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ORIGINAL PAPER

Amyloid-β metabolism in Niemann-Pick C disease models and patients

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Abstract Niemann-Pick type C (NPC) is a progressive neurodegenerative lysosomal disease with altered cellular lipid trafficking. The metabolism of amyloid-β (Aβ) - previously mainly studied in Alzheimer's disease - has been suggested to be altered in NPC. Here we aimed to perform a detailed characterization of metabolic products from the amyloid precursor protein (APP) in NPC models and patients. We used multiple analytical technologies, including immunoassays and immunoprecipitation followed by mass spectrometry (IP-MS) to characterize Aβ peptides and soluble APP fragments (sAPP-α/β) in cell media from pharmacologically (U18666A) and genetically (*NPC1*$^{-/-}$) induced NPC cell models, and cerebrospinal fluid (CSF) from NPC cats and human patients. The pattern of Aβ peptides and sAPP-α/β fragments in cell media was differently affected by NPC-phenotype induced by U18666A treatment and by *NPC1*$^{-/-}$ genotype. U18666A treatment increased the secreted media levels of sAPP-α, AβX-40 and AβX-42 and reduced the levels of sAPP-β, Aβ1-40 and Aβ1-42, while IP-MS showed increased relative levels of Aβ5-38 and Aβ5-40 in response to treatment. *NPC1*$^{-/-}$ cells had reduced media levels of sAPP-α and Aβ1-16, and increased levels of sAPP-β. NPC cats had altered CSF distribution of Aβ peptides compared with normal cats. Cats treated with the potential disease-modifying compound 2-hydroxypropyl-β-cyclodextrin had increased relative levels of short Aβ peptides including Aβ1-16 compared with untreated cats. NPC patients receiving β-cyclodextrin had reduced levels over time of CSF Aβ1-42, AβX-38, AβX-40, AβX-42 and sAPP-β, as well as reduced levels of the axonal damage markers tau and phosphorylated tau. We conclude that NPC models have altered Aβ metabolism, but with differences across experimental systems, suggesting that NPC1-loss of function, such as in *NPC1*$^{-/-}$ cells, or NPC1-dysfunction, seen in NPC patients and cats as well as in U18666A-treated cells, may cause subtle but different effects on APP degradation pathways. The preliminary findings from NPC cats suggest that treatment with cyclodextrin may have an impact on APP processing pathways. CSF Aβ, sAPP and tau biomarkers were dynamically altered over time in human NPC patients.

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Introduction

Niemann-Pick type C (NPC) is a rare autosomal recessive disease caused by mutations in either of the two genes encoding for the lysosome-associated lipid trafficking proteins NPC1 and NPC2 (Swardfager et al. 2010). The mutations cause intracellular accumulation and altered distribution of different lipid species. The major clinical feature is progressive neurological deterioration, with onset typically in childhood. Treatment for NPC is limited, but the glucosylceramide synthase inhibitor miglustat (Zavesca, Actelion Inc.) may have an effect on amelioration of neurological symptoms (Patterson et al. 2007; Pineda et al. 2009). Recently, the cholesterol modulating compound β-cyclodextrin was proposed for NPC treatment (Rosenbaum and Maxfield 2011; Peake and Vance 2012).

NPC may also have late onset in adulthood and is often confused with more common neurological or psychiatric diseases, such as psychosis, depression, or other neurodegenerative diseases (Patterson et al. 2012). Interestingly, NPC shares several pathologic features with Alzheimer's disease (AD), including neurofibrillary tangles (Auer et al. 1995), trafficking abnormalities in endosomes and lysosomes (Nixon et al. 2008), disease exacerbation by the *APOE ε4* allele (Saito et al. 2002) and cellular lipid dysregulation (Grimm et al. 2007). Also, *NPC1* polymorphisms are associated with late-onset AD (Erickson et al. 2008), and AD patients have increased expression of NPC1 in hippocampus and frontal cortex (Kagedal et al. 2010). Notably, in NPC there are indications of abnormal metabolism of the peptide amyloid-β (Aβ) (Jin et al. 2004), which has been proposed to drive AD pathogenesis (Selkoe 1991; Hardy and Higgins 1992). AD patients have extracellular deposits of Aβ and reduced cerebrospinal fluid (CSF) levels of the 42 amino acid isoform Aβ1-42. In contrast, NPC patients accumulate intracellular Aβ (Jin et al. 2004) and may have elevated CSF Aβ levels (Mattsson et al. 2011). Aβ is formed by proteolysis from the amyloid precursor protein (APP) through complicated enzymatic pathways that depend both on the cellular lipid environment and on vesicular trafficking within the cell, ultimately giving rise to a large number of different Aβ peptides and soluble APP (sAPP) fragments (Portelius et al. 2011a). The altered lipid homeostasis and the disturbed vesicular trafficking seen in NPC may be expected to affect APP metabolism, which is dependent on both the cellular lipid topography (Holmes et al. 2012) and the endosomal vesicular system (Cirrito et al. 2008). Previous studies have indeed found that cells lacking the NPC1-protein have reduced surface levels of APP, increased release of sAPP-β (Malnar et al. 2010; Kosicek et al. 2010) and intracellular accumulation of Aβ peptides (Malnar et al. 2010; Yamazaki et al. 2001). NPC mice accumulate Aβ peptides in their brains (Olson and Humpel 2010; Burns et al. 2003; Boland et al. 2010; Yamazaki et al. 2001), and have increased brain activity and levels of Aβ generating enzymes (Kodam et al. 2010). Also, APPxPS1 transgenic mice (an established Alzheimer's disease model) have a more rapid accumulation of brain Aβ if they are NPC1-heterozygous than if they are $NPC1^{+/+}$ (Borbon and Erickson 2011).

These studies point to disturbed APP processing pathways in NPC, but detailed data on APP degradation products is needed to understand precisely how NPC changes APP metabolism. Importantly, a careful analysis of this may give general clues to links between lipid metabolism, vesicular trafficking and APP processing, which may be important to understand mechanisms in other diseases, such as AD. We therefore undertook an investigation with the aim to measure a large number of different Aβ and sAPP-α/β species, using multiple orthogonal and complementing technologies. To assess the robustness of the findings, we included both pharmacological and genetical cell models, the major large animal NPC model, which is a cat strain carrying a missense mutation in the *NPC1* gene (2864 G-C), resulting in a phenotype that is biochemically, neuropathologically and symptomatically similar to the human disease (Somers et al. 2003; Vite et al. 2008), and CSF from human NPC patients. The results differed across the systems, emphasizing the need for carefulness when translating findings between models and human patients.

Experimental procedures

U18666A cell model

Human neuroblastoma SH-SY5Y cells (Biedler et al. 1978) over-expressing wild type human APP695 (APP695wt) were cultivated in Dulbecco's modified Eagle's medium/F-12 (Invitrogen) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and 1 % penicillin-streptomycin solution. Cells were treated with the amphiphile U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one) at 3 μg/mL cell media for 24 h to induce the NPC cholesterol storage phenotype. U18666A blocks cellular cholesterol transport and is a well-established method to emulate the NPC phenotype in cell experiments (Runz et al. 2002; Lange et al. 2000; Roff et al. 1991). Filipin staining was used to confirm the presence of cholesterol storage. Cells were also treated with the β-secretase (β-site aspartyl cleaving enzyme 1, BACE1) inhibitor β-secretase inhibitor IV (Calbiochem, Merck, compound 3 in (Stachel et al. 2004)), the enzyme cathepsin B inhibitor Z-FA-FMK (BD Biosciences, San Jose, CA, USA) or DMSO.

NPC1-null cell model

Since U18666A may influence APP processing enzymes directly (Crestini et al. 2006; Sidera et al. 2005), it is difficult to determine how much of its effects that are related to NPC1 inhibition. We therefore tested Chinese Hamster Ovary wild type cells (CHOwt) and CHO NPC1-null cells (CHO *NPC1*$^{-/-}$) (all originally provided by Dr. Daniel Ory to SH) in which the *NPC1* gene was deleted. These were used and prepared essentially as described previously (Kosicek et al. 2010; Malnar et al. 2010). In short, cells were maintained in Dulbecco's modified Eagle's medium/F-12 supplemented with 10 % FBS, 2 mM L-glutamine and antibiotics/antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B). For transient expression, cells were transfected using Lipofectamine LTX (Invitrogen) according to the supplier's instructions. APP695wt, APPswe and C99 6myc-tagged constructs were generated using pCS2 +6MT vector (Hecimovic et al. 2004). Twenty four hours after transfection medium was removed, fresh medium was added, incubated further for 24 h and collected for analysis.

NPC1 cats

To investigate if the NPC-dependent effects seen in cell experiments were present also in mammals, we performed experiments on cats. As opposed to CHO *NPC1*$^{-/-}$ cells which lack the *NPC1* gene, NPC1 cats carry a spontaneous disease causing mutation in the *NPC1* gene. Control and NPC1 cats were raised in the animal colony at the School of Veterinary Medicine at the University of Pennsylvania under NIH and USDA guidelines for the care and use of animals in research, as described previously (Ward et al. 2010). We also included cats treated with 2-hydroxypropyl-β-cyclodextrin (called cyclodextrin below).

NPC patients

Finally, we assessed APP degradation products in human NPC patients by CSF examination. We have previously reported increased levels of CSF AβX-38, AβX-40, AβX-42 and Aβ1-42 in NPC patients compared to controls, with no major differences during 1 year of follow-up (Mattsson et al. 2011, 2012) (for AβX- peptides, quantification was done by a technique with low specificity for the N-terminal amino acid of the Aβ sequence). Here we analyzed serial CSF samples from two NPC1 patients before and during treatment with cyclodextrin. The samples were obtained in conjunction with treatment with intravenous or intrathecal injections of the compound (under approval for compassionate use Investigation New Drugs from the Food and Drug Administration, IND104,114 and IND104,116). The patients were on simultaneous off-label use of miglustat, which is registered for NPC in the European Union. For contrast values, CSF samples collected by lumbar puncture from 9 age-matched patients (Table 1) were obtained from clinical routine samples at Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden. Contrast samples were selected from patients with normal CSF cell counts, albumin CSF to serum ratio, and no signs of intrathecal immunoglobulin production. All CSF samples were centrifuged at 2,000 × *g* at 4 °C for 10 min and stored at −80 °C pending analysis.

Fluorescent bead-based assays

The xMAP assay INNO-BIA AlzBio3 (Innogenetics, Ghent, Belgium) was used for quantifications of Aβ1-42, T-tau, and P-tau, as explained previously (Olsson et al. 2005). The xMAP assay INNO-BIA Aβ forms (Innogenetics) was used for quantifications of Aβ1-40 and Aβ1-42 (format A) and AβX-40 and AβX-42 (format B), as explained previously (Hansson et al. 2010). Both format A and B use the monoclonal antibodies 21F12 and 2G3, which specifically bind Aβ peptides ending at Ala42 and Val40, respectively, as capture antibodies. In format A, 3D6 (Aβ N-terminal neoepitope epitope starting at Asp1) was used as detection antibody, providing specific quantifications of Aβ1-40/42 isoforms. In format B, 4G8 (epitope within Aβ18-22) was used as detection antibody, providing quantification of AβX-40/42 isoforms.

Electrochemiluminescense assays

The MSD Human/Rodent Abeta Triplex assay (Meso Scale Discovery, Gaithersburg, MD, USA) was used for quantifications of AβX-38, AβX-40 and AβX-42, and the MSD sAPPα/sAPPβ Multiplex Assay for quantifications of sAPP-α and sAPP-β, as explained previously (Mattsson et al. 2011).

Table 1 CSF biomarkers at baseline in NPC patients and contrast group

	Patient 1	Patient 2	Contrast group (N=9)
Age (years)	2	2	3.7 (1.6–11)
Sex	F	F	7 M: 2 F
AβX-38 (ng/L)	1,143	1,827	651 (100–1,517)
AβX-40 (ng/L)	10,257	17,243	7,315 (868–13,733)
AβX-42 (ng/L)	1,247	2,026	601 (53–1,396)
Aβ1-42 (ng/L)	552	667	236 (84–408)
sAPP-α (ng/mL)	467	794	313 (110–781)
sAPP-β (ng/mL)	213	326	102 (31–285)
T-tau (ng/L)	1,399	1,981	62 (40–135)
P-tau (ng/L)	121	133	35 (24–61)

Data for the contrast group is median (range). Regarding AβX-38/40/42 peptides, the X refers to the fact that quantification was done by a technique with low specificity for the N-terminal amino acid of the Aβ sequence

Immunoprecipitation and mass spectrometry

Aβ peptides were analyzed by immunoprecipitation and mass spectrometry (IP-MS) by a method previously developed at our laboratory (Portelius et al. 2007). In short, anti-Aβ antibodies coupled to magnetic beads were used for IP. After elution, Aβ isoforms were analyzed by mass spectrometry on an UltraFlextreme matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) instrument or an AutoFlex MALDI TOF (Bruker Daltonics, Bremen, Germany). An in-house developed MATLAB (Mathworks Inc. Natick, MA, USA) program was used for relative quantifications of Aβ isoforms in the spectra. For each peak the sum of the intensities for the three strongest isotopic signals were calculated and normalized against the sum for all the Aβ peaks in the spectrum followed by averaging of duplicates. This method allows for relative quantification of different Aβ isoforms.

Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Aβ isoform identities were confirmed by liquid chromatography (LC) combined with high resolution tandem mass spectrometry (MS/MS) (Portelius et al. 2007). LC-MS/MS analysis was performed on an Ettan MDLC nanoflow chromatographic system (GE Healthcare) using HotSep Kromasil C4 columns (G&T Septech) coupled to a Thermo LTQ FT Ultra electrospray ionization hybrid linear quadrupole ion trap/Fourier transform ion cyclotron resonance (ESI-LQIT/

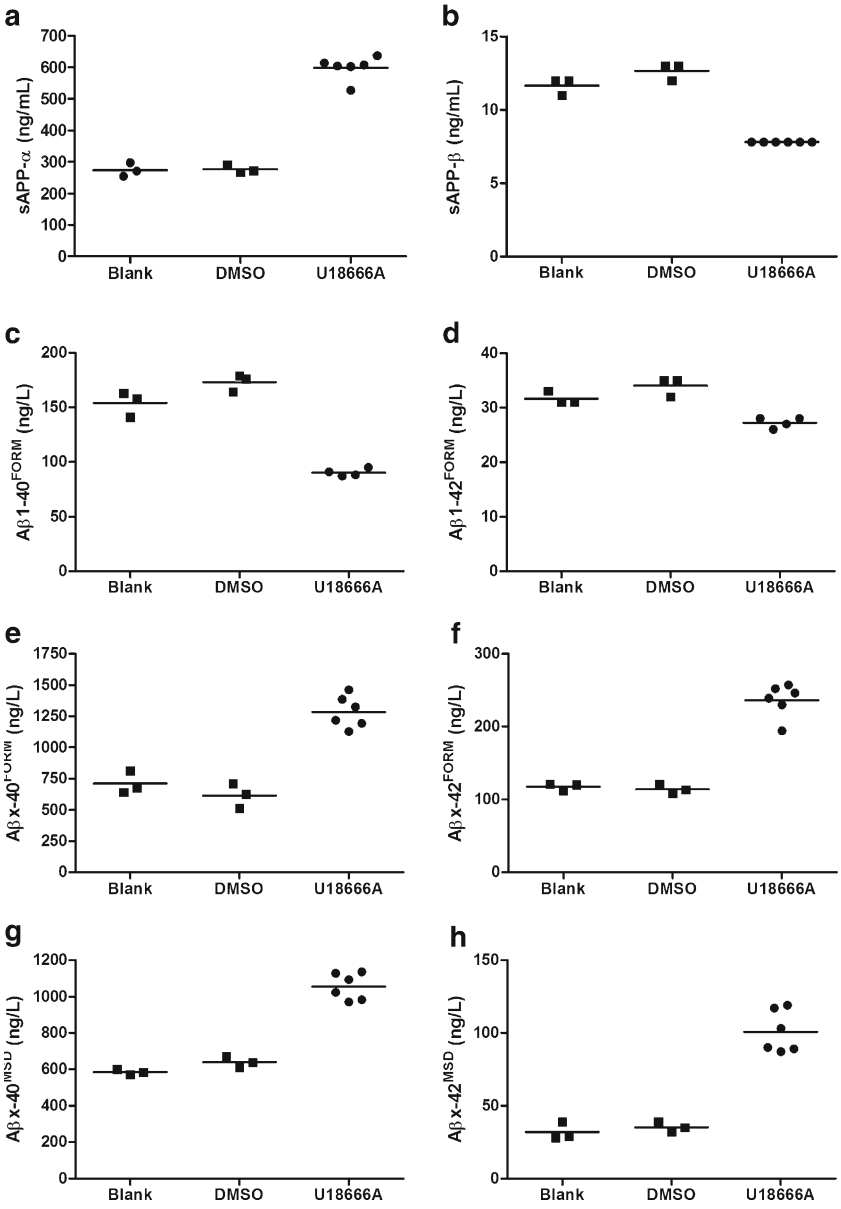

Fig. 1 Cells treated to emulate NPC phenotype have altered release of Aβ- and sAPP-peptides. SH-SY5Y APP695wt cells were treated with *DMSO*, *U18666A*, or untreated (*blank*). Cell media concentrations of sAPP-α (**a**), *sAPP-β* (**b**), *Aβ1-40* (**c**), *Aβ1-42* (**d**), *AβX-40* (**e** and **g**) and *AβX-42* (**f** and **h**), were determined using fluorescent bead-based assay (*FORM*; **c**-**f**) and/or electrochemiluminescent based assay (*MSD*; **a**, **b**, **g** and **h**). Each data point represents individual cell cultures. Lines are means. Two data points are missing from the *U18666A* treatment group in panels **c**-d due to technical error in measurements

FTICR) mass spectrometer (Thermo Fisher Scientific). All spectra were acquired in FTICR mode and collision induced dissociation (CID) as well as electron capture dissociation (ECD) was used to obtain fragment ion data.

Ethics

All subjects or care-givers gave informed and written consent. The cat study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee. The human study was approved by the local Institutional Review Boards at the treating hospitals and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Results

Aβ and sAPP-α/β profile in U18666A treated SH-SY5Y cells

U18666A treatment induced an NPC cholesterol storage phenotype in SH-SY5Y cells with increased concentrations of sAPP-α, AβX-40 and AβX-42 and reduced concentrations of sAPP-β, Aβ1-40 and Aβ1-42 in cell media (experiment repeated twice, Fig. 1). The treatment also increased the AβX-42 to AβX-40 ratio (data not shown), similar to what was previously seen in NPC patients' CSF (Mattsson et al. 2011). Treatment with a BACE1 inhibitor reduced sAPP-β, Aβ1-40 and Aβ1-42 levels (Supplementary Figure 1), confirming an efficient BACE1-inhibition, but did not counter-act the U18666A elevations of AβX-40 and AβX-42, indicating that these originated from BACE1-independent pathways. Inhibition of the enzyme cathepsin B, which has been suggested as an alternative Aβ producing enzyme (Hook et al. 2005) reduced the concentrations of Aβ1-40, Aβ1-42, AβX-40 and AβX-42 in media from both untreated and U18666A treated cells, which made it difficult to determine if this enzyme was involved in any specific APP degradation pathway (Supplementary Figure 2). However, we noted that cathepsin B inhibition reduced AβX-40 and AβX-42 levels in U18666A-treated cells almost to the levels as in vehicle (DMSO)-treated cells. IP-MALDI-MS-TOF analyses showed increased levels of Aβ species starting at position 5 (Aβ5-38 and Aβ5-40) in the U18666A treated cells (Fig. 2), which are peptides known to be formed without BACE1-processing (Mattsson et al. 2012b; Takeda et al. 2004; Portelius et al. 2011b). Such N-truncated Aβ species may account for at least part of the increased concentrations of AβX-40/42 detected by immunoassays after U18666A treatment.

Aβ and sAPP-α/β profile in *NPC1*$^{-/-}$ cells

In contrast to U18666A-treatment, the *NPC1*$^{-/-}$ genotype did not have major effect on media levels of Aβ1-40, Aβ1-42, AβX-40 and AβX-42 (Supplementary Figure 3), but seemed

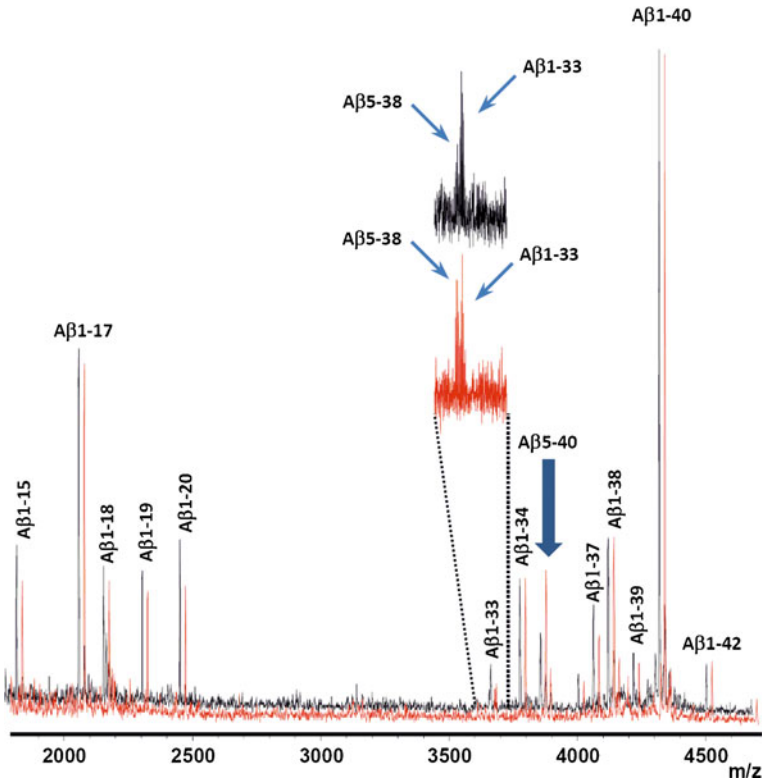

Fig. 2 Cells treated to emulate NPC phenotype have increased relative release of Aβ-peptides starting at position *Aβ5*. The spectra of secreted Aβ peptides in U18666A-treated SH-SY5Y APP695wt cells were analyzed by immunoprecipitation and mass spectrometry. IP-MALDI-MS-TOF showed increased relative signals of *Aβ5-38* and *Aβ5-40* in U18666A treated cells (*red*)

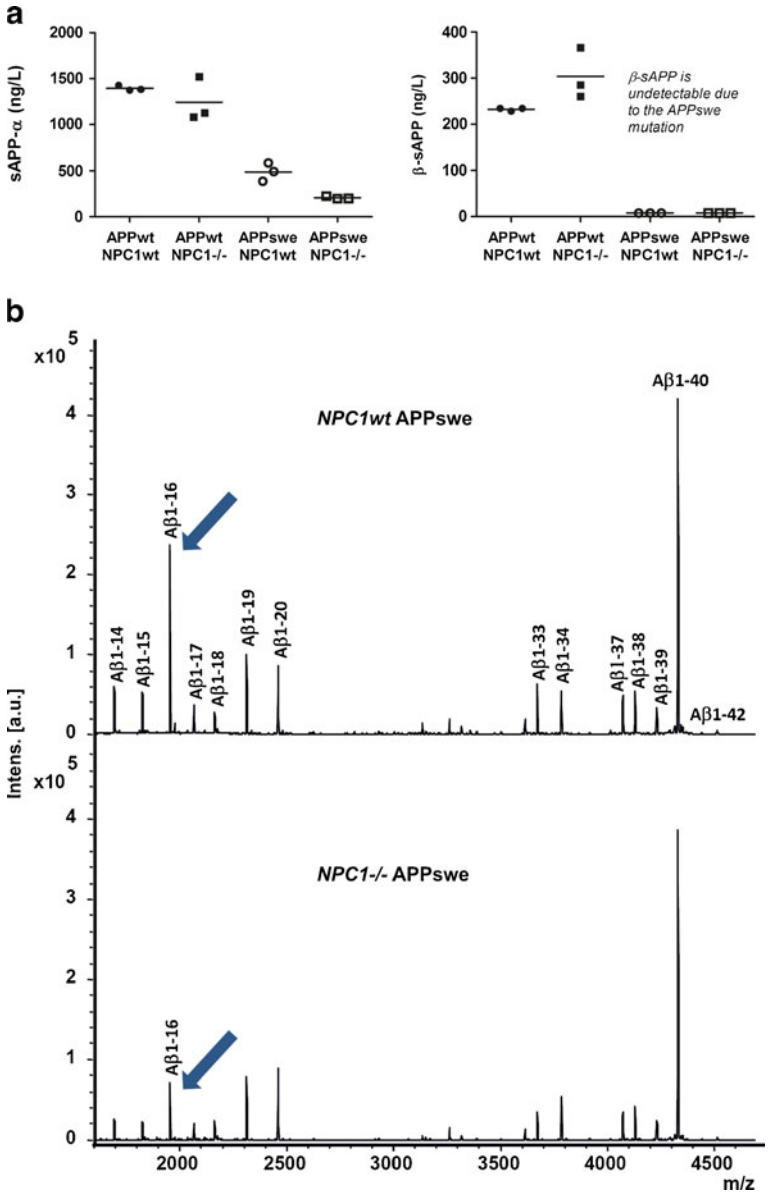

Fig. 3 *NPC1*$^{-/-}$ cells have reduced relative release of *Aβ1-16*. *NPC1wt* and *NPC1*$^{-/-}$ CHO cells were transiently transfected with either *APPwt* or *APPsw* construct. Cell media concentrations of *sAPP-α* and *sAPP-β* were determined using an electrochemiluminescense assay, while Aβ species were analyzed by IP-MALDI-MS-TOF. Each data point represents individual cell cultures. In parallel with decreased *sAPP-α* levels in *NPC1−/−* cells (**a**) we observed reduced relative signal of *Aβ1-16* in the same cells (**b**). Note that in APPsw-transfected cells we were unable to analyze *sAPP-β*, which is expected due to the swe mutation

to reduce sAPP-α levels (at least in APPswe transfected cells) and increase sAPP-β levels (Fig. 3, panel a). Notably, the sAPP-β fragment was not detected from APPswe media which is expected due to the swe mutation (Mullan et al. 1992) (Fig. 3, panel a). Interestingly, IP-MS showed that media from *NPC1*$^{-/-}$ cells had reduced relative levels of Aβ1-16 (Fig. 3, panel b). In combination with reduced sAPP-α this argues for a shift away from α-secretase pathways, since both the formation of sAPP-α and Aβ1-16 requires α-secretase processing (Portelius et al. 2011b). In cells only expressing endogenous APP, the *NPC1*$^{-/-}$ genotype reduced AβX-40 levels (Fig. 4, panel a). Similarly, in cells transfected with C99 the *NPC1*$^{-/-}$ genotype caused a slight reduction of AβX-38, AβX-40 and AβX-42 (Fig. 4). IP-MALDI-MS-TOF unexpectedly showed a major difference in the overall Aβ peptide pattern for C99 transfected cells compared to other cells, with elevated relative Aβ1-41 signals and reduced relative Aβ1-40 and Aβ1-42 signals, and the *NPC1*$^{-/-}$ genotype increased the relative Aβ1-41 signal even more (Supplementary Figure 4).

Aβ profiling in the CSF of NPC1 cat

We analyzed Aβ species in NPC1 cat CSF samples by IP-MALDI-MS-TOF. While the peak profile of Aβ peptides in cat CSF resembled that seen in human CSF, we noted a +14 Da shift of all Aβ species compared to the human variant (Fig. 5, panel a). By MS/MS analysis of *m/z* 4342.175, using MALDI-TOF/TOF, and comparing the fragment ion pattern to that of the human Aβ1-40 analog we assigned this mass difference to an amino acid substitution in the cat Aβ sequence, with an Asp7→Glu replacement compared to the human sequence

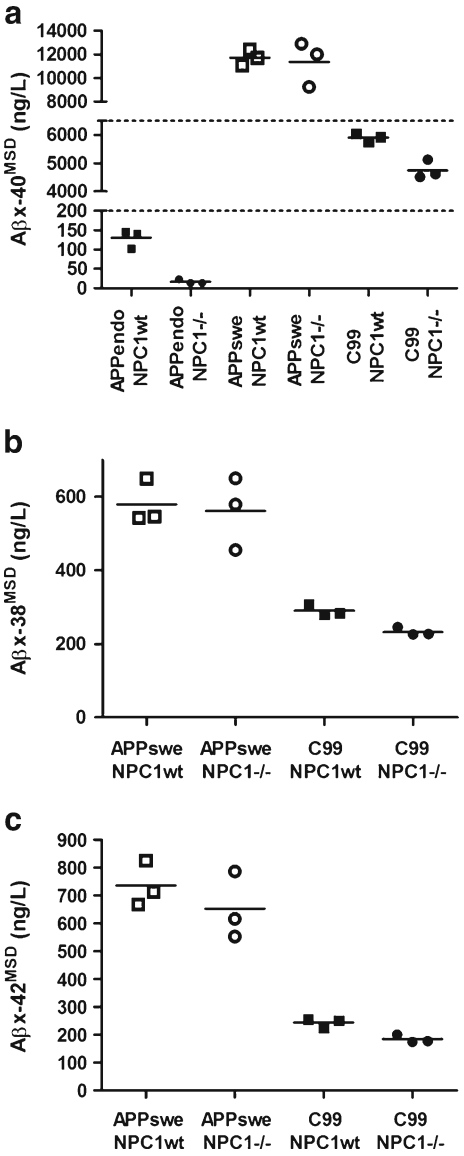

Fig. 4 Effect of *NPC1*$^{-/-}$ on release of *AβX-38*, *AβX-40* and *AβX-42*. *AβX-40* (**a**), *AβX-38* (**b**) and *AβX-42* (**c**) were analyzed in untransfected (*APPendo*) and either *APPsw* or C99-transfected (**a**–**c**) *NPC1wt* and *NPC1*$^{-/-}$ CHO cells by an electrochemiluminescent based assay (*MSD*). Each data point represents individual cell cultures. In untransfected cells expressing only endogenous APP, the *NPC1*$^{-/-}$ genotype reduced *AβX-40* levels (**a**). Similarly, in cells transfected with *C99*, the *NPC1*$^{-/-}$ genotype caused a slight reduction of *AβX-38*, *AβX-40* and *AβX-42* (**a**–**c**)

(Fig. 5, panel b). In combination with homology with human Aβ1-40 it could be established that the cat Aβ1-40 sequence is DAEFRHESGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV. In parallel with this experiment, the cat Aβ sequence was further verified using ECD-MS/MS as described recently (Brinkmalm et al. 2012). The protein sequence data will appear in the UniProt Knowledgebase under the accession number P86906. The amino acid difference at position 7 renders 6E10 non-functional in cat experiments as reflected by no signals corresponding to Aβ when the 6E10 was used in the IP. To include coverage of short C-terminal truncated Aβ peptides, we therefor used the antibodies 82E1 (N-terminal specific) and 4G8. There were some differences in the relative Aβ peptide distribution between NPC1 cats and wild type cats, with lower relative levels of Aβ1-37, Aβ1-38 and Aβ1-39 in NPC1 cats compared to controls. Some of the cats on cyclodextrin had increased relative levels of Aβ1-16, Aβ6-28 and Aβ1-28 compared to the other groups (Fig. 6, Supplementary Figure 5).

Aβ, sAPP-α/β and T-tau/P-tau profile in NPC patient CSF

Finally, we examined serial CSF samples in two NPC patients, twin girls. Measurements on the first two sample rounds have been reported previously (Mattsson et al. 2012, a), but for this study the original samples were re-run together with all follow-up samples. See Table 1 for baseline values and Figs. 7, and 8 for changes over time. As indicated in the figures, the patients started oral miglustat treatment after the first sampling (the first sampling is the baseline value, prior to miglustat or cyclodextrin treatment) and intravenous (IV) cyclodextrin treatment after the second sampling. The patients initiated therapy with low dose (100 mg/kg) IV cyclodextrin and the dose was increased with every drug administration until they reached 2,500 mg/kg. The IV cyclodextrin treatment continued until the 4th sampling when the patients received every 2 week intrathecal (IT) cyclodextrin (175 mg) in addition to continuing the IV dosing at a stable level of 2,500 mg/kg every 2 weeks. After an initial 12 week trial period of IT treatment the FDA placed a clinical hold on the IT treatment until the safety data could be assessed (the IV cyclodextrin treatment and the miglustat treatment were continuous). Following a 6 weeks intermission, the IT dosing was re-initiated and subsequently the IT dosing has been doubled to 375 mg. The patients have not had any deleterious effects of the drugs, either IV or IT. There have been no grade 3 or 4 toxicities and no apparent dose limitation to date. Additionally, the patients have been physically well, with stability of many symptoms (developmental delay, ataxia), improvements (improved hearing threshold, better swallowing), and have shown some progression typically of NPC (difficult control of seizures and gelastic cataplexy). Overall, the patients clinical progression in this pilot study appears to be delayed and some immediate, albeit short lived, effects are apparent after drug administration (increased alertness and energy).

The CSF levels of Aβ1-42, AβX-38, AβX-40, AβX-42 and sAPP-β decreased over time in both patients, suggesting that miglustat and cyclodextrin treatment may have effects on APP and Aβ metabolism. IP-MS analyses (data not shown) revealed no major shifts in the Aβ peptide pattern distribution in the patients compared to a contrast group (the contrast group is described in Table 1).

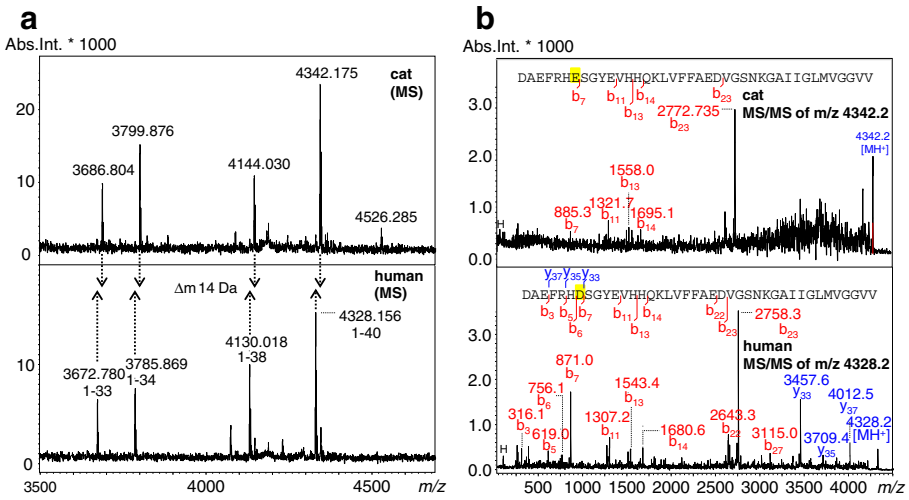

Fig. 5 The cat Aβ sequence. IP-MALDI-TOF/TOF tandem mass spectrum of cat Aβ identifies an amino acid substitution at position 7 compared to the human analog. **a** Analysis in MS mode. A mass shift of +14 Da is observed for all Aβ peptides in cat CSF (*top panel*) compared to human (*bottom panel*). **b** MS/MS analysis of Aβ1-40 from cat (*top panel*) and human (*bottom panel*). For human Aβ1-40, containing Arg at position 5 and Asp at position 7 and 23, MS/MS analysis of singly charged ions is expected to be dominated by charge-remote fragmentation, which is confirmed by the strong b_7, b_{23} signals. In the MS/MS spectrum of the cat Aβ1-40, b_{23} is strong, but b_7 is significantly less intense. This observation together with the 14 Da mass difference indicates that Asp-7 is replaced by Glu in cat Aβ1-40

In addition to APP and Aβ markers we analyzed the axonal damage markers T-tau and P-tau (Table 1 and Fig. 8), both of which decreased over time. Noticeably, P-tau started to decrease later, after introduction of cyclodextrin treatment. The decrease in T-tau and P-tau during treatment might suggest a beneficial effect on the neuronal degeneration, but this has to be examined further in a larger series of patients.

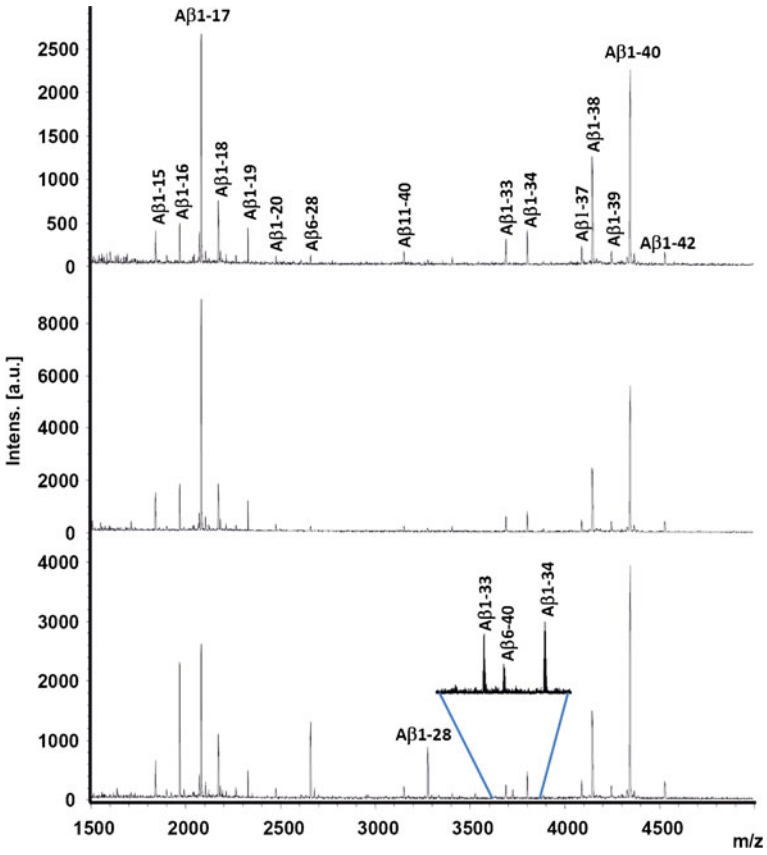

Fig. 6 The effects of NPC and cyclodextrin treatment on CSF Aβ peptides in cats. Aβ peptides were determined by immunoprecipitation and mass spectrometry in CSF from control cats and cyclodextrin treated/untreated NPC1-cats. Untreated NPC1-cats (*middle panel*) had slightly reduced relative CSF levels of *Aβ1-37*, *Aβ1-38*, and *Aβ1-39* compared to controls (*top panel*). Cyclodextrin treated NPC cat (*bottom panel*) show increased relative levels of *Aβ1-16*, *Aβ6-28* and *Aβ1-28* compared to the other groups

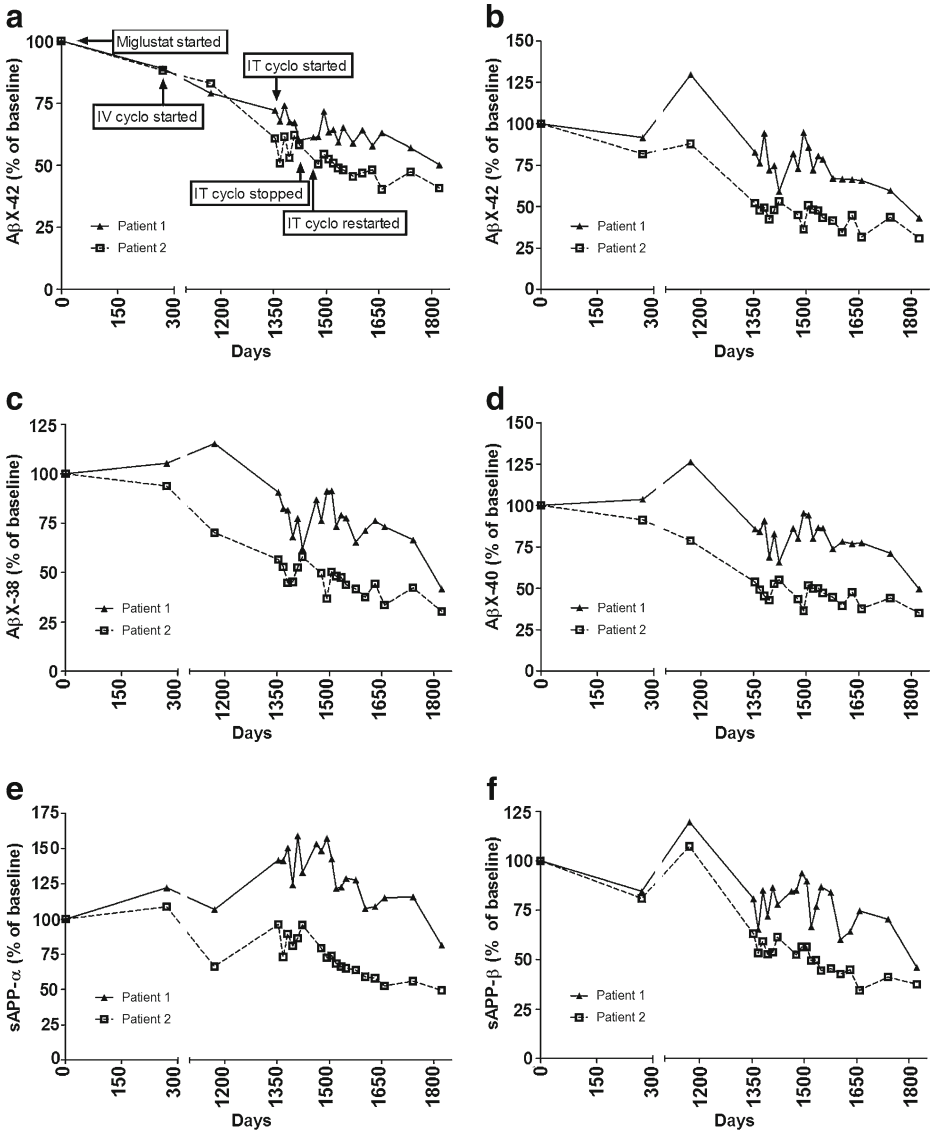

Fig. 7 Longitudinal data on CSF Aβ and sAPP biomarkers in two NPC patients. Biomarkers determined by fluorescent bead-based and electrochemiluminescent assays. X-axes show days from baseline. The patients started oral miglustat treatment after the first sampling and intravenous (IV) cyclodextrin (cyclo) treatment after the second sampling. The IV cyclodextrin treatment continued until the 4th sampling when the patients received intrathecal (IT) cyclodextrin in addition to continuing the IV cyclodextrin dosing. After 12 week trial period IT treatment was stopped for 6 weeks before it was restarted (the IV cyclodextrin treatment and the miglustat treatment were continuous). CSF levels of *Aβ1-42* (**a**), *AβX-42* (**b**), *AβX-38* (**c**), *AβX-40* (**d**) and *sAPP-β* (**f**) were decreased over time to 50 % of the baseline in the two examined NPC patients. Meanwhile, sAPP-α levels were only slightly changed (**e**)

Discussion

Here we report on APP degradation products across multiple NPC models and in human patient CSF. In sum, pharmacologically or genetically induced NPC phenotype have altered patterns of APP degradation products, but the specific results differ between models, which may be a consequence of complex interactions between APP metabolism and NPC-induced pathways. The changes over time in CSF Aβ, sAPP, and tau biomarkers may support the use of these biomarkers to study the disease process in NPC studies and drug trials.

First, we noted that U18666A treatment shifted the APP processing away from the β-secretase dependent pathways with the result that the release of both sAPP-α and Aβ5-X isoforms increased. The latter have previously been shown to be BACE1-independent (Mattsson et al. 2012b) and likely linked to α-secretase activity (Takeda et al. 2004).

Several previous studies have examined Aβ metabolism after U18666A treatment, but the results are difficult to compare due to differences in which cell model that was used and/or methods used for APP and Aβ quantification (e.g. not discriminating between Aβ1-40/42 and AβX-40/42). Runz et al. found that U18666A treatment reduced the release of AβX-40, AβX-42, and cellular levels of β-CTF in SH-SY5Y APP695-transfected cells, arguing for reduced β-secretase processing (Runz et al. 2002). Likewise, Davis found that U18666A increased the release of sAPP-α, concluding that U18666A treatment likely blocks the APP re-endocytosis that is necessary for β-secretase processing in the endocytic pathway in neurons (Davis 2008). In contrast, Yamazaki et al. found no effect of U18666A treatment on secretion of Aβ1-40 or Aβ1-42 from CHO cells, although the treatment lead to intracellular accumulation of Aβ (especially Aβ42) in late endosomes (Yamazaki et al. 2001).

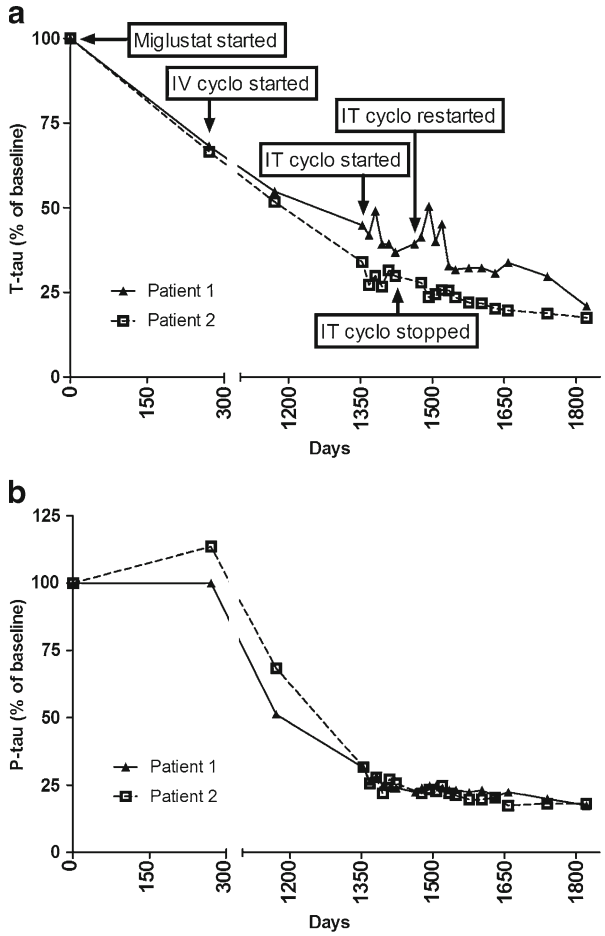

Fig. 8 Longitudinal data on CSF T-tau and P-tau in two NPC patients. T-tau and P-tau were determined in the CSF of treated NPC patients using fluorescent bead-based assay. X-axes show days from baseline. The patients started oral miglustat treatment after the first sampling and intravenous (IV) cyclodextrin (cyclo) treatment after the second sampling. The IV cyclodextrin treatment continued until the 4th sampling when the patients received intrathecal (IT) cyclodextrin in addition to continuing the IV cyclodextrin dosing. After 12 week trial period IT treatment was stopped for 6 weeks before it was restarted (the IV cyclodextrin treatment and the miglustat treatment were continuous). CSF levels of *T-tau* (**a**) and *P-tau* (**b**) were decreased over time in the two

Jin et al. found that U18666A led to accumulation of AβX-42 and CTFs in APP695 transfected primary mouse cortical neurons, but not in C99 transfected cells, suggesting that the treatment increased the activity of other secretases than γ-secretase (Jin et al. 2004). Few studies have explored systems with endogenous APP expression, but Koh et al. found that U18666A treatment reduced the release of Aβ40 and Aβ42 while the intracellular levels increased in mouse cortical neurons only expressing endogenous APP (Koh et al. 2006).

U18666A has several effects, including transcriptional up-regulation of the γ-secretase components presenilin-1 and presenilin-2 (Crestini et al. 2006) and altered glycosylation of BACE1 (Sidera et al. 2005), which makes it difficult to determine if the effects on APP metabolism due to U18666A treatment are directly related to NPC1 inhibition or not. We therefore performed analyses on CHO-*NPC1*$^{-/-}$ cells that were transiently transfected with either *APPwt* or *APPswe* construct. In our experiments on *NPC1*$^{-/-}$ cells, we found the opposite result from U18666A treatment, with increased β-secretase processing and reduced α-secretase processing. This adds to previous data showing that these cells have reduced cell surface APP levels, increased CTF levels in lipid rafts, and increased release of sAPP-β (Malnar et al. 2010; Kosicek et al. 2010). In addition, in accordance with previous findings (Yamazaki et al. 2001; Malnar et al. 2010) we observed no major changes in secreted Aβ species in *NPC1*$^{-/-}$ cells compared to NPCwt cells. However, in future studies it would be interesting to analyze intracellular Aβ by IP-MS since intracellular Aβ has been shown to accumulate in several NPC model cells (Malnar et al. 2010; Yamazaki et al. 2001). Unexpectedly, transfection with C99 increased the relative release of Aβ1-41 and reduced the relative release of Aβ1-40 and Aβ1-42. Also, although the *NPC1*$^{-/-}$ genotype had no effect on secreted Aβ1-40/42 and AβX-38/40/42 in APPswe and APPwt transfected cells, it reduced the release of AβX-38, AβX-40 and AβX-42 in C99 transfected cells, and further increased the relative release of Aβ1-41.

In cats, we found that the Aβ sequence differs from human and dog Aβ at amino acid position Aβ7, although the general CSF Aβ peptide distribution was very similar to what is seen in humans and dogs (Portelius et al. 2006; Portelius et al. 2010; Mattsson et al. 2012b). To our knowledge, this is the first investigation of APP and Aβ metabolism in the NPC1 cat. We found that NPC1-cats had slightly reduced relative CSF levels of Aβ1-37, Aβ1-38, and Aβ1-39 compared to controls. Previous NPC1 animal studies have shown that NPC1 mice accumulate β-CTF, Aβ40, and Aβ42 in their brains (Olson and Humpel 2010; Burns et al. 2003; Boland et al.; Yamazaki et al. 2001), but have unchanged levels of APP, sAPP, PS-1, and BACE protein (Burns et al. 2003). Also, NPC1 mice brain homogenates have slightly increased γ-secretase activity (Burns et al. 2003). In addition, a recent study by Kodam et al. (Kodam et al. 2010) identified increased β-secretase activity along with increased levels of APP, BACE1 and all four components of the γ-secretase complex in NPC mice cerebellum and hippocampus compared to controls.

In the CSF of the two NPC patients studied, we found a decrease over time in Aβ1-42, AβX-38, AβX-40, AβX-42, and sAPP-β (to about 50 % of baseline levels), which may indicate a continuous loss of functional APP-processing synapses or neurons as the disease progresses. This is consistent with a previous study, where CSF AβX-38, AβX-40, AβX-42, and sAPP-β levels were lower in more severely than in less severely affected NPC patients (Mattsson et al.

2011). Extending these observations to other neurodegenerative diseases, such as AD, patients who are severely affected have lower CSF AβX-40, sAPP-α and sAPP-β levels (Rosen et al. 2012), and CSF AβX-40 was recently even found to decline over time in AD patients (Mattsson et al. 2012a). We also found that CSF levels of T-tau and P-tau decreased in the patients (with the baseline levels determined prior to miglustat or cyclodextrin therapy). From this study, it cannot be determined if this reflects the natural course of the disease, or if it represents an effect of miglustat and/or cyclodextrin therapy on the intensity of the axonal degenerative process, which is a proposed interpretation for similar tau changes in AD treatment studies (Blennow et al. 2010; Blennow et al. 2012). Studies with extensive longitudinal CSF sampling in neurodegenerative diseases are still rare. As shown here, such studies may be useful to track changes in CNS metabolism over time.

Cyclodextrin has been proposed as a treatment directed against the lipid alterations in NPC. Lipid metabolism has a complex relationship with APP and Aβ metabolism (Grimm et al. 2007). The α-secretase pathway, which releases the N-terminal ectodomain sAPP-α and prevents Aβ formation, occurs mainly outside cholesterol rich lipid raft membrane domains. In contrast, BACE1 and γ-secretase, which releases Aβ peptides, are most active within lipid rafts. Consequently, cholesterol depletion inhibits Aβ formation (Simons et al. 1998), while cholesterol enrichment may reduce sAPP-α secretion (Bodovitz and Klein 1996) and increase Aβ deposition (Refolo et al. 2000). In cats, cyclodextrin increased the relative CSF levels of Aβ1-16, which is generated by combined cleavage from α- and β-secretase (Portelius et al. 2011b), as well as the levels of Aβ6-28 and Aβ1-28, indicating a shift away from the classical amyloidogenic pathway towards α-secretase dependent pathways, perhaps as a consequence of cellular cholesterol depletion. We could not replicate this finding in human NPC CSF using IP-MS. One limitation to consider, in addition to the very small sample number in this pilot study, is that the patients were in an advanced stage of disease when treatment started, while the cats were treated from birth.

The main findings of this study is that (1) pharmacologically and genetically induced NPC1 models had signs of altered APP processing, but with differences between models, with U18666A treatment shifting APP processing away from β-secretase dependent pathways and *NPC1*$^{-/-}$ genotype instead increasing β-secretase processing, which may partly explain previous differences in results between studies; (2) NPC1-loss of function, such as in *NPC1*$^{-/-}$ cells, may have subtle but different effects on APP processing and Aβ formation compared to NPC1-dysfunction seen in NPC patients and cats as well as in U18666A treated cells; (3) NPC cats had altered CSF distribution of Aβ peptides compared with normal cats and cats treated with cyclodextrin had increased relative levels of short Aβ peptides compared with untreated cats; and (4) NPC patients receiving treatment with miglustat, IV and IT β-cyclodextrin decreased in CSF Aβ, sAPP and tau measurements over time, suggesting that these biomarkers may be useful tools to monitor the disease process and treatment effect. Importantly, although cell models may give support for pathogenetic mechanisms, careful animal studies and patient studies are needed to fully understand the mechanisms of disease in NPC.

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Conflict of interest The authors declare that they have no conflict of interest.

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