological feature. For NPC2 cases, the number of families with affected individuals has been too few for detailed genotype-phenotype studies but overall the same clinical heterogeneity characterizing NPC1 disease also applies [18]. One recent report described adult onset-NPC2 disease in which neuronal storage and cognitive impairment were identified but storage in viscera and bone marrow were absent [20]. Despite this heterogeneity, there are numerous morphological alterations that appear in common across the full spectrum of NPC disease [21]. These include neuronal storage, megal neurite formation and ectopic dendritogenesis, axonal alterations in the form of spheroids, neurofibrillary tangles (NFTs) and other cytoskeletal alterations, fragmentation of Golgi apparatus and neurodegeneration (loss of neurons).

2.1. Neuronal storage

Neuronal storage is consistently observed in NPC disease in humans, although enlarged, swollen neurons are more conspicuous in early-onset than late-onset cases. On routine paraffin sections stained with hematoxylin and eosin (H and
E), the perikarya of affected neurons are packed with fine vacuoles. On frozen section preparation, the neuronal storage material reacts strongly with Periodic Acid Schiff (PAS) and to some extent with filipin, consistent with the presence of glycolipids and cholesterol, respectively, in neuronal perikarya. Biochemical analysis of NPC disease-affected brains has revealed increases of glycolipids (glucosylceramide, lactosylceramide, gangliosides GM3 and GM2) but similar increases of total cholesterol content have not been reported [22]. In situ analysis of gangliosides (GM2, GM3) and unesterified cholesterol using immunocytochemistry and histochemical (filipin) staining, respectively, has documented that these materials accumulate directly within neuronal perikarya [23] (Fig. 1). These storage materials are identified at the ultrastructural level in neurons as unique inclusions consisting of concentrically arranged lamellae resembling membranous cytoplasmic bodies of gangliosidosis [24], although smaller and less compact, and in some cases showing multivesiculated profiles, hence the term polymorphous cytoplasmic bodies [2]. Similar inclusions are found in glial cells of brain as well as in hepatocytes of liver.

Fig. 1. Mammalian neurons deficient in NPC1 accumulate GM2 and GM3 gangliosides and free cholesterol, none of which occurs at conspicuous levels in normal brain. (A) Normal adult mouse brain stained with an antibody to GM2 ganglioside showing its minimal presence. Inset in A: higher magnification of nearby glial cell. (B–C) Mouse and cat cortical neurons in NPC1 disease, respectively, showing the presence of GM2 ganglioside accumulation in neurons (arrows). (D–E) Histochemical (filipin) staining of cerebral cortex from normal mouse (D) and NPC1−/− mouse (E), with only the latter showing cholesterol accumulation in neuronal perikarya (arrows) and in enlarged axon hillocks/meganeurites (arrowheads). (F) Cortical neurons in human NPC1 disease showing the presence of GM3 ganglioside in perikarya (arrows) and in meganeurites (arrowheads). Calibration bar in F equals 25 μm for all.
2.2. Meganeurite formation and ectopic dendritogenesis

The use of the Golgi staining method to evaluate neuronal morphology in a cortical biopsy from a child with an undiagnosed and progressive form of mental retardation in 1975 led to a remarkable discovery. Golgi impregnations of these cortical neurons revealed the presence of elongated swellings at axon hillocks from which projected dendritic spines as well as longer, processes which themselves possessed spines [25]. The swelling at the axon hillock came to be known as a meganeurite, and the associated abnormal dendritic spines and processes as “ectopic dendritogenesis”, due to its peculiar and inappropriate location on the cell. Thus it appeared that these postnatal, mature neurons were undergoing a recapitulation of dendritogenesis normally seen only in early development, and as a result an entirely new basilar dendritic system was being generated below the normal basilar dendrites of the perikaryon. The child in this case was subsequently diagnosed with a rare form of GM2 gangliosidosis (AB variant) [26] in which the degradative enzyme (β-hexosaminidase) for GM2 ganglioside was normal, but failed to function as a consequence of a deficiency in its activator protein [27]. Meganeurites and accompanying neuritic and synaptic changes were subsequently reported in a variety of storage diseases in children, including NPC disease [28,29]. Here meganeurites were often enormous in size, dwarfing adjacent neuronal perikarya, and exhibiting elaborate dendritic processes complete with normal-appearing spines (Fig. 2).

The discovery of ectopic dendritogenesis in a primary ganglioside storage disease was believed to indicate that gangliosides contributed in some manner to dendritic growth regulatory mechanisms and that the resulting altered synaptic connectivity was a basis for brain dysfunction, most notably mental retardation [25,30]. However, it required the discovery and analysis of storage diseases in large animal models (see below) to fully address the underlying reasons for this renewal of dendritogenesis in lysosomal disorders. To this day the phenomenon of ectopic dendritogenesis remains a finding unique to disorders of the endosomal–lysosomal system [31].

2.3. Formation of axonal spheroids

Axonal spheroids [neuroaxonal dystrophy (NAD)] are focal swellings of axons, indicating focal sites of damage. They can be found following acute axonal injury such as Wallerian degeneration as well as in varieties of chronic neurodegenerative disorders such as infantile neuroaxonal dystrophy and Sandhoff disease. However, as pointed out by Elleder et al. [32], formation of axonal spheroids in early-onset NPC disease is a very conspicuous pathological feature. On routine H and E preparations spheroids appear as homogenous or finely granular focal axonal swellings occurring throughout the brain, in particular in the cerebral and cerebellar white matter, brainstem and posterior columns of the spinal cord. At the ultrastructural level, an accumulation of 10-nm filaments is found within the homogeneous spheroids whereas granular spheroids are composed of masses of apparent degenerated organelles, pleiomorphic bodies and amorphous dense substances (Fig. 3). Both patterns have been reported as mixed to various proportions in human NPC disease [32]. The ultrastructural features of spheroids are similar to those of dystrophic axons described in various neurological disorders, including other storage disorders, as well as in experimental studies [33,34]. Axonal spheroids are not as numerous in late-onset cases of NPC disease, in which neurofibrillary tangles are conspicuous, and it appears that axonal spheroids have inverse relation to the presence of neurofibrillary tangles [35]. Spheroids in human NPC disease have also been found to express positive immunoreactivity with antibodies to ubiquitin and phosphorylated neurofilaments.

2.4. Neurofibrillary tangles (NFTs)

Neurofibrillary tangles consist of paired helical filaments (PHFs) formed by phosphorylated microtubular protein, PHFtau, and are considered to be one of the hallmarks in Alzheimer disease (AD). In NPC disease, NFTs are found in almost all cases of juvenile/adult cases, although distribution and numbers of NFT-expressing neurons vary considerably case by case [35,36]. The youngest NPC case with NFTs so far reported in the literature was a 10-year-old child [37] but more recently one of us (KS) has personally observed a few NFTs in the cerebral cortex of a 3-year 10-month-old patient who was previously reported as negative [37]. Immunocytochemically and also by Western blot analysis using a number of different monoclonal antibodies.
(T14, PHF1, AT180, AT8, AT270, 12E8) that bind to AD-brain-derived PHFtau. PHFtau in NPC and AD brains was indistinguishable, suggesting similar mechanisms may play a role in formation of NFTs [38]. Ultrastructural features of NFTs in NPC disease consist of PHFs identical to those of Alzheimer disease (Fig. 4). Apolipoprotein (Apo) E is a major cholesterol transport molecule in the brain and in

Alzheimer disease Apo Eε4 allele is associated with an earlier onset of tangle formation and increased tangle load. Dist et al. [39] recently reported that mean levels of free cholesterol in tangle-bearing neurons were higher than in tangle-free neurons. Tangles are a particularly conspicuous feature of NPC disease patients who are homozygous for the ApoE ε4 allele, in whom deposition of β-amyloid is also detectable [40]. In one of those cases, Lewy bodies were detected in the substantia nigra. The Lewy bodies are the pathological hallmark of Parkinson disease and dementia with Lewy bodies, and the major protein constituent is α-synuclein. More recently, however, Lewy bodies have been reported in Alzheimer disease [41–43] and in other tauopathies [44]. Saito et al. [45] have found expression of phosphorylated α-synuclein as well as tau in neurons in many juvenile NPC cases. In some neurons these two proteins were co-localized. Co-localization of tau and α-synuclein epitopes has been reported in cases of dementia.
with Lewy bodies [46]. Thus, NPC neurons appear to share common mechanisms of neurodegeneration with other brain disorders. Recently, NFTs and axonal spheroids were reported to occur in an NPC patient with a mutation in NPC2 [20]. NFTs have never been detected in the cerebellum of NPC patients, or indeed even in the cerebellum of individuals with AD, although several early markers of NFT formation such as hyperphosphorylated tau and altered cell cycle regulators have been detected immunocytochemically in the cerebellum of NPC1 patients [47].

2.5. Fragmentation of Golgi apparatus

Cholesterol is an essential structural component of the plasma membrane as well as of intracellular membranes such as endosomes, lysosomes, Golgi and trans-Golgi network (TGN). In NPC patients (as well as the NPC1 mouse model, as described later), we have found, by using immunocytochemical staining, apparent fragmentation of the Golgi/TGN in cerebellar Purkinje cells and cerebral cortical neurons (Fig. 5). The antibody used for these studies recognizes a conserved membrane sialoglycoprotein of the medial cistern of the Golgi apparatus, MG160 [48–50] [unpublished data (KS)]. Fragmentation of the Golgi apparatus was first demonstrated in anterior horn cells and later in Betz cells in amyotrophic lateral sclerosis (ALS), and in neurons in AD [51]. Fragmentation of Golgi apparatus has been also detected in the mouse models of ALS prior to the onset of clinical signs [52]. Similar fragmentation has been recognized following depolymerization of microtubules following drug treatment [53] or physiological disassembly of microtubules during mitosis [54,55]. Thus, the fragmentation of the Golgi apparatus is unlikely to represent a specific pathological process but may be related to a more general degenerative process such as, for example, disease-induced disorganization of microtubules. Its presence/absence in other forms of neuronal storage disorders has not yet been explored.

2.6. Neurodegeneration (loss of neurons)

The brains of patients with end-stage NPC disease are often atrophic, suggesting that there has been considerable loss of neuronal elements. Rare TUNEL-positive neurons can be found in the brain of NPC disease patients [unpublished observation (KS)] but necrotic/dying neurons as seen in cerebral ischemia are not reported. How neurons degenerate and what causes neurodegeneration are not well defined in NPC or in other storage disorders. Like many other chronic neurodegenerative disorders, apoptotic death is a likely possibility and further investigation is needed to understand the mechanism of neuronal death in these disorders. As in many other chronic neurodegenerative diseases of humans, it is difficult to assess the mechanism of neuronal death in the postmortem brains because of many factors such as postmortem duration, status of terminal disease state, and so forth. Well-controlled studies with animal models are needed.

3. NPC1 loss of function in cats

Five surviving kittens from a litter of seven were donated to the New York State College of Veterinary Medicine in 1988 due to the presence of unusual neurological symptoms in two of the animals. Subsequent analysis revealed the presence of a neurovisceral storage disease resembling NPC in the affected cats and cultured fibroblasts exhibited decreased ability to esterify exogenous cholesterol [13]. A colony of these animals was established by heterozygote breeding and additional affected kittens have been successfully produced for a variety of studies. Following identification of the NPC1 gene in the human and mouse forms of NPC disease, complementation analysis of the human and feline disorders revealed involvement of the same gene [56],
thus documenting the validity of this important large animal model.

Cats deficient in NPC1 exhibit striking clinical signs [29,57]. Subtle intention tremors first appear at 8–12 weeks of age. Motor signs become progressively conspicuous over the ensuing weeks and involve truncal ataxia, head tremor and dysmetria. Ambulation is compromised by 4–5 months of age although animals remain alert and interested in their environment. By 6–7 months the animals spend more time in sternal recumbency and exhibit severe head and neck tremors. Positional nystagmus and opisthotonus are evident by 9–10 months of age. Few animals survive beyond 11 months.

3.1. Neuronal storage

Cats with NPC disease exhibit accumulation of excess lactosylceramide, glucosylceramide, phospholipids, and unesterified cholesterol in liver and GM2 and GM3 gangliosides in brain [13,23]. Storage within the CNS is extensive and essentially involves all neurons and other cell types beginning during the early postnatal period. Ganglioside accumulation in neurons has been documented using antibodies to GM2 and GM3, and storage, particularly of GM2, is a near-universal feature of the disease in neurons (Fig. 1). Storage bodies are distributed throughout the perikarya of neurons and often extend out into proximal dendrites. Occasionally, large collections of storage inclusions can be found in dendritic profiles. Storage in some types of neurons typically generates the formation of meganeurites at axon hillocks. Ultrastructurally, storage material in neurons consists of heterogeneous populations of vacuoles exhibiting loose membranous swirls and stacks as well as abnormal-appearing multi-vesicular profiles. As such, they appear remarkably similar to the polymembranous cytoplasmic bodies described in human NPC disease (above) and in the mouse models (Fig. 6). The presence of membranous storage materials is consistent with the presence of significant glycolipid storage.

3.2. Meganeurites and ectopic dendritogenesis

The use of Golgi staining and other morphological techniques in cats with NPC1 disease, as well as in a variety of other types of neuronal storage disorders [Niemann–Pick type A, mucopolysaccharidosis (MPS) type I, α-mannosidosis, GM1 and GM2 gangliosidosis], has revealed that meganeurite formation and ectopic dendritogenesis are a widespread feature of lysosomal diseases [58]. Meganeurites have been found to occur just proximal to the axonal initial segment and therefore represent deformations of the axon hillock/lower perikarya region of neurons. This finding, coupled with ultrastructural differences, established that meganeurites and axonal spheroids are distinctly different structures [59]. Meganeurites themselves are found to either be covered with dendritic spines and ectopic dendrites (as seen in the disorders listed above) or to be smooth-surfaced and to lack new spines, neurites or synapses. The presence of the latter so-called aspiny meganeurites on some types of neurons and in some diseases (e.g., the Batten disorders or neuronal ceroid lipofuscinoses) has been interpreted to indicate that meganeurites themselves are volume-accommodators following intraneuronal storage, whereas the growth of new dendritic
membrane is likely due to a disturbance in some specific metabolic process controlling dendritic initiation [58]. For some neurons undergoing storage, particularly in feline models of storage diseases (including NPC1 disease), intraneuronal storage appears to be insufficient to generate prominent meganeurites but axon hillocks nonetheless exhibit striking growth of new dendritic processes (Fig. 2). Ectopic dendrites have been documented as forming synaptic contacts with local axons, with most appearing asymmetrical in form and therefore likely excitatory in function [60].

All lysosomal diseases exhibiting ectopic dendritogenesis have revealed near identical patterns of involvement, with the same cell types (cortical pyramidal neurons, multipolar cells of amygdala and claustrum) appearing vulnerable to this change in each disease. The presence of ectopic dendrites and altered connectivity in cortical and subcortical and limbic system structures is compatible with this change contributing to both cognitive and behavioral deterioration in affected individuals. Overall, when comparing different storage diseases in the feline models, only the relative frequency of neurons in a given brain area varied, e.g., from 95% of cortical pyramidal neurons in GM2 gangliosidosis to 40% in Niemann–Pick type C and to smaller percentages in non-primary glycosphingolipidoses [61]. This was believed to argue that features intrinsic to certain types of neurons, coupled with specific metabolic abnormalities in common between different storage diseases, likely were coalescing to generate this unusual pathological feature. The availability of NPC and other storage diseases in a single species (i.e., cats) has allowed for systematic analysis of the hypothesis that ectopic dendritogenesis was being triggered by abnormal sequestration of gangliosides. These studies revealed that one particular ganglioside (GM2) was consistently present as part of the lysosomal storage process in all neurons characterized by ectopic dendritic sprouting [61]. These studies, coupled with related work showing heightened elevation of this same ganglioside during early development in normal brain when pyramidal neurons sprout dendrites, led to the hypothesis that GM2 ganglioside is functionally allied with mechanisms controlling dendritogenesis in both normal and storage disease-affected pyramidal neurons [62-64].

3.3. Formation of axonal spheroids

Neuroaxonal dystrophy, as described for human NPC disease above, is also a major feature of the feline model. Indeed, many of the storage diseases found in this species [GM1 and GM2 gangliosidosis (Sandhoff), Niemann–Pick disease type A, and α-mannosidosis] exhibit this phenomenon in abundance whereas only a few (e.g., mucopolysaccharidosis type I) do not [58]. Ultrastructurally, most spheroids in cats appear granular in nature and are found in both gray and white matter regions of brain. While they are evident as PAS-negative profiles in PAS-stained plastic sections of brain, their frequency of occurrence typically appears unimpressive unless labeled by specific antibodies. For example, labeling with antibodies to glutamic acid decarboxylase (GAD), the synthetic enzyme for the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), revealed that spheroids were remarkably abundant and specifically affected GABAergic neurons in many brain regions [65]. Only rarely were spheroids labeled significantly with antibodies to neurofilament proteins, a finding consistent with the paucity of neurofilaments in most spheroids in this species.

The overall distribution and incidence of axonal spheroids in different regions of the CNS was found to closely correlate with the type and severity of clinical neurological signs in affected cats. Cats with NPC1 disease were found to exhibit spheroids in all areas of brain containing GABAergic neurons, including cerebral cortex, basal ganglia, cerebellum and brainstem. Spheroids on cerebellar Purkinje cells were particularly evident when labeled with antibodies specific for this type of neuron (calbindin, parvalbumin) as well as with GAD [29]. Spheroids in Purkinje cell axons in NPC disease appeared to form earliest in distal axons and over time enlarged in size and became more conspicuous in proximal portions of axons. Storage disorders in cats that lacked significant spheroid formation, such as MPS I, also lacked significant neurological signs (e.g., motor system dysfunction) in spite of widespread neuronal storage, again consistent with spheroid formation being a major generator of clinical disease.

As mentioned above, spheroids in all types of storage diseases consisted of essentially identical accumulations of tubulovesicular and multivesicular profiles, dense bodies, and mitochondria (Fig. 3). They rarely contained significant amounts of neurofilamentous material, as is characteristic of spheroids in toxic neuropathies and some human storage diseases (see earlier discussion). Importantly, they also do not contain tertiary lysosomes resembling the storage bodies within neuronal perikarya, which differ disease by disease according to the specific lysosomal hydrolase that is defective. Axonal spheroids in feline models of storage diseases also did not appear to be “retraction bulbs” of dying axons. Rather, they are swellings of significant size that occur along the length of an axon with axonal continuities clearly visible on both proximal and distal sides [65]. These structural characteristics of spheroids suggest that they may represent defects in axoplasmic transport. Accumulations of similar heterogeneous organelles have been reported to occur distal to crush or low temperature lesions in axons of experimental animals, indicating that these types of materials are characteristic of a block in retrograde transport [66]. More recent studies using murine NPC disease, reviewed below, suggest that defects in cholesterol transport in axons and/or abnormalities in oligodendroglia may play a role in the generation of axonal spheroids.
3.4. Cytoskeletal abnormalities and Golgi fragmentation

Cats with NPC1 disease have been evaluated for NFTs but the phenomenon has not been observed in this species [T. Mitchell and colleagues, personal communication]. Studies designed to analyze the integrity of the Golgi/TGN have not yet been carried out in cats.

3.5. Neurodegeneration (loss of neurons)

Studies of the feline model of NPC1 disease have not reported significant death of neurons in the CNS of affected animals apart from that of cerebellar Purkinje cells. However, systematic assessment of this issue has not been rigorously pursued and, given the significant amount of neuroaxonal dystrophy found throughout the CNS (as a potential forerunner of neuronal demise), it is conceivable that populations of neurons other than just Purkinje cells are undergoing premature degeneration. In the cerebral cortex there is an indication for thinning of the gray matter and, interestingly, the incidence of ectopic dendritogenesis on cortical pyramidal neurons appears less over time (unlike GM1 and GM2 gangliosidosis in cats in which the incidence of ectopic dendritogenesis on neuronal perikarya as well as distended processes can also be immunostained with the antibody for ubiquitin [72]). The localization of GM2 and GM3 gangliosides and several neutral glycolipids [12,71,73]. The localization of GM2 and GM3 gangliosides in storage neurons has been demonstrated immunocytochemically (Fig. 1) [12,23]. Interestingly, when double-labeled with fluorescent markers and examined by confocal microscopy, the GM2 and GM3 gangliosides were found to largely occur in separate vesicle populations, possibly indicating independent mechanisms to account for their storage [74]. Intracellular storage in NPC mice is most conspicuous in large pyramidal neurons, Purkinje cells, and neurons in the lateral thalamus and brainstem. Many Purkinje cells are lost in mice older than 60 days of age and degenerative changes in dendritic arbors and focal swellings of axons are well recognized in remaining Purkinje cells. These structural changes of Purkinje cells have been particularly well demonstrated using immunocytochemically staining for calbindin. Storage materials in neuronal perikarya as well as distended processes can also be immunostained with the antibody for ubiquitin [72]. The neuronal inclusions consist of concentrically arranged lamellar and multivesicular structures, similar to those of human and feline NPC1 disease (Fig. 6) [12,72,74–76]. Inclusions are also detected in microglia/macrophages, astrocytes and oligodendrocytes [71,72,77]. These inclusions are structurally similar to each other but show some different cell-specific profiles. In addition, as noted in the liver, spleen and lung [78], “cholesterol clefts” indicating the presence of cholesterol are well recognized in macrophages in the brain [72]. “Cholesterol clefts” have not been detected in the neurons. However, filipin staining of brain tissue has revealed numerous positive granules in neurons and other cell types [12,23], which are believed to represent cholesterol-filled endosomes/lysosomes (Fig. 1). Studies using cultured murine NPC sympathetic neurons by Karten et al. [79] have demonstrated altered cholesterol distribution between neuronal cell bodies and axons. In agreement with a previous in vitro study with embryonic striatal neurons [80], they found no differences in cholesterol content in and phospholipid, largely in sphingomyelin content [14]. Major neuropathological changes in these mice are neuronal storage, axonal spheroids, hypomyelination and loss of neurons (neurodegeneration). Unlike human cases, however, no NFTs have been reported in the mouse models of NPC. Hypomyelination is reported in the corpus callosum and anterior commissure [71] but myelination is well advanced in the internal capsules, cerebellar white matter, brain stem and spinal cord. Myelin degeneration becomes apparent after 60 days of age, possibly secondary to axonal degeneration [72].

4. NPC1 and NPC2 loss of function in mice

Two murine models of NPC1 disease, BALB/c $npc^{nih}$ (NPC1−/−) and C57BL/KSJ (sphingomyelinosis or SPM) mice, are known [14,15]. In NPC1−/−, the murine ortholog of NPC1 is mutated [69]. SMP mice are found to be allelic with the NPC1−/− mice by crossing experiments [70] but the exact gene mutation has not yet been identified. Recently, Sleat et al. [12] have reported generating a murine NPC2 hypomorph that expresses 0–4% residual protein in different tissues. In examining its phenotype, it was found to closely resemble the NPC1−/− mouse.

Pathological changes in the NPC1 and NPC2 murine models resemble those observed in late infantile NPC disease in humans. The BALB/c $npc^{nih}$ (NPC1−/−) mice were initially reported as a lysosomal lipid storage disorder characterized by excessive tissue deposition of cholesterol and phospholipid, largely in sphingomyelin content [14]. Major neuropathological changes in these mice are neuronal storage, axonal spheroids, hypomyelination and loss of neurons (neurodegeneration). Unlike human cases, however, no NFTs have been reported in the mouse models of NPC. Hypomyelination is reported in the corpus callosum and anterior commissure [71] but myelination is well advanced in the internal capsules, cerebellar white matter, brain stem and spinal cord. Myelin degeneration becomes apparent after 60 days of age, possibly secondary to axonal degeneration [72].
neurons as a whole between NPC1-deficient and wild-type mice. However, by filipin staining, cholesterol was found sequestered in neuronal perikarya and the cholesterol content of the distal axons was significantly less, suggesting defective transport of cholesterol in NPC1-deficient neurons [79]. Intraneuronal storage of free cholesterol has been demonstrated in vivo in the mouse model by filipin staining [23,81] as well as by a novel cholesterol-binding reagent known as BC-theta [82]. In the latter study, the authors reported that neuronal accumulation of cholesterol occurred as early as 9–10 days of age and preceded evidence of detectable accumulation of GM1 and other gangliosides analyzed. In other studies of ganglioside elevations in the NPC mice, neuronal accumulation of GM2 was found to precede that of GM3 and to be detectable during this same time frame (1–2 weeks of age) (Gondre´-Lewis and Walkley, unpublished data).

There is evidence for significant interaction between cholesterol and glycosphingolipids in neurons, most notably through co-localization in specialized membrane microdomains known as lipid rafts [83]. The significance of glycosphingolipid accumulation in NPC disease and its relationship to NPC1 and NPC2 function, and to the sequestration of cholesterol, are controversial. Glycosphingolipids residing in lipid rafts in the plasma membrane are believed to be imported into the endosomal–lysosomal system for degradation and recycling via the Golgi/TGN. One recent finding suggests that the high level of accumulating cholesterol in the perikarya in NPC1-deficient neurons blocks the endosome/lysosome to Golgi transport and this event then leads to an accumulation of glycosphingolipids [84,85]. This would argue, consistent with long-held beliefs about NPC1 function, and more recently about NPC2 as a cholesterol-binding protein, that ganglioside storage occurs secondary to cholesterol sequestration. However, studies in which the synthesis of complex gangliosides in NPC1 mice was limited by producing double mutant animals lacking both NPC1 and GalNAc-transferase, the enzyme responsible for synthesis of GM2, GD2 and higher order gangliosides, suggest otherwise [9]. While, as expected, double mutant mice accumulated GM3 ganglioside in a manner equivalent to NPC1−/− mice and lacked storage of GM2, they also exhibited dramatic reduction in storage of unesterified cholesterol. Neurons that did store cholesterol consistently showed storage of GM3, whereas some GM3-accumulating neurons lacked detectable staining for cholesterol. Neurons exhibiting cholesterol accumulation but lacking evidence of GM3 staining were not observed, an important finding that strongly suggests that cholesterol accumulation in NPC1−/− neurons is in some manner dependent on the storage of complex gangliosides [9]. These findings are consistent with the possibility that NPC1 plays a key role in ganglioside metabolism and/or trafficking, and possibly in the homeostatic control of glycosphingolipid synthesis in a manner originally proposed for cholesterol [23]. The generation of NPC2−/− mice [12] means that the function of NPC2 can be similarly tested in genetic crosses with mice lacking specific enzymes in the glycosphingolipid synthetic pathway.

4.2. Meganeurite formation and ectopic dendritogenesis

Golgi studies of murine NPC1 disease have revealed the presence of small meganeurites decorated with occasional dendritic spines on some cortical pyramidal neurons [23]. However, the extensive ectopic neuritogenensis and large dendritic spine/ectopic dendrite-covered meganeurites observed in feline and human NPC1 disease, respectively, have not been observed in mice (Fig. 2). Meganeurites without any evidence of dendritic spines or processes (“aspy” types) are often observed in affected mice revealing the presence of significant storage but suggesting an absence of dendritogenic factors (or sensitivity to such factors) that in affected cat and human brain must be prominent. Interestingly, Golgi staining of a wide variety of neuronal storage diseases in mice provides similar findings [62]. That is, ectopic dendritogenesis is not a conspicuous feature even in disorders already documented as exhibiting this phenomenon in humans (e.g., Tay-Sachs disease). The degree of ectopic spines and processes found in NPC1 mice, in fact, is the most observed to date in any murine storage disease model. One interpretation of the lack of significant ectopic dendritogenesis in murine storage disease models is that postnatal mechanisms of dendritic plasticity in rodent brain may be more limited than those in carnivore (cat) and primate (human). That murine NPC1 disease shows the greatest degree of ectopic dendritic membrane production and is also the earliest onset and most rapidly progressive of the known murine storage diseases is consistent with this view. Determining why ectopic dendritogenesis is limited in mouse neurons may eventually be revealing as to important regulatory factors controlling postnatal dendritogenesis in all mammalian species.

4.3. Axonal spheroids

Focal swellings within axons (spheroids) are very conspicuous in NPC1−/− mice just as in young human NPC patients and in the feline NPC1 disease [23,76]. They are recognized throughout the brain as early as 15 days of age and are gradually increased in number with progression of the disease process. Predilection sites include internal capsule, substantia nigra, medial lemniscus, cerebellar nuclei and white matter, pontine base, dorsal nuclei in medulla and spinal white matter. Topographically, the extent of neuronal storage does not necessarily coincide with the presence of axonal spheroids. The topographic distribution of axonal spheroids appears to be influenced by the background strain. In the NPC−/− mice that are backcrossed to C57BL/6, heavy concentrations of axonal spheroids are observed in the fimbria [unpublished observation by KS]. They can be stained with antibodies to amyloid precursor protein.
protein, parvalbumin, ubiquitin and phosphorylated or non-phosphorylated neurofilaments. At the ultrastructural level, three types of spheroids have been identified in the NPC1 mouse model: (1) focally swollen axons with centrally displaced bundles of neurofilaments and peripherally displaced inclusions consisting of abnormal mitochondria, lamellar and or dense bodies, (2) enlarged inner tongue of oligodendrocytes with numerous inclusions ensheathing apparently normal axons, and (3) accumulation of abnormal inclusions in both axolemma and inner tongue processes [72]. Bu et al. [86] have reported increases of cyclin-dependent kinase 5 (cdk5) and its potent activator p25, a proteolytic fragment of p35, in the axonal spheroids, together with hyperphosphorylated cytoskeletal protein. They suggested that focal deregulation of cdk5/p25 in axons leads to cytoskeletal abnormalities and eventual neurodegeneration in NPC disease. Cholesterol deficiency in cultured neurons is reported to result in hyperphosphorylation of microtubular protein tau accompanied by focal axonal degeneration [87], and cultured sympathetic neurons of NPC1−/− mice showed deficient cholesterol in distal axons [79]. Axonal spheroids in NPC1−/− mice are generally negative for filipin or BC-theta stain [unpublished observation by KS]. These studies are supportive of the hypothesis by Bu and co-workers in that distal axonal degeneration due to deficient cholesterol may be the initial event of neurodegeneration in NPC. As will be described below, Fas ligand (Fas-L), the molecule involved in Fas/Fas-L mediated cell death pathway, is expressed in macrophages in areas where axonal spheroids are numerous. Axonal spheroids are commonly found together with the degeneration of distal oligodendroglial processes. Degeneration of distal oligodendroglial cell processes has been well documented as distal oligodendrogliopathy in myelin disorders [88,89], suggesting that perturbed cholesterol trafficking in oligodendrocytes may be an additional contributory factor for the formation of axonal spheroids.

4.4. Cytoskeletal abnormalities

As reported above, NFTs are a hallmark of Alzheimer disease and consist of hyperphosphorylated aggregations of microtubular protein tau, PHFtau. NFTs are commonly associated neuropathological changes in the brain of human patients with NPC [35–37] but not detected histologically in the mouse model of NPC [90]. However, site-specific hyperphosphorylation of tau (at Ser-396 and Ser-404) accompanied by an activation of MAP kinase has been reported in the brain of NPC1−/− mice and some of the cerebellar granular neurons and cerebral cortical neurons were reported to be immunostained with antibody PHF-1 [91]. Bu et al. [86] found that hyperphosphorylation of neurofilaments (NF-M isoform), MAP2 and tau was associated with a significant increase in activity of the cyclin-dependent kinase 5 (cdk5) and some PHF-1 immunoactive neurons. Immunoreactivity to phosphorylated neurofilaments is detected in many axonal spheroids as stated above.

4.5. Golgi fragmentation

As noted in human NPC1 disease, Golgi fragmentation was also detected in neurons in the cerebral cortex as well as in cerebellar Purkinje cells in NPC1−/− mice (Fig. 5). The numbers of Purkinje cells with fragmented Golgi apparatus appear to increase prior to disappearance of the cells. Fragmented Golgi apparatus was observed in the brainstem neurons in NPC1−/− mice but also in the brainstem neurons in twitcher mice, a murine model of globoid cell leukodystrophy [unpublished observation by KS]. In contrast, fragmentation of the Golgi apparatus was not detected in the GM1 gangliosidosis model mice. Thus, further investigation is needed to clarify the significance of this intriguing phenomenon.

4.6. Neurodegeneration (loss of neurons)

The brains of NPC1−/− and SPM mice are small (approximately 2/3 of wild type at 40 days) and become atrophic with loss of neurons, most pronounced in the cerebellar Purkinje cells and thalamus [75,76,90,92]. Clinical course and neurodegeneration could not be prevented by pharmacological treatments and genetic manipulations aimed at alleviation of cholesterol storage [93,94]. However, reduction of glycosphingolipid storage in NPC1−/− mice through the use of a glycosphingolipid synthesis inhibitor delayed Purkinje cell death and clinical onset [95]. Similar increases in longevity were observed in some (though not all) double mutant mice lacking both NPC1 and GalNAc-transferase [9] (see also Ref. [96]). Genetic cross studies which removed low-density lipoprotein receptor [97] or that introduced the human Bel-2 transgene into NPC1−/− mice [98] failed to reveal clinical benefit. The loss of Purkinje cells was most dramatically abated by introduction of wild-type NPC1 protein to the NPC1−/− mice, proving that mutation of NPC1 gene is responsible for neurodegeneration in NPC1−/− mice [99].

Loss of Purkinje cells in NPC1−/− mice follows a well-defined pattern [76,100]. Zebrin II-negative Purkinje cells disappear first and degeneration of other Purkinje cells follows this event. At the terminal stage, the majority of Purkinje cells disappear with exception of those in lobules IX and X of the posterior vermis. Surviving cells expressed ectopic tyrosine hydroxylase and the small heat shock protein HSP25 [100]. This orderly pattern of Purkinje cell loss in NPC1−/− mice suggests that the loss of neurons in NPC1 is not resulting from an accidental (necrotic) event but is consistent with programmed (apoptotic) cell death. Scattered TUNEL-positive cerebral cortical neurons and cerebellar Purkinje cells and also the presence of active caspase 3-immunoreactive Purkinje cells are consistent with cell death being due to apoptosis [101]. Since overexpres-
sion of Bcl-2 failed to prevent neurodegeneration [98], cell death in NPC1−/− mice is unlikely to be through Bcl-2 mediated mitochondrial cell death pathway. Numerous Fas-L immunoreactive macrophages/microglia appeared in NPC1 mice with close association to axonal spheroids [101]. Since Fas/Fas-L induced apoptosis cannot be blocked by Bcl-2 [102], involvement of the Fas/Fas-L signaling pathway is a possibility in the cell death in NPC1−/− mice. Using amino cupric silver stain, Ong and co-workers reported widespread degeneration of nerve fibers followed by degeneration of nerve cell bodies as early as day 9. They suggested that the earliest structure involved was the nerve terminal and axons and functional disruption of perisynaptic astrocytes may contribute to neurodegeneration [103]. The pattern of Purkinje cell loss in NPC2 mice has recently been reported to closely resemble that observed in NPC1 disease [12]. Indeed, double mutant mice lacking both NPC1 and NPC2 exhibited the same pattern of cerebellar pathology in a time frame equivalent to NPC1 disease.

4.7. Function of NPC1 vs. NPC2 in mice

Development of a murine model of NPC2 disease created the opportunity to cross the two disease models to produce NPC1;NPC2 double mutants [12]. It was reasoned that if the NPC1 and NPC2 proteins functioned in independent metabolic pathways, the loss of both proteins in the same animal would likely lead to a more severe phenotype than that observed with either alone. If however, both proteins co-operated in carrying out the same function, the disease phenotype would likely resemble that of the more severe of the two mutants, i.e., the NPC1 mice. Results showed the latter, that is, double mutants appeared essentially indistinguishable phenotypically from NPC1 mice in terms of disease onset and progression, pathology, neuronal storage and biochemistry of lipid accumulation [12]. These findings strongly suggest that NPC1 and NPC2 proteins function in concert to facilitate the intracellular retro-transport of lipids from endosomes/lysosomes to other cellular sites.

5. Summary

Neuropathological consequences of NPC1 and NPC2 deficiency in neurons of humans and in feline and murine models of this important lysosomal disease are briefly reviewed. Basic structural alterations as they occur in NPC disease in the three species are remarkably similar. Several important interspecies distinctions, however, were observed. The phenomenon of ectopic dendritogenesis, which is a conspicuous feature of many cases of human NPC disease, shows attenuated involvement in the feline disease and is even less evident in the mouse models. This may reflect important species differences in postnatal mechanisms of dendritic plasticity as opposed to actual differences in the function(s) of NPC1 and NPC2 in each species. Likewise, neurons in many cases of human NPC disease, but not mouse or cat, exhibit NFT similar to AD. This again may possibly reflect species differences in disease response (e.g., the effects of cholesterol dysregulation on specific tau isoforms in neurons) as opposed to actual differences in the function of NPC1/NPC2 proteins. In future studies it will be important to expand the analysis of NPC1 and NPC2 loss of function to neurons in other species, including Drosophila and C. elegans, to more closely analyze the ultimate role of these proteins in retroendocytic lipid trafficking and other possible functions. While genotype–phenotype analysis and detailed neuropathological studies in humans with NPC disease should be pursued, determining the consequences of NPC1 and NPC2 loss of function in simpler cells and organisms can provide critical insights into cell biological mechanisms of NPC disease presently unrecognized in humans, and may ultimately hold the key to delineating pathogenic cascades and developing new and successful therapeutic measures.

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