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Cholesterol accumulation in Niemann Pick type C (NPC) model cells causes a shift in APP localization to lipid rafts

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Abstract

It has been suggested that cholesterol may modulate amyloid- β (A β) formation, a causative factor of Alzheimer's disease (AD), by regulating distribution of the three key proteins in the pathogenesis of AD (β -amyloid precursor protein (APP), β -secretase (BACE1) and/or presenilin 1 (PS1)) within lipid rafts. In this work we tested whether cholesterol accumulation upon NPC1 dysfunction, which causes Niemann Pick type C disease (NPC), causes increased partitioning of APP into lipid rafts leading to increased CTF/A β formation in these cholesterol-rich membrane microdomains. To test this we used CHO NPC1^{-/-} cells (NPC cells) and parental CHOwt cells. By sucrose density gradient centrifugation we observed a shift in fl-APP/CTF compartmentalization into lipid raft fractions upon cholesterol accumulation in NPC vs. wt cells. Furthermore, γ -secretase inhibitor treatment significantly increased fl-APP/CTF distribution in raft fractions in NPC vs. wt cells, suggesting that upon cholesterol accumulation in NPC1-null cells increased formation of APP-CTF and its increased processing towards A β occurs in lipid rafts. Our results support that cholesterol overload, such as in NPC disease, leads to increased partitioning of APP/CTF into lipid rafts resulting in increased amyloidogenic processing of APP in these cholesterol-rich membranes. This work adds to the mechanism of the cholesterol-effect on APP processing and the pathogenesis of Alzheimer's disease and supports the role of lipid rafts in these processes.

Keywords

Alzheimer's disease; Amyloid- β ; APP; cholesterol; lipid rafts; Niemann Pick type C disease; NPC1

Introduction

Formation of amyloid- β peptide (A β) is considered to be a central event in the pathogenesis of Alzheimer's disease (AD) [1;2]. A β is generated through the β -secretase pathway that involves sequential cleavage of β -amyloid precursor protein (APP) by β -secretase (BACE1) followed by γ -secretase (consisting of presenilin 1 (PS1), nicastrin, Aph1 and Pen2). Processing of APP by β -secretase generates a membrane bound C-terminal APP-fragment C99/

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CTF β that is further cleaved by γ -secretase to generate A β . In parallel, APP can be processed through the non-amyloidogenic α -secretase pathway which involves a sequential cleavage of APP by α - and γ -secretase. Although the details of APP processing are well understood, it is still not known how the partitioning of APP processing through the α - and β -secretase pathway is regulated and what may trigger APP-cleavage. Indeed, it has been postulated that increasing α -secretase processing of APP and/or decreasing β -secretase cleavage in parallel, may be considered for designing novel therapies against Alzheimer's disease.

Recently, lipid rafts, cholesterol and sphingolipid rich membrane microdomains, have been implicated in the pathogenesis of Alzheimer's disease [3]. It has been shown that lipid rafts serve as a site of A β production [4] and that all three key proteins involved in A β formation (APP, BACE1 and γ -secretase complex components) are localized in lipid rafts [5-8]. The role of lipid rafts in AD has also been supported by evidence that cholesterol may contribute to the pathogenesis of Alzheimer's disease [9]. It has been hypothesized that cholesterol levels may modulate formation of A β by regulating partitioning of APP, BACE1 and/or PS1 to lipid rafts. Indeed, it has been shown that cholesterol depletion disrupts APP [5], BACE1 [6] and PS1 [7] compartmentalization within lipid rafts, leading to decreased A β . In contrast, increased lipid raft localization of APP/BACE1 by either antibody co-patching [4] or GPI-anchoring [10] caused increased β -secretase processing of APP and A β formation. Overall, these studies suggest that lipid raft compartmentalization of APP, BACE1 and/or PS1 may be an important event in regulating amyloidogenic processing of APP leading to A β .

The link between cholesterol and A β has been revealed in Niemann Pick Type C (NPC) disease as well as AD [11-16]. NPC1 dysfunction causes accumulation of free cholesterol in late endosomal/lysosomal compartments that leads to increased C99/A β formation [13;14;16] and a shift in PS1 localization towards early/late endosomes [14;15]. The aim of this work was to assess whether the cholesterol-effect on C99/A β in NPC disease is mediated through lipid rafts. We hypothesized that increased levels of C99/A β upon cholesterol accumulation in NPC disease are due to increased compartmentalization of APP in lipid rafts. Indeed, it has been shown that excessive storage of cholesterol in lysosomes of NPC1-mutant cells/NPC fibroblasts was accompanied by increased partitioning of cholesterol in lipid rafts [17]. Through these studies we aim to elucidate the role of lipid rafts on APP processing and C99/A β production in NPC disease. Our findings may also shed light on the link between cholesterol, lipid rafts and the pathogenesis of Alzheimer's disease.

Materials and Methods

Cell lines, cell tissue culture and transient transfection

Chinese hamster ovary wild type cells (CHOwt) and CHO NPC1-null cells (NPC1^{-/-}, kindly provided by Dr. Daniel Ory) were grown in DMEM:F12 medium (1:1) containing 0.5 mM Na-pyruvate supplemented with 10% FBS, 2 mM L-glutamine and antibiotic/antimycotic solution (all from Invitrogen).

For experiments with γ -secretase inhibitor, cells were treated with 1 μ M DAPT in media 24 hours before lipid raft isolation.

For transient expression, cells were transfected with APPsw 6myc-tagged construct [18] using GeneJuice (Novagen) according to the supplier's instructions. To monitor transfection efficiency between the cell lines, cells were co-transfected with secreted alkaline phosphatase (SEAP) construct (kindly provided by Dr. Stefan Lichtenthaler and Dr. Raphael Kopan). Forty-eight hours after transfection alkaline phosphatase activity was measured in the medium as described previously [19] and corrected for the protein concentration in the cell lysate. Forty-eight hours after transfection cells were collected as indicated in lipid raft isolation protocol.

Lipid raft isolation

Lipid rafts were isolated by centrifugation in a discontinuous sucrose density gradient (5%/30%/40% w/v) [6-8] using zwitterionic (2% CHAPSO) or nonionic (1% Triton X-100, 1% Lubrol-WX) detergent. Briefly, the cells (from five Ø15cm-plates for endogenous APP or from three Ø15cm-plates for overexpressed APP) were washed two times in ice cold PBS and homogenized in 2 ml of MBS buffer (25mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA) containing detergent (1% Triton X-100, 1% Lubrol-WX or 2% CHAPSO) and 1 × protease inhibitor cocktail (Roche Applied Science) by passing 15 times through a 25G needle. After 30 minutes incubation on ice, cell homogenates containing 40% (w/v) sucrose were placed at the bottom of the centrifuge tube and were overlaid with 30% and 5% (w/v) sucrose in MBS buffer. The total volume of gradient was 12 ml (3,5 ml 5%, 5 ml 30% and 3,5 ml of 40% sucrose in MBS). After centrifugation (3h at 175,000 × g, 4°C) 1 ml fractions were collected from the top and lipid raft (flotillin1-positive) and non-raft (transferrin receptor-positive) fractions [20] as well as APP compartmentalization was monitored by western blotting.

Protein and cholesterol levels

Protein (DC Protein Assay, BioRad) and cholesterol levels (AmplexRed Cholesterol Assay, Molecular Probes) were measured in each fraction as indicated in the manufacturer's protocols.

Western blotting

Fraction aliquots were mixed with 6× sample buffer (60% glycerol, 12% SDS, 3.1% DTT, 1/8 v/v 0.5 M Tris pH 6.8, bromphenol blue), heated at 70°C for 10 min and subjected to SDS-PAGE. Lipid raft fractions were identified using a positive (anti-flotillin 1, Sigma-Aldrich) and a negative (anti-transferrin-receptor, Zymed Laboratories Inc.) lipid raft marker. To detect endogenous or exogenous levels of full-length APP (fl-APP) we used 22C11 (Chemicon Int.) or 9E10 (Sigma-Aldrich) antibody, respectively.

Endogenous APP-CTF levels were detected by immunoprecipitation in a co-IP buffer (50mM Tris-HCl, pH 7.6, 150mM NaCl, 2mM EDTA 1% NP40, 0,5% Triton X-100) using a polyclonal C-terminal APP 6687 antibody (kindly provided by Dr. Christian Haass) and Protein A Sepharose at 4°C overnight with constant rocking. The immunoprecipitates were washed twice with co-IP buffer and once with PBS buffer. Samples were heated at 70°C for 10 min in 2× sample buffer and subjected to SDS-PAGE on 10%/16.5% Tris-tricine gels [21]. Proteins were visualized by chemiluminescence using POD chemiluminescence blotting substrate (Roche Applied Science) or SuperSignal West Femto chemiluminescent substrates (Pierce). Western blots were quantified using ImageJ software (National Institutes of Health, USA).

Filipin staining of free cholesterol in cultured cells

The cells were grown on coverslips. They were rinsed twice in PBS, following fixation in 4% PFA (Sigma-Aldrich) in PBS for 20 min at room temperature. The cells were rinsed in PBS again and incubated with filipin (Sigma-Aldrich) working solution (100 µg/ml filipin in 10% FBS in PBS) for 1 h in the dark at room temperature [22]. After washing the cells twice in PBS and once in deionized water, the cells were mounted (Polyvinyl alcohol mounting medium with DABCO antifading, Fluka) and were viewed by fluorescence microscopy (Olympus BX51).

Glycosphingolipid staining in cultured cells

Glycosphingolipid (GSL) staining was performed using BODIPY-LacCer [23] following the protocol available at http://mayoresearch.mayo.edu/mayo/research/pagano_lab/protocols.cfm. After fixation, the

cells were mounted and viewed by confocal microscope Leica TCS SP2 AOBS (excitation 450-490nm, emission 520-560 nm).

Results

Lipid raft isolation – 1% Triton X-100 enables clear separation of lipid raft from non-raft fractions

To investigate whether the cholesterol-effect on APP processing upon NPC1 dysfunction is mediated through lipid rafts we analyzed APP compartmentalization in lipid rafts by sucrose density gradient in CHOwt and CHO NPC1^{-/-} cells. In agreement with previous studies [23; 24], CHO NPC1^{-/-} cells exert NPC-like phenotype (Fig. 1): they show 2-fold increase in free cholesterol levels (Fig. 1A), cholesterol accumulation in punctuate endocytic structures (Fig. 1B) and mistrafficking of glycosphingolipids (GSLs) to late/endosome lysosome compartments compared to its Golgi staining in CHOwt cells (Fig. 1B).

Since lipid rafts are biochemically characterized as detergent-resistant membranes (DRMs) to isolate lipid rafts we employed three different detergents: 1% Triton X-100 (that has been the most widely used), 1% Lubrol-WX and 2% CHAPSO (Fig. 2A). These detergents were previously used to analyze APP, BACE1 and/or PS1 localization in lipid rafts [5-8]. We, firstly, tested which of the detergents enables clear separation between raft (flotilin1-positive) and non-raft fractions (transferrin receptor-positive, TfR) and, secondly, using different detergents we wanted to be sure that APP compartmentalization to lipid rafts is not detergent-specific. After centrifugation in a sucrose density gradient fractions were collected from the top and were analyzed by western blotting. Flotilin1, a lipid raft marker, was mainly localized in fraction 4 using all three detergents in both CHOwt and NPC1^{-/-} cells. In NPC1^{-/-} cells we also observed a strong flotilin1 signal in fraction 5 (among all three detergents), suggesting that cholesterol accumulation upon NPC1 loss may lead to increased lipid raft formation in NPC1^{-/-} cells. Indeed, Lusa *et al.* [17] have previously reported enhanced association of cholesterol with lipid rafts in NPC mutant cells and NPC fibroblasts. A negative lipid raft marker, transferrin-receptor (TfR), was mainly localized in non-raft fractions (fractions 9-12) when 1% Triton X-100 was used, while in 1% Lubrol-WX or 2% CHAPSO detergent it was distributed in both raft (fraction 4) and non-raft fractions (fractions 9-12). These results showed that among the three detergents used, 1% Triton X-100 enabled clear separation between lipid raft and non-raft fractions.

By measuring protein and total cholesterol levels in each fraction we further confirmed that fraction 4 is, indeed, a lipid raft fraction. As shown in Fig. 2B, fraction 4 contained high cholesterol levels and low protein levels compared to other fractions in both CHOwt and NPC1^{-/-} cells. In addition, total cholesterol levels in fraction 4 of NPC1^{-/-} cells were markedly increased compared to the levels in fraction 4 of CHOwt cells, indicating that increased cholesterol in late endosomal/lysosomal compartments upon NPC1 loss is mainly associated with lipid rafts, consistent with findings by Lusa *et al.* [17]. When 1% Lubrol-WX or 2% CHAPSO were used we also observed a substantial increase of cholesterol in fraction 3 in NPC1^{-/-} vs. CHOwt cells (Fig. 2B).

In CHO NPC1^{-/-} cells more APP cofractionates with flotilin positive lipid raft fractions

Next, we analyzed lipid raft distribution of endogenous full-length APP (fl-APP) between CHOwt and CHO NPC1^{-/-} cells. We hypothesized that increased cholesterol levels in NPC1^{-/-} cells cause increased partitioning of APP in lipid rafts. Surprisingly, we did not detect any endogenous fl-APP levels in lipid raft fractions (fraction 4) in both CHOwt and NPC1^{-/-} cells when using all three different detergents (Fig. 2A). Fl-APP was localized in non-raft fractions (fractions 9-12) in both CHOwt and in NPC1^{-/-} cells (Fig. 2A). To further test whether

non-raft distribution of fl-APP in CHO cells was due to its low endogenous levels, unabling the detection of a small proportion of APP in lipid rafts, we overexpressed APP in CHOwt and CHO NPC1^{-/-} cells and monitored its lipid raft distribution as described. Indeed, in APPsw-6myc transfected cells a small fraction of fl-APP was observed in the lipid raft fraction (fraction 4) in both CHOwt and NPC1^{-/-} cells (Fig. 3). Longer exposure revealed that in NPC1^{-/-} cells fl-APP distribution in a sucrose gradient ranged from fraction 3-6, while in CHOwt cells it was mainly localized in fraction 4. This shift in fl-APP redistribution towards cholesterol-rich fractions was paralleled with increased flotilin1 staining in these fractions in NPC1^{-/-} cells compared to CHOwt (Fig. 3). Furthermore, in APP-overexpressing cells we observed slightly increased compartmentalization of APP C-terminal fragments (CTFs) in lipid rafts in NPC1^{-/-} cells vs. CHOwt (Fig. 3). As observed for fl-APP, in NPC1^{-/-} cells CTFs were redistributed to fractions 3-6, while in CHOwt cells they cofractionated with fraction 4 mainly. These results indicated that under cholesterol-loaded conditions, such as in NPC1^{-/-} cells, a small portion of fl-APP/CTFs could be redistributed to lipid rafts. These findings were further confirmed on endogenous levels by immunoprecipitating APP/CTFs in lipid raft (fraction 4) and non-raft (fraction 11) fractions (Fig. 4, see next paragraph)

Increased formation of APP-CTF upon cholesterol accumulation in CHO NPC1^{-/-} cells involves lipid rafts

To further analyze whether increased formation of C99 and, thus, A β upon cholesterol accumulation in NPC1^{-/-} cells involves lipid rafts, we analyzed the levels of endogenous APP-CTF (C83/C99) fragments, a direct γ -secretase substrates, in raft (fraction 4) and non-raft (fraction 11) fractions of CHOwt and CHO NPC1^{-/-} cells both under normal (DAPT-untreated) as well as under γ -secretase inhibited conditions (DAPT-treated) (Fig. 4). Endogenous fl-APP and APP-CTF levels (C83/C99) were immunoprecipitated using C-terminal antibody 6687 (kindly provided by dr. C. Haass). Since hamster N-terminal C99 sequence differs from human, we were not able to use 6E10 antibody in order to differentiate between endogenous C83 and C99 levels. We observed increased levels of immunoprecipitated full-length APP in the lipid raft fraction (fraction 4) in NPC1^{-/-} vs. wt cells both under DAPT-untreated and DAPT-treated ($p < 0.01$) conditions (Figs. 4A and B). As expected, and in accordance with previous studies, we observed increased levels of endogenous APP-CTFs in NPC1^{-/-} vs. CHOwt cells (fractions 4 and 11, Fig. 4A). Significantly increased distribution of APP-CTFs towards lipid rafts (fraction 4) in NPC1^{-/-} cells vs. CHOwt was observed both under DAPT-untreated ($p < 0.01$) and DAPT-treated ($p < 0.05$) conditions (Figs. 4A and B), indicating that cholesterol accumulation upon NPC1 loss causes a shift in amyloidogenic processing of APP towards lipid rafts. Importantly, under γ -secretase inhibitor treatment (DAPT, 1 μ M) NPC1^{-/-} cells showed a marked increase of APP-CTFs in lipid raft fraction (fraction 4) compared to DAPT-treated CHOwt cells (Figs. 4A and B), suggesting that upon cholesterol accumulation in NPC1-null cells increased formation of APP-CTF and its increased processing towards A β likely occurs in lipid rafts. Overall, these results show that cholesterol accumulation upon NPC1 dysfunction leads to increased partitioning of fl-APP/CTF into lipid rafts.

Discussion

Lipid rafts could be a missing link between cholesterol and Alzheimer's disease (AD). The importance of these cholesterol-rich microdomains on APP processing has been highlighted by the fact that three key proteins in the pathogenesis of AD (APP, BACE1 and PS1) are found in lipid rafts and that cholesterol depletion disrupts this association leading to decreased A β [5-8]. Niemann Pick type C disease (NPC), a lysosomal storage disorder in which cholesterol accumulation in late endosomal/lysosomal compartments due to NPC1/2 dysfunction leads to increased C99/A β formation [13;14;16], has become an important model to study the link between cholesterol, APP processing and Alzheimer's disease. In this work we show that

cholesterol accumulation upon NPC1 dysfunction leads to increased partitioning of APP/CTF to lipid rafts. Our results suggest that upon cholesterol-loaded conditions, such as those seen in NPC disease, lipid rafts are likely to be the site of increased CTF/A β formation, raising the possibility that the cholesterol-effect on APP processing and AD may be mediated through lipid rafts.

While previous studies have analyzed the effect of cholesterol depletion (using different drug treatments) on lipid raft association of APP, BACE1 and/or PS1 proteins [5-7], we employed NPC disease, in which disturbed cholesterol homeostasis leads to cholesterol accumulation and increased C99/A β formation [13;14;16], to study the link between cholesterol, lipid rafts and APP processing. In our analyses we used NPC model cells: CHO cells in which *NPC1* gene has been deleted (CHO NPC1^{-/-} cells). Lusa *et al.* [17] have previously observed increased cholesterol association in lipid raft fractions in NPC mutant cells and NPC fibroblasts. We hypothesized that under these conditions cholesterol mediates raft oligomerization (or raft co-patching) that may lead to increased APP/BACE1/PS1 compartmentalization within the same lipid raft, resulting in increased C99/A β . Indeed, we have recently found that increased C99/A β formation in CHO NPC1^{-/-} cells is related to cholesterol levels (Malnar *et al.*, unpublished results). In this work we tested whether altered amyloidogenic processing of APP upon cholesterol accumulation in NPC1^{-/-} cells involves lipid rafts and whether this may be due to altered partitioning of APP/CTF within these cholesterol-rich membrane microdomains upon NPC1 dysfunction. In contrast to previous studies that analyzed compartmentalization to lipid rafts of overexpressed APP, BACE1 and/or PS1 proteins [5-8], we monitored lipid raft distribution of endogenous as well as exogenous APP/CTFs in CHOwt and CHO NPC1^{-/-} cells. Using sucrose density gradient centrifugation we show that under cholesterol-loaded conditions (upon NPC1 dysfunction) both fl-APP and CTFs are redistributed to lipid rafts in CHO NPC1^{-/-} cells compared to wt cells. Indeed, immunoprecipitation of endogenous fl-APP/CTFs revealed that more fl-APP/CTFs cofractionated with raft fractions in NPC1^{-/-} cells vs. CHOwt, suggesting that cholesterol accumulation shifts their compartmentalization to lipid rafts. Although only a small portion of fl-APP/CTFs was observed in lipid raft fractions either when APP/CTFs was immunoprecipitated or when APP was overexpressed, probably because it is further processed, using γ -secretase inhibitor DAPT we showed that CTFs grossly accumulate in lipid raft fractions in NPC1^{-/-} cells. This finding confirms that lipid rafts are likely the site of increased amyloidogenic processing of APP leading to increased A β upon NPC1 dysfunction. Indeed, antibody co-patching or GPI-anchoring studies have shown that increased APP and BACE1 targeting to lipid rafts leads to increased A β [4;10], suggesting that lipid rafts may be a major site of A β production. Our findings support this and show that under cholesterol loaded conditions, such as in NPC disease, APP/CTFs are shifted towards lipid rafts resulting in increased processing of CTFs in these cholesterol-rich membrane microdomains. In agreement with our observation, Yamazaki *et al.* have previously shown that increased A β in NPC model cells (U18666A-treated CHO cells) is found in cholesterol-rich (a lipid raft) fraction [13]. Whether cholesterol accumulation upon NPC1 dysfunction leads to enhanced APP/BACE1/PS1 colocalization in lipid rafts remains to be determined.

Conclusions

We show that cholesterol accumulation upon NPC1 dysfunction leads to increased partitioning of APP/CTFs to lipid rafts. Finding significantly increased levels of CTFs in lipid rafts of NPC1^{-/-} cells vs. CHOwt under γ -secretase inhibitor treatment further indicates that aberrant production of A β upon NPC1 dysfunction most likely occurs in lipid rafts. This work adds to the mechanism of the cholesterol-effect on APP processing and the pathogenesis of Alzheimer's disease and supports the role of lipid rafts in these processes.

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Figure 1. CHO NPC1^{-/-} cells exert characteristic NPC phenotype - cholesterol accumulation in punctuate endocytic structures and mistrafficking of glycosphingolipids to late/endosome lysosome compartments

A, Cholesterol levels were measured using Amplex Red Cholesterol Assay kit (Molecular probes). Free cholesterol levels were significantly increased ($p < 0.001$) in NPC1^{-/-} cells vs. CHOwt. **B**, NPC1^{-/-} cells show a characteristic accumulation of free cholesterol and glycosphingolipids (GSLs) in punctuate endocytic structures compared to CHOwt cells. Accumulation of free cholesterol was monitored by filipin staining, while GSLs were analyzed using BODIPY-LacCer as indicated in Materials and Methods.

Figure 2. Lipid raft isolation using 1% Triton X-100 enables clear separation of lipid raft from non-raft fractions

A, Isolation of lipid rafts by centrifugation in a sucrose density gradient using different detergents (1% Triton X-100, 1% Lubrol WX or 2% CHAPSO). After centrifugation in a discontinuous sucrose density gradient (5%/ 30%/40% w/v), 12 fractions were collected from the top. Equal volume fractions were analyzed by immunoblotting using the following antibodies: Flotilin1 (Flt1 Sigma-Aldrich) – a positive lipid raft marker, transferrin-receptor (TfR, Zymed) – a negative lipid raft marker and 22C11 (Chemicon) for full-length APP (fl-APP). Flt1, a lipid raft marker, was mainly detected in fraction 4 using all three detergents. In CHO NPC1^{-/-} cells Flt1 staining in fraction 5 was observed as well. When 1% Triton X-100

was used TfR was localized in non-raft fractions (fractions 9-12), while in 1% Lubrol-WX and 2% CHAPSO detergent TfR was detected in lipid raft and non-raft fractions. Endogenous fl-APP was localized in non-raft fractions (fractions 9-12) using all three detergents. **B**, Analysis of cholesterol and protein levels. Protein levels (marked dark grey) were determined using DC Protein Assay (BioRad), while cholesterol levels (marked light grey) were analyzed using AmplexRed Cholesterol Assay kit (Molecular Probes/Invitrogen). Note that in fraction 4 we observed the highest cholesterol levels and lower protein levels compared to other fractions, proving that fraction 4 is, indeed, a lipid raft fraction. All experiments were performed three times and similar cholesterol/protein patterns were observed.

Figure 3. Overexpression of APP reveals increased partitioning of fl-APP/CTFs into lipid raft fractions of CHO NPC1^{-/-} vs. CHOwt cells

Cells were transiently transfected with APPsw-6myc construct. Lipid raft isolation using 1% Triton X-100 was performed as in Fig.2. Equal volume fractions were analyzed by immunoblotting using the following antibodies: Flotilin1 (Flt1 Sigma-Aldrich) – a positive lipid raft marker, transferrin-receptor (TfR, Zymed) – a negative lipid raft marker and 9E10 (Sigma-Aldrich) for full-length APP (fl-APP) and APP C-terminal fragments (CTFs). We observed increased cofractionation of fl-APP and CTFs with flotilin1 lipid raft marker in CHO NPC1^{-/-} compared to CHOwt cells.

Figure 4. Cholesterol accumulation upon NPC1 loss of function leads to increased distribution of endogenous fl-APP/CTFs towards lipid rafts

A. Lipid raft (fraction 4) and non-raft fractions (fraction 11) of DAPT-untreated and DAPT-treated CHOwt and CHO NPC1^{-/-} cells were immunoprecipitated using the C-terminal APP antibody 6687. As observed previously CTF levels were markedly increased in NPC1^{-/-} cells vs. CHOwt. In addition, we observed increased levels of both fl-APP and CTFs in lipid raft fraction (fraction 4) of NPC1^{-/-} cells vs. CHOwt under DAPT-untreated and DAPT-treated conditions. **B.** Western blots were quantified using ImageJ software. Portion of APP/CTFs in lipid raft fraction for each cell line under normal and DAPT-treated conditions is shown. Fl-APP was significantly increased ($p < 0.01$) in lipid raft fraction of DAPT-treated CHO NPC1^{-/-} cells, while CTF distribution within lipid raft fractions was significantly increased in both DAPT-untreated and DAPT-treated CHO NPC1^{-/-} cells ($p < 0.01$ and $p < 0.05$, respectively).