Characterization of lipid-linked oligosaccharide accumulation in mouse models of Batten disease

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The neuronal ceroid lipofuscinoses (NCLs, also known collectively as Batten disease) are a group of lysosomal storage disorders characterized by the accumulation of autofluorescent storage material in the brain and other tissues. A number of genes underlying various forms of NCL have been cloned, but the basis for the neurodegeneration in any of these is unknown. High levels of dolichol pyrophosphoryl oligosaccharides have previously been demonstrated in brain tissue from several NCL patients, but the specificity of the effect for the NCLs has been unclear. In the present study, we examine eight mouse models of lysosomal storage disorders by modern FACE and found striking lipid-linked oligosaccharide (LLO) accumulation in NCL mouse models (especially CLN1, CLN6, and CLN8 knockout or mutant mice) but not in several other lysosomal storage disorders affecting the brain. Using a mouse model of the most severe form of NCL (the PPT1 knockout mouse), we show that accumulated LLOs are not the result of a defect in LLO synthesis, extension, or transfer but rather are catabolic intermediates derived from LLO degradation. LLOs are enriched about 60-fold in the autofluorescent storage material purified from PPT1 knockout mouse brain but comprise only 0.3% of the autofluorescent storage material by mass. The accumulation of LLOs is postulated to result from inhibition of late stages of lysosomal degradation of autophagosomes, which may be enriched in these metabolic precursors.

Key words: Batten disease/dolichol/lipofuscinosis/lysosome/storage disorders

Introduction

The neuronal ceroid lipofuscinoses (NCLs, also known collectively as Batten disease) are progressive inherited neurodegenerative disorders characterized by the accumulation of autofluorescence storage material in brain and other tissues. Interest in the disorders has predominantly been fueled by the similarity of the pathological storage material (lipofuscin) to that occurring with normal aging. The physiologically important pathology in the NCLs is limited to the central nervous system, and affected individuals suffer progressive blindness, dementia, seizures, loss of motor skills, and premature death. To date, the NCLs have been systematically classified into eight different subtypes based on characteristic age of onset and appearance of the storage material by electron microscopy. Six of the NCL genes have been cloned (CLN1/PPT1, CLN2/TPPI, CLN3, CLN5, CLN6, and CLN8), and the others have shown distinctive clinical features as members of the NCLs (Hofmann and Peltonen, 2001; Mitchison and Mole, 2001; Mole, 1999; Wisniewski, 2001). Demonstration that the underlying genes encode either soluble lysosomal hydrolases (palmitoyl-protein thioesterase-1, PPT1, in CLN1 and tripeptidyl peptidase-1 in CLN2) or intrinsic lysosomal membrane proteins (CLN3p and CLN5p) has resulted in the recognition of NCLs as true lysosomal storage disorders (Hofmann and Peltonen, 2001).

In contrast to the classical lysosomal storage disorders, biochemical analyses of the storage material in the NCLs did not provide clues as to the underlying defects, due either to the heterogeneous nature of the metabolites (proteins or lipid-modified proteins) or perhaps because the metabolites are toxic. One striking observation from the pregenomic era was abnormally high levels of dolichol phosphate (Dol-P) in brain tissue of several NCL patients, reaching concentrations 10- to 20-fold higher than those of age-matched controls (Hall et al., 1989, Keller et al., 1984; Sakakihara et al., 1992; Wolfe et al., 1988). Because periodic acid–Schiff reagent staining had suggested that the storage material contains carbohydrate, high-performance liquid chromatography (HPLC) analysis was performed to show that the bulk of the Dol-P found in these cases was present as dolichol pyrophosphoryl oligosaccharides (referred to here as lipid-linked oligosaccharides, LLOs) (Daniel et al., 1992; Hall and Patrick, 1985, 1987, 1988; Hall et al., 1992). Furthermore, oligosaccharide structure analysis suggested that the LLOs were derived from catabolic rather than synthetic intermediates. However, these studies were limited by uncertainties as to the genetic diagnosis in the clinical specimens, the use of autopsy material, the paucity of samples available for analysis, inability to study the process with age, and technical limitations imposed by more cumbersome analytical methods.

Five well-defined genetic mouse models corresponding to human NCLs are available to date. Three of these models were generated by gene targeting—CLN1/PPT1 (Gupta et al.,...
2001), CLN2/TPP1 (Sleat et al., 2004), and CLN3 (Mitchison et al., 1999; Wheeler et al., 2002)—whereas the other two were isolated as spontaneous mutations in CLN6 (Gao et al., 2002) and CLN8 (Ranta et al., 1999). We have also recently created a form of NCL in a mouse model through a knock-out of PPT2, a homolog of PPT1 with lysosomal thioesterase activity with a specificity that overlaps that of PPT1 (Soyombo and Hofmann, 1997). This mouse model also develops autofluorescent storage material in the brain and other tissues, yet has some pathology outside the central nervous system (Gupta et al., 2003).

We examined brain and tissue samples from these and other mouse models of lysosomal storage disorders with central nervous system neurodegenerative phenotypes using a newly developed fluorophore-assisted carbohydrate electrophoresis (FACE) technique. The FACE analysis allows study of true steady-state LLO compositions, as well as molar quantitation of each intermediate (Gao and Lehrman, 2002a). Detailed structural characterization revealed that the LLOs are consistent with catabolic products, and the increase in LLOs in the PPT1 knockout mouse brain is confined to nonglucosylated intermediates containing Man5–9GlcNAc2. We suggest that the accumulation of LLOs in the NCLs is related to inhibition of lysosomal function in the late stages of autophagy, because the proposed source of autophagic membranes (rough endoplasmic reticulum, RER) are rich in these metabolites.

**Results**

**LLO accumulation in the PPT1 knockout mouse**

LLOs corresponding to the structure Glc3Man9GlcNAc2-P-dolichol are typically the precursors of asparagine-linked glycans in mammalian cells. The steady-state concentrations of LLOs are normally low in biological systems, in the range of 1 nmol/g tissue. FACE analysis allows for the sensitive detection and structural characterization of monosaccharides and oligosaccharides in biological samples without the need for radioactive metabolic labeling (Gao and Lehrman, 2002a), and therefore we applied this technique to the characterization of LLOs extracted from PPT1 knockout mouse tissues, a mouse model representing the most severe form of NCL. Briefly, tissues were homogenized rapidly in methanol, dried under nitrogen, and the dried pellets extracted with chloroform/methanol 2:1 (v/v) and water sequentially to remove contaminants. The resulting pellets were extracted again with chloroform:methanol:water (CMW) 10:10:3 (v/v/v) to recover the LLOs, and the LLOs were treated with mild acid to cleave the pyrophosphate linkage. The released oligosaccharides were then reacted with the fluorophore 7-amino-1,3-naphthalenedisulfonic acid (ANDS), resolved by gel electrophoresis, and visualized with a fluorescence scanner (Lehrman and Gao, 2003).

As shown in Figure 1, striking accumulations of LLO intermediates were observed in PPT1 knockout mouse tissues, kidney, and liver (the three tissues chosen for examination). In brain tissue (Figure 1A, lanes 1 and 2), the accumulation appeared to be relatively specific for LLOs migrating as Man5–9GlcNAc2, with little or no increase in Glc3Man9GlcNAc2 species. In contrast, in the kidneys of PPT1 knockout mice, all species of LLO were increased to about the same extent (Figure 1A, lanes 3 and 5 and lanes 2, 4 and 6 in each panel corresponds to the ANDS-labeled LLOs from wild-type and PPT1 knockout mice, respectively. One-tenth of the total derivatized extract from one organ was loaded. The positions of LLO standards Glc3Man9GlcNAc2-ANDS, Man9GlcNAc2-ANDS, and Man6GlcNAc2-ANDS are indicated as G3M9, M9, and M6, respectively. Results shown represent one of three experiments giving similar results.

**Fig. 1.** (A) LLOs accumulate in PPT1 knockout mouse tissues. Lanes 1, 3, and 5 and lanes 2, 4 and 6 in each panel corresponds to the ANDS-labeled LLOs from wild-type and PPT1 knockout mice, respectively. One-tenth of one whole brain. The result shown is one of two yielding similar results.

To estimate the composition of accumulated LLOs from PPT1 knockout and normal mouse brains, LLOs were derivatized with ANDS and quantitated in comparison with known standards on FACE gels. Of note, the dry weight of PPT1 knockout mouse brain (75.9 ± 4.0 mg) was 23% less than that of wild-type brain (98.0 ± 5.0 mg), presumably...
due to massive neuronal loss during the progress of the disease (Bible et al., 2004). We found that the increase in LLOs in the PPT1 knockout mouse brain is confined to the nonglucosylated intermediates (Man\(_{5–9}\)GlcNAc\(_2\)), which accumulated to an overall level that was 14.5-fold higher compared to wild-type brain (data not shown). The mature species (Glc\(_2\)-Man\(_9\)GlcNAc\(_2\)), normally the major species in the wild-type brain, was found to be somewhat decreased (to 47% of the wild-type level) in PPT1 knockout brain.

The accumulation of LLO intermediates was age-dependent as assessed in the brains of 2-week to 6-month-old PPT1 knockout mice (Figure 1B). Mannosylated LLO intermediates were the predominant component of the accumulation and started to accumulate as early as 2 weeks of age. Greater than 10-fold accumulation of LLOs as compared to the wild type were observed by the age of 6 months, an age at which 50–75% of mice develop neurological abnormalities, such as clamping and myoclonic jerks (Gupta et al., 2001). A similar time-dependent increase in LLOs derived from CLN3 knockout mice was also observed (data not shown).

**Structural characterization of ANDS-labeled oligosaccharides from PPT1 knockout mouse brain**

To characterize the composition and structure of LLOs that accumulate in the tissues of PPT1 knockout mice, several analytical enzyme treatments were performed. Jack bean \(\alpha\)-mannosidase hydrolyzes exposed \(\alpha\)-mannosyl residues on oligosaccharides. One or more glucosyl residues on the \(\alpha\)-1,3 arm of the oligosaccharide have two effects: They block digestion of the \(\alpha\)-1,3 arm by mannosidases, and they retard digestion of exposed mannosyl residues on the \(\alpha\)-1,6 arm by jack bean \(\alpha\)-mannosidase (Beeley, 1985; Cacan et al., 2001). Therefore, the enzyme may be used to test for the presence of glucosylated Glc\(_1\)–3Man\(_{5–9}\)GlcNAc\(_2\) species. As shown in Figure 2B, ANDS-labeled oligosaccharides from a standard LLO mix prepared from normal mouse liver (which appears largely as Glc\(_3\)-Man\(_9\)GlcNAc\(_2\) plus various nonglucosylated LLOs) was converted to the relatively protected Glc\(_3\)Man\(_9\)GlcNAc\(_2\)-ANDS (Figure 2B, lane 2, asterisk) and Man\(_9\)GlcNAc\(_2\)-ANDS (containing a \(\beta\)-mannosyl residue as the result of digestion of the unglucosylated species (Lehrman and Gao, 2003). The mannosyl residues most likely removed by jack bean mannosidase to generate Glc\(_3\)Man\(_9\)GlcNAc\(_2\)-ANDS are indicated in Figure 2A (cleavage site labeled \(a\)). In contrast, all of the ANDS-labeled LLOs from the PPT1 knockout mouse brain were converted to a product that migrated as Man\(_9\)GlcNAc\(_2\)-ANDS after digestion with jack bean \(\alpha\)-mannosidase (Figure 2B, compare lanes 4 and 5). This result suggests that LLOs from the PPT1 knockout brain consist of oligomers of mannosic units that are not capped by glucose units.

Golgi endomannosidase specifically converts Glc\(_1\)–3Man\(_9\)GlcNAc\(_2\)-ANDS to Man\(_9\)GlcNAc\(_2\)-ANDS (Figure 2A, cleavage site marked \(b\)) (Gao and Lehrman, 2002a; Spiro and Spiro, 2000). The result of enzyme treatment of the normal mouse liver LLO mix was consistent with this specificity, shifting the glucosylated species to Man\(_9\)GlcNAc\(_2\)-ANDS (Figure 2B, compare lanes 1 and 3) without affecting other intermediates. Treatment of LLOs from the PPT1 knockout...
brain caused no changes in migration (Figure 2B, compare lanes 4 and 6); again suggesting that the accumulated LLO intermediates lack glucosyl residues.

LLO biosynthesis occurs in a well-ordered series of reactions in which mannose residues are added sequentially to the growing oligosaccharide chain (Figure 2A). Endoglycosidase H (endo H) recognizes a particular mannose residue (Figure 2A, shaded circle) that is added only after the core five mannose residues have been added and cleaves the growing chain at the β1→6 linkage between two GlcNAc molecules (Figure 2, cleavage site marked c). Therefore, \( \text{Man}_4\text{GlcNAc}_2 \) (M₄) derived from the biosynthetic pathway is insensitive to endo H, because it does not contain the sixth mannose residue recognized by the enzyme (Figure 2A, shaded circle). Figure 2C shows that \( \text{Man}_4\text{GlcNAc}_2 \) prepared from Chinese hamster ovary K1 mutant Lec35.1 cells (which lack a factor required for the addition of the sixth mannose) (Anand et al., 2001; Camp et al., 1993) is shown to be resistant to endo H treatment (lanes 1 and 2). In contrast, the species migrating as \( \text{Man}_4\text{GlcNAc}_2 \) in PPT1 knockout brain (Figure 2B, lane 3) as well as the species migrating as \( \text{Man}_4\text{GlcNAc}_2 \) were sensitive to endo H (lane 4). This result suggests that the \( \text{Man}_4\text{GlcNAc}_2 \) (M₄/M₅) LLO in PPT1 knockout brain contain the sixth mannose residue, and that rather than being intermediates in the biosynthetic pathway, they are atypical LLOs that have gained the sixth mannose and subsequently lost residues via degradation by a catabolic pathway.

Because endo H treatment removes the ANDS group from labeled oligosaccharides (thereby precluding an examination of the oligosaccharide products of the endo H reaction), we performed experiments in which we incubated the LLOs with endo H prior to ANDS labeling. In these experiments, we demonstrated that endo H causes a shift of oligosaccharides corresponding to a loss of one GlcNAc residue (Figure 2C, lanes 7 and 8), confirming the presence of the expected products of the endo H reaction.

The absence of glucose (as indicated by α-mannosidase and endomannosidase digestion results) together with the observation of endo H sensitivity provide strong evidence that the LLOs accumulating in PPT1 knockout mouse brain are derived from a catabolic rather than the normal biosynthetic pathway.

**Normal LLO synthesis in PPT1 knockout mouse brain microsomes**

To determine whether LLO synthesis proceeds normally in PPT1 knockout mouse brain, incorporation of GDP-[2-3H]mannose into LLOs was measured using an in vitro quantitative assay as previously described (Gao and Lehrman, 2002). Microsomal fractions were prepared from PPT1 knockout and wild-type brain and liver as described in Material and methods. In short, brain (Figure 3A), and liver (Figure 3B) microsomes were incubated with 0.2 µCi/ml GDP-[2-3H]mannose, 1 µM UDP-GlcNAc for 30 min at 37°C, and LLOs were recovered by extraction into CMW (10:10:3). GDP-[2-3H]mannose incorporated into LLOs was counted, and assays were performed with acceptor (Ac-Asn-Tyr-Thr-CONH₂) tripeptide and nonacceptor (Ac-Gln-Tyr-Thr-CONH₂) peptides for oligosaccharyltransferase (OST) to measure the rate of discharge as well. OST is an ER luminal enzyme that transfers oligosaccharide from the mature LLO (Glc₃Man₉GlcNAc₂-P-P-dolichol) to appropriate asparaginyl residues of newly synthesized polypeptides. OST, however, also transfers immature LLO intermediates to the target peptide if excess acceptor peptides are available (Gao and Lehrman, 2002b). As shown in Figure 3, there were no significant differences in LLO synthesis between wild-type and PPT1 knockout brain and liver microsomes. As anticipated, liver microsomes showed higher OST activity than brain microsomes (Figure 3C). In addition, no significant differences in rates of discharge of LLOs onto the target peptide between wild-type and PPT1 knockout

![Fig. 3. Normal LLO synthesis in liver and brain of PPT knockout mice. The rate of incorporation of GDP-[2-3H]mannose into LLOs was measured in PPT1 knockout or age-matched wild-type control brain (A) or liver (B) microsomes in the presence of 400 µM of acceptor or nonacceptor tripeptide for oligosaccharyltransferase. (C) Percent discharge of oligosaccharide intermediates onto the target peptides were not different between knockout and control microsomes. The results shown are typical of two independent experiments.](image-url)
microsomes were observed. These results suggest that LLO synthesis, extension, and transfer are normal in the PPT1 knockout mouse.

**Accumulated LLOs in PPT1 knockout mouse brain are not discharged by OST**

The experiments presented so far suggest that the accumulated LLOs in PPT1 knockout mouse brain are catabolic products. Extracted LLOs from *CLN1/PPT1* -/- brain and liver microsomes were also analyzed by FACE after treatment with acceptor and nonacceptor peptides for OST (Figure 4). LLOs in wild-type brain (Figure 4A, lanes 1–3 versus lanes 4–6) and liver (Figure 4A, lanes 1–3 versus lanes 4–6) were efficiently discharged, whereas almost none of accumulated LLOs in both PPT1 knockout brain (Figure 4A, lanes 7–9 and 10–12) and liver (Figure 4B, lanes 7–9 and 10–12) were discharged, implying either that the accumulated LLOs are not substrates for OST or that they are present in a different subcellular localization that is inaccessible to OST. As noted earlier, appreciable deglucosylation of preformed Glc₃Man₉GlcNAc₂-P-P-dolichol can occur during the incubation. This explains the predominance of Man₉GlcNAc₂-P-P-dolichol in the liver LLO samples incubated with control peptide (Figure 4B, lanes 1–3 and 7–9).

**Enrichment of LLOs in storage material from PPT1 knockout mouse brain**

One of the distinctive pathological characteristics of infantile Batten disease is the accumulation of autofluorescence storage material (also known as granular osmiophilic deposits, or GRODs) in brain and other tissues. To determine whether storage deposits are enriched in LLOs, we isolated GRODs by CsCl density centrifugation and analyzed these by epifluorescence microscopy and FACE (Figure 5). The crude and CsCl purified storage material was visualized under Nomarski optics (Figure 5A, a and b) and these images were compared with images visualized under the fluorescence microscope (Figure 5A, c and d). Autofluorescence in

![Fig. 4. LLOs accumulating in the PPT1 knockout mouse are not competent for transfer to the acceptor peptide. Brain (A) and liver (B) microsomes were incubated in the presence of acceptor or control peptides for 30 min at 37°C, and the extracted LLOs were resolved and analyzed by FACE. Shown are results from incubations done in triplicate.](image1)

![Fig. 5. Enrichment of autofluorescent storage material and LLOs from PPT1 mouse brain by CsCl density gradient centrifugation. (A) Autofluorescent storage material was purified on a 16.75% CsCl density gradient (ρ = 1.13 g/cm³) and a suspension of pelleted material was analyzed by Nomarski optics (a and b) and epifluorescence microscopy (c and d). (B) LLOs extracted from crude unfractionated normal brain (lane 1), the CsCl-purified pellet (lane 2), and supernatant (lane 3) were analyzed by FACE. An identical purification procedure was applied to PPT1 knockout brain (lanes 4–6). Extracts from equivalent amounts of brain (based on wet weight) were loaded. Results shown represent one of two independent experiments yielding similar results.](image2)
storage material purified from PPT1 knockout brain covered nearly 100% of the area seen in the bright field (b and d), whereas in crude homogenates it represented a minor fraction (a and c), indicating that significant enrichment of autofluorescent material was accomplished by CsCl centrifugation. LLOs were extracted directly from the CsCl-purified storage material pellet and analyzed by FACE (Figure 5B). As a control, LLOs were extracted directly from the unfraccionated PPT1 knockout brain (Figure 5B, lane 4) and wild-type brain subjected to the same procedures (Figure 5B, lanes 1–3). Extracts from equivalent amounts of brain (wet weight) were loaded. As can be seen in Figure 5B, most of the truncated LLOs in PPT1 knockout brain that were present in the crude homogenate were recovered in the CsCl-purified storage material, indicating that accumulated LLOs are enriched in the storage material in the PPT1 knockout mouse (57.3-fold enrichment, data not shown). Furthermore, we calculate that LLOs constitute 0.3% of the dry weight of storage material from PPT1 knockout mouse brain (data not shown).

Note that in normal and PPT1 knockout tissue, the mature species Glc3Man9GlcNAc2 was recovered from the CsCl supernatant rather than from the pellet (compare Figure 5B, lanes 4 and 6 versus lanes 1 and 3). Taken together with the results shown in Figure 4, these data confirm that the accumulated LLOs in the storage material are associated with a subcellular compartment distinct from those LLOs that are utilized in asparagine-linked glycosylation.

To further elucidate the association of the LLOs with autofluorescent storage material in PPT1 knockout brain, differential centrifugation of brain homogenates from knockout and age-matched wild-type mice was performed, and the resulting pellets were observed under Nomarski and epifluorescence microscopy and subjected to FACE and analysis of LLO synthesis (Figure 6). Consistent with the known high density of the storage material, the majority of autofluorescent material sedimented at 6000 × g or lower (Figure 6A, f–h), whereas the material sedimenting at higher g forces displayed little autofluorescence (Figure 6A, i and j). FACE analysis showed that in wild-type brain, the bulk of LLOs sedimented in the 100,000 × g microsomal fraction (Graham, 1984) and appeared as the mature LLO, Glc3Man9GlcNAc2-ANDS (Figure 6B, lane 5). However, in the PPT1 knockout brain, most of the accumulated LLOs were sedimented between 1000 × g and 6000 × g in fractions that were also highly enriched in autofluorescence (Figure 6B, lanes 6–8). As a metabolic marker for each fraction, the rate of incorporation of GDP-[2-3H]mannose into LLOs was monitored (Figure 6C). As expected, GDP-[2-3H]mannose incorporation into LLO was predominantly observed in the 100,000 × g fractions from wild-type and PPT1 knockout brain, which is consistent with the known subcellular localization of LLO synthesis in the ER. Interestingly, the major band observed in 100,000 × g pellet of PPT1 knockout brain was Man9GlcNAc2-ANDS (asterisk) rather than Glc3Man9GlcNAc2-ANDS, and the level of Glc3Man9GlcNAc2-ANDS was reduced as compared with wild type. The explanation for this finding is unclear but may indicate somewhat elevated glucosidase action on pre-existing G3M9Gn2-P-P-Dol in PPT1 brain microsomes.

Fig. 6. Cofractionation of LLOs accumulating in PPT1 knockout mouse brain with autofluorescent storage material and non-ER fractions. Brain homogenates from age-matched wild-type and PPT1 knockout brains were fractionated by differential centrifugation. (A) The fractionated homogenates from PPT1 knockout brain were analyzed by Nomarski optics (lanes a–e) and by epifluorescence microscopy (lanes f–j). (B) The resulting fractions from wild type (lanes 1–5) and CLN1/PPT1−/− (lanes 6–10) brains were subjected to FACE analysis. (C) An aliquot of each fraction was incubated with GDP-[2-3H]mannose for 30 min at 37°C to monitor the rate of incorporation of GDP-[2-3H]mannose into LLOs. Results shown are one of two independent experiments yielding similar results. The asterisk (*) denotes Man9GlcNAc2-ANDS. (D) Normal LLO biosynthesis in wild-type versus PPT1 knockout mouse brains. Homogenates from two mouse brains (normal, upper; PPT1 knockout, lower) were cleared at 6000 × g for 10 min, and microsomes were collected from the supernatants by centrifugation at 100,000 × g for 1 h. Microsomes (10 mg of microsomal protein in 1 ml) were incubated under conditions similar to those for B, and included 100 µM UDP-GlcNAc, 200 nM GDP-[3-3H]mannose, 200 µM UDP-glucose, and 200 µg/ml castanospermine. Positions of glycan standards are shown. HPLC analysis was performed as described (Anand et al., 2001).
To establish that LLO synthesis itself was not abnormal, normal and PPT1 knockout microsomes (obtained at 100,000 × g after discarding material collected at 6000 × g) were incubated with GDP-[3H]mannose in the presence of castanospermine to block any glucosidase activity, and [3H]-labeled glycans obtained from the LLO fraction were examined by HPLC (Figure 6D). A limiting concentration of GDP-mannose, 200 nM, was used to increase the likelihood that abnormalities in LLO synthesis could be detected. LLO synthesis by brain microsomes is not considered to be robust (for example, see Figure 3 for comparison with liver). Consequently, the HPLC signals were weak and partially obscured by detector noise. However, glycans from M5GlcNAc2 to G3M9GlcNAc2 were detectable, and the profile for normal microsomes was highly similar to the PPT1 profile. Thus, there is no indication of an LLO biosynthetic abnormality in PPT1 brain.

Accumulation of LLO intermediates in mouse models of lysosomal storage disease

LLO profiles of six homozygous NCL mouse models (CLN1/PPT1-/-, CLN2/TPP1-/-, CLN3-/-, CLN6/nclf, CLN8/mnd, and PPT2-/-) and three non-NCL models (Niemann Pick Type C, designated NPC-/-, Sanfilippo Type B, NAGLU-/-, and Krabbe disease, GALC-/-) were analyzed by FACE (Figure 7). Characteristics of the animal models and the ages of mice used in the experiment are summarized in Tables I and II. In each case, brains from at least three clearly symptomatic animals were examined. (Of note, all mouse models represent complete loss of function mutations, with the exception of the CLN6/nclf, and CLN8/mnd. In these two models, the impact of underlying missense mutations on protein function is unknown.) Marked LLO accumulation was observed in the brains of CLN1/PPT1-/- (Figure 7A), CLN6-/- (Figure 7D) and CLN8-/- (Figure 7E).

Fig. 7. LLO profiles of brain tissue from mouse models of NCL and non-NCL lysosomal storage disorders. All mice demonstrated overt neurological signs at the time of analysis. The mouse models analyzed and their corresponding human disorders are CLN1/PPT1-/- (infantile NCL), CLN2/TPP1 (late infantile NCL), CLN3-/- (juvenile NCL), CLN6/nclf (variant late infantile NCL), CLN8/mnd (Northern epilepsy), PPT2-/- (unknown), NPC-/- (Niemann Pick Type C), NAGLU-/- (Sanfilippo B), and GALC-/- (Krabbe disease). Three brains from each model (and an equal number of littermate controls) were analyzed. Quantitation of LLO profiles were determined by densitometric scanning of FACE gels. Values shown represent mean ± SEM.
mice. These models have all been shown to demonstrate very large amounts of autofluorescent storage material in brain tissue (Bolivar et al., 2002; Bronson et al., 1993, 1998; Gao et al., 2002; Gupta et al., 2001; Ranta et al., 1999; Wheeler et al., 2002). Lesser but significantly increased LLOs were seen in CLN2-/- (Figure 7B), and CLN3-/- (Figure 7C) mouse brains. Accumulation of LLOs in PPT2 knockout mice was modest (Figure 7F) consistent with the scant amounts of storage material in brains of mice at this age (Gupta et al., 2003). Quantitation of LLOs by densitometric scanning of gels in comparison with standards revealed that brains from symptomatic mice with NPC, NAGLU, GALC were not significantly different from normal (Figure 7G–I). These results, taken together with the demonstration of LLOs in the autofluorescent storage material of PPT1 knockout mice, support a relative specificity for LLO accumulation in the neuronal ceroid lipofuscinoses.

**Discussion**

In the present study, we demonstrated a 15-fold elevation of LLO intermediates in the brains (and lesser increases in other tissues) of mice representing models of NCL. The LLOs represent intermediates in a catabolic pathway, as determined by structural characterization through enzymatic digestion and FACE analysis. The rate of incorporation of GDP-[3H]mannose into new LLO synthesis in brain and liver microsomes of PPT1 knockout mice was normal, as was the rate of oligosaccharide discharge from LLO to an OST acceptor peptide, again supporting the idea that the accumulation of LLOs is related to a defect in LLO degradation rather than synthesis or transfer. Excess LLOs colocalized with autofluorescent CsCl-purified storage material from PPT1 knockout mouse brain and sedimented in the dense fraction rather than the microsomal fraction. Quantitative analysis showed that the LLOs constituted only a minor proportion of the autofluorescent storage material (0.3%) by mass. The accumulation of LLOs was age-dependent and specific for mouse models of NCL (CLN1, CLN2, CLN3, CLN6, and CLN8) and not seen in three mouse models of other lysosomal storage disorders (Niemann Pick type C, Sanfilippo syndrome type B, and Krabbe disease).

In several respects, our studies confirm and extend earlier studies performed on brain material obtained at autopsy from NCL patients in the era before NCL genetics were defined. The quantitation of LLOs in the storage material measured in brain tissue from human (Daniel et al., 1992)

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**Table I. Characteristics of NCL mouse mutants in this study**

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Defective gene</th>
<th>Affected protein</th>
<th>Mouse phenotype</th>
<th>Age at this study</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infantile NCL</td>
<td>CLN1/Ppt1</td>
<td>PPT1</td>
<td>Spasticity by age 5 months; myoclonic jerking and seizures; fatal by 10 months</td>
<td>7 months</td>
<td>Gupta et al., 2001, 2003</td>
</tr>
<tr>
<td>Late infantile NCL</td>
<td>CLN2/Tpp1</td>
<td>Tripeptidyl peptidase-I</td>
<td>Tremor at 7 weeks and then ataxia; median survival 4.5 months</td>
<td>3.5–4 months</td>
<td>Sleet et al., 2004</td>
</tr>
<tr>
<td>Juvenile NCL</td>
<td>CLN3</td>
<td>Lysosomal transmembrane protein</td>
<td>Neuropathological abnormalities. Motor coordination deficits at 6 months</td>
<td>6 months and 20 months</td>
<td>Mitchison et al., 1999; Weimer and Pearce, unpublished data</td>
</tr>
<tr>
<td>Variant late-infantile NCL</td>
<td>CLN6(Nclf)</td>
<td>Putative transmembrane protein</td>
<td>Progressive retinal atrophy; gait disturbance and weakness in limbs evident by 6 months and severe paralysis by 9 months</td>
<td>6 months</td>
<td>Bronson et al., 1998; Gao et al., 2002; Wheeler et al., 2002</td>
</tr>
<tr>
<td>Epilepsy with mental retardation</td>
<td>CLN8(Mnd)</td>
<td>Putative transmembrane protein</td>
<td>Hindlimb weakness and ataxia at 6–7 months; behavioral abnormalities</td>
<td>7 months</td>
<td>Bolivar et al., 2002; Bronson et al., 1993; Ranta et al., 1999</td>
</tr>
<tr>
<td>Unknown</td>
<td>PPT2</td>
<td>Palmitoyl protein thioesterase 2</td>
<td>Spasticity and ataxia by 13 months; scant neuronal storage; bone marrow infiltration and extramedullary hematopoiesis; death by 17 months</td>
<td>14 months</td>
<td>Gupta. et al., 2003</td>
</tr>
</tbody>
</table>

**Table II. Characteristics of non-NCL LSD mouse mutants in this study**

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Defective gene</th>
<th>Affected protein</th>
<th>Mouse phenotype</th>
<th>Age at this study</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Niemann Pick type C</td>
<td>NPC1</td>
<td>Transmembrane protein with sterol-sensing domain</td>
<td>Weight loss, tremor and ataxia by 7 weeks</td>
<td>2 months</td>
<td>Loftus et al., 1997; Miyawaki et al., 1982; Patel et al., 1999</td>
</tr>
<tr>
<td>Sanfilippo Type B</td>
<td>NAGLU</td>
<td>α-N-acetylgalcosaminidase</td>
<td>Ganglioside accumulation in brain.</td>
<td>9 months</td>
<td>Li et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heparan sulfate in liver and kidney.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Death at 8–12 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krabbe disease</td>
<td>GALC</td>
<td>Galactocerebrosidase</td>
<td>Normal at birth; progressive tremor, weakess and wasting at 3 weeks; death at 3 months</td>
<td>1 month</td>
<td>Biswas et al., 2002; Duchen et al., 1980; Luzi et al., 2001</td>
</tr>
</tbody>
</table>
and mouse (this study) were remarkably similar (13.7 nmol/g versus 17.2 ± 1.4 nmol/g and 2.1 ± 1.9 nmol/g versus 1.2 ± 0.7 nmol/g for infantile NCL/CLN1 and normal brain, respectively). The Dp-L content measured in two infantile NCL brain samples (0.3% and 0.37% by mass) (Hall et al., 1989) agree well with our estimation of LLO from Csl-purified storage material from CLN1 mouse brain (0.3%). Our results confirm that LLOs do not constitute a major portion of the mass of the material. In addition, we were able to demonstrate that the accumulation of LLO in PPT1 and CLN3 knockout mice is age-dependent, an observation that was not feasible in human studies but supports the idea that the LLOs arise through accumulation of metabolic products with time.

Our structural characterization of LLOs in mouse CLN1 brain was also consistent with experiments performed by others (Daniel et al., 1992; Hall et al., 1992) and support the conclusion that these LLOs represent catabolic rather than synthetic intermediates. In our study, we were also able to cleanly separate the abnormal catabolic products from the normal synthetic products by differential centrifugation, with the catabolic intermediates pelleting in the dense fraction and the normal intermediates fractionating as expected in the 100,000 x g microsomal fraction.

In previous studies, no differences in [3H]-mannose incorporation or OST activity were found in cells derived from the juvenile form of NCL (CLN3) as compared to control cells (Hall et al., 1992; Van Dessel et al., 1992). We also observed the normal incorporation of [3H]mannose into brain and liver microsomes prepared from CLN1 mice and in addition showed that there was no block in transfer of the oligosaccharide from Dol-PP to nascent protein, because the discharge rate of LLOs onto an acceptor peptide was normal. Thus we ruled out failure to transfer as a mechanism for accumulation of LLO in this form of NCL.

Why might catabolic LLOs accumulate in NCL tissues? Any model must account for the appearance of LLOs in the dense fraction, the complete absence of glucosyl residues, and the partial loss of mannansyl residues. A potential explanation for the appearance of LLOs in dense membranes is that they might arise through the process of autophagy. The lipofuscin pigments and residual storage bodies observed in NCL have structural properties reminiscent of autophagolysosomes, sometimes containing clear morphological evidence of partially autolyzed mitochondria or other organelles. These tertiary lysosomes are believed to form from the fusion of autophagosomes and mature lysosomes as a late step in the process of autophagy (Cuervo and Dice, 1998; Dunn, 1990; Kim and Klionsky, 2000). In the NCLs, the metabolism or removal of these autophagolysosomes appears to be inhibited. Although the origin of autophagocytic membrane is not yet clear (Dunn, 1990; Klionsky and Emr, 2000), it has been suggested that the formation of autophagic vacuoles is initiated from the membranes of RER (Dunn, 1990), which is also the site of LLO synthesis and transfer to proteins. Therefore, it is likely that catabolic LLOs accumulating in NCLs are derived from autophagocytic membranes, and that the accumulation of LLO in the NCLs may result from overrepresentation in membranes (such as RER) destined for autophagocytic removal. An alternative explanation may be that a small fraction of the LLO pool is always mis-sorted to lysosomes and rapidly degraded. However, due to a lysosomal dysfunction in NCL (see following discussion) these LLOs would accumulate as storage material.

Degradation of Man9GlcNAc2-P-P-dolichol would normally be expected to be carried out by lysosomal α-mannosidase, a housekeeping enzyme. The optimal physiological pH for lysosomal α-mannosidase is 4.5 (Merkle et al., 1997; Stinchfield et al., 1999). However, it has been reported that lysosomal pH in fibroblasts derived from NCLs is elevated by as much as 1 pH unit (Holopainen et al., 2001). Perhaps the lysosomal pH is insufficient to ensure complete degradation of LLOs at this step. Alternatively, it is possible that the Man5,9GlcNAc2-P-P-dolichol forms aggregates, or are in some other way inaccessible to lysosomal α-mannosidase. It is also plausible that one of the other storage products (e.g., in the case of PPT1 a palmitoylated peptide) acts as a mannosidase inhibitor.

The absence of glucosyl residues from the accumulated LLOs in PPT1 defective tissue may simply indicate that deglucosylation occurs efficiently in the dense membranes. Alternatively, for some reason the LLOs in dense membranes may be selectively derived from nonglucosylated LLOs generated from the pool of Man9GlcNAc2-P-P-dolichol that is normally present in equilibrium with Glc3Man9GlcNAc2-P-P-dolichol as a result of the glucose-1-phosphate-glucosidase shuttle in the ER (Spiro and Spiro, 1991).

The accumulation of dolichol in brain has been proposed as a phenotype of the aging process (Hall and Patrick, 1988; Ng Ying Kin et al., 1983). Therefore, a most pressing question is whether dolichol or its metabolites contribute to the cellular and physiologic dysfunction that accompanies NCL or aging. The link between accelerated lipofuscin formation, LLO accumulation, and neurodegeneration suggests that this is a possibility that should be further explored.

Materials and methods

Rodent tissues

Gene-targeted CLN1/PPT1-/-, PPT2-/-, and littermate control mice were maintained on a mixed C57BL/6J × 129S6/SvEv background in the animal facility of the University of Texas Southwestern Medical Center (Dallas) as described previously (Gupta et al., 2001, 2003). The CLN1/PPT1-/- and PPT2-/- mice used in this study resulted from four to six generations of backcrosses from 129S6/SvEv onto C57BL6J. Frozen brain tissues derived from CLN3 knockout mice (20 months of age) were kindly provided by Dr. Beverly Davidson (University of Iowa College of Medicine). Frozen brain tissues derived from CLN2/TPPI knockout mice (129SvEv) were kindly provided by Drs. Peter Lobel and David Sleet (University of Medicine and Dentistry of New Jersey). Mouse models of juvenile-onset NCL/CLN3 (7 months of age, 129SvEv−Cln3tm1Nbm/J, stock #: 000472); variant late-infantile NCL, CLN6/nclf (B6.Cg-Chs5mclf/J, stock #: 003605); Northern epilepsy, CLN8/nmd (B6.KB2-Cln8omd/Jsri, stock #: 001612); Niemann Pick Type C/Npc1Ipm (C57BLKS/ J-Npc1Ipm/J, stock #: 002760) (Loftus et al., 1997); Sanfilippo
Type Bl/Naglu (B6.129S6-Naglu<sup>Em1Efn</sup>/J, stock #: 003827) (Li et al., 1999); and Krabbe disease/GalC<sup>Em</sup> (B6.129S6-GalC<sup>Em1</sup>/J, stock #: 000845) (Luzzi et al., 2001) were obtained from Jackson Laboratory stocks and maintained at the University of Rochester. All of the animals were euthanized in accordance with NIH and institutional guidelines. Tissues were harvested immediately and frozen under liquid nitrogen, except tissues used for the microsomal preparation and subcellular localization studies, which were freshly prepared.

**FACE analysis**

FACE analysis of LLOs derived from mouse tissues was performed essentially as described (Gao and Lehrman, 2002a; Lehrman and Gao, 2003). Briefly, tissues were homogenized in ice-cold methanol using a Polytron (Brinkmann) and dried under a stream of nitrogen. The dried pellet was extracted sequentially with chloroform/methanol 2:1(v/v), water, and CMW 10:10:3 (v/v/v). The organic phase from the final extraction was loaded onto a column containing 1 ml DEAE-cellulose (Whatman) in the acetate form equilibrated with CMW. The column was washed to remove contaminating glycogen fragments with 10 ml CMW followed by 10 ml CMW containing 3 mM ammonium acetate. LLOs were eluted with 10 ml CMW containing 300 mM ammonium acetate. Chloroform (4.4 ml) and water (1.4 ml) were then added to remove ammonium acetate and the lower phase (containing LLOS) was recovered after centrifugation and dried under nitrogen gas. The oligosaccharides were removed from dolichol lipid by mild acid treatment with 0.1 N HCl in 50% isopropanol for 1 h at 50°C. Residual ammonium acetate was removed by desalting with Dowex (BioRad, Hercules, CA) ion exchange resins AG50-X8 (hydrogen form) and AG1-X8 (formate form). The released oligosaccharides were labeled with 150 mM ANDS (Molecular Probes, Eugene, OR) and 1 M sodium cyanoborohydride solution and then resolved on a oligosaccharide profiling gel as described previously (Lehrman and Gao, 2003). The gel was imaged with a Biorad Fluor-S MultiImager using a 530DF60 filter. Electronic gel images were generated, and individual ANDS-positive species were quantified using software supplied with the scanner. The linearity and sensitivity of oligosaccharide detection by FACE was determined with known quantities (Glyko, Novato, CA) or dissolved in 100 mM Na–2-(N-morpholino)ethanesulfonic acid (pH 6.5) containing 0.2% Triton X-100 and incubated with 270 ng recombinant Golgi endomannosidase (a gift of Dr. Robert Spiro) at 37°C for 18 h. For endo H digestions, ANDS-oligosaccharides (or unlabeled oligosaccharides) were incubated with 0.1 U endo H (New England Biolabs, Beverly, MA) in 50 mM sodium citrate (pH 5.0) containing 5% glycerol and 0.1% Triton X-100 at 37°C for 30 min to 18 h as indicated in the figure legends.

**Microsomal preparation, LLO synthesis, and oligosaccharide transfer assays**

To prepare microsomes, fresh tissues from CLN1/PPT1−/− and age-matched wild-type mice were pooled (two animals from each group) and homogenized in ice-cold buffer containing 50 mM Tris–HCl (pH 7.5) and 250 mM sucrose using a motor-driven Dounce homogenizer. The homogenate was centrifuged at 100,000 × g for 15 min to remove nuclei and cell debris. The supernatant was subjected to centrifugation at 100,000 × g at 4°C for 1 h to sediment the microsomal pellet. The microsomes were resuspended in buffer A (50 mM K-HEPES [pH 7.5], 25 mM KCl, and 5 mM MgCl<sub>2</sub>) and incubated with 0.2 µCi per ml of GDP-[2-3H]mannose, 1 µM UDP-GlcNAC, and 400 µM acceptor peptide (Ac-Asn-Tyr-Thr-CONH<sub>2</sub>) or control peptide (Ac-Gln-Tyr-Thr-CONH<sub>2</sub>). The reaction mixture was incubated for 30 min at 37°C, and LLOs were recovered by extraction into CMW (10:10:3, v/v/v) as described previously (Gao and Lehrman, 2002a; Pan and Elbein, 1990; Zeng and Lehrman, 1990). The incorporation of GDP-[2-3H]mannose into newly synthesized LLOs was determined by counting a portion (5%) of the organic phase from the CMW extraction. The remaining organic phase was dried under nitrogen gas, and the oligosaccharides were released by mild acid, labeled with ANDS, and analyzed by FACE as described.

**Isolation of autofluorescent storage material**

Storage material was isolated as described previously (Palmer et al., 1986; Tynela et al., 1993) with modifications. Briefly, PPT1 knockout and age-matched wild-type mouse brains were homogenized in Milli-Q water using a Polytron (Brinkmann, Westburg, NY). The homogenate was filtered through glass wool and sonicated for 1 min. The resulting homogenate was centrifuged at 500 × g for 10 min, 3000 × g for 20 min, and the pellet was resuspended in 16.75% CsCl (w/v, ρ = 1.13 g/cm<sup>3</sup>) and centrifuged at 1500 × g for 20 min. The pellet was washed twice with CsCl and resuspended with Milli-Q water. The isolation was monitored visually using a Zeiss Axioshot epifluorescence microscope.

**Differential fractionation of mouse brain tissue**

Microsomes were prepared from fresh brain tissue from CLN1/PPT1−/− and age-matched wild-type mice (two mice per group) were prepared by Dounce homogenization in sucrose buffer as described and subjected to a series of stepwise centrifugations (1000 × g for 10 min, 3000 × g for 10 min, 6000 × g for 10 min, 9000 × g for 15 min, and 100,000 × g for 1 h). Each pellet was washed once with buffer A and subjected to fluorescence microscopy and FACE analysis.

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Abbreviations
ANDS, 7-amino-1,3-naphthalenedisulfonic acid; CMW, chloroform/methanol/water; CLN1, ceroid-lipofuscinosis, neuronal-1; endo H, endoglycosaminidase H; FACE, fluorophore-assisted carbohydrate analysis; GROD, granular osmiophilic deposit; HPLC, high-performance liquid chromatography; LLO, lipid-linked oligosaccharide; PPT1, palmitoyl-protein thioesterase-1; NCL, neuronal ceroid lipofuscinosis; OST, oligosaccharyltransferase; RER, rough endoplasmic reticulum.

References


