

Neuronal membrane cholesterol loss enhances amyloid peptide generation

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Recent experimental and clinical retrospective studies support the view that reduction of brain cholesterol protects against Alzheimer's disease (AD). However, genetic and pharmacological evidence indicates that low brain cholesterol leads to neurodegeneration. This apparent contradiction prompted us to analyze the role of neuronal cholesterol in amyloid peptide generation in experimental systems that closely resemble physiological and pathological situations. We show that, in the hippocampus of control human and transgenic mice, only a small pool of endogenous APP and its β -secretase, BACE 1,

are found in the same membrane environment. Much higher levels of BACE 1-APP colocalization is found in hippocampal membranes from AD patients or in rodent hippocampal neurons with a moderate reduction of membrane cholesterol. Their increased colocalization is associated with elevated production of amyloid peptide. These results suggest that loss of neuronal membrane cholesterol contributes to excessive amyloidogenesis in AD and pave the way for the identification of the cause of cholesterol loss and for the development of specific therapeutic strategies.

Introduction

A large body of evidence indicates that changes in cholesterol homeostasis affect APP metabolism. More than a decade ago Roses and colleagues (Corder et al., 1993) demonstrated the existence of a genetic link between the risk of AD and the $\epsilon 4$ allele of apolipoprotein E, a protein involved in cholesterol homeostasis (Myers and Goate, 2001). In cells in culture, overexpressed APP and its β and γ cleaving enzymes have been found in cholesterol-rich regions of cell membranes, known as detergent-resistant membrane (DRM) microdomains or rafts (Burns and Duff, 2002; Eehalt et al., 2003). In agreement with such colocalization having a functional relevance, treatment of these cells with cholesterol synthesis inhibitors and membrane cholesterol extracting drugs results in a drastic reduction of A β production (Simons et al., 1998; Fassbender et al., 2001; Eehalt et al., 2003). These studies have led to

the view that the principal site of A β production in the cell are the cholesterol rich membrane areas and that high brain cholesterol can contribute to AD by increasing the number of sites where A β generation can occur. This conclusion is supported by retrospective clinical studies showing that people with high blood-cholesterol levels treated with the cholesterol-lowering drugs statins present a reduced incidence of AD (Jick et al., 2000; Wolozin et al., 2000; Austen et al., 2002).

Contrary to the above view, *in vivo* evidence implies that lowering brain cholesterol can lead to AD. Rodents treated with the cholesterol-synthesis inhibitor lovastatin, the statin that is most permeable to the blood brain barrier, have increased amyloid production and senile plaque deposition (Park et al., 2003). Moreover, A β aggregation has been also found in Niemann Pick disease type C (Burns and Duff, 2002), a disorder characterized by the accumulation of cholesterol in late endosomes and lysosomes and a reduction of cholesterol on the axonal membrane (Karten et al., 2002). In the genetic disorder known as RSH/Smith-Lemli-Opitz syndrome, an accumulation of 7-dehydrocholesterol and a paucity of cholesterol cause severe neurodegeneration and early death (Porter, 2000). The altered gene in this disease, which is responsible for the conversion of 7-dehydrocholesterol into cholesterol, is homolo-

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Abbreviations used in this paper: A β , amyloid peptide; AD, Alzheimer's disease; β -CTF, β -COOH-terminal fragment; CNS, central nervous system; DRM, detergent-resistant membrane; MCD, methyl- β -cyclodextrin; SFV-APP, Semliki Forest virus/human APP.

gous to seladin-1, which is abnormally down-regulated in the neurons of AD patients in areas of the brain showing the highest levels of amyloid deposition (Greeve et al., 2000; Iivonen et al., 2002). This finding is consistent with the recent observation that the hippocampus of certain AD patients present a moderate, yet significant, reduction in membrane cholesterol (Ledezma et al., 2003). The precise role of neuronal membrane cholesterol in A β peptide production in the physiological scenario of constitutive protein expression in neuronal cells therefore remains to be determined.

Results

Endogenous BACE 1 and APP copartition in detergent-soluble membrane fractions of human and rodent brain membranes

To determine the possible involvement of membrane cholesterol in APP processing in physiological conditions, we started by analyzing the buoyant flotation density of this protein and that of its β -secretase BACE 1 in human brain hippocampal membranes. Sucrose gradient centrifugation of membranes extracted with the detergents Lubrol, CHAPS, or Triton X-100, followed by SDS-PAGE and Western blotting with NH₂ terminus antibody (see Materials and methods), revealed that most APP is in heavy, detergent-soluble membrane fractions (Fig. 1 A; Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200404149/DC1>). By contrast, a significant amount of BACE 1 is also present in light, detergent-resistant fractions, similarly to the DRM marker flotilin 1 (Fig. 1 A). The existence of a pool of BACE 1 in DRMs and another in non-DRMs, similarly to other canonical DRM markers (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200404149/DC1>), confirms that the protein is in a dynamic balance on the membrane. On the other hand, the almost complete absence of APP in DRMs suggests that this protein is largely restricted to the most fluid environment of non-DRMs.

To establish if endogenous APP is undetected in DRMs because of true natural low cholesterol-partitioning affinity or because of secondary events that affected its biochemical characteristics, i.e., due to the conservation process, age or associated diseases, the flotation density of APP was analyzed in freshly prepared hippocampal membranes from human APP transgenic mice, that express moderate levels of the protein without plaque or tangle pathology (Mucke et al., 2000). The results obtained were similar to those with the human membranes: APP is present in heavy fractions and undetectable in DRMs (Fig. 1 B).

To rule out that APP is undetected in DRMs because of the low concentration in our preparation of membranes from Golgi stacks or endosomes, that have been postulated as the sites where β -cleavage takes place (Xu et al., 1997; Daugherty and Green, 2001) membranes from these organelles were purified from hAPP mice brains (see Materials and methods). As with the total membrane extract only a significant pool of BACE 1, but not APP, is present in DRMs (Fig. 1 C).

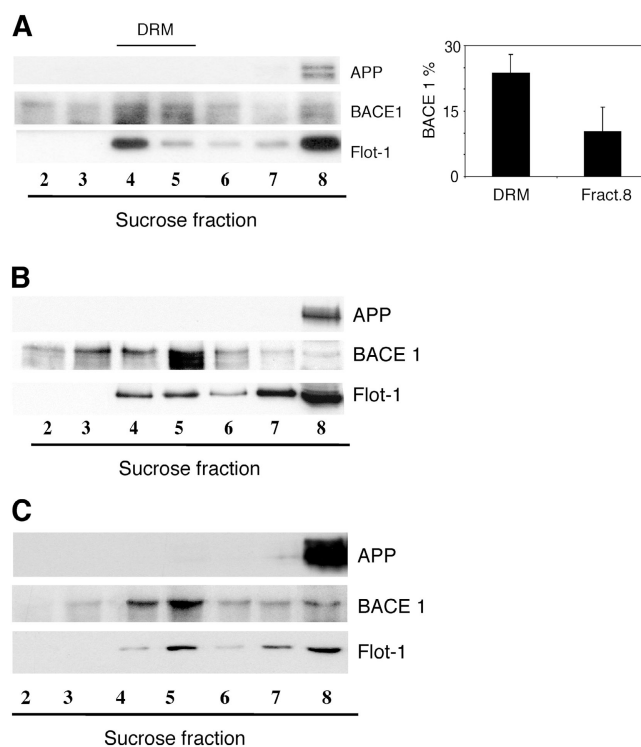


Figure 1. BACE 1 floats in the DRM fraction from control human and mice brain membranes, whereas APP remains in non-DRM heavy fractions. (A) Immunoblots for APP, BACE 1, and flotilin1 in a representative control human hippocampal sample after Lubrol WX extraction and sucrose gradient centrifugation. Note that BACE 1 is enriched in fractions 4 and 5 corresponding to DRMs as indicated by the presence of the DRM marker flotilin 1 (Flot-1). APP, on the contrary, is only detected in heavy fraction 8. The percentages of total BACE 1 in DRMs and fraction 8 are shown in the graph as means and SDs from 10 control human samples. (B) Immunoblots for APP, BACE 1, and flotilin 1 of hippocampal extracts from mice expressing the human APP after Lubrol WX extraction and sucrose gradient centrifugation. As for the human brain, BACE 1 is enriched in fractions 4 and 5 corresponding to DRMs as indicated by the presence of the DRM marker flotilin 1 (Flot-1), whereas APP is only detected in heavy fraction 8. (C) Immunoblots for APP, BACE 1, and flotilin 1 of Golgi-endosomal-enriched brain membranes from mice expressing the human APP after Lubrol WX extraction and sucrose gradient centrifugation. Although BACE 1 floats to DRM light fractions similar to flotilin 1, APP remains in the heavy fractions of the gradient.

Considering that a pool of APP has been reported to partition in DRMs in a number of cell types under overexpression conditions (Ehehalt et al., 2003), we analyzed if increasing APP levels in neurons would suffice for its incorporation into these domains. Thus, human APP was expressed transiently via the Semliki Forest virus vector (De Strooper et al., 1995) in pseudo-neuronal and primary neurons in culture. Fig. 2 A shows that only a small percentage of the protein is in DRMs (<5%) in overexpressing undifferentiated N2A rodent neuroblastoma cells. On the other hand, APP remains undetectable in DRMs of both overexpressing SH-SY5Y human neuroblastoma cells and rat hippocampal neurons in primary culture (Fig. 2, B–D).

Together, these first series of results point in the direction that non-DRM domains of brain cells are the preferred sites for the interaction between BACE 1 and its substratum APP.

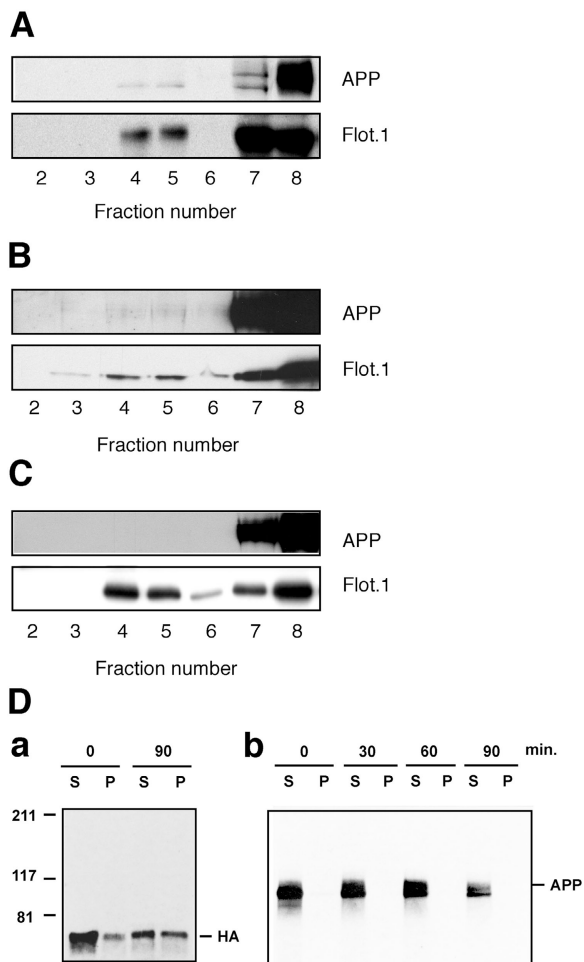


Figure 2. Overexpression of human APP leads to the incorporation of the protein in DRMs from rodent neuroblastoma N2A cells but not in human neuroblastoma SH-SY5Y cells or rat hippocampal neurons in primary culture. Nondifferentiated N2A cells (A), nondifferentiated SH-SY5Y cells (B) and mature primary rat hippocampal neurons (C) were infected with SFV-APP for 8 h. Cell extracts were detergent extracted at 4°C and centrifuged in sucrose gradients. DRMs were obtained in fractions 4 and 5 as indicated by the enrichment of the DRM marker flotillin1. Although a small amount of human APP (<5%) appears in DRMs from overexpressing N2A cells, no significant amount of the protein was found in the DRM fractions from SH-SY5Y or primary neurons even when the levels of APP overexpression are very high (see fractions 7 and 8 of the gradients). The absence of overexpressed APP in detergent insoluble membranes of primary hippocampal neurons was further confirmed along the biosynthetic pathway with a pulse-chase experiment after metabolic labeling (D). Mature neurons were infected either with Fowl plague virus to express the DRM marker HA (D, a) or with SFV-APP (D, b) and extracted with 20 mM CHAPS at 4°C. CHAPS-soluble material (S) and insoluble (P) was resolved in SDS-PAGE (6%) and the images obtained by autoradiography. Although HA CHAPS insolubility increases during the biosynthetic pathway (D, a) and is evident after 90-min chase, when the protein has already reached the plasma membrane, APP remains CHAPS soluble along the biosynthetic pathway and transport to the membrane (D, b).

A minor pool of BACE 1 and APP colocalize on the surface of hippocampal neurons in culture

Because differences in detergent partitioning cannot be used as the sole parameter to conclude that a given protein is present or excluded from a particular domain of the membrane (Zurzolo et al., 2003), we investigated next the distribution of

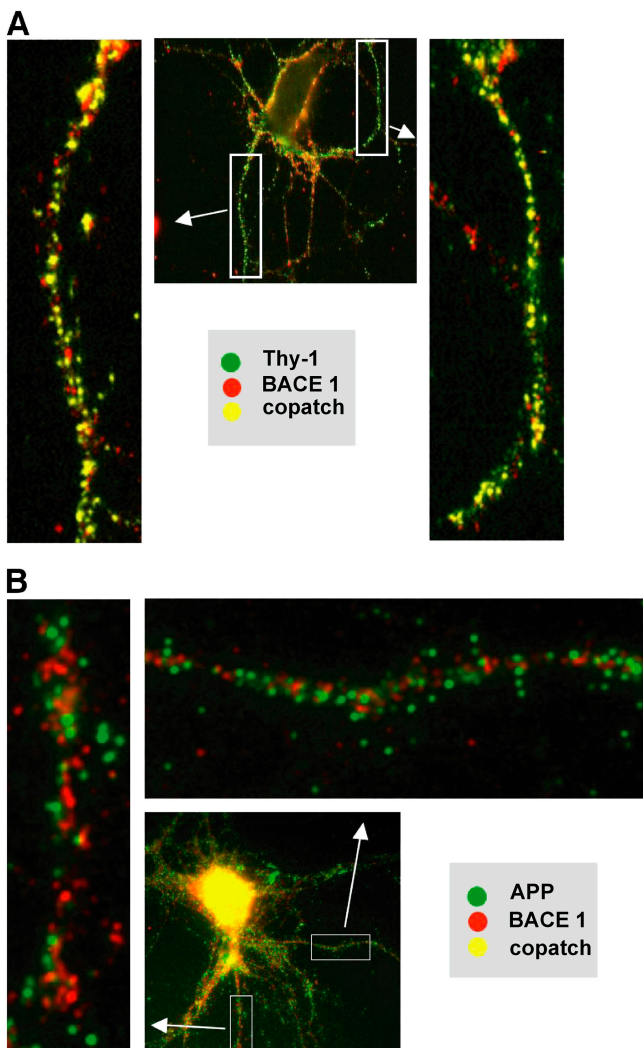


Figure 3. BACE 1 copatches with the DRM marker Thy-1 but is largely segregated from APP in the neuronal plasma membrane. Hippocampal neurons were cultured for 10 d and pairs of membrane proteins were studied by the copatching technique (see Materials and methods). (A) BACE 1 and the DRM marker Thy-1 copatch extensively, indicating that both molecules are located in membrane DRM domains (note yellow dots in the enlarged images). (B) APP and BACE 1, in contrast, appear extensively segregated.

endogenous APP and BACE 1 on the plasma membrane of living neurons in culture. Thus, antibody “copatching” (Harder et al., 1998; Ehehalt et al., 2003) for these molecules was performed in live rodent hippocampal neurons in culture (see Materials and methods). This work revealed that only 7% of APP positive dots exactly colocalize with BACE 1 clusters (Fig. 3 B; Fig. 5 for details and quantitation; Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200404149/DC1>). Because APP is not detected in DRMs by biochemistry (Fig. 1), it is quite likely that the few coclusters revealed in the microscopy assay reflect non-DRM coexistence. Further confirming the biochemical differences, a large proportion of BACE 1 on the neuronal surface is in DRMs, as judged from the extensive copatching with Thy-1 (Fig. 3 A). Together, with the previously demonstrated paucity of APP in the DRMs of Golgi and endosomal membranes (Fig. 1 C), this first series of results is

consistent with the view that non-DRM domains of the plasma or internal membranes are the preferred sites for BACE 1–APP interaction.

APP and BACE 1 biochemical and spatial association is enhanced in conditions of low membrane cholesterol

To determine if the cleavage of APP can be affected by changes in membrane cholesterol content we analyzed the flotation density of BACE 1 and APP in hippocampal membranes from the brains of a group of AD patients that present a moderate, still significant, reduction of brain membrane cholesterol (30% less than controls; Ledesma et al., 2003). Fig. 4 A shows that in these membranes APP remains concentrated in detergent-soluble fractions, like in the control situation. Quite differently from controls, BACE 1 is significantly reduced from the light detergent-resistant fractions and increased in the soluble fractions (Fig. 4 A, compare the partitioning profile of both proteins with Fig. 1 A). Densitometric quantification reveals that in the low cholesterol membranes there is a 50% increase ($P < 0.005$) in the amount of BACE 1 present in the soluble fractions where APP also concentrates (compare Fig. 1 A with Fig. 4 A). The fact that the total amount of BACE 1, measuring both insoluble and soluble fractions, is similar in low cholesterol AD samples and controls, indicates that the loss of BACE 1 from DRMs is not the consequence of changes in its rates of synthesis or degradation but results from a change in DRM organization. In agreement, a significant pool of flotilin 1 is displaced to the soluble fractions in the low cholesterol human membranes (Fig. 4 A).

To determine if BACE 1 reduction from DRM in the low cholesterol human membranes is the direct consequence of cholesterol loss, we studied the partitioning characteristics of BACE 1 and APP in rodent hippocampal neurons in culture induced to lose 30% of membrane cholesterol (Ledesma et al., 2003), similar to the constitutive loss of the AD membranes. Identical to the human membranes, moderate membrane cholesterol reduction in rodent neurons results in a significant loss of BACE 1 from DRMs, and increase in the soluble, APP-containing fractions (Fig. 4 B).

To test whether enhanced BACE 1–APP copartitioning due to cholesterol reduction results in higher spatial association, living hippocampal neurons with low and normal cholesterol membrane levels (see Materials and methods; Ledesma et al., 2003), were incubated with specific antibodies and the pattern of colocalization analyzed by immunofluorescence microscopy (Fig. 5, A and B; Fig. S3). Quantitative analysis showed that in low cholesterol neurons 18% of APP clusters colocalize completely with those containing BACE 1, whereas an additional 21% of APP positive areas partially colocalize (Fig. 5 C). In cells with normal-cholesterol levels only 7% completely copatch and 12% partially copatch ($P < 0.005$). The enhanced colocalization cannot be attributed to membrane shrinkage, due to the reduction of cholesterol, as clusters of the non-DRM membrane protein transferrin receptor remain segregated from those of APP or BACE 1 (unpublished data). Although antibody incubation is performed at low tempera-

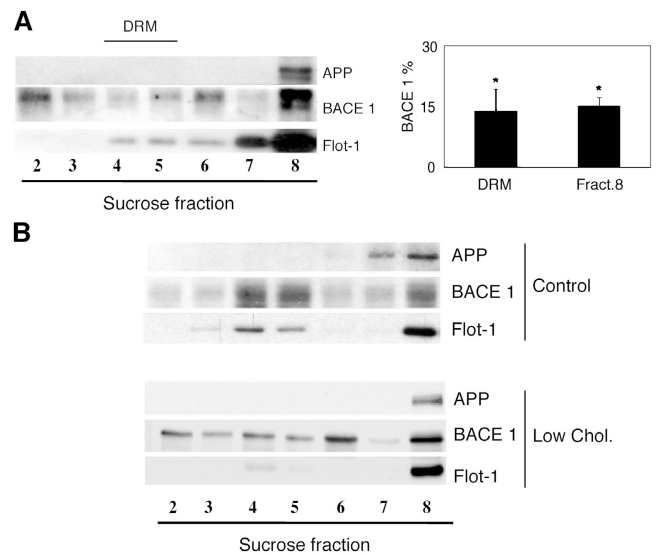


Figure 4. BACE 1 is displaced from DRMs and cofractionates with APP in heavier membrane fractions of low membrane cholesterol AD hippocampal and hippocampal neurons in culture. (A) Immunoblots for APP, BACE 1, and flotilin 1 in representative low membrane cholesterol AD hippocampal sample after Lubrol WX extraction and sucrose gradient centrifugation. Note that BACE 1 migration is shifted to the heavy APP-containing fraction 8. DRM modification is shown by a similar shift in the flotation characteristics of the DRM marker flotilin 1 (compare with Fig. 1 A). For quantification, the amount of BACE 1 in fractions 4 and 5 of sucrose gradients (DRM fraction) and fraction 8 (heavy APP-containing fraction) was measured by densitometry. The percentage of BACE 1 in DRMs is significantly reduced to 14% (graph, * indicates $P < 0.005$) compared with 24% in control samples (Fig. 1 A, graph). Conversely in the APP-containing fraction 8 BACE 1 content is increased to 15% compared with 10% in control samples (Fig. 1 A, graph). Data are means and SDs from 10 low cholesterol AD samples. (B) Moderate membrane cholesterol reduction in vitro displaces BACE 1 from DRMs. Hippocampal neurons grown for 5 d in culture were treated (low chol.) or not (control) with low concentrations of mevilonin and MCD for 5 d (see Materials and methods). This treatment induced $<30\%$ reduction in membrane cholesterol. Sucrose gradient fractionations after Lubrol WX extraction and Western blotting for APP and BACE 1 show that in control neurons BACE 1 peaks in fractions 4 and 5 (DRMs), whereas in low cholesterol neurons BACE 1 is spread along the gradient, with a relative enrichment in the APP-containing fraction 8. Disruption of DRMs is shown by the almost complete absence of flotilin 1 in fractions 4 and 5 and the relative enrichment in heavy fraction 8 (compare Flot-1 lines in control and low chol. samples).

ture, that should prevent internalization, we cannot rule out that some of the APP–BACE 1 coclusters observed in the low cholesterol samples occur in early endosomes. In any event, whether surface or endosomal, the fact that higher BACE 1 and APP colocalize in low cholesterol membranes moved us to test how this condition would affect APP β -cleavage.

Moderate cholesterol loss enhances APP β -cleavage and amyloid peptide production

To determine whether the enhanced biochemical and spatial association between BACE 1 and APP resulting from moderate cholesterol reduction is functionally relevant, the production of the β -COOH-terminal fragment (β -CTF) of APP and of A β was measured in APP constitutively expressing hippocampal neurons and in APP stably transfected fibroblast-like cells (CHO) in culture, with normal or a moderate to high membrane

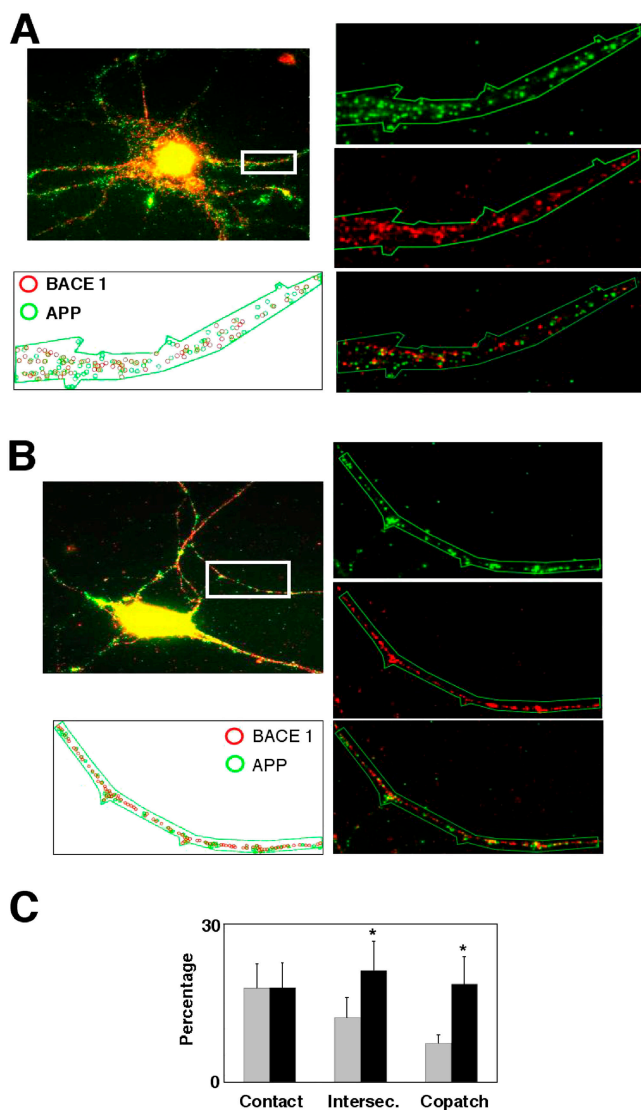


Figure 5. Moderate cholesterol reduction induces coclustering of BACE 1 and APP on the plasma membrane of cultured hippocampal neurons. BACE 1-APP colocalization was studied in 10 d *in vitro* hippocampal neurons using the copatching technique (see Materials and methods). (A) In control neurons BACE 1 (red clusters) and APP (green clusters) are extensively segregated to different membrane domains. (B) In contrast, low cholesterol neurons (treated as indicated in Materials and methods to lower the membrane cholesterol up to 30%) show a clear enhancement of BACE 1-APP colocalization. (C) For quantification, the degree of intersection among APP and BACE 1 clusters was considered as "copatching" (intersection >80%), "partial copatching" (intersection between 30% and 50%), "contact" (intersection between 0% and 30%), or random (no intersection; not depicted). For control cells 7% of APP clusters copatch and 12% partially copatch with BACE 1 (gray bars). These values are significantly increased to 19% and 21%, respectively (* indicates $P < 0.005$) in low cholesterol neurons (black bars). Data are means and SDs of three independent experiments.

cholesterol reduction. The level of β -CTF in hippocampal neurons with a 25–30% reduction of membrane cholesterol is 39% higher than in cells with normal membrane cholesterol content ($P < 0.005$; Fig. 6 A). To test if the increased production is the direct consequence of the loss of cholesterol different from a secondary, unspecific effect of the added drugs, cholesterol was replenished to the treated neurons in the form of cyclodex-

trin-cholesterol inclusion complexes (Simons et al., 1998). Such treatment for 15 min restores membrane cholesterol levels (110% of control). Notably, the level of APP β -cleavage in this condition is reestablished to the nontreated situation (91% of control; Fig. 6 A).

To confirm that mild reduction of membrane cholesterol leading to higher β cleavage results in higher $A\beta$ production, the amount of peptide was measured in low and normal membrane cholesterol CHO cells constitutively expressing human APP. These cells were chosen because they release much more peptide into the medium than the primary neurons, still APP is confined to non-DRM domains (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200404149/DC1>), like in the hippocampal neurons. In CHO cells, membrane cholesterol reduction of <25% results in a significant increase in the amount of peptide into the medium 47%, Fig. 6 B compared with cells with normal membrane cholesterol levels. This is paralleled by an increase in the production of β -CTF (30.6%). Contrary to the mild cholesterol loss situation, cells with a reduction of >35% of membrane cholesterol release less $A\beta$ than control cells (Fig. 6 B). This last effect is most likely due to overall disruption of membrane integrity.

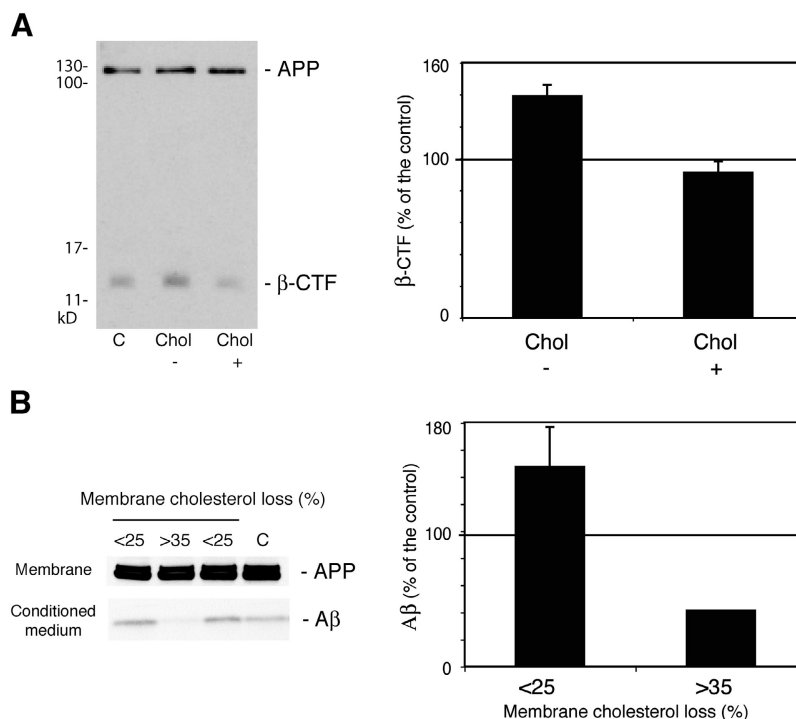
Discussion

The demonstration that in neuronal cells both APP and BACE 1 colocalize in the more fluid areas of membranes, as judged from detergent-solubility criteria, strongly indicates that a fluid milieu offers the optimal conditions for enzyme-substratum interaction. This finding is supported by the observation that APP is largely, if not fully, excluded from the less fluid cholesterol-rich DRMs. Future work is needed to define whether the fluid membrane domains where β -cleavage occurs are those of the plasma membrane or of the Golgi and endosomes, which have been postulated as preferred cleavage sites (Xu et al., 1997; Daugherty and Green, 2001).

The above scenario raises the question of the role played by BACE 1 in the cholesterol-rich membrane domains. One possibility is that this pool of BACE 1 is involved in the cleavage of substrates other than APP (Kitazume et al., 2003). Alternatively, BACE 1 in DRMs may cleave the few APP molecules that gain access to such environment. Although the latter scenario cannot be ruled out, a number of results indicate that it is unlikely. First, APP in neurons does not seem to have the capacity to become incorporated into cholesterol-rich environments, even at high expression levels (Fig. 2). Second, and most relevant, treatments leading to a moderate reduction of neuronal cholesterol result in increased cleavage, which is inconsistent with the occurrence of BACE 1 cleavage of APP in cholesterol-rich territories. Thus, BACE 1 in cholesterol-rich domains may represent an inactive pool of the protein, at least in terms of APP cleavage, which can be delivered when required to soluble domains where APP resides. In support of this view, a mild reduction of membrane cholesterol results in more BACE 1 in the soluble fractions, higher BACE 1-APP colo-

Figure 6. Moderate membrane cholesterol reduction in vitro enhances APP- β -cleavage and A β production.

(A) 10 d in vitro hippocampal neurons were not treated (C) or treated as indicated in Materials and methods to lower the membrane cholesterol up to 30% (Chol-). Cholesterol was added back to some of the treated cells for 15 min (Chol+) as cholesterol-MCD inclusion complexes. The amount of total APP and APP- β -CTF fragment in the different cell extracts was determined by Western blot. Densitometry of the β -CTF fragment normalized to the amount of total APP revealed a significant 39% increase in low cholesterol neurons with respect to untreated neurons. This effect was reverted by cholesterol replenishment. Thus, a 15-min treatment with the cholesterol inclusion complexes results in the production of similar amount of β -CTF fragment than control neurons (91% of control). Data shown in the graph are means and SDs from three different experiments. (B) Crude membrane pellets and conditioned media of control or low membrane cholesterol CHO-7w cells (stably expressing human APP) were submitted to PAGE-SDS in 10% Bis-Tris NuPage gels. Western blot detection was performed with an anti-APP COOH-terminal antibody to visualize holo-APP and with anti-APP mAb (WO2) to detect the amyloid peptide. A β peptide production was increased in cases of moderate low cholesterol an average of 47% over the control (lanes 1 and 3 in the blot and <25% bar in the graph, $n = 6$). Confirming previous data from other groups, extensive cholesterol loss over 35% leads to a strong decrease in A β production (lane 2 in the blot and >35% bar in the graph, $n = 2$).



calization and enhanced β processing. We observed that β -processing returns to control values when cholesterol is added back. Given this evidence, it is reasonable to postulate that neuronal cholesterol-rich membrane domains, and thus cholesterol, directly affect APP cleavage efficacy by acting as membrane “basins” from which BACE 1 can exit or enter to initiate or arrest, respectively, APP cleavage in nearby domains.

Our data and conclusions contradict previous work in which acute and drastic reduction of membrane cholesterol resulted in decreased amyloid production (Simons et al., 1998; Fassbender et al., 2001; Ehehalt et al., 2003). One explanation for the discrepancy is that these previous results were obtained using cells that overexpress APP and thus have higher amounts of the protein in DRMs. Because endogenous BACE 1 is present in DRMs, “addition” of APP into these domains by means of overexpression may increase β cleavage in a cholesterol-dependent manner. Alternatively, in the overexpressing cells more cleavage may have occurred as a result of excess APP in non-DRM domains, an event that could certainly be affected by a drastic cholesterol reduction, as we have now shown.

Based on the observation that overexpression of a GPI-form of BACE 1 in undifferentiated pseudo-neuronal cells (SH-SY5Y) enhances amyloid production, and that this is blocked by membrane cholesterol depletion, Cordy and colleagues (Cordy et al., 2003) suggested that BACE 1 cleaves APP within DRMs. However, the fact that a significant amount of overexpressed GPI-BACE 1 is present in non-DRMs is equally consistent with the cleavage of APP having taken place in such domains. This interpretation is sup-

ported by the authors’ demonstration that cholesterol reduction also decreases A β production in cells that overexpress the non-GPI form of BACE 1 and by our demonstration that the cells used in the work, SH-SY5Y, have little, if any, capacity to include APP in DRMs, even after overexpression (Fig. 2).

Our data, together with genetic evidence (Introduction), imply that lowering CNS cholesterol would be deleterious to neuronal function. Paradoxically, chronic treatments with cholesterol-reducing statins currently in clinical use have been shown to prevent AD (Jick et al., 2000; Wolozin et al., 2000). The most commonly used statins, however, do not penetrate the CNS significantly (Sparks et al., 2002), and neither patients nor experimental animals treated with statins show major changes in brain membrane cholesterol (Fassbender et al., 2001); only circulating cholesterol is lowered. Thus, it is unlikely that these drugs prevent AD by reducing neuronal membrane cholesterol. The preventive effects of these drugs may be due to their well-known antioxidant and anti-inflammatory properties (Cucchiara and Kasper, 2001; Zamvil and Steinman, 2002) or to the improvement of brain oxygenation secondary to low peripheral cholesterol levels. Care should be taken not to administer to patients statins that can cross the blood-brain barrier and directly inhibit cholesterol synthesis in brain cells, as one likely consequence may be the perturbation of the numerous physiological functions performed in cholesterol-rich membrane areas, including the restriction of APP cleavage. Therefore, we find it necessary to develop drugs to prevent neuronal cholesterol loss. Simultaneously, we need to learn more about the causes of such deficit.

Materials and methods

Cell culture

Primary cultures of rat embryo hippocampal neurons were prepared as described previously (Goslin, 1991). For biochemical analysis, 100,000 cells were plated onto 3-cm plastic dishes coated with poly-L-lysine (0.1 mg/ml) and containing MEM with N2 supplements. For morphological studies, 150,000 cells were plated onto poly-L-lysine glass coverslips in 6-cm dishes containing MEM with N2 supplements. Neurons were kept under 5% CO₂ at 36.5°C.

Human brain samples

Samples correspond to the hippocampus of 10 control brains and 10 AD brains with the 4 allele of apolipoprotein E and low levels of membrane cholesterol (Ledesma et al., 2003). The samples had mean ages 87 ± 11 and 79 ± 6 yr old, respectively. Postmortem delays were ranging from 5 to 25 h and the causes of death were variable. The following parameters were analyzed as described by Delacourte (2001): Braak stage for neurofibrillary tangles ranged from stage 0 to stage III in controls and from stage IV to VI in AD samples. Braak stage for amyloid deposition varied from stage 0 to stage A in controls, whereas AD samples showed stage B in one case and the rest were stage C. The amount of A β 42 deposition in the hippocampus was 0–34 μ g/g for control brains and 130–412 μ g/g for AD brains. The clinical dementia rating scored 0–0.5 in control individuals and 3 for all AD cases except for one, which showed 0.5. Finally, the mean duration of dementia was 8.6 ± 3.9 yr for AD brains.

Mice expressing human APP

Mice expressing wild-type human APP (Mucke et al., 2000) were donated by L. Mucke (University of California, San Francisco, San Francisco, CA). These mice express moderate levels of human APP in neurons that do not result in the formation of amyloid plaques.

Total and membrane hippocampal extracts

Human or mice hippocampal tissues were homogenized in PBS containing 9% sucrose and protease inhibitors (CLAP: pepstatin, antipain, chymostatin, each at a final concentration of 25 μ g/ml) using a dounce homogenizer and 10 passages through a 22-gauge syringe. The samples were centrifuged for 10 min at 2,500 rpm and the supernatants were considered as total extracts. A further centrifugation was performed at 100,000 g for 1 h at 4°C to pellet the membrane fraction. Protein concentration was quantified by the BCA method (Bio-Rad Laboratories).

Preparation of Golgi-endosomal-enriched membranes

Golgi-endosomal membranes were isolated from total mice brain as described previously (Fath and Burgess, 1993) with several modifications. Total brains were homogenized in ice-cold 0.5 M sucrose-PKM buffer (100 mM potassium phosphate, pH 6.5, 5 mM MgCl₂, and 3 mM KCl) with a hand-held tissue grinder. Samples were centrifuged for 10 min at 2,500 rpm. The postnuclear supernatant was centrifuged again two more times for 10 min at 8,000 rpm each to get a lysosomal and mitochondrial-free fraction. The last supernatant was layered onto a step gradient containing 1.3 M sucrose-PKM and 0.7 M sucrose-PKM, then centrifuged at 17,500 rpm in a SW40 rotor for 60 min. Membranes that concentrated at the 0.7/1.3 sucrose interface were collected and adjusted to 1.25 M sucrose-PKM. The membranes were overlaid with 1.1 M sucrose-PKM, 0.5 M sucrose-PKM, and centrifuged at 15,000 rpm in a SW40 rotor for 90 min. Golgi-endosomal membranes were collected at the 0.5/1.1 M interface.

DRM isolation on sucrose gradients

Hippocampal samples (100 μ g of total protein) or membrane pellets from cell cultures were extracted in MBS buffer (25 mM MES, 150 mM NaCl, pH 6.5) containing CLAP and 1% Lubrol WX (Serva), 1% Triton X-100 or 20 mM CHAPS, depending on the experiment. After 60 min of incubation at 4°C, the suspensions were brought to 60% sucrose in MBS and a sucrose step gradient was overlaid (35% and 5% sucrose). After centrifugation at 35,000 rpm for 18 h at 4°C, fractions were collected from the top of each tube. Fractions 4–5 were identified as the raft fraction by the presence of the raft marker flotillin 1 in the control samples.

Viral infection and metabolic labeling

Nondifferentiated N2A and SH-SY5Y cell lines and primary rat hippocampal neurons were infected for 8 h with Semliki Forest virus/human APP (SFV-APP; De Strooper et al., 1995) and extracted for 1 h at 4°C in 20 mM CHAPS before sucrose gradient fractionation. For metabolic label-

ing experiments, hippocampal neurons were infected with Fowl plague virus or SFV-APP for 8 h and pulse labeled during 30 or 10 min, respectively, with ³⁵S-methionine (200 μ Ci/ml). The cells were chased in fresh medium for 0–90 min as indicated.

Partial membrane cholesterol reduction and conditioned media preparation

For low membrane cholesterol experiments 5 d *in vitro* hippocampal neurons were treated with 0.4 μ M mevilonin and 0.5 mM methyl- β -cyclodextrin (MCD) and the cells were processed at day 10. CHO-7w cells were treated during 48 h with 0.4 μ M mevilonin and 1 mM MCD for moderate cholesterol loss (<25%) or with 0.4 μ M mevilonin and 1.5 mM MCD for high cholesterol loss (>35%). Cells were scraped with PBS-CLAP at 4°C, lysed by sonication, and centrifuged at 1,000 g. Postnuclear supernatants were further centrifuged at 100,000 g, 1 h at 4°C to get the membrane pellet. After resuspension in PBS-CLAP 0.1% Triton X-100, protein and cholesterol concentrations were measured. Conditioned media were prepared by incubating the cultured cells with Locke's solution for 8 h in 5% CO₂ at 36.5°C.

Cholesterol-MCD inclusion complexes

Cholesterol-MCD inclusion complexes were prepared as described previously (Klein et al., 1995). These complexes containing 0.3 mM complexed cholesterol were added to the medium at a final 1:10 dilution together with 2 μ g/ml free cholesterol to neurons treated with mevilonin and MCD as described above.

Cholesterol determination

We measure total cholesterol in samples containing equal amount of protein using Ecoline 25 (Merck). Ecoline relies on the enzymatic oxidation by cholesterol oxidase, producing H₂O₂. This is converted into a colored quinonimine in a reaction with 4-aminoantipyrine and salicylic alcohol catalyzed by peroxidase. The OD was measured at 500 nm and we used pure cholesterol (Sigma-Aldrich) solutions as standards.

Antibodies, Western blots, quantification, and statistical analysis

Sucrose gradient fractions were submitted to 10% PAGE-SDS electrophoresis and probed with monoclonal anti-APP (clone 22C11; Roche), monoclonal anti-flotillin 1 (clone 18; Transduction Laboratories), and polyclonal chicken anti-BACE 1 (raised against Fc-Asp 2-fusion protein). The specificity of the antibody was controlled by both Western blotting and immunohistochemistry in samples from BACE 1 knockout mice (Riddell et al., 2001). Species-specific peroxidase-conjugated secondary antibodies and the ECL method (Amersham Biosciences) were subsequently used. Quantification was done by densitometry of the autoradiograms using the NIH-image software. Results were checked with *t* test and P values <0.005 were considered statistically significant.

Quantification of peptides from APP cleavage

Cell pellets from mature cultured hippocampal neurons (control, low cholesterol, or replenished cholesterol) were submitted to 8–15% gradient PAGE-SDS, transferred to nitrocellulose, and blotted with monoclonal anti-APP (clone 6E10; Sigma-Aldrich) for β -CTF.

Crude membrane pellets of control CHO-7w cells (stably expressing human APP) and CHO-7w cells treated to lower membrane cholesterol, were resuspended in 0.2% SDS. 30 μ g of these membrane extracts were applied on a 10% Bis-Tris NuPage gel (Invitrogen) using MES as running buffer and transferred to nitrocellulose membrane. Western blot detection was performed with an anti-APP COOH-terminal antibody (B63.1) to visualize holo-APP. The conditioned media of the same samples were resolved on a 10% Bis-Tris NuPage gel, with MES as running buffer and transferred to nitrocellulose membrane. The amyloid peptide was detected directly using mAb WO2 (ABETA GmbH). The quantity of conditioned medium applied on the gel was normalized to the protein concentration of the membrane pellet. Species-specific peroxidase-conjugated secondary antibodies and the ECL method (Amersham Biosciences) were subsequently used. Quantification was done by densitometry of the autoradiograms using the NIH-image software.

Antibody-induced patching

Cultured neurons were first washed with PBS and incubated with 50 mM 2-mercaptoethanol for 5 min at 12°C. The cells were briefly washed with CO₂-independent MEM medium and incubated (45 min, 12°C) with pairs of primary antibodies using: monoclonal anti-NH₂-terminal APP (22c11; Roche), chicken anti-BACE 1 and rabbit polyclonal anti-Thy1 (Biotrend). After another brief washing the cells were incubated under the same con-

ditions with fluorophore-coupled antispecies antibodies (AlexaFluor 488 and 568; Molecular Probes). Coverslips were fixed, mounted, and pictures were acquired (objectives 40×/1.25, 63×/1.32) on a fluorescence microscope (model DMIRE2; Leica) equipped with a digital camera (model DC250; Leica) using the acquisition software (model Q550; Leica).

Quantification of copatching was performed as described previously (Harder et al., 1998; Ehehalt et al., 2003). In brief, 10 fields from 10 different pictures were taken. APP and BACE 1 clusters were converted to colored circles (red for BACE 1 and green for APP). The degree of intersection among red and green circles was considered as “copatching” (intersection >80%), “partial copatching” (intersection between 30% and 50%), “contact” (intersection between 0% and 30%), or random distribution. At least three independent experiments were performed for each cell treatment. NIH image and Adobe Photoshop software were used to perform these analyses.

Online supplemental material

Fig. S1 shows that the absence of endogenous APP in DRMs does not depend on the detergent used to isolate these microdomains (DRM isolation on sucrose gradients). Fig. S2 shows the validation of the specificity of DRMs fractionation from cultured neurons or mice brains through the use of antibodies against flotilin 1, Prion protein for DRMs and transferrin receptor for non-DRMs. Fig. S3 shows the specificity of 22C11 anti-APP antibody in copatching experiments after epitope exposure with β-mercaptoethanol. Similar APP-BACE 1 copatching results are obtained with other anti-APP antibodies without epitope exposure. Fig. S4 shows that APP is not detected in DRMs of CHO cells constitutively expressing human APP. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200404149/DC1>.

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