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Cholesterol depletion inhibits the generation of β-amyloid in hippocampal neurons

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ABSTRACT **The amyloid precursor protein (APP) plays a crucial role in the pathogenesis of Alzheimer's disease. During intracellular transport APP undergoes a series of proteolytic cleavages that lead to the release either of an amyloidogenic fragment called β-amyloid (Aβ) or of a nonamyloidogenic secreted form consisting of the ectodomain of APP (APP_{sec}). It is Aβ that accumulates in the brain lesions that are thought to cause the disease. By reducing the cellular cholesterol level of living hippocampal neurons by 70% with lovastatin and methyl-β-cyclodextrin, we show that the formation of Aβ is completely inhibited while the generation of APP_{sec} is unperturbed. This inhibition of Aβ formation is accompanied by increased solubility in the detergent Triton X-100 and is fully reversible by the readdition of cholesterol to previously depleted cells. Our results show that cholesterol is required for Aβ formation to occur and imply a link between cholesterol, Aβ, and Alzheimer's disease.**

The amyloid precursor protein (APP) is a transmembrane protein containing a large N-terminal ectodomain and a small C-terminal cytoplasmic domain (1). Although its physiological function remains unknown, several findings suggest a crucial role for APP in the pathogenesis of Alzheimer's disease (2, 3). During intracellular transport APP undergoes a series of proteolytic cleavages by as yet unidentified proteases, which lead to the release either of an amyloidogenic fragment called β-amyloid (Aβ) or of a nonamyloidogenic secreted form consisting of the ectodomain of APP (APP_{sec}) (4). Aβ generation occurs in two steps (5). The first cleavage occurring in the luminal domain of APP (β-cleavage) generates a 10-kDa fragment that is further cleaved within the transmembrane domain (γ-cleavage) to produce Aβ. The generation of APP_{sec} by α-cleavage in the luminal domain leaves an 8-kDa transmembrane fragment in the cell membrane, which subsequently is a substrate for γ-cleavage producing nonamyloidogenic p3. It is Aβ that accumulates in the brain lesions that are thought to cause the disease (5).

Cholesterol metabolism and Alzheimer's disease are genetically linked. The *apoE4* allele of the apolipoprotein E gene is associated with higher cholesterol levels (6) and has been shown to increase the risk of developing the disease (7). Also, atherosclerosis for which hypercholesterolemia is considered a risk factor is associated with Alzheimer's disease (8). These genetic links between cholesterol metabolism and Alzheimer's disease lead us to address the question whether APP processing is cholesterol dependent. Another possible link between cholesterol and APP processing is the finding that a fraction of APP is Triton X-100 insoluble in neurons and present in a low-density membrane fraction consisting of sphingolipid–cholesterol rafts (9). It could be this pool of APP that would be susceptible to β-cleavage generating Aβ. If this were the case then cholesterol depletion could affect generation of Aβ. To study the influence of cholesterol on APP metabolism we used primary cultures of rat hippocampal neurons infected with recombinant Semliki Forest virus (SFV) carrying APP (10). This system has been used successfully to study the intracellular transport and processing of human APP (11–13). Infection of mature and polarized neurons with recombinant SFV allows expression of relatively high amounts of APP without disturbing the polar organization and the viability of the cells within the time course of the experimental window chosen (3–7 hr after infection).

Cholesterol depletion of the cultured neurons was achieved by a combination of lovastatin treatment and methyl-β-cyclodextrin extraction. Lovastatin in the presence of low amounts of mevalonate prevents the new synthesis of cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (14). β-Cyclodextrins have been shown to selectively and rapidly extract cholesterol from the plasma membrane, in preference to other lipids (15–17). By combining lovastatin and methyl-β-cyclodextrin we previously have shown that total cellular cholesterol levels can be reduced by 60–70% without significantly affecting cell viability and integrity (18). Under these conditions we observed a dramatic reduction of transport of influenza virus hemagglutinin from the trans-Golgi network to the cell surface of nonpolarized baby hamster kidney cells as well as to the apical surface of polarized Madin–Darby canine kidney cells. The transport of the vesicular stomatitis virus glycoprotein to the basolateral surface was not affected under these conditions. These data provided us with direct evidence for the functional significance of cholesterol and sphingolipid-cholesterol rafts as sorting platforms for inclusion of protein cargo destined for delivery to the apical membrane (9).

We now have applied the cholesterol depletion conditions previously established for baby hamster kidney and Madin–Darby canine kidney cells to hippocampal neurons, and we show that these conditions lead to a 70% reduction of cellular cholesterol levels while not affecting neuronal viability. Under these conditions Aβ formation is inhibited while the generation of APP_{sec} is unperturbed. Our results show that cholesterol is required for Aβ formation to occur and imply a link between cholesterol, Aβ, and Alzheimer's disease.

MATERIALS AND METHODS

Cell Culture. Hippocampal neurons were prepared from 18-day-old fetal rats as described (19). After the hippocampi

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Abbreviations: APP, amyloid precursor protein; Aβ, β-amyloid fragment of APP; APP_{sec}, nonamyloidogenic secreted form consisting of the ectodomain of APP; DIGs, detergent-insoluble low-density glycosphingolipid-enriched complexes; SFV, Semliki Forest virus.

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were dissected and dissociated, the cells were plated on either polylysine-coated dishes or on glass coverslips and kept in MEM supplemented with 10% horse serum. The cells were maintained under serum-free conditions in MEM with N2-supplement (maintenance medium) at 5% CO_2 and 36.5°C. The proliferation of nonneuronal cells was prevented by adding 5 mM cytosine arabinonucleoside. After 5–7 days in culture, 4 μM lovastatin (MSD Sharp & Dohme, Haar, Germany) and 0.25 mM mevalonate (Sigma) were added for 4 days. Control cells were left untreated.

Antibodies. Polyclonal antibodies used were antibody FdAPP directed against APP695 (20), antibody B12/4 directed against the 20 C-terminal amino acids of APP (12), and antibody B7/6 recognizing amino acids 1–16 of human Aβ synthetic peptide 1–40 (12).

Cholesterol Depletion and Metabolic Labeling of Infected Neurons. Recombinant SFV encoding human APP695 was prepared as previously described (10, 21). Neurons were infected for 1 hr at 37°C and 5% CO_2 with recombinant SFV. The virus solution was replaced by maintenance medium containing lovastatin/mevalonate, and the cells were incubated for an additional 2 hr. After a treatment for 5–20 min with 5 mM methyl-β-cyclodextrin (Sigma) in methionine-free labeling medium (MEM with 1/10 N2-supplement), the cells were labeled for 2.5 hr with 150 μCi of [^{35}S]methionine (Amersham; 1 Ci = 37 GBq). Control cells were processed in parallel in the absence of lovastatin/mevalonate and methyl-β-cyclodextrin.

Cholesterol–Methyl-β-Cyclodextrin Inclusion Complexes. Cholesterol–methyl-β-cyclodextrin inclusion complexes were prepared as described (22). These complexes containing 0.3 mM complexed cholesterol were added together with 2 μg/ml free cholesterol for 15 min to neurons grown, infected, and treated with methyl-β-cyclodextrin as described above.

Flotation of Triton X-100 Extracts. Lovastatin-treated neurons grown, infected, and treated with methyl-β-cyclodextrin as described above were pulse-labeled for 20 min and then chased or not for 100 min in maintenance medium. The cells were extracted for 30 min on ice with 1% Triton X-100 in TEX (150 mM NaCl/50 mM Tris·HCl, pH 7.4/2 mM EDTA/2 mM DTT containing 25 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A). The extracts then were mixed with an equal volume of OptiPrep (Nycomed, Oslo), and overlayered in a TLS 55 centrifugation tube (Beckman) with a step gradient of 30%, 25%, and 3% OptiPrep in TEX. After a centrifugation for 3 hr at 4°C and 50,000 rpm in a TL-100 ultracentrifuge (Beckman), fractions were collected and APP was immunoprecipitated.

Immunoprecipitation and Quantitation. After metabolic labeling the culture medium was collected and cell extracts were prepared in 2% Nonidet P-40/0.2% SDS/5 mM EDTA, supplemented with protease inhibitors. Immunoprecipitates were recovered on protein A-Sepharose (Boehringer Mannheim), and were analyzed on 10–20% Tris-Tricine polyacrylamide gels (NOVEX, San Diego). Radioactivity in the individual bands was determined by using a PhosphorImager (Molecular Dynamics).

Quantitation of Cholesterol Depletion. [1a,2a(N)-^{3}H]Cholesterol (6.25 μCi; Amersham) was added for 8 hr to neurons grown on glass coverslips in the presence of lovastatin/mevalonate. After an equilibration for 16 hr with the cellular cholesterol pool, the cells were treated for 20 min with 5 mM methyl-β-cyclodextrin. [^{3}H]Cholesterol released into the medium and remaining in the cells, respectively, was determined by scintillation counting. Control cells were processed in parallel in the absence of lovastatin/mevalonate and methyl-β-cyclodextrin.

Staining of Hippocampal Neurons with Filipin. Neurons grown on glass coverslips in the presence of lovastatin/mevalonate were treated for 20 min with 5 mM methyl-β-cyclodextrin. After fixation on ice with 4% paraformaldehyde, they were stained with 125 μg/ml filipin (Sigma) in PBS. Digital images were taken with a Zeiss Axioskop microscope equipped with a three-chip color camera (Photonic Sciences, Millham, U.K.).

RESULTS AND DISCUSSION

Cholesterol depletion of the cultured neurons was achieved by a combination of lovastatin treatment and methyl-β-cyclodextrin extraction as described (18). Lovastatin in the presence of low amounts of mevalonate inhibits cholesterol biosynthesis (14). Methyl-β-cyclodextrin specifically removes cellular cholesterol (15–17). We measured the extent of cholesterol removal using filipin, a fluorescent polyene antibiotic that forms complexes with cholesterol that can be visualized by fluorescence microscopy (23). After 4 days of treatment with 4 μM lovastatin, followed by extraction of the cells for 20 min with 5 mM methyl-β-cyclodextrin a dramatic reduction of the filipin staining as compared with control cells was seen (Fig. 1). Lovastatin treatment alone did not lead to a significant decrease of staining. However, lovastatin and methyl-β-cyclodextrin treated neurons labeled with [^{3}H]cholesterol were depleted of $\approx$70% of the cellular [^{3}H]cholesterol as compared with control cells.

We used these conditions for the subsequent experiments. Prolonging the lovastatin treatment or increasing the concentration of methyl-β-cyclodextrin or the length of treatment started to affect the viability of the neuronal cultures.

To monitor the amount of APP_{sec} being secreted in cholesterol-depleted cells, hippocampal neurons, treated for 4 days with lovastatin, were infected for 1 hr with SFV carrying the

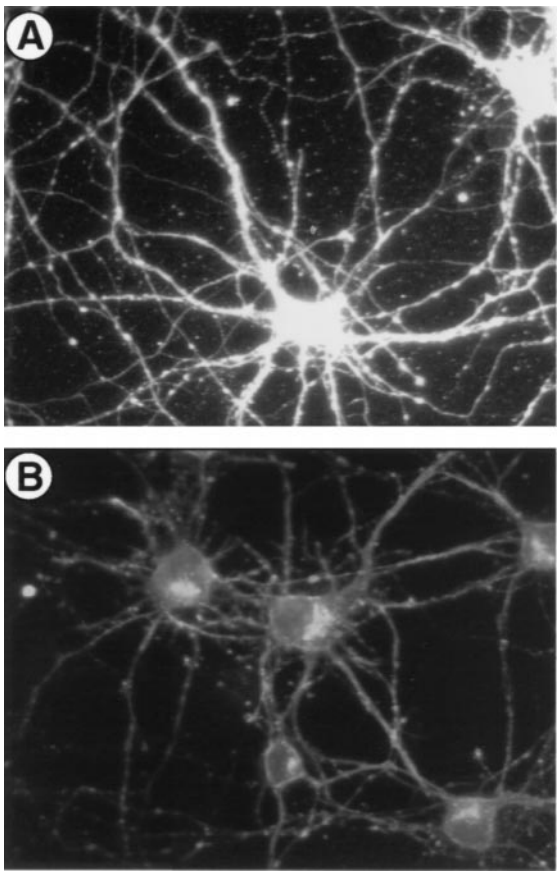

FIG. 1. Staining of cholesterol-depleted hippocampal neurons with filipin. (*A*) Untreated neurons stained with filipin. (*B*) Neurons stained with filipin after treatment for 4 days with lovastatin/mevalonate and for 20 min with 5 mM methyl-β-cyclodextrin.

gene for human APP. Two hr after infection, methyl-β-cyclodextrin was added for 20 min and the cells were subsequently labeled for 2.5 hr with [^{35}S]methionine. APP was immunoprecipitated from cell lysates and from the culture media using a polyclonal anti-APP antibody. A prominent 115-kDa band (full-length APP) was revealed by SDS/PAGE in similar amounts in both cholesterol-depleted and in control cells (Fig. 2). The immunoprecipitation demonstrated that a 110-kDa fragment (APP_{sec}) was secreted by the neurons and that the amount secreted was not affected by cholesterol depletion (Fig. 2) as quantified by PhosphorImager analysis. The ratio of full-length APP to APP_{sec} being 7.4 ± 4.2 and 5.9 ± 1.6 in cholesterol-depleted and control cells (mean of 10 experiments), respectively. These data indicate that the generation of APP_{sec} that is the main processed form of APP in neurons and other cells (5) is not significantly affected by reduction of cellular cholesterol levels.

Next we compared Aβ secretion in the same cholesterol-depleted and control neurons that we studied for APP_{sec} formation. A striking inhibition of Aβ generation was seen (Fig. 3*A*). Even a shorter treatment with methyl-β-cyclodextrin (5 min) was sufficient to reduce Aβ secretion. The lovastatin treatment protocol alone did not cause a significant difference in Aβ secretion, suggesting that fairly large amounts of cholesterol need to be removed before effects on Aβ secretion can be observed. This is in agreement with the observation that the same lovastatin treatment protocol reduced the total cellular cholesterol levels by only ≈10% in baby hamster kidney cells and had no effect on biosynthetic transport in these cells (18).

To demonstrate that the inhibition of the production of Aβ was due to cholesterol depletion and not to any other effects of methyl-β-cyclodextrin we performed two control experiments (Figs. 3 *B* and *C*). First we used a cholesterol-methyl-β-cyclodextrin complex instead of methyl-β-cyclodextrin alone. Under these conditions there was no reduction of Aβ formation. Second, we attempted to replete the cholesterol-depleted cells with cholesterol by adding back the cholesterol-methyl-β-cyclodextrin complex to the neurons after the 20 min extraction of cholesterol by methyl-β-cyclodextrin. The repletion incubation was for 15 min. Under these conditions the production of Aβ was fully restored. These data convincingly demonstrate that cholesterol depletion by a combination of lovastatin and methyl-β-cyclodextrin causes inhibition of Aβ secretion in hippocampal neurons. On the other hand, cholesterol depletion of COS cells, stably transfected with human APP695 did not lead to a significant decrease of Aβ production (data not shown).

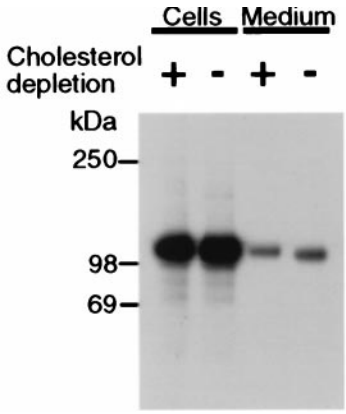

FIG. 2. Cholesterol depletion does not change APP biosynthesis and secretion. Hippocampal neurons were grown for 4 days in the presence (+) or absence (−) of lovastatin/mevalonate. After infection with SFV/APP, cells were treated (+) or not (−) for 20 min with 5 mM methyl-β-cyclodextrin, and metabolically labeled for 2.5 hr. Cellular and secreted APP were recovered by immunoprecipitation.

Next we analyzed which Aβ cleavage is inhibited. Aβ generation occurs in two steps (5). The first cleavage (β-cleavage) generates a 10-kDa fragment from APP that is further cleaved within the transmembrane domain (γ-cleavage) to produce Aβ. The generation of APP_{sec} by α-cleavage leaves an 8-kDa transmembrane fragment in the cell membrane. When antibodies recognizing the C-terminal end of APP were used to immunoprecipitate APP fragments from the cell lysates of cholesterol-depleted and control neurons a dramatic inhibition of the β-cleavage was detected by SDS/PAGE, while the production of the fragment generated by α-cleavage was unperturbed (Fig. 3*D*). Under these conditions there was also no change in the production of nonamyloidogenic p3 that is generated by α- and γ-cleavage (data not shown). Thus our combined results suggest that cholesterol-depletion interferes with the β-cleavage but not with α-cleavage that produces APP_{sec} (5). The γ-cleavage is probably inhibited indirectly because it requires prior β-cleavage (24). We conclude that depletion of cholesterol affects amyloidogenic processing while allowing nonamyloidogenic cleavage to proceed.

One reason to believe that APP-processing might be cholesterol-dependent comes from the genetic links between cholesterol metabolism and Alzheimer's disease. The apoE4 allele is associated with higher cholesterol levels (6) and increases the risk of developing the disease (7). Also, atherosclerosis for which hypercholesterolemia is considered a risk factor is associated with Alzheimer's disease (8). All these findings led us to test the hypothesis that Aβ production might be dependent on the cholesterol level in neurons.

However, the major reason why we initiated these studies was the observation that a fraction of APP in neurons is insoluble in Triton X-100 at 4°C (25). This is a property shared by glycosyl-phosphatidylinositol-anchored proteins and transmembrane proteins associating with sphingolipid–cholesterol rafts (9). These proteins are in detergent-insoluble low-density glycosphingolipid-enriched complexes (DIGs). Recent studies demonstrated that cholesterol plays an essential role in raft assembly (9): cholesterol depletion abolishes the association of proteins with rafts (18, 26, 27). Therefore we wanted to check the relation between APP insolubility in Triton X-100 and cholesterol-depletion. After infection with recombinant SFV carrying APP we extracted cholesterol-depleted and control neurons with detergent. The extract was mixed with an equal volume of OptiPrep and overlayered with 30%, 25%, and 3% OptiPrep. After centrifugation a fraction of the cellular APP was found to float to lower density in the extract from control neurons (Fig. 4). DIG association was greatly reduced in cholesterol-depleted cells. Thus under conditions in which Aβ formation is inhibited APP does not associate with DIGs. Moreover the pulse-chase experiment (Fig. 4) demonstrated that APP does not associate with DIGs immediately after synthesis, but presumably after the protein has entered the Golgi complex as is the case for glycosyl-phosphatidylinositol-anchored proteins (28) and for influenza virus hemagglutinin (29, 30).

This finding raises the question of how cholesterol depletion affects Aβ formation. One possibility is that reduction in membrane cholesterol changes the intracellular transport of APP so that the protein does not reach the cellular site(s) where β-cleavage takes place. Influenza virus hemagglutinin that is routed apically in epithelial cells is missorted to the basolateral surface in cholesterol-depleted Madin–Darby canine kidney cells (18). Alternatively, the protease responsible for β-cleavage is active only in intact rafts that depend on cholesterol for integrity. Interestingly, conversion of the glycosyl-phosphatidylinositol-anchored prion protein to the protease-resistant form is reduced by cholesterol depletion (31), suggesting that the raft environment is important for the generation of the disease-causing form of prions. One factor

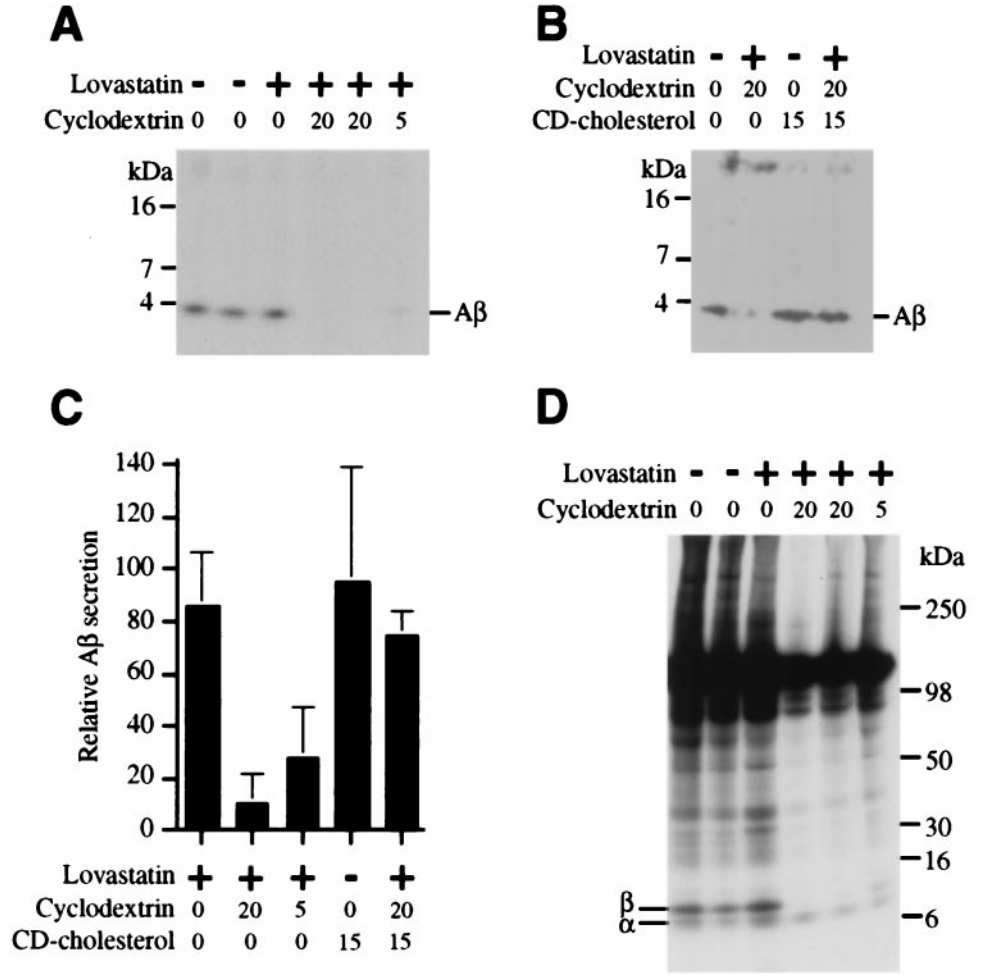

FIG. 3. Cholesterol depletion inhibits production and secretion of Aβ. Hippocampal neurons were grown for 4 days in the presence (+) or absence (−) of lovastatin/mevalonate and after infection with SFV/APP were treated with 5 mM methyl-β-cyclodextrin for the indicated times (min) and metabolically labeled for 2.5 hr. Cholesterol was added back for the indicated times (min) as a cholesterol-methyl-β-cyclodextrin inclusion complex (CD-cholesterol). (*A*) Aβ secreted from control and cholesterol-depleted cells (immunoprecipitated using antibody B7/6). (*B*) Aβ secreted from cholesterol-depleted/repleted cells. (*C*) Aβ secretion in cholesterol-depleted cells as compared with untreated control cells (mean of 3–11 experiments). (*D*) Immunoprecipitation of APP and its proteolytic fragments from cell homogenates using antibody B12/4. Fragments generated by α- and β-cleavage are indicated.

influencing raft association of APP could be the binding of the Aβ fragment to the raft lipid ganglioside, GM1 (32). These intriguing relationships raise the hopes of new strategies to influence the progression of Alzheimer's disease.

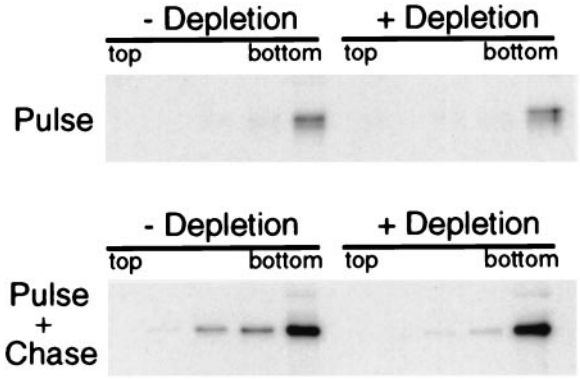

FIG. 4. Cholesterol depletion reduces association of APP with detergent-insoluble low-density DIGs. Hippocampal neurons treated or not for 4 days with lovastatin/mevalonate and 20 min with 5 mM methyl-β-cyclodextrin were extracted on ice with 1% Triton X-100 immediately after pulse labeling for 20 min or after an additional chase for 100 min. After flotation in an OptiPrep step-gradient, APP was immunoprecipitated from the collected fractions.

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