

## **Amyloid- $\beta$ 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- $\beta$  ( $A\beta$ ) - 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  $A\beta$  peptides and soluble APP fragments (sAPP- $\alpha/\beta$ ) in cell media from pharmacologically (U18666A) and genetically (*NPCI*<sup>-/-</sup>) induced NPC cell models, and cerebrospinal fluid (CSF) from NPC cats and human patients. The pattern of  $A\beta$  peptides and sAPP- $\alpha/\beta$  fragments in cell media was differently affected by NPC-phenotype induced by U18666A treatment and by *NPCI*<sup>-/-</sup> genotype. U18666A treatment increased the secreted media levels of sAPP- $\alpha$ ,  $A\beta$ X-40 and  $A\beta$ X-42 and reduced the levels of sAPP- $\beta$ ,  $A\beta$ 1-40 and  $A\beta$ 1-42, while IP-MS showed increased relative levels of  $A\beta$ 5-38 and  $A\beta$ 5-40 in response to treatment. *NPCI*<sup>-/-</sup> cells had reduced media levels of sAPP- $\alpha$  and  $A\beta$ 1-16, and increased levels of sAPP- $\beta$ . NPC cats had altered CSF distribution of  $A\beta$  peptides compared with normal cats. Cats treated with the potential disease-modifying compound 2-hydroxypropyl- $\beta$ -cyclodextrin had increased relative levels of short  $A\beta$  peptides including  $A\beta$ 1-16 compared with untreated cats. NPC patients receiving  $\beta$ -cyclodextrin had reduced levels over time of CSF  $A\beta$ 1-42,  $A\beta$ X-38,  $A\beta$ X-40,  $A\beta$ X-42 and sAPP- $\beta$ , as well as reduced levels of the axonal damage markers tau and phosphorylated tau. We conclude that NPC models have altered  $A\beta$  metabolism, but with differences across experimental systems, suggesting that NPC1-loss of function, such as in *NPCI*<sup>-/-</sup> 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  $A\beta$ , sAPP and tau biomarkers were dynamically altered over time in human NPC patients.

**Keywords:** Niemann-Pick type C; amyloid- $\beta$ ; amyloid precursor protein; biomarker; cerebrospinal fluid.

## 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  $\beta$ -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*  $\epsilon 4$  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- $\beta$  ( $A\beta$ ) (Jin et al. 2004), which has been proposed to drive AD pathogenesis (Selkoe 1991; Hardy and Higgins 1992). AD patients have extracellular deposits of  $A\beta$  and reduced cerebrospinal fluid (CSF) levels of the 42 amino acid isoform  $A\beta_{1-42}$ . In contrast, NPC patients accumulate intracellular  $A\beta$  (Jin et al. 2004) and may have elevated CSF  $A\beta$  levels (Mattsson et al. 2011a).  $A\beta$  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  $A\beta$  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- $\beta$  (Malnar et al. 2010; Kosicek et al. 2010) and intracellular accumulation of  $A\beta$  peptides (Malnar et al. 2010; Yamazaki et al. 2001). NPC mice accumulate  $A\beta$  peptides in their brains (Olson and Humpel 2010; Burns et al. 2003; Boland et al. ; Yamazaki et al. 2001), and have increased brain activity and levels of  $A\beta$  generating enzymes (Kodam et al. 2010). Also, APPxPS1 transgenic mice (an established Alzheimer's disease model) have a more rapid accumulation of brain  $A\beta$  if they are NPC1-heterozygous than if they are NPC1<sup>+/+</sup> (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  $A\beta$  and sAPP- $\alpha/\beta$  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 (2864G-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- $\beta$ -[2-(diethylamino)ethoxy]androst-5-en-17-one) at 3  $\mu$ g/mL cell media for 24 hours 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  $\beta$ -secretase ( $\beta$ -site aspartyl cleaving enzyme 1, BACE1) inhibitor  $\beta$ -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*<sup>-/-</sup>) (all originally provided by Dr. Daniel Ory to SH) in which *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, APP<sup>swe</sup> 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*<sup>-/-</sup> 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- $\beta$ -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 A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42 and A $\beta$ 1-42 in NPC patients compared to controls, with no major differences during one year of follow-up (Mattsson et al. 2011a, b) (for A $\beta$ X-peptides, quantification was done by a technique with low specificity for the N-terminal amino acid of the A $\beta$  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 x 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 A $\beta$ 1-42, T-tau, and P-tau, as explained previously (Olsson et al. 2005). The xMAP assay INNO-BIA A $\beta$  forms (Innogenetics) was used for quantifications of A $\beta$ 1-40 and A $\beta$ 1-42 (format A) and A $\beta$ X-40 and A $\beta$ X-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 A $\beta$  peptides ending at Ala42 and Val40, respectively, as capture antibodies. In format A, 3D6 (A $\beta$  N-terminal neopeptide epitope starting at Asp1) was used as detection antibody, providing specific quantifications of A $\beta$ 1-40/42 isoforms. In format B, 4G8 (epitope within A $\beta$ 18-22) was used as detection antibody, providing quantification of A $\beta$ X-40/42 isoforms.

#### Electrochemiluminescence assays

The MSD Human/Rodent Abeta Triplex assay (Meso Scale Discovery, Gaithersburg, MD, USA) was used for quantifications of A $\beta$ X-38, A $\beta$ X-40 and A $\beta$ X-42, and the MSD sAPP $\alpha$ /sAPP $\beta$  Multiplex Assay for quantifications of sAPP- $\alpha$  and sAPP- $\beta$ , as explained previously (Mattsson et al. 2011a).

### Immunoprecipitation and mass spectrometry

A $\beta$  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-A $\beta$  antibodies coupled to magnetic beads were used for IP. After elution, A $\beta$  isoforms were analyzed by mass spectrometry on an UltraFlex extreme 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 A $\beta$  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 A $\beta$  peaks in the spectrum followed by averaging of duplicates. This method allows for relative quantification of different A $\beta$  isoforms.

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

A $\beta$  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 Septeck) coupled to a Thermo LTQ FT Ultra electrospray ionization hybrid linear quadrupole ion trap/Fourier transform ion cyclotron resonance (ESI-LQIT/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

### A $\beta$ and sAPP- $\alpha/\beta$ profile in U18666A treated SH-SY5Y cells

U18666A treatment induced an NPC cholesterol storage phenotype in SH-SY5Y cells with increased concentrations of sAPP- $\alpha$ , A $\beta$ X-40 and A $\beta$ X-42 and reduced concentrations of sAPP- $\beta$ , A $\beta$ 1-40 and A $\beta$ 1-42 in cell media (experiment repeated twice, Figure 1). The treatment also increased the A $\beta$ X-42 to A $\beta$ X-40 ratio (data not shown), similar to what was previously seen in NPC patients' CSF (Mattsson et al. 2011a). Treatment with a BACE1 inhibitor reduced sAPP- $\beta$ , A $\beta$ 1-40 and A $\beta$ 1-42 levels (Supplementary figure 1), confirming an efficient BACE1-inhibition, but did not counter-act the U18666A elevations of A $\beta$ X-40 and A $\beta$ X-42, indicating that these originated from BACE1-independent pathways. Inhibition of the enzyme cathepsin B, which has been suggested as an alternative A $\beta$  producing enzyme (Hook et al. 2005) reduced the concentrations of A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ X-40 and A $\beta$ X-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 A $\beta$ X-40 and A $\beta$ X-42 levels in U18666A-treated cells almost to the levels as in vehicle (DMSO)-treated cells. IP-MADLI-TOF MS analyses showed increased levels of A $\beta$  species starting at position 5 (A $\beta$ 5-38 and A $\beta$ 5-40) in the U18666A treated cells (Figure 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 A $\beta$  species may account for at least part of the increased concentrations of A $\beta$ X-40/42 detected by immunoassays after U18666A treatment.

#### A $\beta$ and sAPP- $\alpha/\beta$ profile in *NPCI*<sup>-/-</sup> cells

In contrast to U18666A-treatment, the *NPCI*<sup>-/-</sup> genotype did not have major effect on media levels of A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ X-40 and A $\beta$ X-42 (Supplementary figure 3), but seemed to reduce sAPP- $\alpha$  levels (at least in APP<sub>swe</sub> transfected cells) and increase sAPP- $\beta$  levels (Figure 3, panel A). Notably, the sAPP- $\beta$  fragment was not detected from APP<sub>swe</sub> media which is expected due to the swe mutation (Mullan et al. 1992) (Figure 3, panel A). Interestingly, IP-MS showed that media from *NPCI*<sup>-/-</sup> cells had reduced relative levels of A $\beta$ 1-16 (Figure 3, panel B). In combination with reduced sAPP- $\alpha$  this argues for a shift away from  $\alpha$ -secretase pathways, since both the formation of sAPP- $\alpha$  and A $\beta$ 1-16 requires  $\alpha$ -secretase processing (Portelius et al. 2011b). In cells only expressing endogenous APP, the *NPCI*<sup>-/-</sup> genotype reduced A $\beta$ X-40 levels (Figure 4, panel A). Similarly, in cells transfected with C99 the *NPCI*<sup>-/-</sup> genotype caused a slight reduction of A $\beta$ X-38, A $\beta$ X-40 and A $\beta$ X-42 (Figure 4). IP-MALDI-TOFMS unexpectedly showed a major difference in the overall A $\beta$  peptide pattern for C99 transfected cells compared to other cells, with elevated relative A $\beta$ 1-41 signals and reduced relative A $\beta$ 1-40 and A $\beta$ 1-42 signals, and the *NPCI*<sup>-/-</sup> genotype increased the relative A $\beta$ 1-41 signal even more (Supplementary Figure 4).

#### A $\beta$ profiling in the CSF of NPC1 cat

We analyzed A $\beta$  species in NPC1 cat CSF samples by IP-MALDI-TOFMS. While the peak profile of A $\beta$  peptides in cat CSF resembled that seen in human CSF, we noted a +14 Da shift of all A $\beta$  species compared to the human variant (Figure 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 A $\beta$ 1-40 analog we assigned this mass difference to an amino acid substitution in the cat A $\beta$  sequence, with an Asp7  $\rightarrow$  Glu replacement compared to the human sequence (Figure 5, panel b). In combination with homology with human A $\beta$ 1-40 it could be established that the cat A $\beta$ 1-40 sequence is DAEFRHESGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV. In parallel with this experiment, the cat A $\beta$  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 A $\beta$  when the 6E10 was used in the IP. To include coverage of short C-terminal truncated A $\beta$  peptides, we therefore used the antibodies 82E1 (N-terminal specific) and 4G8. There were some differences in the relative A $\beta$  peptide distribution between NPC1 cats and wild type cats, with lower relative

levels of A $\beta$ 1-37, A $\beta$ 1-38 and A $\beta$ 1-39 in NPC1 cats compared to controls. Some of the cats on cyclodextrin had increased relative levels of A $\beta$ 1-16, A $\beta$ 6-28 and A $\beta$ 1-28 compared to the other groups (Figure 6, Supplementary fig 5).

#### A $\beta$ , sAPP- $\alpha/\beta$ 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. 2011b, a), but for this study the original samples were re-run together with all follow-up samples. See Table 1 for baseline values and Figures 7-8 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 2500 mg/kg. The IV cyclodextrin treatment continued until the 4<sup>th</sup> 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 2500 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 A $\beta$ 1-42, A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42 and sAPP- $\beta$  decreased over time in both patients, suggesting that miglustat and cyclodextrin treatment may have effects on APP and A $\beta$  metabolism. IP-MS analyses (data not shown) revealed no major shifts in the A $\beta$  peptide pattern distribution in the patients compared to a contrast group (the contrast group is described in Table 1).

In addition to APP and A $\beta$  markers we analyzed the axonal damage markers T-tau and P-tau (Table 1 and Figure 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.

## 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 A $\beta$ , 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  $\beta$ -secretase dependent pathways with the result that the release of both sAPP- $\alpha$  and A $\beta$ 5-X isoforms increased. The latter have previously been shown to be BACE1-independent (Mattsson et al. 2012b) and likely linked to  $\alpha$ -secretase activity (Takeda et al. 2004).

Several previous studies have examined A $\beta$  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 A $\beta$  quantification (e.g. not discriminating between A $\beta$ 1-40/42 and A $\beta$ X-40/42). Runz et al. found that U18666A treatment reduced the release of A $\beta$ X-40, A $\beta$ X-42, and cellular levels of  $\beta$ -CTF in SH-SY5Y APP695-transfected cells, arguing for reduced  $\beta$ -secretase processing (Runz et al. 2002). Likewise, Davis found that U18666A increased the release of sAPP- $\alpha$ , concluding that U18666A treatment likely blocks the APP re-endocytosis that is necessary for  $\beta$ -secretase processing in the endocytic pathway in neurons (Davis 2008). In contrast, Yamazaki et al. found no effect of U18666A treatment on secretion of A $\beta$ 1-40 or A $\beta$ 1-42 from CHO cells, although the treatment lead to intracellular accumulation of A $\beta$  (especially A $\beta$ 42) in late endosomes (Yamazaki et al. 2001). Jin et al. found that U18666A led to accumulation of A $\beta$ X-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  $\gamma$ -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 A $\beta$ 40 and A $\beta$ 42 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  $\gamma$ -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*<sup>-/-</sup> cells that were transiently transfected with either *APP*<sup>wt</sup> or *APP*<sup>swe</sup> construct. In our experiments on *NPC1*<sup>-/-</sup> cells, we found the opposite result from U18666A treatment, with increased  $\beta$ -secretase processing and reduced  $\alpha$ -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- $\beta$  (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 A $\beta$  species in *NPC1*<sup>-/-</sup> cells compared to NPCwt cells. However, in future studies it would be interesting to analyze intracellular A $\beta$  by IP-MS since intracellular A $\beta$  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 A $\beta$ 1-41 and reduced the relative release of A $\beta$ 1-40 and A $\beta$ 1-42. Also, although the *NPC1*<sup>-/-</sup> genotype had no effect on secreted A $\beta$ 1-40/42 and A $\beta$ X-38/40/42 in *APP*<sup>swe</sup> and *APP*<sup>wt</sup> transfected cells, it reduced the release of A $\beta$ X-38, A $\beta$ X-40 and A $\beta$ X-42 in C99 transfected cells, and further increased the relative release of A $\beta$ 1-41.

In cats, we found that the A $\beta$  sequence differs from human and dog A $\beta$  at amino acid position A $\beta$ 7, although the general CSF A $\beta$  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 A $\beta$  metabolism in the NPC1 cat. We found that NPC1-cats had slightly reduced relative CSF levels of A $\beta$ 1-37, A $\beta$ 1-38, and A $\beta$ 1-39 compared to controls. Previous NPC1 animal studies have shown that NPC1 mice accumulate  $\beta$ -CTF, A $\beta$ 40, and A $\beta$ 42 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  $\gamma$ -secretase activity (Burns et al. 2003). In addition, a recent study by Kodam et al. (Kodam et al. 2010) identified increased  $\beta$ -secretase activity along with increased levels of APP, BACE1 and all four components of the  $\gamma$ -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 A $\beta$ 1-42, A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42, and sAPP- $\beta$  (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 A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42, and sAPP- $\beta$  levels were lower in more severely than in less severely affected NPC patients (Mattsson et al. 2011a). Extending these observations to other neurodegenerative diseases, such as AD, patients who are severely affected have lower CSF A $\beta$ X-40, sAPP- $\alpha$  and sAPP- $\beta$  levels (Rosen et al. 2012), and CSF A $\beta$ X-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 A $\beta$  metabolism (Grimm et al. 2007). The  $\alpha$ -secretase pathway, which releases the N-terminal ectodomain sAPP- $\alpha$  and prevents A $\beta$  formation, occurs mainly outside cholesterol rich lipid raft membrane domains. In contrast, BACE1 and  $\gamma$ -secretase, which releases A $\beta$  peptides, are most active within lipid rafts. Consequently, cholesterol depletion inhibits A $\beta$  formation (Simons et al. 1998), while cholesterol enrichment may reduce sAPP- $\alpha$  secretion (Bodovitz and Klein 1996) and increase A $\beta$  deposition (Refolo et al. 2000). In cats, cyclodextrin increased the relative CSF levels of A $\beta$ 1-16, which is generated by combined cleavage from  $\alpha$ - and  $\beta$ -secretase (Portelius et al. 2011b), as well as the levels of A $\beta$ 6-28 and A $\beta$ 1-28, indicating a shift away from the classical amyloidogenic pathway towards  $\alpha$ -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  $\beta$ -secretase dependent pathways and *NPC1*<sup>-/-</sup> genotype instead increasing  $\beta$ -secretase processing,

which may partly explain previous differences in results between studies; (2) NPC1-loss of function, such as in *NPC1*<sup>-/-</sup> cells, may have subtle but different effects on APP processing and A $\beta$  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 A $\beta$  peptides compared with normal cats and cats treated with cyclodextrin had increased relative levels of short A $\beta$  peptides compared with untreated cats; and (4) NPC patients receiving treatment with miglustat, IV and IT  $\beta$ -cyclodextrin decreased in CSF A $\beta$ , 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.

### **Conflict of interest**

The authors declare that they have no conflict of interest.

### **Acknowledgments**

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

	<b>Patient 1</b>	<b>Patient 2</b>	<b>Contrast group (N=9)</b>
<b>Age (years)</b>	2	2	3.7 (1.6-11)
<b>Sex</b>	F	F	7 M: 2 F
<b>A<math>\beta</math>X-38 (ng/L)</b>	1143	1827	651 (100-1517)
<b>A<math>\beta</math>X-40 (ng/L)</b>	10257	17243	7315 (868-13733)
<b>A<math>\beta</math>X-42 (ng/L)</b>	1247	2026	601 (53-1396)
<b>A<math>\beta</math>1-42 (ng/L)</b>	552	667	236 (84-408)
<b>sAPP-<math>\alpha</math> (ng/mL)</b>	467	794	313 (110-781)
<b>sAPP-<math>\beta</math> (ng/mL)</b>	213	326	102 (31-285)
<b>T-tau (ng/L)</b>	1399	1981	62 (40-135)
<b>P-tau (ng/L)</b>	121	133	35 (24-61)

Data for the contrast group is median (range). Regarding A $\beta$ X-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 A $\beta$  sequence

## Figure legends

**Fig. 1 Cells treated to emulate NPC phenotype have altered release of A $\beta$ - and sAPP-peptides.** SH-SY5Y APP695wt cells were treated with DMSO, U18666A, or untreated (blank). Cell media concentrations of sAPP- $\alpha$  (A), sAPP- $\beta$  (B), A $\beta$ 1-40 (C), A $\beta$ 1-42 (D), A $\beta$ X-40 (E and G) and A $\beta$ X-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.

**Fig. 2 Cells treated to emulate NPC phenotype have increased relative release of A $\beta$ -peptides starting at position A $\beta$ 5.** The spectra of secreted A $\beta$  peptides in U18666A-treated SH-SY5Y APP695wt cells were analyzed by immunoprecipitation and mass spectrometry. IP-MALDI-TOF MS showed increased relative signals of A $\beta$ 5-38 and A $\beta$ 5-40 in U18666A treated cells (red).

**Fig. 3 NPC1<sup>-/-</sup> cells have reduced relative release of A $\beta$ 1-16.** NPC1wt and NPC1<sup>-/-</sup> CHO cells were transiently transfected with either APPwt or APPsw construct. Cell media concentrations of sAPP- $\alpha$  and sAPP- $\beta$  were determined using an electrochemiluminescence assay, while A $\beta$  species were analyzed by IP-MALDI-MS TOF. Each data point represents individual cell cultures. In parallel with decreased sAPP- $\alpha$  levels in NPC1<sup>-/-</sup> cells (A) we observed reduced relative signal of A $\beta$ 1-16 in the same cells (B). Note that in APPsw-transfected cells we were unable to analyze sAPP- $\beta$ , which is expected due to the swe mutation.

**Fig. 4 Effect of NPC1<sup>-/-</sup> on release of A $\beta$ X-38, A $\beta$ X-40 and A $\beta$ X-42.** A $\beta$ X-40 (A), A $\beta$ X-38 (B) and A $\beta$ X-42 (C) were analyzed in untransfected (APPendo) and either APPsw or C99-transfected (A-C) NPC1wt and NPC1<sup>-/-</sup> CHO cells by an electrochemiluminescent based assay (MSD). Each data point represents individual cell cultures. In untransfected cells expressing only endogenous APP, the NPC1<sup>-/-</sup> genotype reduced A $\beta$ X-40 levels (A). Similarly, in cells transfected with C99, the NPC1<sup>-/-</sup> genotype caused a slight reduction of A $\beta$ X-38, A $\beta$ X-40 and A $\beta$ X-42 (A-C).

**Fig. 5 The cat A $\beta$  sequence.** IP-MALDI-TOF/TOF tandem mass spectrum of cat A $\beta$  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 A $\beta$  peptides in cat CSF (top panel) compared to human (bottom panel). (b) MS/MS analysis of A $\beta$ 1-40 from cat (top panel) and human (bottom panel). For human A $\beta$ 1-40, 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<sub>7</sub>, b<sub>23</sub> signals. In the MS/MS spectrum of the cat A $\beta$ 1-40, b<sub>23</sub> is strong, but b<sub>7</sub> is significantly less intense. This observation together with the 14 Da mass difference indicates that Asp-7 is replaced by Glu in cat A $\beta$ 1-40.

**Fig. 6 The effects of NPC and cyclodextrin treatment on CSF A $\beta$  peptides in cats.** A $\beta$  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 A $\beta$ 1-37, A $\beta$ 1-38, and A $\beta$ 1-39 compared to controls (top panel). Cyclodextrin treated NPC cat (bottom panel) show increased relative levels of A $\beta$ 1-16, A $\beta$ 6-28 and A $\beta$ 1-28 compared to the other groups.

**Fig. 7 Longitudinal data on CSF A $\beta$  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 4<sup>th</sup> 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 A $\beta$ 1-42 (A), A $\beta$ X-42 (B), A $\beta$ X-38 (C), A $\beta$ X-40 (D) and sAPP- $\beta$  (F) were decreased over time to 50% of the baseline in the two examined NPC patients. Meanwhile, sAPP- $\alpha$  levels were only slightly changed (E).

**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 4<sup>th</sup> 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 to the baseline levels in the two examined NPC patients.

**Supplementary fig. 1 Effects of BACE1-inhibition on NPC phenotype cells.** SH-SY5Y APP695wt cells were treated with DMSO, BACE1 inhibition with  $\beta$ -secretase inhibitor IV (Calbiochem, Merck), and/or U18666A. Cell media concentrations of A $\beta$ 1-40 (A), A $\beta$ 1-42 (B), A $\beta$ X-40 (C) and A $\beta$ X-42 (D) were determined using fluorescent bead-based assay. Each data point represents individual cell cultures. Treatment with a BACE1 inhibitor reduced A $\beta$ 1-40 and A $\beta$ 1-42 levels.

**Supplementary fig. 2 Effects of cathepsin B-inhibition on NPC phenotype cells.** SH-SY5Y APP695wt cells were treated with DMSO, cathepsin B inhibition with Z-FA-FMK (BD Biosciences, San Jose, CA, USA), and/or U18666A. Cell media concentrations of A $\beta$ 1-40 (A), A $\beta$ 1-42 (B), A $\beta$ X-40 (C) and A $\beta$ X-42 (D) were determined using fluorescent bead-based assay. Each data point represents individual cell cultures. Cathepsin B inhibition reduced A $\beta$ X-40 and A $\beta$ X-42 levels in U18666A-treated cells almost to the levels as in vehicle (DMSO)-treated cells.

**Supplementary fig. 3 Effects of NPC1<sup>-/-</sup> on release of A $\beta$  peptides.** NPC1wt and NPC1<sup>-/-</sup> CHO cells were transiently transfected with either APPwt or APPsw construct. Cell media concentrations of A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ X-40 and A $\beta$ X-42 were determined using fluorescent bead-based assay (FORM) and/or electrochemiluminescent based assay (MSD). Each data point represents individual cell cultures. NPC1<sup>-/-</sup> genotype did not have a major effect on media levels of A $\beta$ 1-40, A $\beta$ 1-42, A $\beta$ X-40 and A $\beta$ X-42.

**Supplementary fig. 4 Release of A $\beta$  peptides from C99 transfected cells.** NPC1wt and NPC1<sup>-/-</sup> CHO cells were transiently transfected with either APPsw or C99 construct. Signals of A $\beta$ 1-40, A $\beta$ 1-41 and A $\beta$ 1-42 were determined in the cell media with IP-MALDI-TOFMS. Each data point represents individual cell cultures. IP-MALDI-TOFMS analysis showed elevated relative A $\beta$ 1-41 signals and reduced relative A $\beta$ 1-40 and A $\beta$ 1-42 signals in C99 transfected cells compared to APPsw transfected cells. NPC1<sup>-/-</sup> genotype increased the relative A $\beta$ 1-41 signal even more.

**Supplementary fig. 5 A $\beta$  peptides in cat CSF.** A $\beta$ 1-16, A $\beta$ 6-28, A $\beta$ 1-28, A $\beta$ 6-40, A $\beta$ 1-34, A $\beta$ 1-37, A $\beta$ 1-38 and A $\beta$ 1-39 peptides were determined by IP-MALDI-TOFMS in CSF from control and cyclodextrin treated/untreated NPC1-cats. Each data point represents individual animals. Some of the cats treated with cyclodextrin showed increased relative levels of A $\beta$ 1-16, A $\beta$ 6-28 and A $\beta$ 1-28 compared to the other groups.

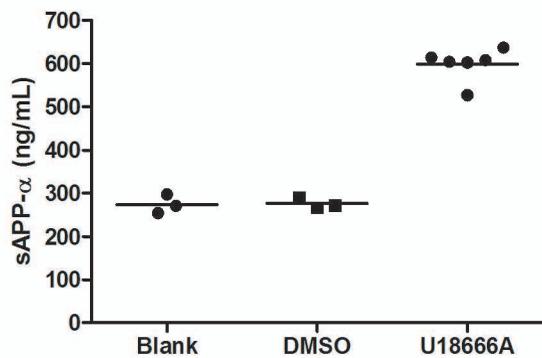
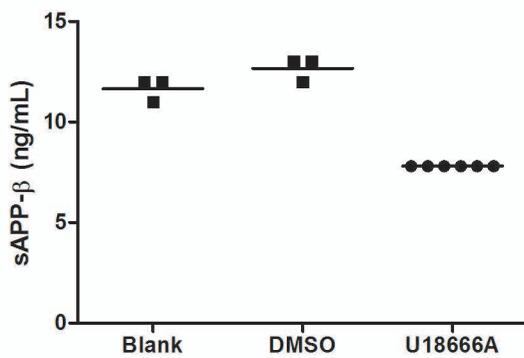
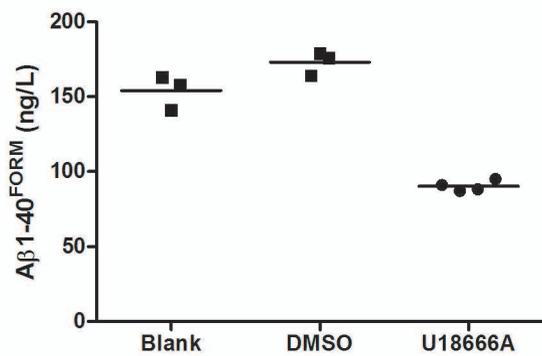
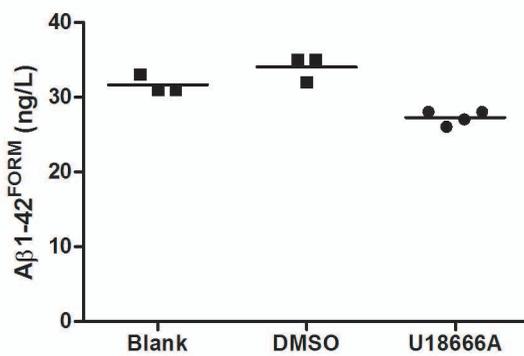
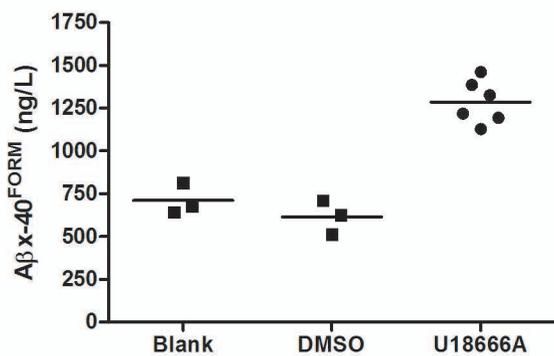
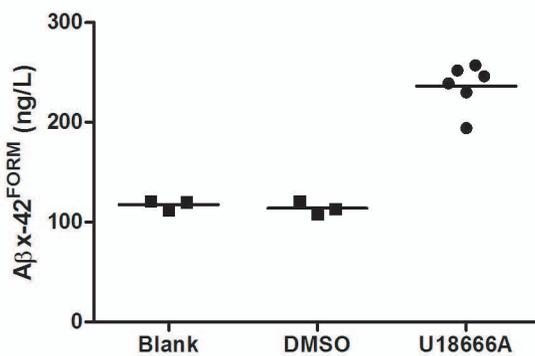
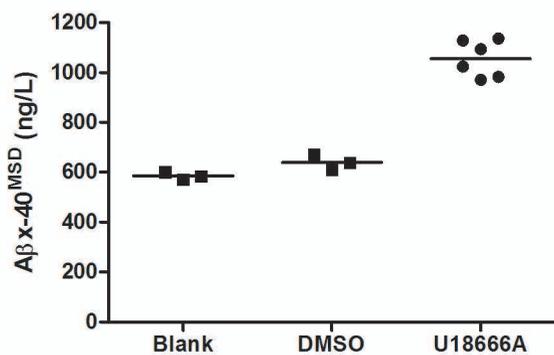
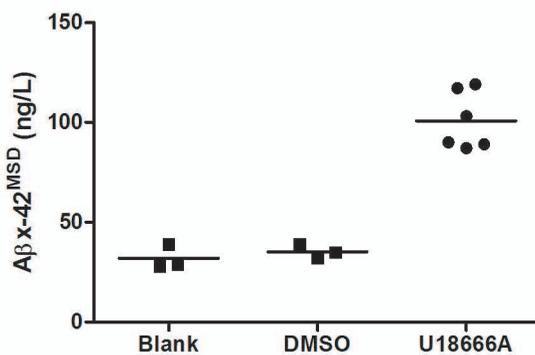
## References

- Auer IA, Schmidt ML, Lee VM, Curry B, Suzuki K, Shin RW, Pentchev PG, Carstea ED, Trojanowski JQ (1995) Paired helical filament tau (PHFtau) in Niemann-Pick type C disease is similar to PHFtau in Alzheimer's disease. *Acta Neuropathol* 90 (6):547-551.
- Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res* 38 (11 Pt 1):3751-3757.
- Blennow K, Hampel H, Weiner M, Zetterberg H (2010) Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. *Nat Rev Neurol* 6 (3):131-144.
- Blennow K, Zetterberg H, Rinne JO, Salloway S, Wei J, Black R, Grundman M, Liu E, for the AABI (2012) Effect of Immunotherapy With Bapineuzumab on Cerebrospinal Fluid Biomarker Levels in Patients With Mild to Moderate Alzheimer Disease. *Arch Neurol*.
- Bodovitz S, Klein WL (1996) Cholesterol modulates alpha-secretase cleavage of amyloid precursor protein. *J Biol Chem* 271 (8):4436-4440.
- Boland B, Smith DA, Mooney D, Jung SS, Walsh DM, Platt FM Macroautophagy is not directly involved in the metabolism of amyloid precursor protein. *J Biol Chem* 285 (48):37415-37426.
- Borbon IA, Erickson RP (2011) Interactions of Npc1 and amyloid accumulation/deposition in the APP/PS1 mouse model of Alzheimer's. *Journal of applied genetics* 52 (2):213-218.
- Brinkmalm G, Portelius E, Ohrfelt A, Mattsson N, Persson R, Gustavsson MK, Vite CH, Gobom J, Mansson JE, Nilsson J, Halim A, Larson G, Ruetschi U, Zetterberg H, Blennow K, Brinkmalm A (2012) An online nano-LC-ESI-FTICR-MS method for comprehensive characterization of endogenous fragments from amyloid beta and amyloid precursor protein in human and cat cerebrospinal fluid. *J Mass Spectrom* 47 (5):591-603.
- Burns M, Gaynor K, Olm V, Mercken M, LaFrancois J, Wang L, Mathews PM, Noble W, Matsuoka Y, Duff K (2003) Presenilin redistribution associated with aberrant cholesterol transport enhances beta-amyloid production in vivo. *J Neurosci* 23 (13):5645-5649.
- Cirrito JR, Kang JE, Lee J, Stewart FR, Verges DK, Silverio LM, Bu G, Mennerick S, Holtzman DM (2008) Endocytosis is required for synaptic activity-dependent release of amyloid-beta in vivo. *Neuron* 58 (1):42-51.
- Crestini A, Napolitano M, Piscopo P, Confaloni A, Bravo E (2006) Changes in cholesterol metabolism are associated with PS1 and PS2 gene regulation in SK-N-BE. *J Mol Neurosci* 30 (3):311-322.
- Davis W, Jr. (2008) The cholesterol transport inhibitor U18666a regulates amyloid precursor protein metabolism and trafficking in N2aAPP "Swedish" cells. *Curr Alzheimer Res* 5 (5):448-456.
- Erickson RP, Larson-Thome K, Weberg L, Szybinska A, Mossakowska M, Styczynska M, Barcikowska M, Kuznicki J (2008) Variation in NPC1, the gene encoding Niemann-Pick C1, a protein involved in intracellular cholesterol transport, is associated with Alzheimer disease and/or aging in the Polish population. *Neurosci Lett* 447 (2-3):153-157.
- Grimm MO, Grimm HS, Hartmann T (2007) Amyloid beta as a regulator of lipid homeostasis. *Trends Mol Med* 13 (8):337-344.
- Hansson O, Zetterberg H, Vanmechelen E, Vanderstichele H, Andreasson U, Londos E, Wallin A, Minthon L, Blennow K (2010) Evaluation of plasma Aβ(40) and Aβ(42) as predictors of conversion to Alzheimer's disease in patients with mild cognitive impairment. *Neurobiol Aging* 31 (3):357-367.
- Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256 (5054):184-185.
- Hecimovic S, Wang J, Dolios G, Martinez M, Wang R, Goate AM (2004) Mutations in APP have independent effects on Aβ and CTFγ generation. *Neurobiol Dis* 17 (2):205-218.
- Holmes O, Paturi S, Ye W, Wolfe MS, Selkoe DJ (2012) Effects of membrane lipids on the activity and processivity of purified gamma-secretase. *Biochemistry* 51 (17):3565-3575.
- Hook V, Toneff T, Bogyo M, Greenbaum D, Medzihradzsky KF, Neveu J, Lane W, Hook G, Reisine T (2005) Inhibition of cathepsin B reduces beta-amyloid production in regulated secretory

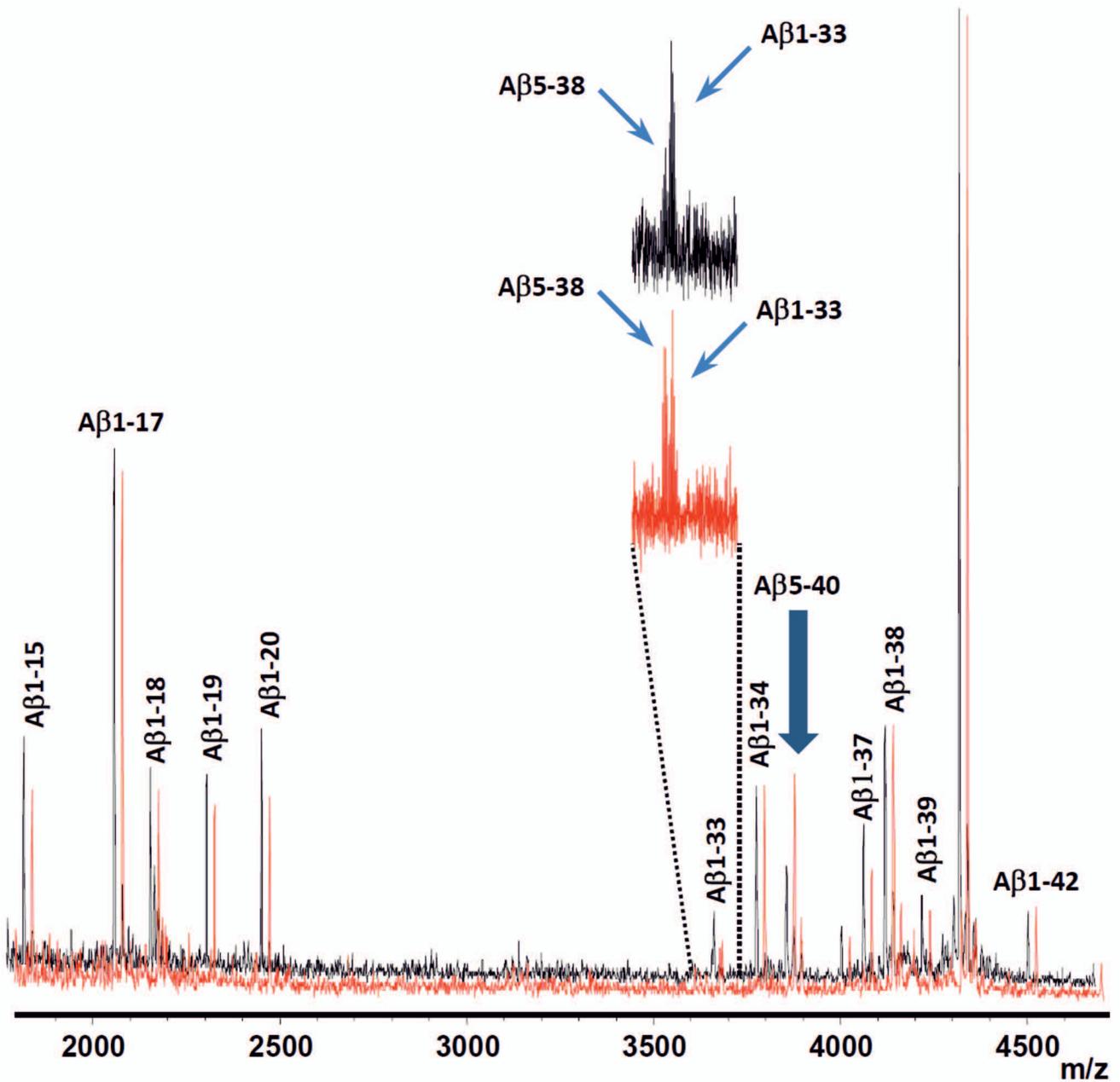
- vesicles of neuronal chromaffin cells: evidence for cathepsin B as a candidate beta-secretase of Alzheimer's disease. *Biol Chem* 386 (9):931-940.
- Jin LW, Shie FS, Maezawa I, Vincent I, Bird T (2004) Intracellular accumulation of amyloidogenic fragments of amyloid-beta precursor protein in neurons with Niemann-Pick type C defects is associated with endosomal abnormalities. *Am J Pathol* 164 (3):975-985.
- Kagedal K, Kim WS, Appelqvist H, Chan S, Cheng D, Agholme L, Barnham K, McCann H, Halliday G, Garner B (2010) Increased expression of the lysosomal cholesterol transporter NPC1 in Alzheimer's disease. *Biochim Biophys Acta* 1801 (8):831-838.
- Kodam A, Maulik M, Peake K, Amritraj A, Vetrivel KS, Thinakaran G, Vance JE, Kar S (2010) Altered levels and distribution of amyloid precursor protein and its processing enzymes in Niemann-Pick type C1-deficient mouse brains. *Glia* 58 (11):1267-1281.
- Koh CH, Whiteman M, Li QX, Halliwell B, Jenner AM, Wong BS, Laughton KM, Wenk M, Masters CL, Beart PM, Bernard O, Cheung NS (2006) Chronic exposure to U18666A is associated with oxidative stress in cultured murine cortical neurons. *J Neurochem* 98 (4):1278-1289.
- Kosicek M, Malnar M, Goate A, Hecimovic S (2010) Cholesterol accumulation in Niemann Pick type C (NPC) model cells causes a shift in APP localization to lipid rafts. *Biochem Biophys Res Commun* 393 (3):404-409.
- Lange Y, Ye J, Rigney M, Steck T (2000) Cholesterol movement in Niemann-Pick type C cells and in cells treated with amphiphiles. *J Biol Chem* 275 (23):17468-17475.
- Malnar M, Kosicek M, Mitterreiter S, Omerbasic D, Lichtenthaler SF, Goate A, Hecimovic S (2010) Niemann-Pick type C cells show cholesterol dependent decrease of APP expression at the cell surface and its increased processing through the beta-secretase pathway. *Biochim Biophys Acta* 1802 (7-8):682-691.
- Mattsson N, Portelius E, Rolstad S, Gustavsson M, Andreasson U, Stridsberg M, Wallin A, Blennow K, Zetterberg H (2012a) Longitudinal Cerebrospinal Fluid Biomarkers over Four Years in Mild Cognitive Impairment. *J Alzheimers Dis* 30 (4):767-778.
- Mattsson N, Rajendran L, Zetterberg H, Gustavsson M, Andreasson U, Olsson M, Brinkmalm G, Lundkvist J, Jacobson LH, Perrot L, Neumann U, Borghys H, Mercken M, Dhuyvetter D, Jeppsson F, Blennow K, Portelius E (2012b) BACE1 Inhibition Induces a Specific Cerebrospinal Fluid beta-Amyloid Pattern That Identifies Drug Effects in the Central Nervous System. *PLoS One* 7 (2):e31084.
- Mattsson N, Zetterberg H, Bianconi S, Yanjanin NM, Fu R, Mansson JE, Porter FD, Blennow K (2011a) Gamma-secretase-dependent amyloid-beta is increased in Niemann-Pick type C: a cross-sectional study. *Neurology* 76 (4):366-372.
- Mattsson N, Zetterberg H, Bianconi S, Yanjanin NM, Fu R, Mansson JE, Porter FD, Blennow K (2011b) Miglustat treatment may reduce cerebrospinal fluid levels of the axonal degeneration marker tau in Niemann-Pick type C. *JIMD Reports* Accepted for publication.
- Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nat Genet* 1 (5):345-347.
- Nixon RA, Yang DS, Lee JH (2008) Neurodegenerative lysosomal disorders: a continuum from development to late age. *Autophagy* 4 (5):590-599.
- Olson L, Humpel C (2010) Growth factors and cytokines/chemokines as surrogate biomarkers in cerebrospinal fluid and blood for diagnosing Alzheimer's disease and mild cognitive impairment. *Exp Gerontol* 45 (1):41-46.
- Olsson A, Vanderstichele H, Andreasen N, De Meyer G, Wallin A, Holmberg B, Rosengren L, Vanmechelen E, Blennow K (2005) Simultaneous measurement of beta-amyloid(1-42), total tau, and phosphorylated tau (Thr181) in cerebrospinal fluid by the xMAP technology. *Clin Chem* 51 (2):336-345.
- Patterson MC, Hendriksz CJ, Walterfang M, Sedel F, Vanier MT, Wijburg F, on behalf of the NPCGWG (2012) Recommendations for the diagnosis and management of Niemann-Pick disease type C: An update. *Mol Genet Metab*.

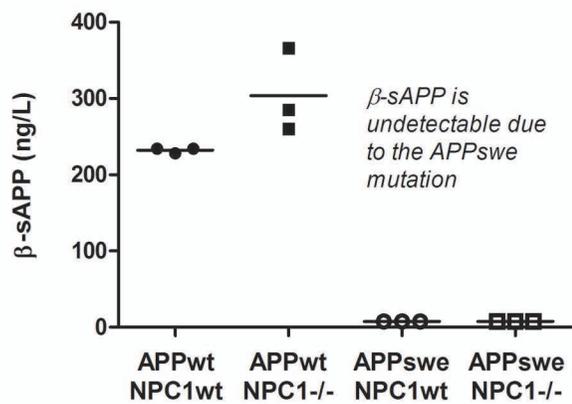
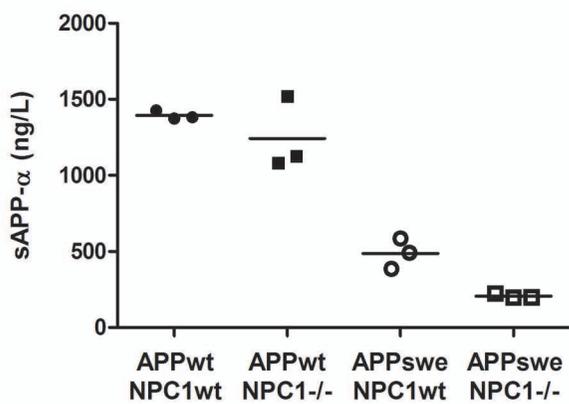
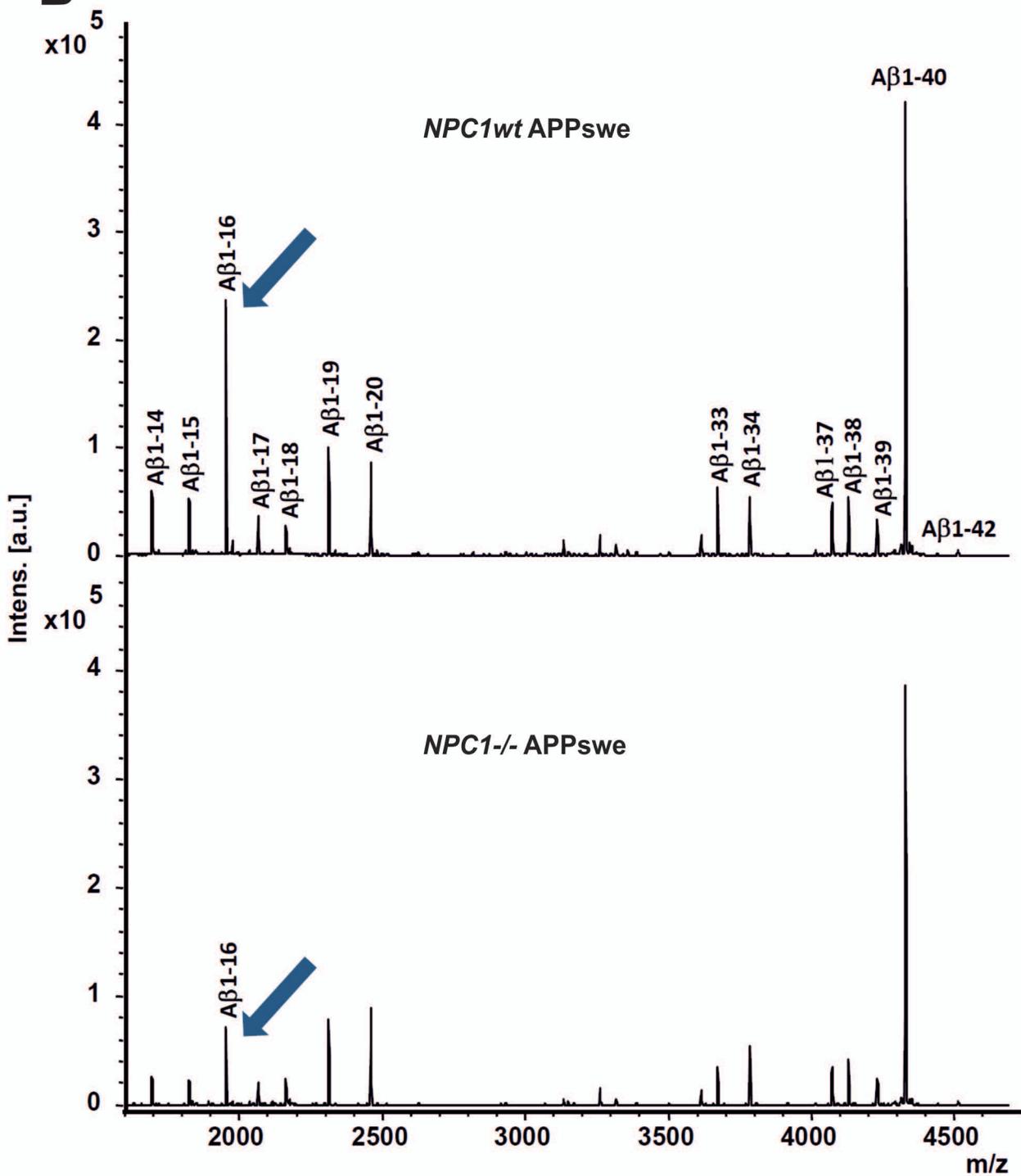
- Patterson MC, Vecchio D, Prady H, Abel L, Wraith JE (2007) Miglustat for treatment of Niemann-Pick C disease: a randomised controlled study. *Lancet Neurol* 6 (9):765-772.
- Peake KB, Vance JE (2012) Normalization of cholesterol homeostasis by 2-hydroxypropyl-beta-cyclodextrin in neurons and glia from Niemann-Pick C1-deficient mice. *J Biol Chem*.
- Pineda M, Wraith JE, Mengel E, Sedel F, Hwu WL, Rohrbach M, Bembi B, Walterfang M, Korenke GC, Marquardt T, Luzy C, Giorgino R, Patterson MC (2009) Miglustat in patients with Niemann-Pick disease Type C (NP-C): a multicenter observational retrospective cohort study. *Mol Genet Metab* 98 (3):243-249.
- Portelius E, Mattsson N, Andreasson U, Blennow K, Zetterberg H (2011a) Novel abeta isoforms in Alzheimer's disease - their role in diagnosis and treatment. *Curr Pharm Des* 17 (25):2594-2602.
- Portelius E, Price E, Brinkmalm G, Stiteler M, Olsson M, Persson R, Westman-Brinkmalm A, Zetterberg H, Simon AJ, Blennow K (2011b) A novel pathway for amyloid precursor protein processing. *Neurobiol Aging* 32 (6):1090-1098.
- Portelius E, Tran AJ, Andreasson U, Persson R, Brinkmalm G, Zetterberg H, Blennow K, Westman-Brinkmalm A (2007) Characterization of amyloid beta peptides in cerebrospinal fluid by an automated immunoprecipitation procedure followed by mass spectrometry. *J Proteome Res* 6 (11):4433-4439.
- Portelius E, Van Broeck B, Andreasson U, Gustavsson MK, Mercken M, Zetterberg H, Borghys H, Blennow K (2010) Acute effect on the Abeta isoform pattern in CSF in response to gamma-secretase modulator and inhibitor treatment in dogs. *J Alzheimers Dis* 21 (3):1005-1012.
- Portelius E, Westman-Brinkmalm A, Zetterberg H, Blennow K (2006) Determination of beta-Amyloid Peptide Signatures in Cerebrospinal Fluid Using Immunoprecipitation-Mass Spectrometry. *J Proteome Res* 5 (4):1010-1016.
- Refolo LM, Malester B, LaFrancois J, Bryant-Thomas T, Wang R, Tint GS, Sambamurti K, Duff K, Pappolla MA (2000) Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. *Neurobiol Dis* 7 (4):321-331.
- Roff CF, Goldin E, Comly ME, Cooney A, Brown A, Vanier MT, Miller SP, Brady RO, Pentchev PG (1991) Type C Niemann-Pick disease: use of hydrophobic amines to study defective cholesterol transport. *Developmental neuroscience* 13 (4-5):315-319.
- Rosen C, Andreasson U, Mattsson N, Marcusson J, Minthon L, Andreasen N, Blennow K, Zetterberg H (2012) Cerebrospinal fluid profiles of amyloid beta-related biomarkers in Alzheimer's disease. *Neuromolecular Med* 14 (1):65-73.
- Rosenbaum AI, Maxfield FR (2011) Niemann-Pick type C disease: molecular mechanisms and potential therapeutic approaches. *J Neurochem* 116 (5):789-795.
- Runz H, Rietdorf J, Tomic I, de Bernard M, Beyreuther K, Pepperkok R, Hartmann T (2002) Inhibition of intracellular cholesterol transport alters presenilin localization and amyloid precursor protein processing in neuronal cells. *J Neurosci* 22 (5):1679-1689.
- Saito Y, Suzuki K, Nanba E, Yamamoto T, Ohno K, Murayama S (2002) Niemann-Pick type C disease: accelerated neurofibrillary tangle formation and amyloid beta deposition associated with apolipoprotein E epsilon 4 homozygosity. *Ann Neurol* 52 (3):351-355.
- Selkoe DJ (1991) The molecular pathology of Alzheimer's disease. *Neuron* 6 (4):487-498.
- Sidera C, Parsons R, Austen B (2005) The regulation of beta-secretase by cholesterol and statins in Alzheimer's disease. *J Neurol Sci* 229-230:269-273.
- Simons M, Keller P, De Strooper B, Beyreuther K, Dotti CG, Simons K (1998) Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. *Proc Natl Acad Sci U S A* 95 (11):6460-6464.
- Somers KL, Royals MA, Carstea ED, Rafi MA, Wenger DA, Thrall MA (2003) Mutation analysis of feline Niemann-Pick C1 disease. *Mol Genet Metab* 79 (2):99-103.
- Stachel SJ, Coburn CA, Steele TG, Jones KG, Loutzenhiser EF, Gregro AR, Rajapakse HA, Lai MT, Crouthamel MC, Xu M, Tugusheva K, Lineberger JE, Pietrak BL, Espeseth AS, Shi XP, Chen-

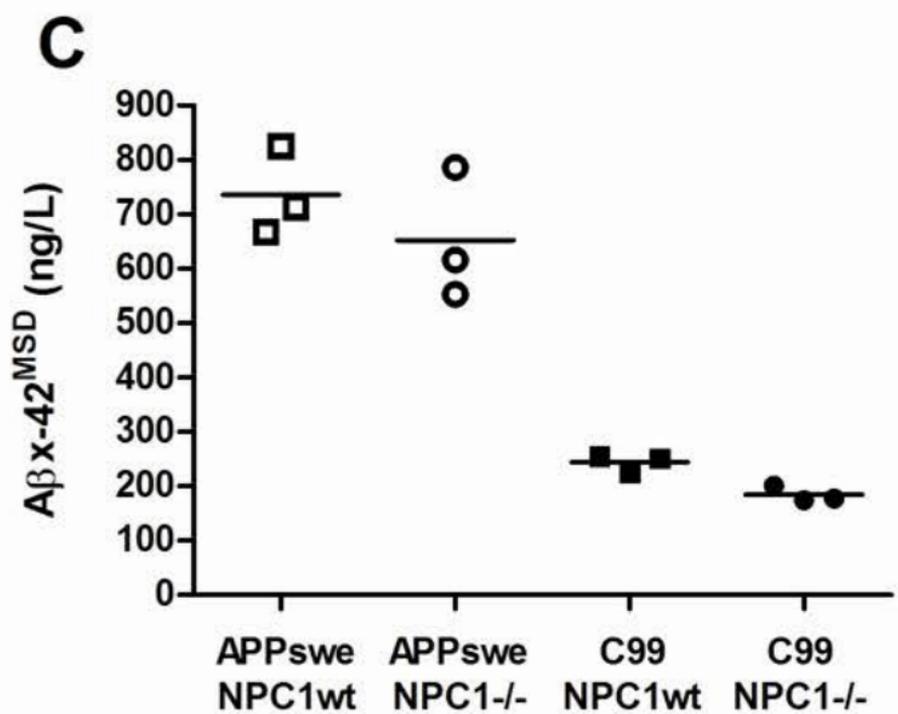
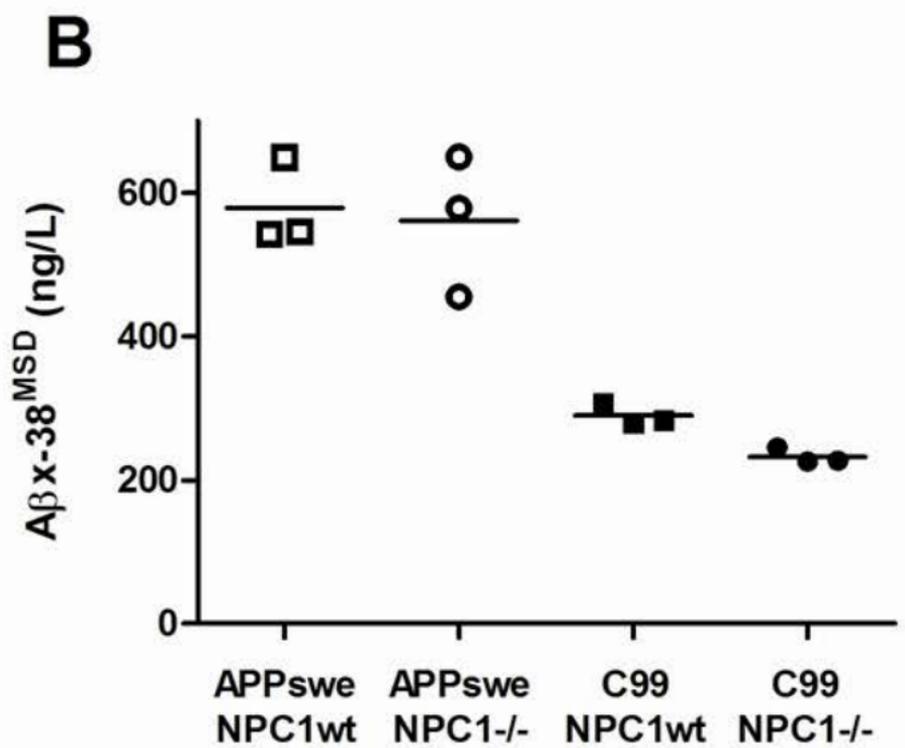
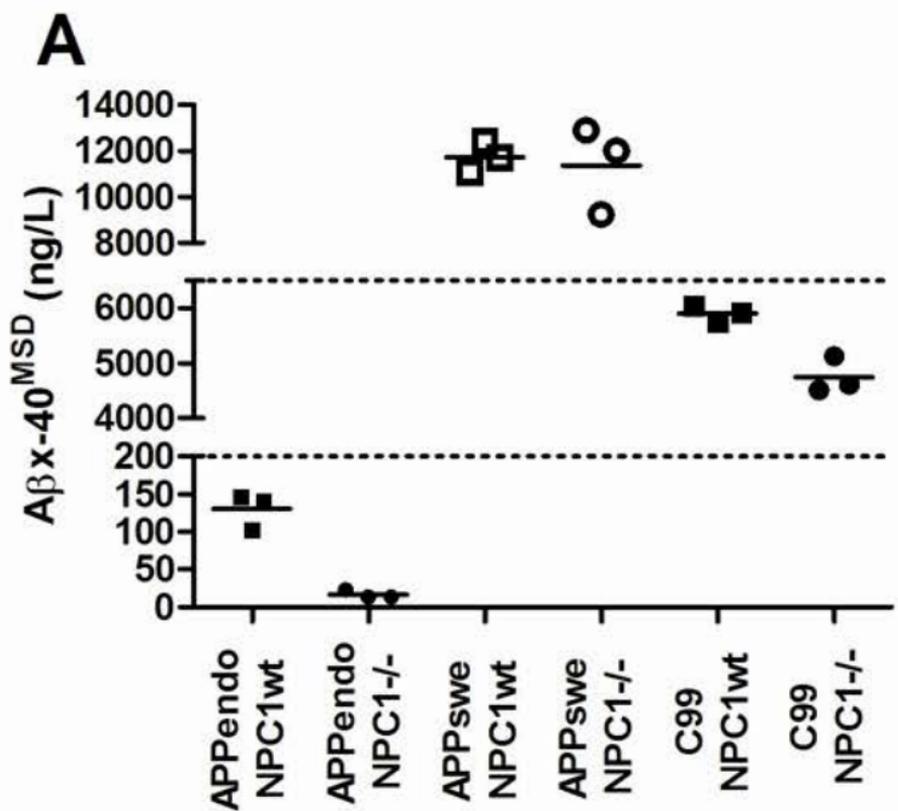
- Dodson E, Holloway MK, Munshi S, Simon AJ, Kuo L, Vacca JP (2004) Structure-based design of potent and selective cell-permeable inhibitors of human beta-secretase (BACE-1). *J Med Chem* 47 (26):6447-6450.
- Swardfager W, Lanctot K, Rothenburg L, Wong A, Cappell J, Herrmann N (2010) A meta-analysis of cytokines in Alzheimer's disease. *Biol Psychiatry* 68 (10):930-941.
- Takeda K, Araki W, Akiyama H, Tabira T (2004) Amino-truncated amyloid beta-peptide (A $\beta$ 5-40/42) produced from caspase-cleaved amyloid precursor protein is deposited in Alzheimer's disease brain. *FASEB J* 18 (14):1755-1757.
- Ward S, O'Donnell P, Fernandez S, Vite CH (2010) 2-hydroxypropyl-beta-cyclodextrin raises hearing threshold in normal cats and in cats with Niemann-Pick type C disease. *Pediatr Res* 68 (1):52-56.
- Vite CH, Ding W, Bryan C, O'Donnell P, Cullen K, Aleman D, Haskins ME, Van Winkle T (2008) Clinical, electrophysiological, and serum biochemical measures of progressive neurological and hepatic dysfunction in feline Niemann-Pick type C disease. *Pediatr Res* 64 (5):544-549.
- Yamazaki T, Chang TY, Haass C, Ihara Y (2001) Accumulation and aggregation of amyloid beta-protein in late endosomes of Niemann-pick type C cells. *J Biol Chem* 276 (6):4454-4460.

**A****B****C****D****E****F****G****H**

A $\beta$ 1-40

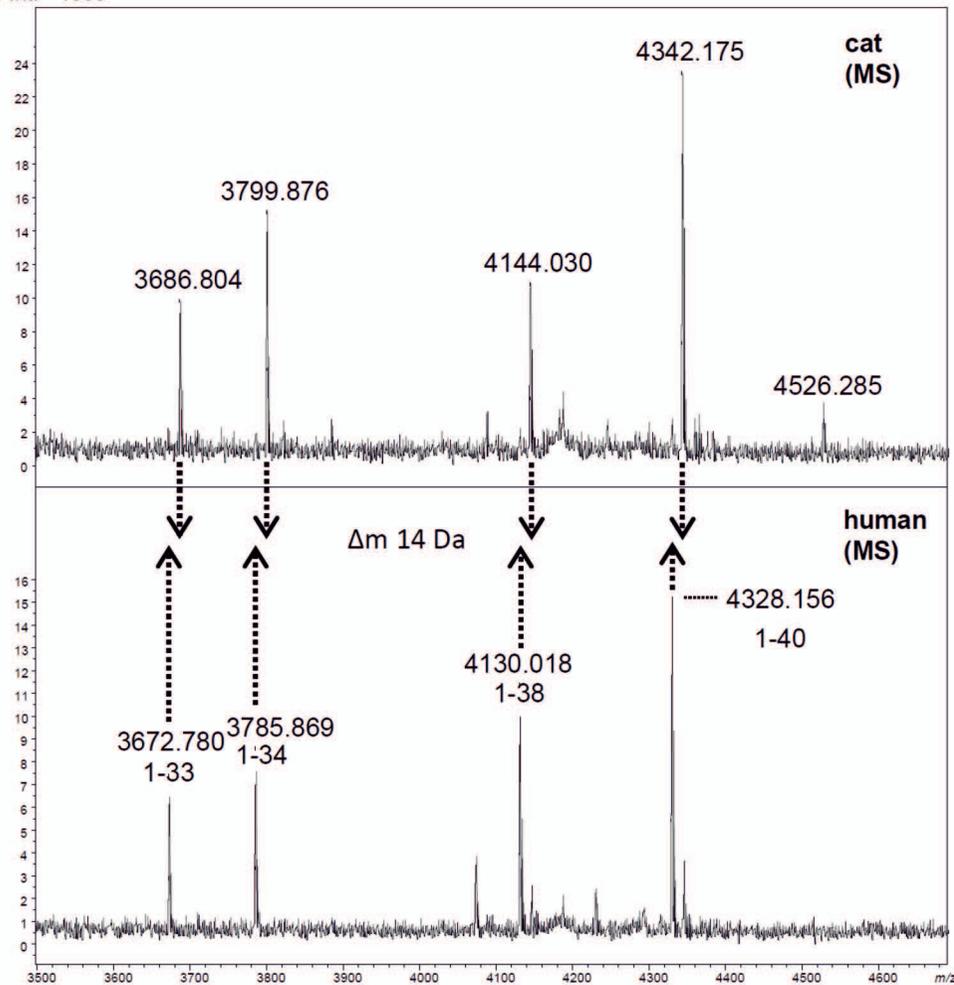


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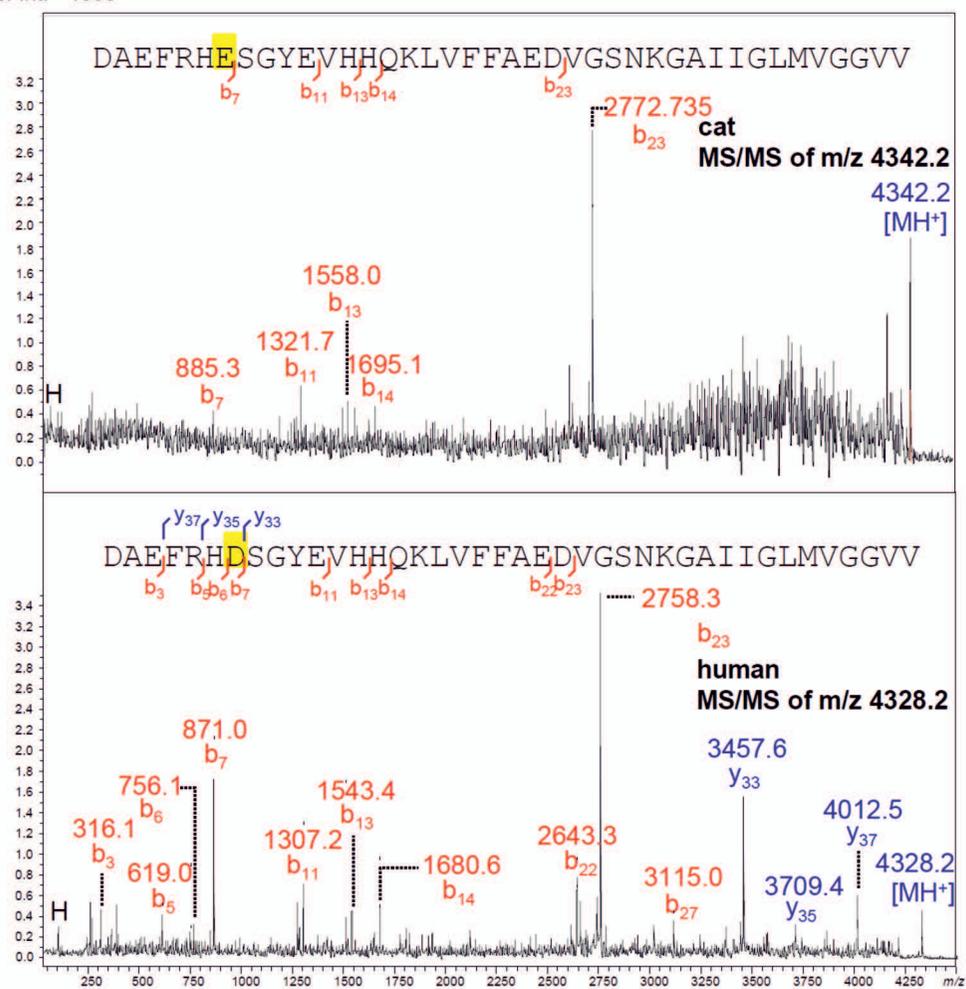


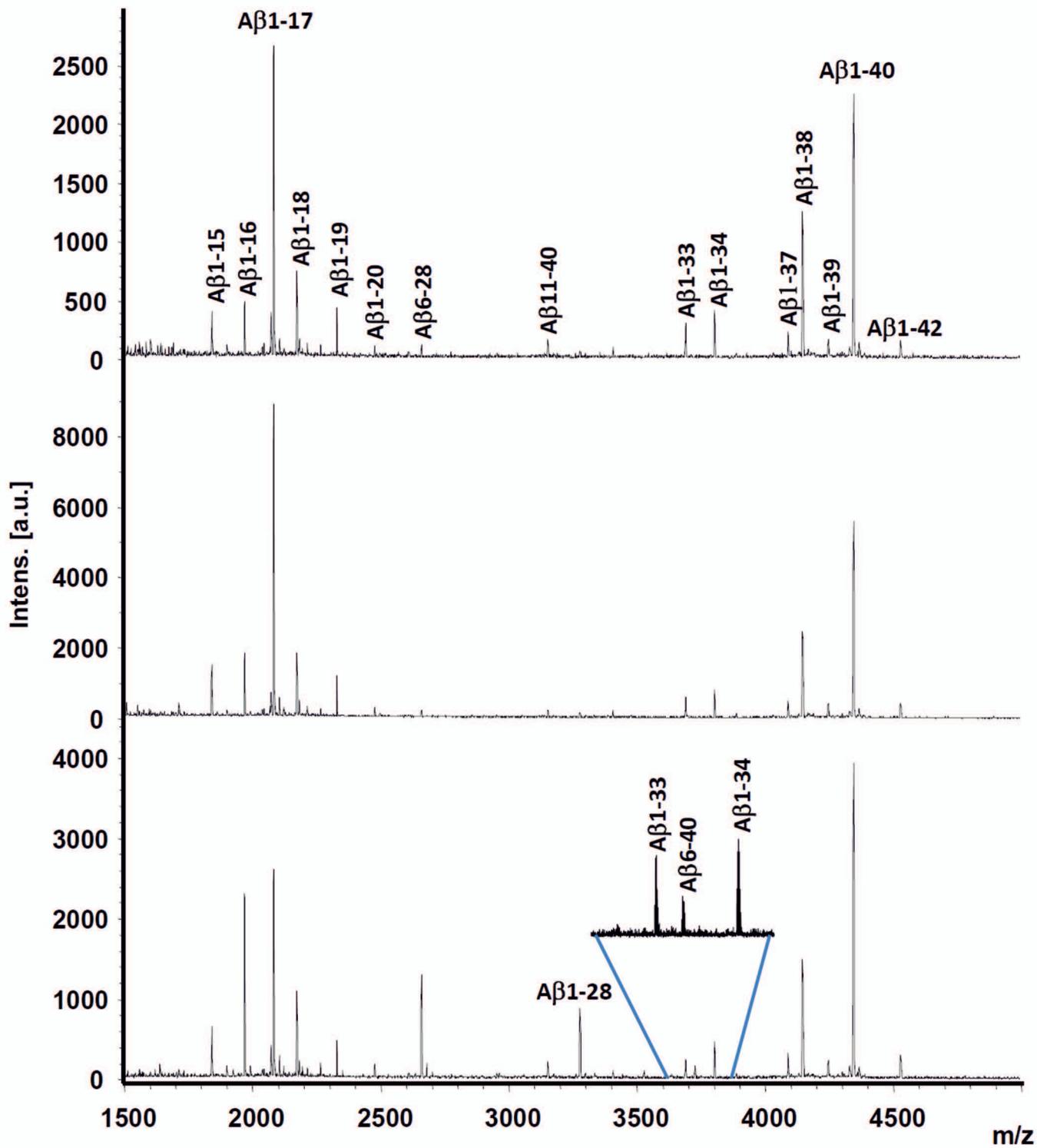
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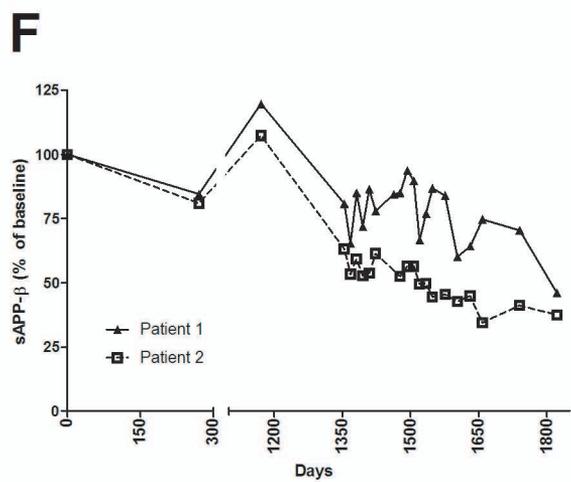
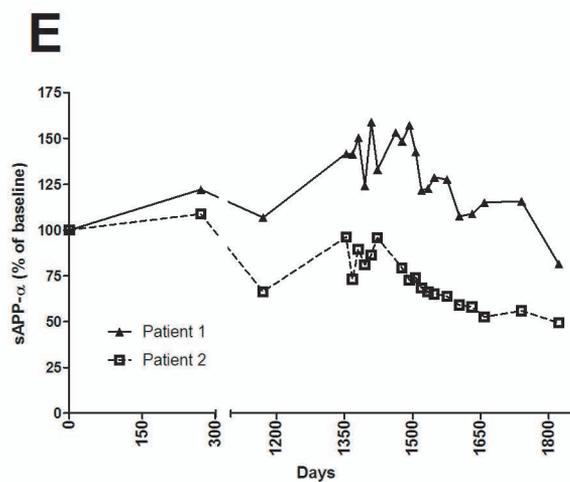
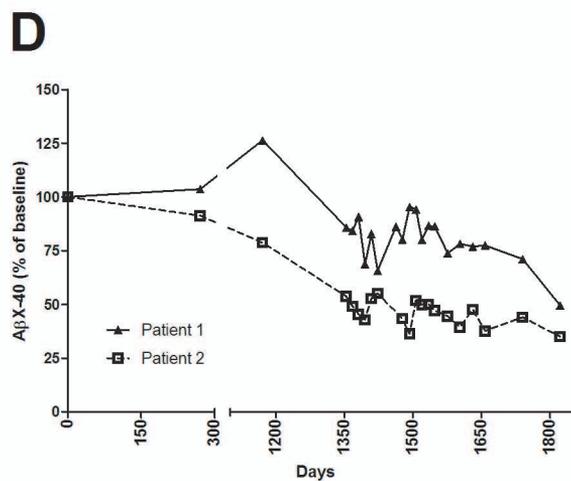
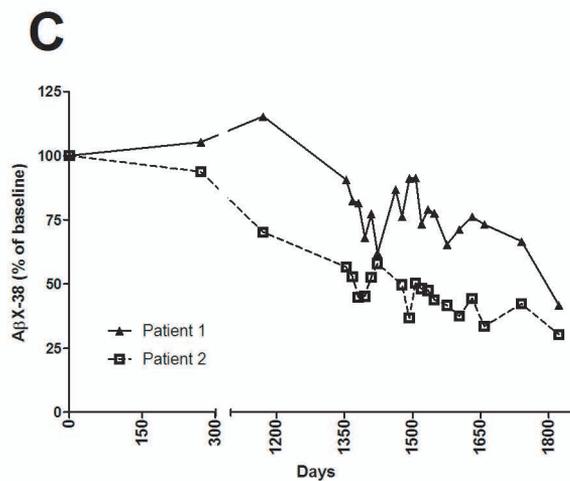
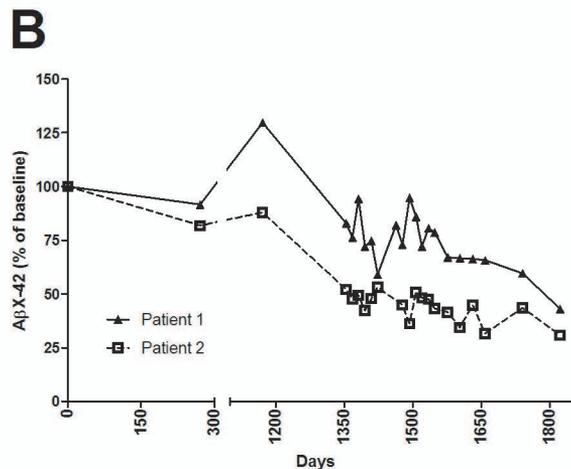
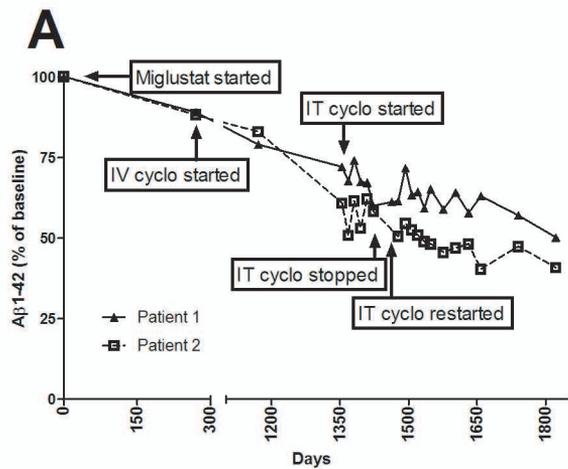
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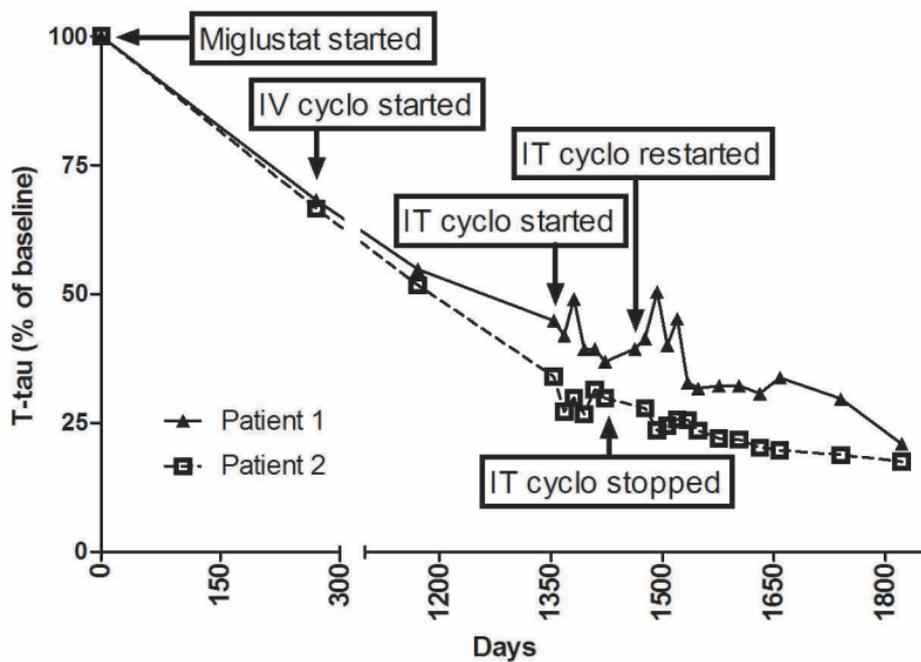
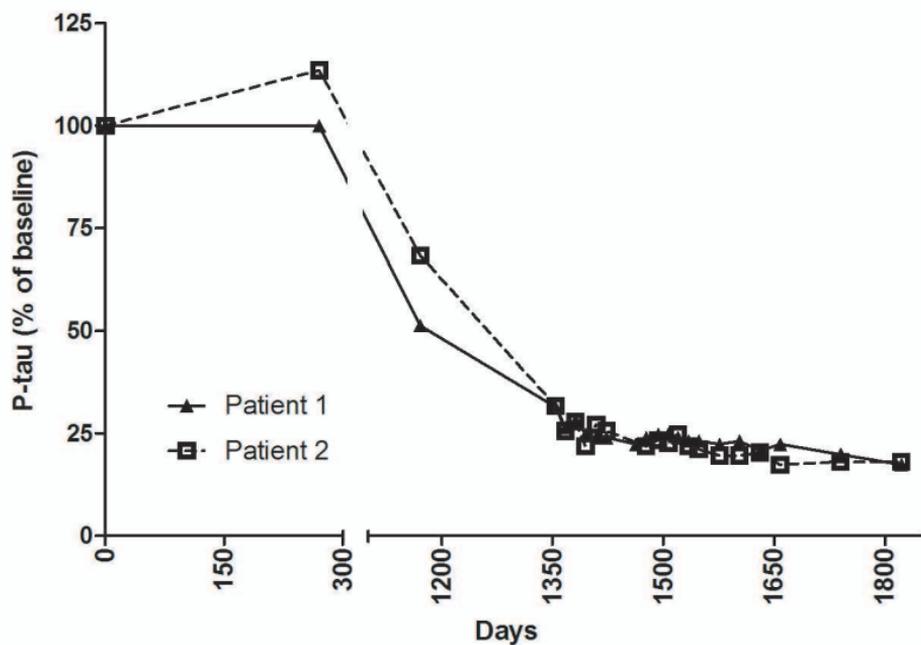
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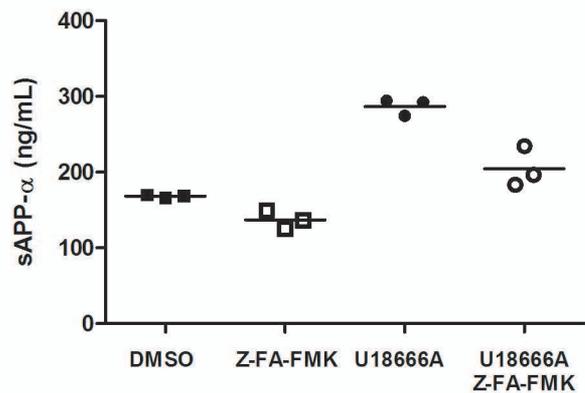
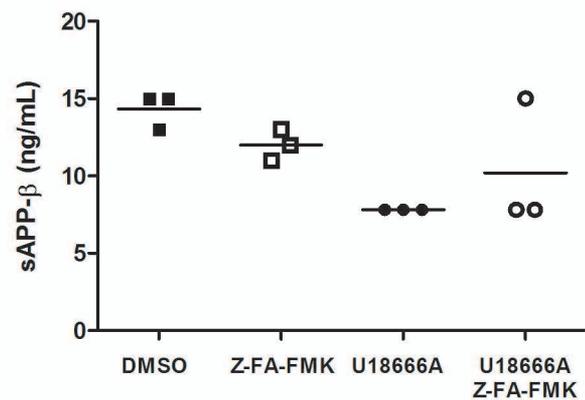
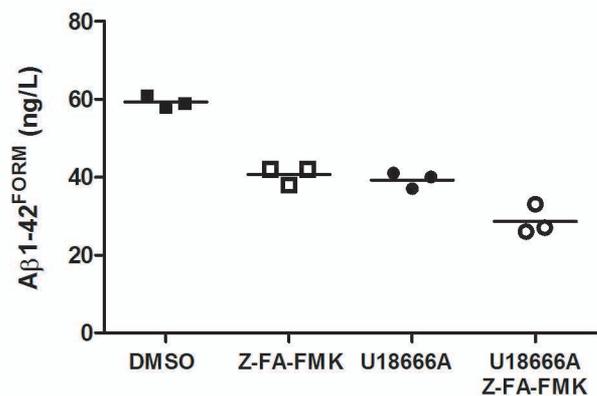
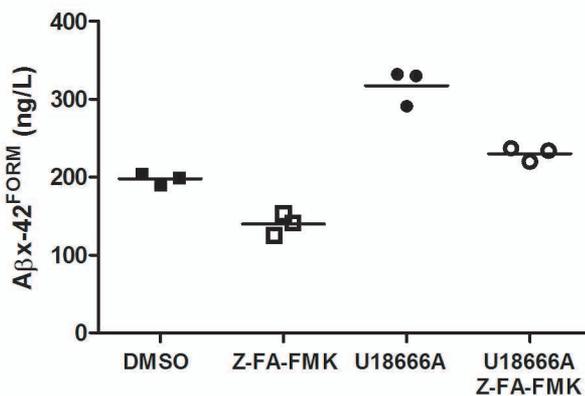
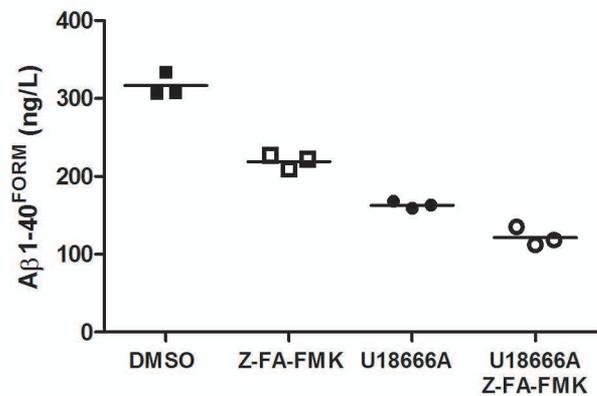
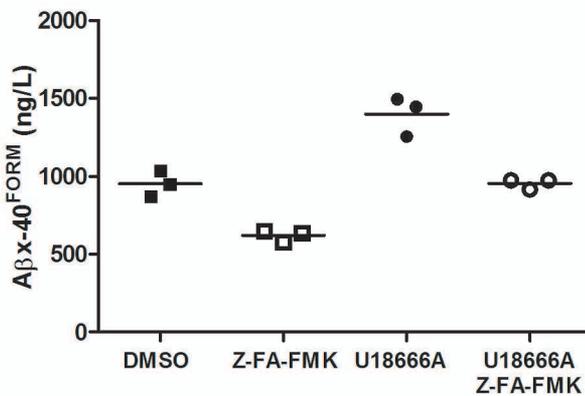


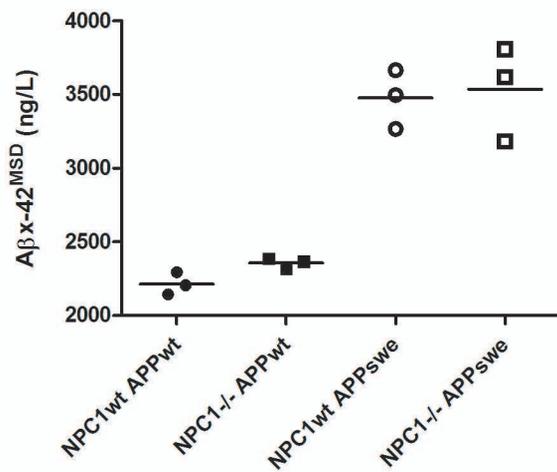
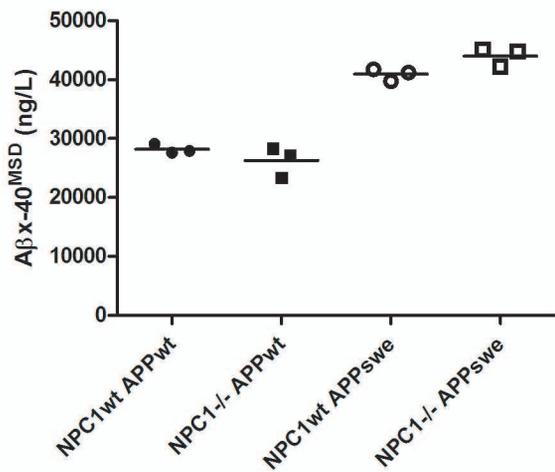
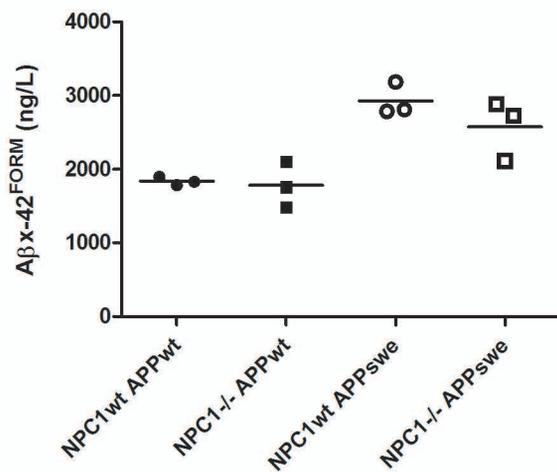
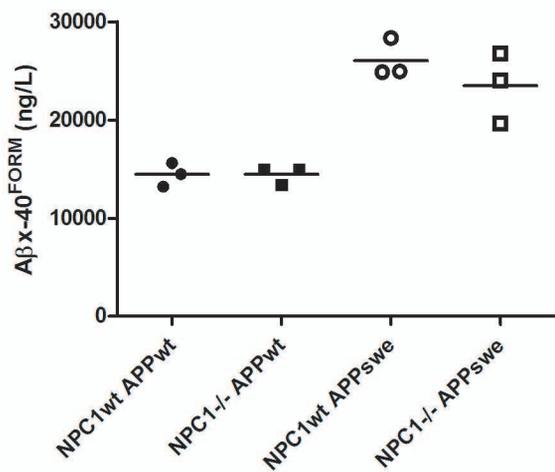
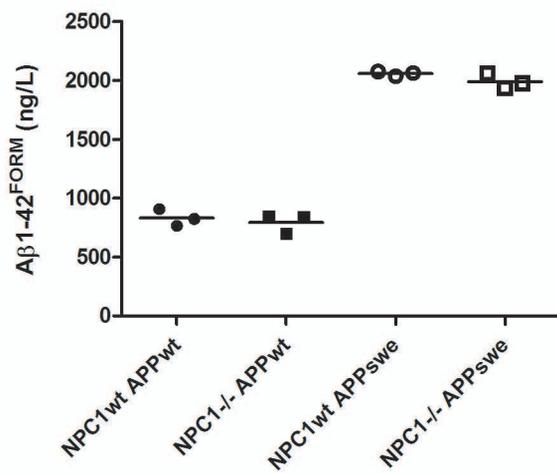
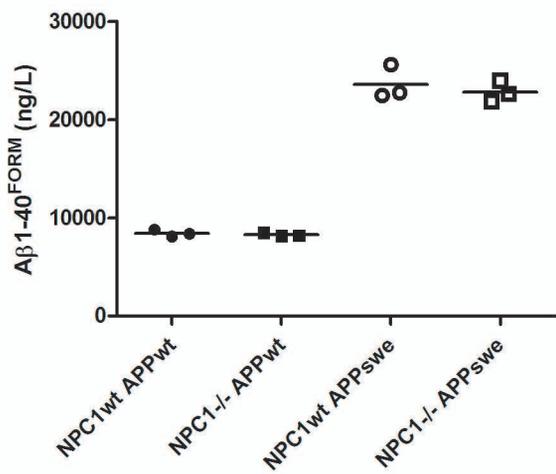


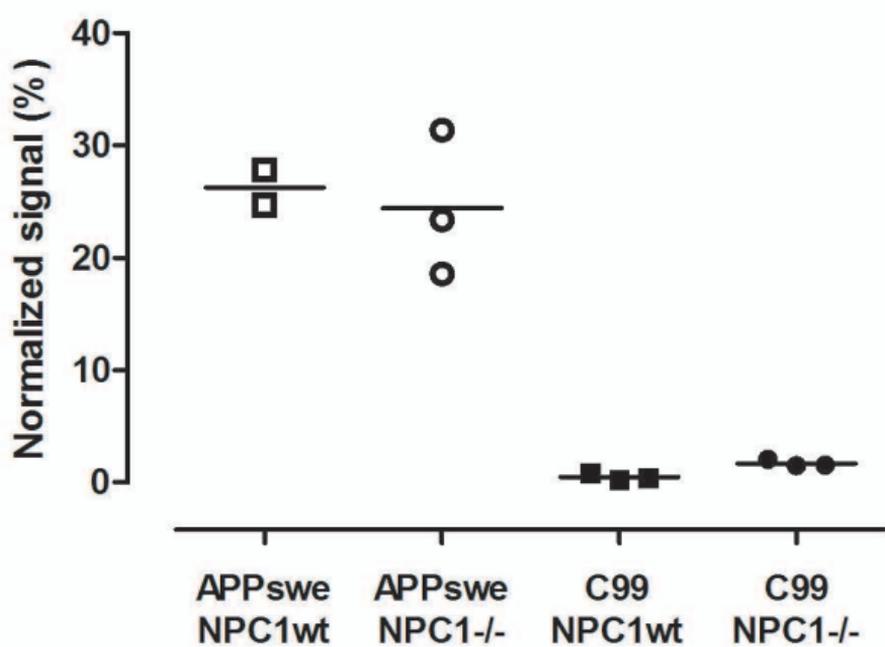
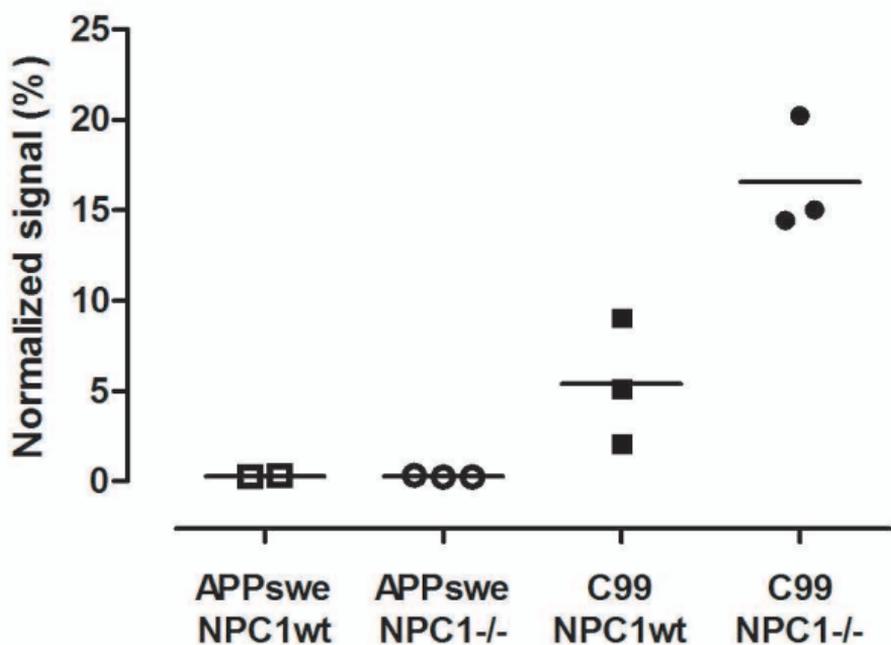
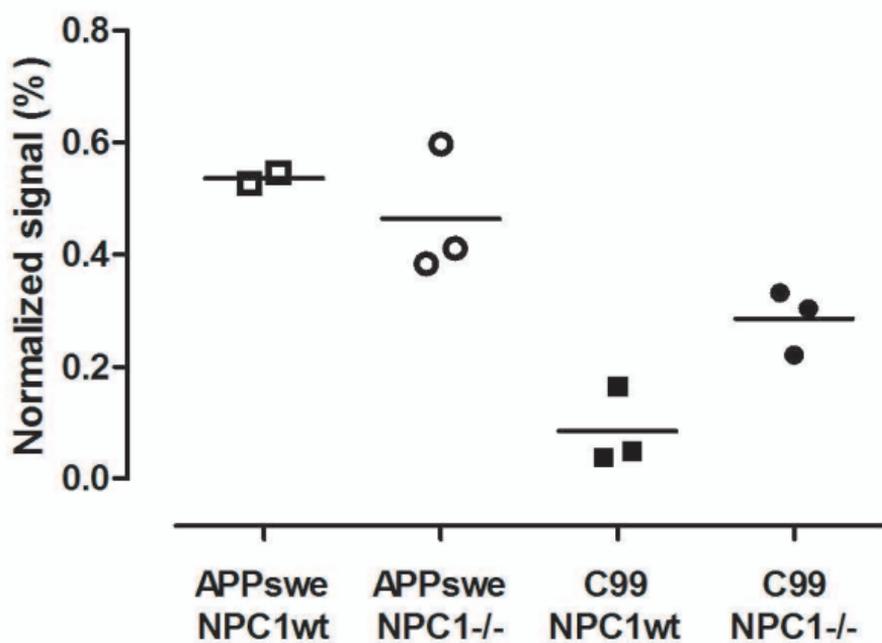


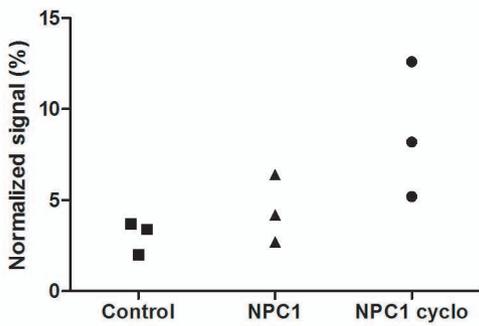
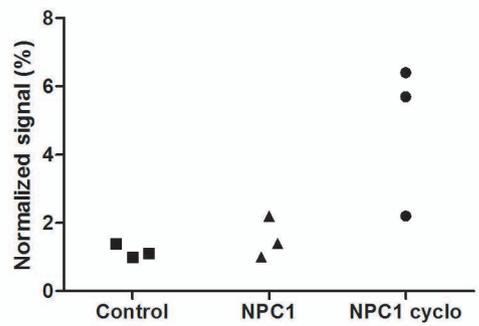
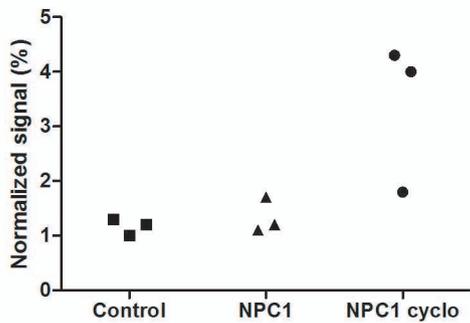
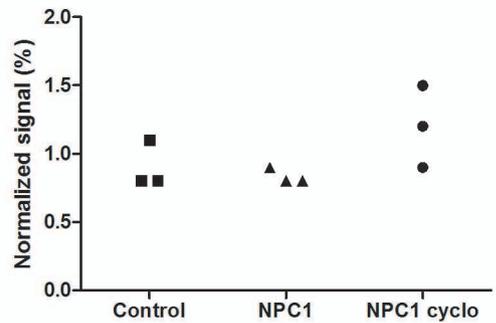
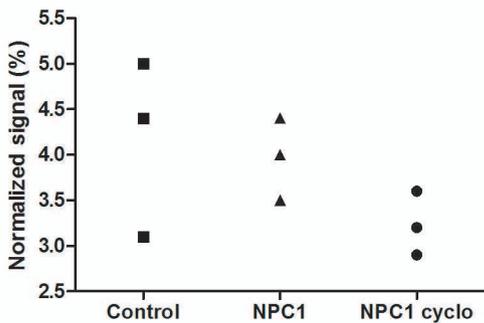
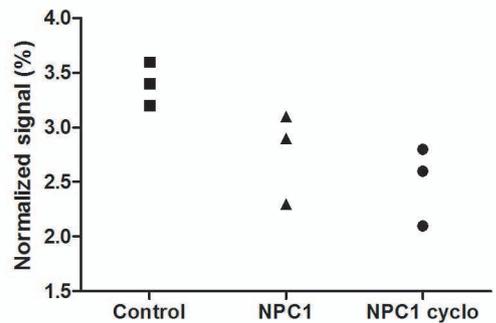
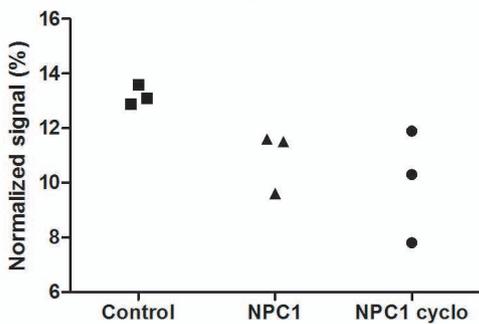
**A****B**



**A****B****C****D****E****F**



**A****A $\beta$ 1-40****B****A $\beta$ 1-41****C****A $\beta$ 1-42**

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