

A Ganglioside-induced Toxic Soluble A β Assembly

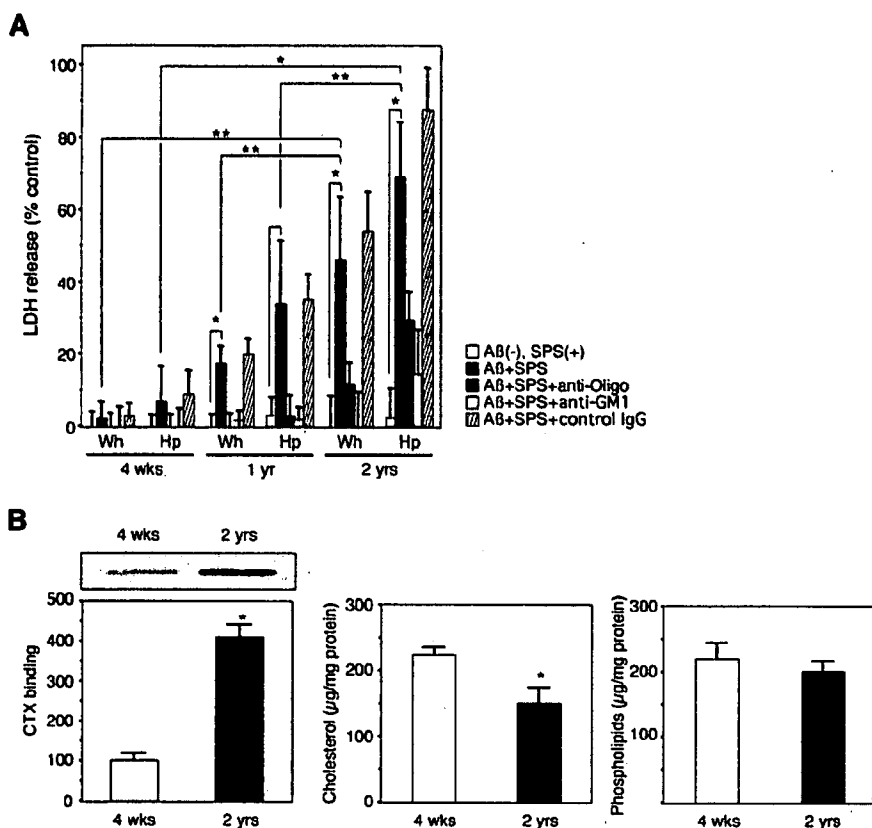


FIGURE 5. TA β formation from Arctic-type A β incubated in the presence of synaptosomes. A, TA β formation was assessed by LDH release assay of PC12N cell cultures treated at 37 °C for 48 h with incubation mixtures containing Arctic-type A β (A β 40) at a final concentration of 25 μ M, which had been preincubated at 50 μ M and 37 °C for 2 h in the absence or presence of synaptosomes (SPS) prepared from brains of mice of three different age groups with or without anti-Oligo or an antibody specific to GM1 ganglioside (Calbiochem). Wh, whole brain minus hippocampus; Hp, hippocampus. Each column indicates the average of four values \pm S.D. *, $p < 0.0001$; **, $p < 0.005$. B, lipid composition of synaptosomes prepared from young (1-month-old) and aged (2-year-old) mouse brains. GM1 ganglioside levels were determined by densitoscanning the blot following incubation with cholera toxin. Levels of cholesterol and phospholipids were determined using Determiner L (Kyowa, Tokyo, Japan) and phospholipids C (Wako, Osaka, Japan), respectively. Each column indicates the average of four values \pm S.D. *, $p < 0.0001$.

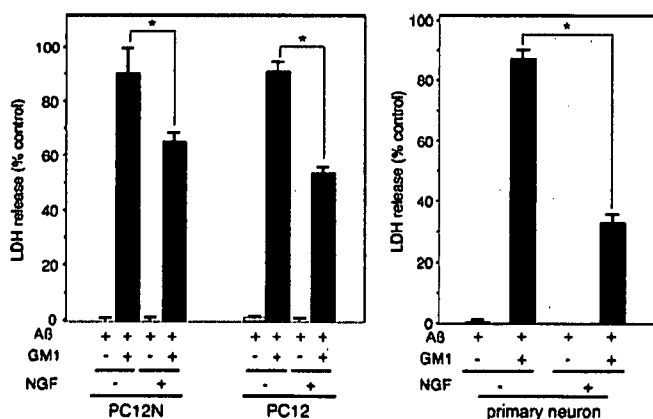


FIGURE 6. Suppression of TA β toxicity by the addition of exogenous NGF. NGF-treated PC12 (PC12N), native PC12 cells, and primary neurons were treated with the incubation mixture containing Arctic-type A β (A β 40) at a final concentration of 25 μ M, which had been preincubated at 50 μ M and 37 °C for 2 h in the absence or presence of 500 μ M GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%) and exogenous NGF (100 ng/ml). TA β toxicity was assessed by an LDH release assay in these cultures. Each column indicates the average of three values \pm S.D. *, $p < 0.0001$.

cell bodies, and the condensation and fragmentation of nuclei in conjunction with an increase in the level of LDH released from TA β -treated PC12N cells (data not shown). To determine if TA β toxicity is mediated by NGF receptors, we first treated PC12N cells, native PC12 cells, and primary neurons with TA β in the presence of exogenous NGF. In these cultures, cell death was markedly prevented (Fig. 6). We then knocked down the NGF receptors, including TrkA and p75^{NTR}, of PC12 cells, SY5Y cells, and primary neurons using specific siRNAs. The knockdown of p75^{NTR} or TrkA markedly suppressed the cell death induced by TA β in these cultures (Fig. 7).

DISCUSSION

Here, we show that a highly toxic soluble A β assembly (TA β) can be formed more rapidly and to a greater extent from Arctic-type A β than from wild-type A β . Notably, TA β formation requires GM1 ganglioside at certain densities. TA β is probably formed via a pathway different from one that leads to amyloid fibril formation. Biophysical and structural analyses by AFM and size exclusion chromatography revealed that TA β is spherical with diameters of 10–20 nm and molec-

ular masses of 200–300 kDa. The most striking feature of TA β is its unique toxicity. Our results suggest that TA β induces the NGF receptor-mediated apoptosis of cultured cells.

Accumulating evidence suggests that soluble A β assemblies are formed as intermediates en route to amyloid fibril formation. This scenario is mainly supported by the formation of soluble A β assemblies early during the incubation period *in vitro*, which is frequently followed by the appearance of mature fibrils (5, 6, 8, 13). Indeed, certain inhibitors of A β fibrillogenesis are potent for blocking the generation of A β oligomers (46). In this study, TA β was preferably formed in the presence of GM1 ganglioside at lower densities than those required for amyloid fibril formation (36). Furthermore, a monoclonal antibody specific to a seed for amyloid fibril formation (40) failed to inhibit TA β formation. These results suggest that TA β is formed via a pathway different from a straightforward pathway leading to amyloid fibril formation, as was previously suggested in the formation of other soluble A β assemblies (11, 12).

In this study, monomeric Arctic-type A β was converted to TA β more rapidly and to a greater extent than wild-type A β . The propensity of Arctic-type A β to form toxic nonamyloid A β assemblies has recently attracted interest (13, 30, 34); however,

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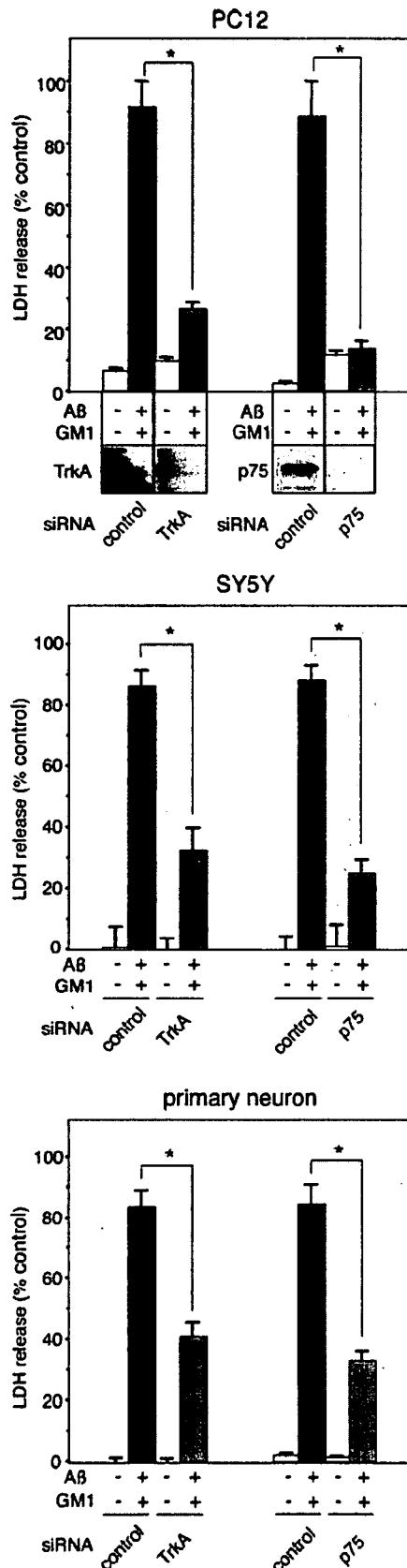


FIGURE 7. TA β toxicity mediated by NGF receptors. PC12 cells, SY5Y cells, and primary neurons, which had been treated with siRNAs against TrkA or p75^{NTR}, were exposed to incubation mixtures containing Arctic-type A β

it remains to be clarified how the assembly of Arctic-type A β is accelerated compared with that of wild-type A β . We previously found that A β fibrillogenesis from Arctic-type A β is also enhanced in the presence of SDS as well as GM1 ganglioside (36). Thus, taken together with the results of this study, it is likely that the negatively charged membrane surface is a preferred environment for Arctic-type A β to form soluble and insoluble assemblies. A previous study suggested that the lateral distribution of GM1 ganglioside affects the spatial arrangements of the oligosaccharide chain of a molecule (47). Thus, the conformation of GM1 ganglioside may be modulated at certain densities, providing a favorable microenvironment for TA β formation.

Results of this study imply that GM1 ganglioside potentially accelerates the formation of not only amyloid fibrils but also the soluble A β assembly. It has recently been reported that A β oligomerization is induced in the presence of lipid rafts isolated from brain tissues and cultured cells in a ganglioside-dependent manner (48). Although further studies are necessary, it may be assumed that GM1 ganglioside-rich membrane microdomains, such as lipid rafts, provide a favorable environment that facilitates the formation of soluble A β assemblies, including A β oligomers and dimers (49).

In this study, the incubation of Arctic-type A β with synaptosomes prepared from aged mouse brains markedly induced TA β formation. Furthermore, the level of GM1 ganglioside significantly increased, whereas that of cholesterol significantly decreased with age. Our observation of an age-dependent alteration in lipid composition of neuronal membranes is in agreement with the result of a recent study of cerebral cortices of AD brains (38). Taking this together with our recent observation that the level of GM1 ganglioside in synaptosomes increases not only with age but also with the expression of apolipoprotein E4 (37), it is possible that TA β can be formed in the brain in association with the risk factors for AD development.

It was previously reported that A β -derived diffusible ligands potentially alter NGF-mediated signaling in cultured cells (11). Moreover, many previous studies suggested that A β toxicities emerge through the association with p75^{NTR} (50–56) (for a review, see Refs. 57–59). In particular, it is noteworthy that A β toxicity mediated by p75^{NTR} depends on a death domain (60) in the cytoplasmic part of p75^{NTR} molecules (56). Evidence indicates the dual function of p75^{NTR}: one for survival and the other for death (61) (for a review, see Refs. 57 and 58). Furthermore, a previous study revealed that heteromeric TrkA-p75^{NTR} complexes have different functions from homo-oligomeric TrkA or p75^{NTR} alone (62). Notably, the knockdown of either TrkA or p75^{NTR} is sufficient for suppressing TA β toxicity. Thus, it may be assumed that the function of heteromeric TrkA-p75^{NTR} complexes is

(A β 40) at a final concentration of 25 μ M, which had been preincubated at 50 μ M and 37 $^{\circ}$ C for 2 h in the absence or presence of 500 μ M GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%). TA β toxicity, which was assessed by LDH release assay, was markedly suppressed by the knockdown of TrkA or p75^{NTR}. Decreases in TrkA and p75^{NTR} expression levels were confirmed by Western blotting of cell lysates using anti-TrkA and anti-p75^{NTR} antibodies, respectively. Each column indicates the average of three values \pm S.D. *, $p < 0.0001$.

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perturbed by TA β binding to p75^{NTR} or TrkA, leading to apoptosis through the activation of the death domain of p75^{NTR} (for a review, see Ref. 58). However, it should be noted that conflicting evidence also exists; the expression of p75^{NTR} protects against the toxicity of soluble A β assembly or extracellular A β (63, 64). These opposite conclusions imply that the signaling pathways of p75^{NTR} are complicated and that the functions of p75^{NTR} vary depending on cell type and context (for a review, see Ref. 57).

To date, various soluble A β assemblies with diverse structural features have been detected in a broad range of *in vitro* and *in vivo* studies, which employed different techniques in preparing or isolating such assemblies. As previously reported (11, 65), A β assembles into multiple alternative structures. Thus, at this point, it is difficult to determine whether TA β is identical to or distinct from previously identified soluble A β assemblies. However, on the basis of its biophysical features, including its SDS disaggregatability and unsuccessful detection on a carbon-coated grid by EM, TA β probably differs from previously reported A β assemblies, particularly protofibrils, because most protofibrils appear to adsorb equally onto carbon-coated grids (65); moreover, no TA β is detected by EM under conditions in which protofibrils are readily detected. One interesting soluble A β assembly is A β *56 (25). A β *56 may be a candidate A β assembly responsible for plaque-independent cognitive decline in AD; however, its biophysical features, including molecular mass and marked stability in SDS-PAGE, make it distinct from TA β .

Finally, this study indicates a novel pathological implication of soluble A β assemblies. It is well documented that early and severe neuronal loss in the cholinergic basal forebrain in AD is probably responsible for cognitive decline in AD patients. Previous studies suggested that cholinergic phenotype alone is unlikely to be a sufficient condition for inducing neuronal death in AD. Certain cholinergic neurons, such as those in the pontomesencephalon, are unaffected in AD (66). Notably, cholinergic neurons in the pontomesencephalon are free of NGF receptors, whereas those in the basal forebrain, which are early and severely affected in AD, have NGF receptors (67). Taken together, our results suggest that soluble A β assemblies, such as TA β , are responsible for the loss of NGF-dependent neurons in the cholinergic basal forebrain in AD. A future challenge is the production of a monoclonal neutralizing antibody against TA β toxicity, which would provide promising therapeutic strategies, as suggested by *in vitro* and *in vivo* studies that selectively targeted A β oligomers (68, 69).

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Research article

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Novel action of apolipoprotein E (ApoE): ApoE isoform specifically inhibits lipid-particle-mediated cholesterol release from neurons

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Abstract

Background: Since the majority of apolipoprotein E (apoE) existing in the cerebrospinal fluid is associated with high-density lipoprotein (HDL), one should focus on the role of the apoE-HDL complex rather than on that of free apoE in cholesterol metabolism in the central nervous system. However, the apoE-isoform-specific effect of apoE-HDL on cholesterol transport remains unclarified.

Results: Here we show that apoE3-HDL induced a marked cholesterol release from neurons, while apoE4-HDL induced little. To elucidate the mechanism underlying this phenomenon, we used a complex of lipid emulsion (EM) with recombinant apoE3 or apoE4 (apoE-EM) at various apoE concentrations. When a small number of apoE molecules were associated with EM, apoE3- and apoE4-EM, induced a marked cholesterol release to a level similar to that induced by EM alone. However, when apoE at given concentrations was incubated with EM, apoE3-EM induced a marked cholesterol release, while apoE4-EM induced little. Under these conditions, a greater number of apoE4 molecules were associated with EM than apoE3 molecules. When an increasing number of apoE molecules were associated with EM, both apoE3-EM and apoE4-EM induced little cholesterol release. Preincubation with β -mercaptoethanol increased the number of apoE3 molecules associated with EM similar to that of apoE4 molecules, indicating that the presence (apoE3) or absence (apoE4) of intermolecular disulfide bond formation is responsible for the association of a greater number of apoE4 molecules to EM than apoE3 molecules.

Conclusion: These results suggest that although apoE and a lipid particle are lipid acceptors, when apoE and a lipid particle form a complex, apoE on the particle surface inhibits the lipid particle-mediated cholesterol release from cells in an apoE-concentration-dependent manner.

Background

It has been shown that the prevalence of Alzheimer's disease (AD) is associated with the polymorphisms of genes related to cholesterol metabolism, including *apolipoprotein E (apoE)* [1], *ATP-binding cassette transporter A1 (ABCA1)* [2], and *CYP46*, the gene encoding cholesterol 24-hydroxylase [3,4]. However, before discussing the association of altered cholesterol metabolism with AD pathogenesis, one should delineate mutual interaction between cholesterol metabolism in the circulation and that in the central nervous system across the blood-brain barrier, and also determine how cholesterol is transported within the central nervous system and how altered cholesterol metabolism induces AD pathologies. In the central nervous system, apoE is one of the major lipid acceptors [5,6] and interacts with ABCA1 [7] to remove cholesterol from cells and generate HDL particles [8] in an apoE-isoform-specific manner [9-11]. This isoform-specific action of free apoE to remove cholesterol and to generate HDL would be a possible cause for the altered cholesterol metabolism in an AD brain. On the other hand, it was shown that the majority of apoE existing in cerebrospinal fluid (CSF) and culture media is associated with HDL and the free form of apoE is at a very low level in the CSF [5,6] and culture media [10,12]. Thus, to determine the apoE-isoform-specific cholesterol transport in the central nervous system, one should focus on the role of the apoE-HDL complex rather than on that of free apoE.

Many studies have shown that HDL stimulates cholesterol release from cultured cells [13-15]. It is believed that this removal of cellular cholesterol induced by HDL involves at least two different mechanisms working cooperatively. One involves the biochemical pathway mediated by apolipoproteins [16,17]. The other involves the physicochemical pathway for the bidirectional movement of cholesterol mediated by aqueous diffusion mechanism [18,19]. However, how these two acceptors contribute and modulate the cholesterol release remains to be clarified. Our recent finding that the apoE-isoform-specific ability to generate HDL is associated with an apoE-isoform-specific ratio of apoE molecules per HDL particle [10] led us to examine the effect of apoE3- and apoE4-containing HDLs or lipid emulsions (EMs) at different apoE ratios on cholesterol release from neurons. Here we show that apoE3-HDL induces a strong cholesterol release, while apoE4-HDL induces a very weak release, and that this isoform-specific effect of apoE associated with lipid particle (HDL or EM) is due to the finding that (1) apoE4 has a higher affinity to lipid particles and thus a greater number of apoE4 molecules bind to lipid particles than apoE3, and (2) with increasing number of apoE molecules covering the surface of lipid particle, both apoE3 and apoE4 inhibit the lipid-particle-mediated cholesterol release. These results suggest that both apoE and a

lipid particle are strong lipid acceptors; however, when apoE forms a complex with a lipid particle, apoE on the particle surface inhibits the lipid-particle-mediated cholesterol release by covering its surface.

Results

ApoE-isoform-specific lipid release mediated by apoE3- and apoE4-containing HDL

Human apoE3- and apoE4-containing HDL (apoE3-HDL and apoE4-HDL, respectively) were obtained from the conditioned media of each culture as described in the "Experimental Procedures". As many previous studies demonstrated, apoE3-HDL promoted cholesterol and phosphatidylcholine (PC) release from neurons in an HDL-dose-dependent manner (Fig. 1A). In contrast, surprisingly, the amounts of cholesterol and PC released from cultured neurons in the presence of apoE4-HDL remained very low at any HDL-cholesterol concentrations examined (Fig. 1A). Because our previous study demonstrated that apoE4-HDL contains apoE molecules twofold those in apoE3-HDL per particle [10], we determined the amount of apoE molecules in each HDL fraction added and plotted against the amount of cholesterol and PC released at various apoE concentrations. As shown in Fig. 1B, even when a comparable or a greater amount of apoE molecules was included in the apoE4-HDL than that in the apoE3-HDL, it did not promote lipid release, either (Fig. 1B). Analysis of the time dependence of lipid release mediated by apoE-HDL showed that lipid release mediated by apoE-HDL reached the peak 60 min following the addition of apoE3-HDL and apoE4-HDL (Fig. 2).

Effect of apoE-emulsion complex on cholesterol release from cultured neurons

To elucidate the mechanism underlying the apoE-isoform-specific effect on cholesterol release mediated by apoE-HDL, we used a complex consisting of lipid emulsion (EM) and recombinant human apoE3 or apoE4, because one cannot modulate the number of apoE molecules associated with HDL, but one can modulate apoE number associated with EM. Using this system, we can investigate the effect of apoE on EM-mediated lipid efflux. EM is generated using phosphatidylcholine and triolein and its diameter was determined as described in the Experimental Procedures, and the apoE-EM complexes were then re-isolated before use for the various assays performed. When EM was added to the neuronal cultures in which cholesterol was labeled with ^{14}C -acetate, cholesterol and PC were released in a time- and an EM-dose-dependent manner (Fig. 3), showing that EM serves as a lipid acceptor to release cholesterol. Time-dependent kinetics in terms of lipid release showed that the level of lipids released into the conditioned media saturated 30 min following the commencement of treatment. We determined the amounts of cholesterol and PC released

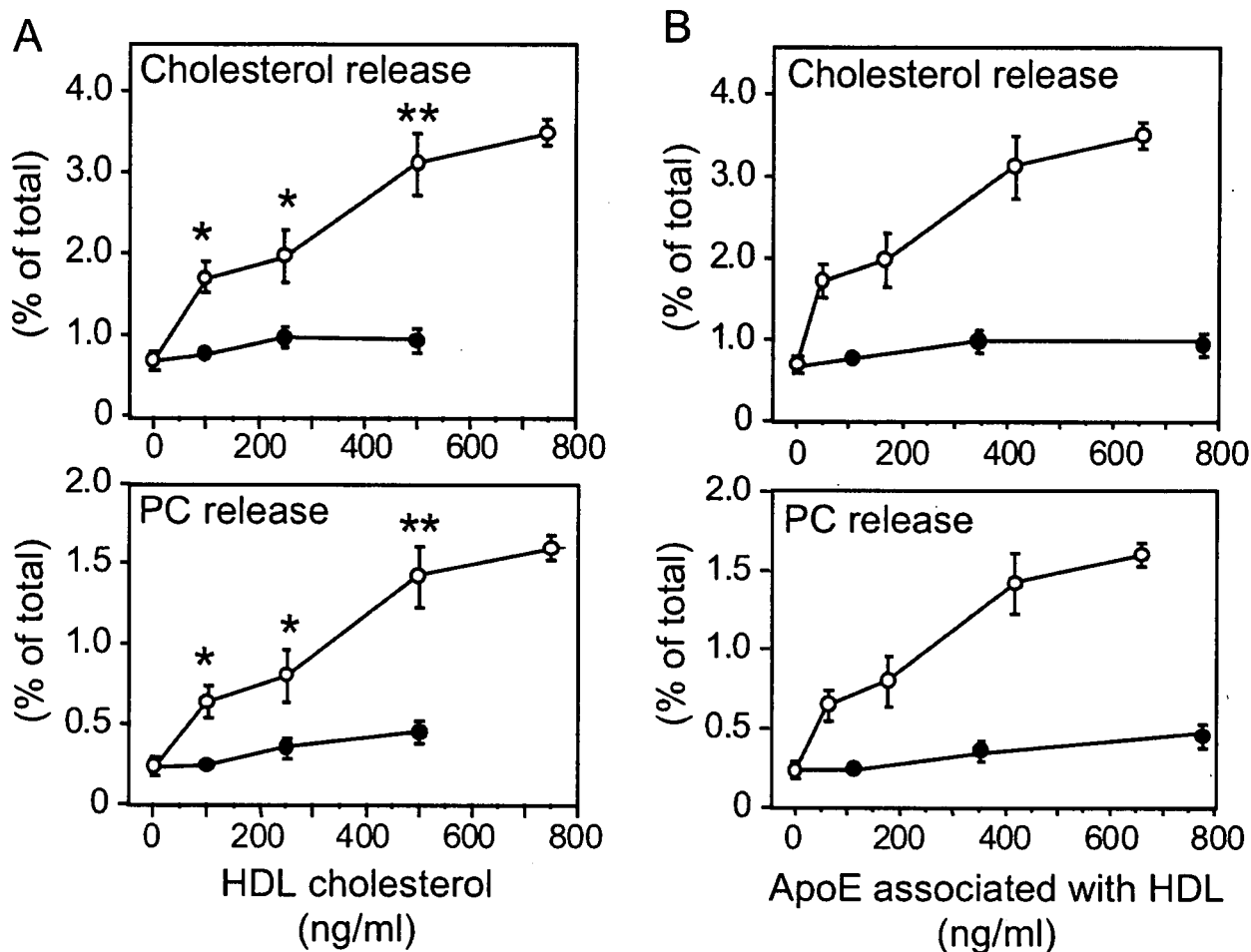


Figure 1
Characterization of cholesterol and PC release from cultured neurons in the presence of apoE3-HDL or apoE4-HDL. Neurons labeled with 37 Bq/ml [14 C] acetate were cultured for 3 days at 37°C. These neurons were washed in DMEM three times and incubated in DMEM for 1 h in the presence of apoE3-HDL or apoE4-HDL at various ApoE or HDL cholesterol concentrations, and the amounts of released [14 C]-labeled cholesterol and PC were determined. **A)** The amounts of cholesterol and PC released, as induced by apoE3-HDL (O), were significantly greater than those of cholesterol and PC released, as induced by apoE4-HDL (●). **B)** The amounts of cholesterol and PC are plotted against the concentrations of apoE associated with HDL. Data are means \pm S.E. of four samples. * $p < 0.05$ and ** $p < 0.001$ vs apoE4-HDL at various cholesterol concentrations. Six independent experiments show similar results.

using EM particles at a PC concentration of 5 μ g/ml 60 min following the commencement of treatment in the following experiments performed.

ApoE-dose-dependent inhibition of EM-mediated lipid release

We examined the dose-dependent effect of apoE associated with EM on apoE-EM-mediated lipid release. EM alone and the apoE3- and apoE4-EM complexes induced lipid release from neurons; however, with increasing concentration of apoE incubated with EM, the levels of lipid

released induced by both apoE3- and apoE4-EM complexes decreased (Fig. 4). However, when the apoE-EM complexes were generated by the incubation of apoE at concentrations of 0.1 and 1 μ g/ml with EM (at a PC concentration of 50 μ g/ml) and were applied at a PC concentration of 5 μ g/ml, apoE3-EM strongly induced cholesterol release, whereas apoE4-EM induced little. The weight ratio of PC per apoE calculated in each treatment is shown in Table 1. The ratio of ratio of PC per apoE at apoE concentrations of 0.1, 1, 10, and 30 μ g/ml was greater in the apoE3-EM complex than in the apoE4-EM complex,

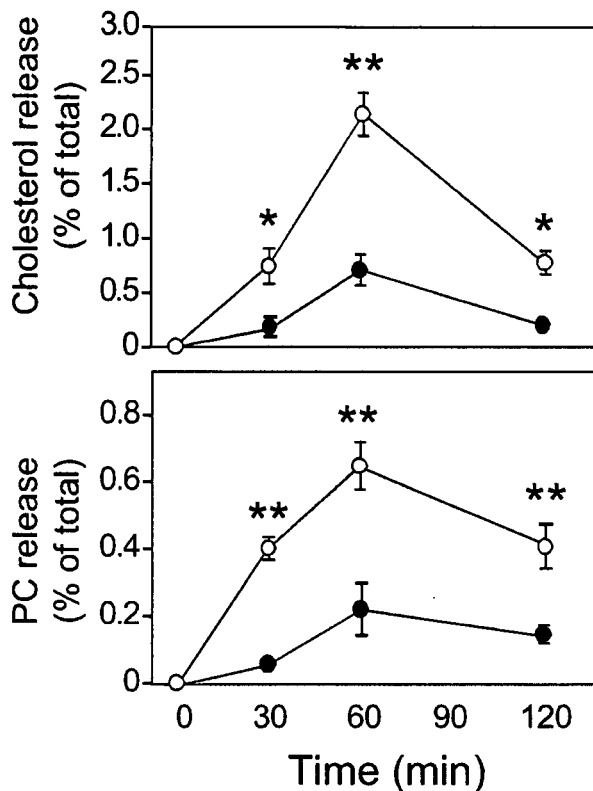


Figure 2
Time-dependent release of cholesterol and PC from cultured neurons in the presence of apoE3-HDL or apoE4-HDL. The kinetics of cholesterol and PC release in the presence of apoE3- or apoE4-HDL at a cholesterol concentration of 500 ng/ml were determined. Both apoE3- (○) and apoE4-HDL (●) induced cholesterol and PC release in a time-dependent manner. Data are means ± S.E. of four samples. *p < 0.001 and **p < 0.0001 vs apoE4-HDL. Six independent experiments showed similar results.

indicating that apoE4 has higher binding affinity to EM than apoE3. Interestingly, when the ratios (PC/apoE) were (a) 496 ± 97 (for apoE3) and 269 ± 85 (for apoE4), and (b) 23 ± 6 (for apoE3) and 16 ± 6 (for apoE4), which were obtained by incubating EM with apoE at concentrations of (a) 0.1 and (b) 1.0 μg/ml, respectively (Table 1), the apoE-isoform-specific cholesterol release was observed (Fig. 4) as was the case for apoE-HDL (Fig. 1), that is, apoE3-EM and apoE3-HDL induced cholesterol release from neurons, whereas apoE4-EM and apoE4-HDL induced little release.

We next examined whether the formation of the apoE-EM complex is required for apoE to attenuate the EM-mediated lipid release. When apoE3 and apoE4 at 10 μg/ml were preincubated with 5 μg/ml EM, lipid release in the

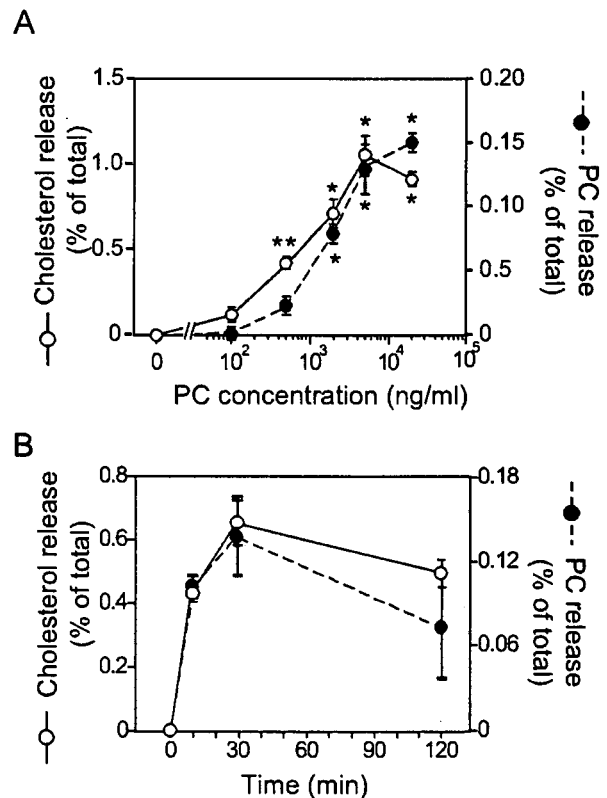


Figure 3
EM mediated cholesterol and PC release from cultured neurons. Neurons labeled with 37 Bq/ml [¹⁴C]acetate were cultured for 3 days at 37°C. These neurons were washed in DMEM three times and further incubated in DMEM for 1 h (a) or various times (b) in the presence of EM at various PC concentrations. The conditioned media were then collected and the amounts of [¹⁴C]-labeled cholesterol and PC were determined. (a) EM-induced cholesterol (○) and PC (●) release from cultured neurons occurs in an EM-PC-dose-dependent manner. (b) The time-dependent release of cholesterol (○) and PC (●) from cultured neurons in the presence of EM at PC concentration of 5 μg/ml is shown. Data are means ± S.E. of four samples. *p < 0.0001 and **p < 0.001 vs PC at 0 ng/ml and time at 0 h. Four independent experiments showed similar results.

presence of apoE3-EM or apoE4-EM was significantly reduced compared with that in the presence of EM. On the other hand when they were added into the culture medium without preincubation, they did not inhibit lipid release mediated by EM (Fig. 5).

Dimerization of apoE3 via disulfide bonds alters binding affinity of apoE3 to be similar to that of apoE4

Because apoE3 differs from apoE4 by one amino acid at residue 152 having cysteine instead of arginine, we exam-

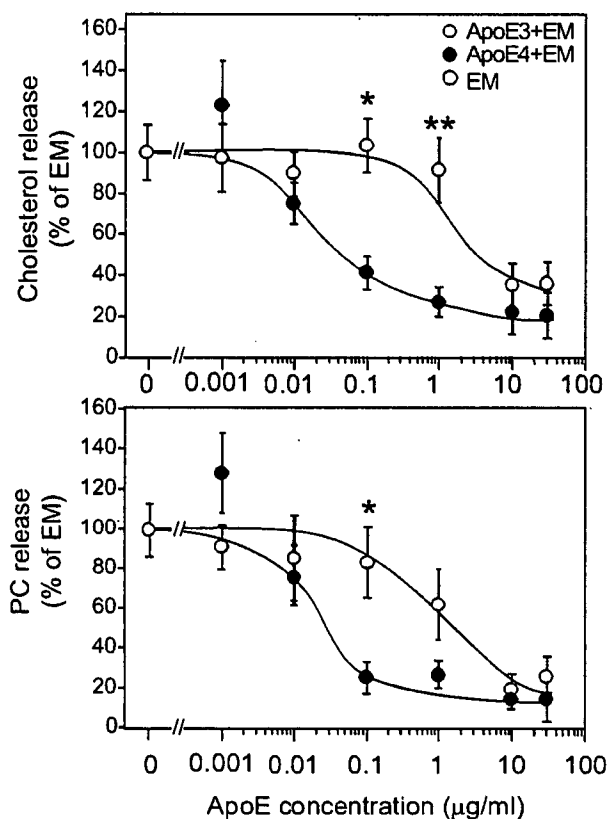


Figure 4
Effect of apoE associated with EM on EM-mediated cholesterol and PC release. Cultured neurons were incubated with serum-free N2 medium containing 37 Bq/ml [¹⁴C]acetate for 3 days at 37°C. These neurons were washed in DMEM three times and further incubated in DMEM for 1 h in the presence of the apoE-EM complex at various apoE concentrations. The conditioned media were then collected and the amounts of [¹⁴C]-labeled cholesterol and PC released were determined. The amounts of cholesterol and PC released into the conditioned media in the presence of apoE3-EM (○) or apoE4-EM (●) are shown. Data are means ± S.E. of four samples. *p < 0.01 and **p < 0.005 vs apoE4-EM. Six independent experiments showed similar results.

ined whether the dimerization of apoE3 molecules via disulfide bonds is responsible for the apoE-isoform-specific binding affinity to lipids. In the presence of 5% β-mercaptoethanol, the binding affinity of apoE3 increased to a level similar to that of apoE4 (Fig. 6). The next question is whether EM associated with apoE3 preincubated with β-mercaptoethanol, loses its ability to release cholesterol. However, this experiment was difficult to perform due to the toxic effect of β-mercaptoethanol on neurons.

Discussion

Previous studies showed that HDL induces cholesterol release from various cell types [15,18,20]. The present

Table 1: Binding of apoE3 and apoE4 molecules to emulsion particles

apoE concentration (µg/ml)	0.1	1	10	30
Weight ratio of PC per apoE				
apoE3	496 ± 97	23 ± 6	7 ± 2	8 ± 3
apoE4	269 ± 85	16 ± 6	6 ± 1	7 ± 1

Lipid emulsions were prepared by the method described previously [36] using a high-pressure emulsifier. The emulsion used had a particle size of 34.7 ± 5.2 nm and a weight ratio of TO/PC was 1.63 ± 0.07. For the generation of apoE-EM complex, apoE at various concentrations, from 0.1 to 30 µg/ml, was incubated with EM at PC concentration of 5.0 µg/ml in a 5-ml solution for 1 hr at room temperature. The apoE-EM complex was isolated and assayed by the ultracentrifugation method as previously reported [24, 37]. The weight ratio of apoE and PC were determined as described in the Experimental Procedures. Data are means ± S.E. of four samples.

study also showed that apoE3-HDL induced a strong cholesterol release, whereas apoE4-HDL induced a weak cholesterol release from neurons. As a mechanism underlying this apoE-isoform specificity, we showed a novel action of apoE, that is, although apoE is a lipid acceptor, when apoE is associated with lipid particles such as HDL and EM, apoE inhibits lipid-particle-mediated cholesterol release in an apoE-dose-dependent manner. We also found that more apoE4 molecules are associated with HDL or EM than apoE3. This may explain why apoE4 associated with HDL or EM inhibits HDL- or EM-mediated cholesterol release, whereas apoE3 does not. We also found that the dimerization of apoE3 by disulfide bonds causes the apoE-isoform dependence of the apoE binding affinity to lipids.

Cellular cholesterol release is mediated by two distinct mechanisms. One is the reaction of lipid-free apolipoproteins, such as apoE or apoAI, and any other molecules containing a certain amount of amphipathic α-helix with a cellular surface protein, ABCA1, resulting in the removal of cellular lipids and the subsequent generation of HDL-like particles [16,21]. The other one is a nonspecific physicochemical interaction causing cholesterol diffusion and its exchange between plasma membrane and lipid particles mediated by EM and liposomes. The cellular cholesterol release mediated by HDL likely involves both mechanisms, because HDL in the central nervous system contains at least two potential acceptors, apoE and lipids. One may reasonably raise questions as to which apoE isoform and to what extent lipids contribute to the HDL-mediated cholesterol release. Previous studies showed that apolipoprotein AI (apoAI) dissociated from HDL cooperatively induces cholesterol release [21,22] and that cholesterol release mediated by HDL is, in part, mediated by apoAI dissociated from HDL in an ABCA1-dependent manner [23]. However, our previous study showed that the level of free apoE in conditioned media and physiological fluid is underdetectable and almost all of the apoE is recovered from the HDL fraction [10].

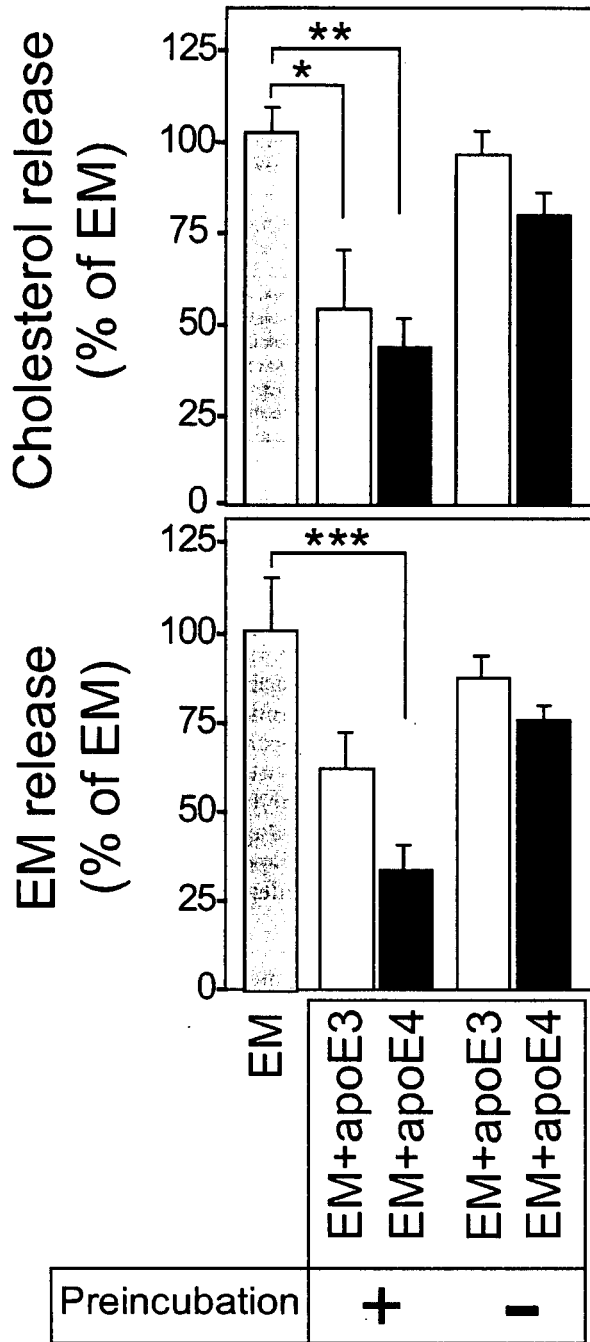


Figure 5
ApoE inhibits EM-mediated lipid release when apoE is associated with EM particles. ApoE-EM complexes were prepared as described in the Experimental Procedures. Neurons were prepared as described in Figure 4 until treatment. Neurons were treated with EM (5 µg/ml); the apoE3-EM complex (10 µg/ml, and 5 µg/ml, respectively) and the apoE4-EM complex (10 µg/ml, and 5 µg/ml, respectively), which was preincubated and isolated by centrifugation; apoE3 (10 µg/ml) and EM (5 µg/ml) or apoE4 (10 µg/ml) and EM (5 µg/ml), which were added separately. Data are means ± S.E. of four samples. *p < 0.05, **p < 0.002, and ***p < 0.02 between the values indicated. Three independent experiments showed similar results.

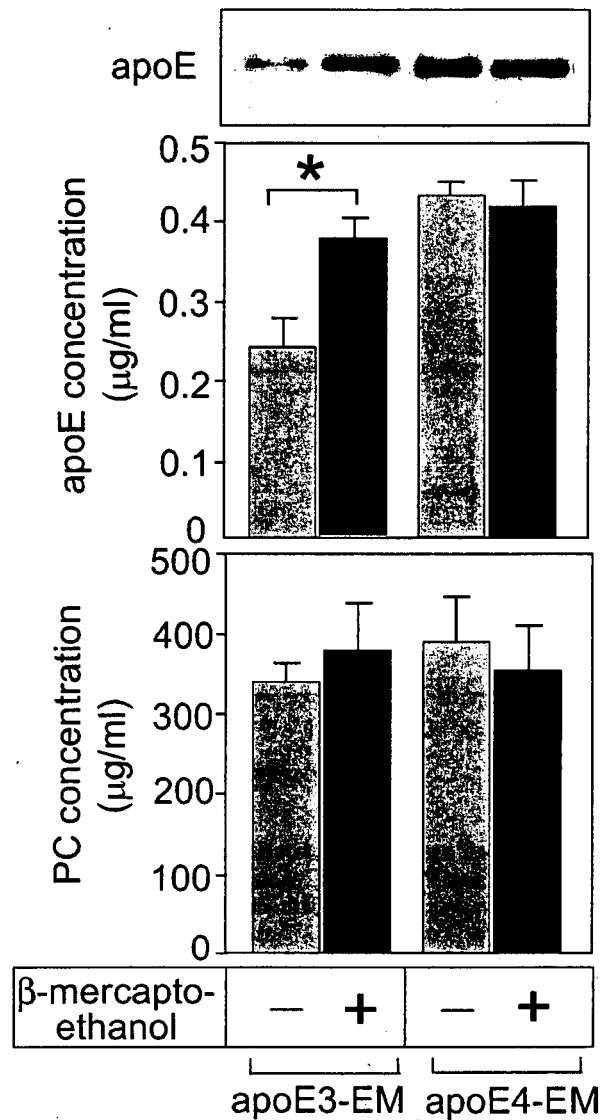


Figure 6
Dimerization of apoE3 by disulfide bonds causes the difference in apoE isoform-specific binding affinity. EM and apoE3 (or apoE4) were incubated in the presence or absence of 5% β-mercaptoethanol for 1 h and EM-PC concentrations and the number of apoE molecules in the apoE-EM complex was determined as described in the Experimental Procedures. Data are means ± S.E. of three samples. *p < 0.001. Three independent experiments showed similar results.

Moreover, as we previously reported [10], apoE4-HDL contains number of apoE molecules per particle twofold that of apoE3; however, apoE4-HDL induced a very-weak cholesterol release, whereas apoE3-HDL induced a strong cholesterol release (Figs. 1A and 1B). These results suggest that HDL harboring apoE4 on its surface does not induce cholesterol release. That is, apoE4 on the HDL surface inhibits cholesterol release mediated by HDL. This notion is confirmed by our experiments performed to determine the effect of apoE on EM-induced cholesterol release using the apoE-EM complex at various apoE concentrations on the EM surface, that is, with increasing number of apoE molecules associated with EM, not only apoE4-EM, but also apoE3-EM lose their ability to induce cholesterol release, suggesting that apoE associated with EM strongly inhibits EM-mediated cholesterol release. Interestingly, the apoE-EM complex at certain apoE concentrations and apoE/EM particle ratios induces cholesterol release in an apoE-isoform-specific manner; apoE-EM3 induces cholesterol release, but apoE4-EM does not (Fig. 4), as observed in the cases of apoE3-HDL and apoE4-HDL (Fig. 1). As shown previously [24], our data confirm that more apoE4 molecules are associated with HDL or EM than apoE3 (Table 1). These results lead us to propose a novel hypothesis that apoE molecules covering the surface of lipid particles inhibit the physicochemical interaction occurring between lipids and cell membrane. There are greater numbers of apoE4 molecules associated with EM and HDL, which in turn inhibit the EM- and HDL-mediated physicochemical exchange of cholesterol, whereas in the case of apoE3, the smaller number of apoE3 molecules on the EM or HDL surface allows cholesterol influx to EM or HDL surface from the cell membrane (Fig. 7b).

One may raise the issue that apoE-HDL/EM internalization via several apoE receptors expressed on neurons and recycled-apoE-mediated cholesterol efflux may explain apoE-isoform-dependent cholesterol efflux induced by apoE-HDL/EM. The recycle of apoE4 has been shown to be significantly decreased, resulting in a decreased level of cholesterol efflux [25]. Thus, it is possible that the poor recycling of internalized apoE4 is responsible for the reduced ability of apoE4-containing particles to release cholesterol from neurons. However, the observation that a greater amount of apoE3 associated with EM induces a smaller amount of cholesterol efflux compared with EM without apoE3 (Fig. 4) indicates that this is not the case.

It has been shown that HDL in the central nervous system contains apoE and that neurons express apoE receptors [26]. Thus, different from HDL in systemic circulation, HDL in the central nervous system is internalized into cells via apoE receptors to supply cholesterol to neurons (Fig. 7a). One may question, which is the net cholesterol transport, release from or supply to cells, in the presence

of apoE-HDL and apoE-EM? It has been shown that HDL serves as the net cholesterol supplier to neurons. Previous studies showed that HDL promotes synaptogenesis [27], synaptic plasticity [28], and elongation of axons [29], and strongly suppresses cholesterol synthesis (unpublished data), indicating that HDL-cholesterol is taken up by neurons and used for axonal elongation and synaptogenesis.

If HDL, as a whole, functions as a net cholesterol supplier to neurons, what is the biological significance of apoE-HDL-mediated cholesterol release from neurons? Because physicochemical interaction causes a bidirectional cholesterol exchange (nonspecific cholesterol diffusion) between HDL cholesterol and cholesterol in the plasma membrane, it is reasonable to assume that cholesterol exchange may contribute to the maintenance of a fresh supply of cholesterol in the plasma membrane by replacing accumulated oxidized cholesterol in the membrane. Thus, the isoform-specific apoE-HDL action on cholesterol exchange suggests that the apoE3-HDL complex has a greater ability to maintain a fresh supply of cholesterol in the plasma membrane (Fig. 7b). The lower ability of apoE4-HDL may result in the accumulation of oxysterols in the plasma membrane, leading to altered membrane functions e.g., signal transduction, enzyme activities, and ion channel properties.

The last issue to be addressed is the cause of the apoE-isoform dependence of the preferential association of apoE with HDL and EM particles. Previous studies showed that apoE3 forms a disulfide-linked homodimer in plasma [30] and in culture media [10]. Thus, we determined whether this dimerization of apoE3 is responsible for the lesser number of apoE3 molecules associated with EM. The treatment of apoE3 with β -mercaptoethanol significantly increased the number of apoE3 molecules associated with EM (Fig. 6), indicating that when apoE3 remains as a monomer, more apoE3 monomers can associate with EM than apoE3 dimers. The next question is whether EM associated with apoE3, which is treated with β -mercaptoethanol, loses its ability to induce strong cholesterol release. However, this experiment is difficult to perform due to toxic effect of β -mercaptoethanol on neurons. Regarding the effect of apoE polymorphism on lipid interaction, it has been suggested that domain interaction mediated by a salt bridge between Arg-61 in the N-terminus and Glu-255 in the C-terminus, leading to a compact structure, results in the preferential binding of apoE4 to very low density lipoproteins [11,31,32]. The apoE4 domain interaction has been observed in vivo in Arg-61 knock-in mice [33]. Taken all together, it is possible that in addition to structural differences within an apoE molecule such as domain interaction, dimerization also has an effect on the apoE structure (which may also affect domain interaction), leading to the enhancement of the

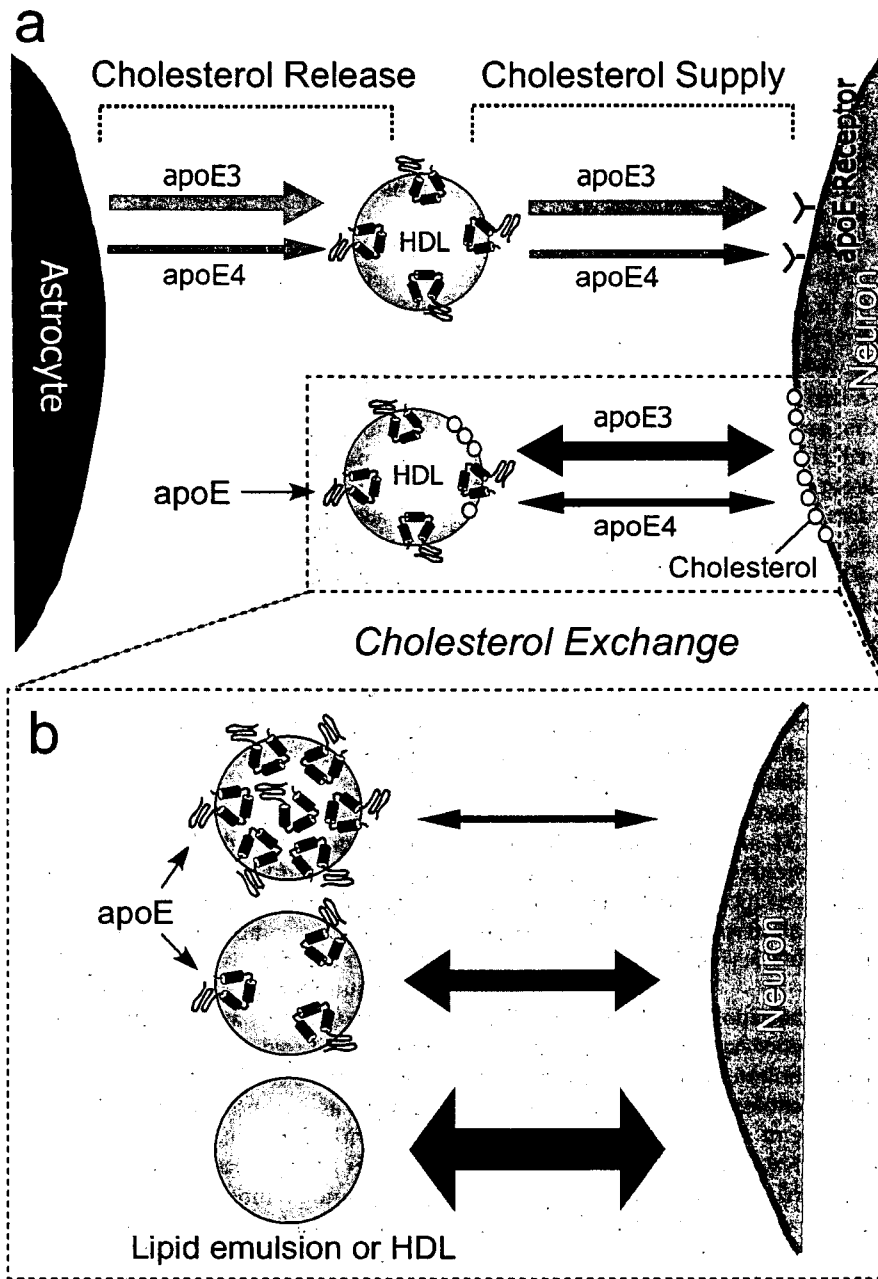


Figure 7

Schema showing the apoE-isoform-specific effect of apoE-HDL and apoE-EM complex on HDL- and EM-mediated cholesterol release. Lipid-free apoE3 released from astrocytes generates a greater number of HDL particles than apoE4 with a similar number of apoE molecules [10], indicating that apoE3-expressing astrocytes can supply more cholesterol as HDL to neurons via apoE receptors (a). Lipid particles such as HDL and EM have another role in cholesterol metabolism, that is, physicochemical and nonspecific cholesterol exchange between lipid particles and the cell membrane. Our present results show that with increasing number of apoE molecules on the particle surface, apoE inhibits the particle-mediated cholesterol exchange (b). Because a greater number of apoE4 molecules bind to lipid particles (HDL and EM) than apoE3 molecules, apoE4 inhibits cholesterol exchange (release) by covering the lipid surface, whereas a smaller number of apoE3 molecules on lipid particle surface allows apoE3-HDL- or apoE3-EM-mediated cholesterol release (b).

apoE-isoform-specific effect on the interaction between apoE and lipids.

Conclusion

Here we have shown that although apoE and a lipid particle such as EM are lipid acceptors, when apoE and a lipid particle form a complex, apoE inhibits the lipid particle-mediated cholesterol release from cells in an apoE-dose-dependent manner, probably owing to the occupation of the surface of the lipid particle, thereby inhibiting lipid diffusion and exchange. The observation that a greater number of apoE4 molecules are associated with EM than apoE3 may explain the apoE-isoform-dependent lipid release induced by the apoE-lipid complex. Because lipid particles such as HDL and EM induce physicochemical and nonspecific cholesterol exchange between lipid particles and the cell membrane, the lower ability of the apoE4-lipid complex to release lipids may result in the lower lipid replacement and accumulation of oxysterols in the plasma membrane, leading to altered membrane functions, e.g., signal transduction, enzyme activities, and ion channel properties.

Methods

Animals

The animal care and the experiments using animals were carried out in accordance with institutional guidelines. Mice expressing human apoE4 in place of mouse apoE were generated by the gene-targeting technique taking advantage of homologous recombination in embryonic stem cells (knock-in) as previously described [34]. ApoE3 knock-in mice were generated in the same manner except that the transgene carried apoE3 cDNA in place of apoE4 cDNA. Postnatal day 2 mice that possess the homozygous epsilon 3 (3/3) or epsilon 4 (4/4) allele, and correctly expressing human apoE3 or apoE4 proteins, respectively, were used in this study.

Cell culture

Highly astrocyte-rich cultures were prepared according to a previously described method [10]. In brief, the brains of postnatal day 2 mice were removed under anesthesia. The cerebral cortical fragments were incubated in 0.25% trypsin and 20 mg/ml DNase I in phosphate-buffered saline (PBS) (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl and 2.7 mM KCl, pH 7.4) at 37°C for 20 min. The fragments were then dissociated into single cells by pipetting. The dissociated cells were seeded in 75-cm² dishes at a cell density of 1 × 10⁷ in Dalbecco's modified essential medium (DMEM) containing 10% FBS. After 10 days of incubation in vitro, astrocytes in the monolayer were trypsinized (0.1%) and reseeded onto six-well dishes and maintained in DMEM containing 10% FBS until use.

Neuron-rich cultures were prepared from rat cerebral cortices as previously described [35]. Dissociated cells were suspended in the feeding medium and plated onto poly-D-lysine-coated twelve-well plates at a cell density of 2 × 10⁵/cm². The feeding medium consisted of DMEM nutrient mixture (DMEM/F12; 50%: 50%) and N2 supplements. More than 99% of the cultured cells were identified as neurons by immunocytochemical analysis using a monoclonal antibody against microtubule-associated protein 2, a neuron-specific marker, on day 3 of culture.

Preparation of HDL released into conditioned media of astrocytes expressing apoE3 or apoE4

Astrocytes in 75-cm² dishes were washed in DMEM three times and incubated in 12 ml of DMEM for 5 days at 37°C. After incubation, the astrocyte culture medium was collected, centrifuged at 1,600 × g for 15 min in a 50-ml plastic tube to exclude cell debris, and adjusted to a discontinuous sucrose gradient, which was prepared in a 14 × 89 mm ultracentrifuge tube (Ultraclear, Beckman, Palo Alto, CA) from the bottom to the top, with 1.5 ml of sucrose at a density of 1.30 g/ml, 3 ml at 1.20 g/ml, 4.5 ml at 1.10 g/ml, and 3 ml at 1.006 g/ml medium. The sample in the sucrose gradient was then centrifuged in an SW41-Ti swing rotor (Beckman, Palo Alto, CA) at 16°C for 48 h at 160,000 × g. Following density gradient centrifugation, twelve 1.0-ml fractions were collected with a micropipette from the top gradient. The final fraction was stirred to resuspend the pellet. The density of each fraction was determined using a density meter, DMA35N (Anton Paar, Graz, Austria).

Preparation of lipid emulsions (EM) and apoE-EM complex

Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei (Tokyo, Japan). Triolein (TO) was purchased from Sigma (St. Louis, MO). Lipid emulsions were prepared by the method described previously [36] using a high-pressure emulsifier (Nanomizer System YSNM-2000AR; Yoshida Kikai Co., Nagoya, Japan). The mixture of TO and PC at a weight ratio of 1:1 was suspended in 50% glycerol in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA and 0.01% NaN₃, and subsequently emulsified under 140 MPa of pressure at 60°C. Glycerol was exhaustively removed by dialysis against PBS overnight. The contaminating vesicles and larger particles were removed by ultracentrifugation. The weight-averaged particle size of emulsions was 34.7 ± 5.2 nm determined from dynamic light scattering measurements (Photal LPA-3000/3100; Otsuka Electronic Co., Osaka, Japan). The concentrations of TO and PC were determined using enzymatic assay kits purchased from Wako Pure Chemicals (Osaka, Japan). After ultracentrifugation, the weight ratio of TO/PC was 1.63 ± 0.07 (mean

± S.D., n = 5). For the generation of the apoE-EM complex, apoE at various concentrations, from 0.001 to 30 µg/ml, was incubated with EM at a PC concentration of 50 µg/ml in a 5 ml solution for 1 h at room temperature. The apoE-EM complex was isolated and assayed by the ultracentrifugation method as previously reported [24,37].

Lipid analysis

The extraction of lipids and the subsequent determination of the amounts of cholesterol and phospholipids in the HDL fraction were carried out according to previously described methods [9]. Aliquots (1.0 ml) of conditioned culture media were transferred to clean glass tubes containing 5.0 ml of chloroform: methanol (2:1 v/v). The organic phases were removed, evaporated under N₂ gas, followed by redissolution in 50 µl of isopropanol for lipid assay. The amount of total cholesterol was determined using a cholesterol determination kit, LTCII (Kyowa Medex, Tokyo, Japan). The amount of phospholipids was determined using a phospholipid determination kit, PLB (Wako, Osaka, Japan). For the determination of PC level in the apoE+EM complex in the presence of 5% β-mercaptoethanol, PC was extracted with chloroform: methanol (2:1 v/v) as described above. The organic phases were removed, evaporated under N₂ gas. The samples were redissolved in 20 µl of chloroform: methanol (2:1 v/v) and 5 µl of each solution was spotted on a chromarod-SIII quartz rod and analyzed by thin-layer chromatography/flame ionization detector (Iatroscan MK-5 ; Iatron Lab., Inc., Tokyo, Japan).

Determination of amount of cholesterol and phosphatidylcholine released from neurons labeled with [¹⁴C]acetate

Neurons cultured for 2 days were labeled with 37 Bq/ml [¹⁴C] acetate (DuPont NEN) for another 2 days. Two days later, these neurons were washed three times with 1.5 ml of DMEM and incubated in DMEM containing reagents such as apoE3-HDL, apoE4-HDL, apoE3-EM, or apoE4-EM at various concentrations. Aliquots of 1.0 ml each of the conditioned culture media were transferred to clean glass tubes containing 5.0 ml of hexane:isopropanol (3:2 v/v). For the extraction of intracellular lipids, dried cells were incubated in hexane:isopropanol (3:2 v/v) for 1 h at room temperature. The solvent from each sample was evaporated and the organic phases were redissolved in 20 µl (for the condition medium) and 200 µl (for the cells) of chloroform, and 10 µl of each sample was spotted on activated silica gel high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany); the lipids were separated by sequential one-dimensional chromatography using chloroform: methanol: acetic acid: water (25:15: 4: 2, v/v/v/v), followed by another run in hexane: diethylether: acetic acid (80: 30: 1). [¹⁴C]-Cholesterol and [¹⁴C]-phosphatidylcholine were used as stand-

ards. The chromatography plates were exposed to radiosensitive films and each lipid was visualized and quantified with BAS2500 (Fuji Film, Tokyo, Japan).

Immunoblot analysis

Samples of each fraction were dissolved in the sample buffer consisting of 100 mM Tris-HCl (pH 7.4), 10% glycerol, 4% SDS, 10% mercaptoethanol and 0.01% bromophenol blue, and analyzed by 4–20% gradient Tris/tricine SDS-PAGE as previously reported [38]. The separated proteins were transferred onto Immobilon membranes with a semidry electrophoretic transfer apparatus (Nihon Eido, Tokyo, Japan) using a transfer buffer (0.1 M Tris, 0.192 M glycine and 20% methanol). Blots were probed for overnight at 4°C with a goat anti-apoE polyclonal antibody, AB947 (1: 2,000; Chemicon, Temecula, CA). Bands were detected using an ECL kit (Amersham Pharmacia Biotech, UK). For the determination of the concentration of apoE released into the culture medium, signals corresponding to apoE of each sample in the immunoblot membrane were quantified by densitometry using NIH image software, at varying concentrations of synthetic apoE protein (Wako, Tokyo, Japan) as standards. Standard signals were demonstrated to be linear in the range of apoE protein amounts from 0 to 2 µg per lane. ApoE concentrations in the HDL fraction and apoE-EM complex fraction within this range were used for analysis. For immunoblot analysis using anti-ABCA1 antibody, the cultured neurons in a 6-well plate were washed in cold PBS and harvested in 500 µl of 50 mM Tris-HCl (pH 7.4) solution containing 2 mM EGTA. The cell lysates were sonicated and centrifuged at 700 rpm for 10 min at 4°C. The supernatant of each sample was centrifuged at 14,000 rpm for 20 min at 4°C, and the pellet fractions were resuspended in DW containing 0.45 M Urea, 0.1% TritonX-100, and 0.05% Dithiothreitol and used for immunoblot analysis as described above.

Statistical analysis

StatView computer software (Windows) was used for statistical analysis. Statistical significance of differences between samples was evaluated by multiple pairwise comparison among the sets of data using ANOVA and the Bonferoni t-test.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

JSG carried out major part of the experiments. SM and TH prepared microemulsion. MK and SCF generated and provided ApoE3- and ApoE4-knock-in mice. KY contributed to interpret the results and make critical intellectual comments. MM participated in its design and coordination

and was involved in the interpretation of the results and in drafting the manuscript.

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STEM CELLS[®]

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NPC1 Gene Deficiency Leads to Lack of Neural Stem Cell Self-Renewal and Abnormal Differentiation Through Activation of p38 Mitogen-Activated Protein Kinase Signaling

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Key Words. Neural stem cells • Astrocytes • Niemann-Pick type C1 • p38 mitogen-activated protein kinase

ABSTRACT

Neural stem cells (NSCs) are capable of giving rise to neurons, glia, and astrocytes. Although self-renewal and differentiation in NSCs are regulated by many genes, such as Notch and Numb, little is known about the role of defective genes on the self-renewal and differentiation of NSCs from developing brain. The Niemann-Pick type C1 (NPC1) disease is a neurodegenerative disease caused by a mutation of the NPC1 gene that affects the function of the NPC1 protein. The ability of NSC self-renewal and differentiation was investigated using a model of NPC1 disease. The NPC1 disorder significantly affected the self-renewal ability of NSCs, as well as the differentiation.

NSCs from *NPC1*^{-/-} mice showed impaired self-renewal ability compared with the *NPC1*^{+/+} mice. These alterations were accompanied by the enhanced activity of p38 mitogen-activated protein kinases (MAPKs). Further, the specific p38 MAPK inhibitor SB202190 improved the self-renewal ability of NSCs from *NPC1*^{-/-} mice. This indicated that the NPC1 deficiency can lead to lack of self-renewal and altered differentiation of NSCs mediated by the activation of p38 MAPK, impairing the generation of neurospheres from *NPC1*^{-/-}. Thus, the NPC1 gene may play a crucial role in NSC self-renewal associated with p38 MAPK. *STEM CELLS* 2006;24:292–298

INTRODUCTION

Neural stem cells (NSCs) are multipotent cells that are able to renew themselves and differentiate into neural and glial lineages *in vitro*. The NSC clonal aggregates, often called neurospheres, may provide an unlimited source of cells for grafting into patients with neurodegenerative diseases [1, 2].

Many factors, such as the well-known signaling of Notch and Numb, are crucial in determining the fate of NSCs [3–5]. Notch signaling is often referred to as a gatekeeper against differentiation because it blocks primary differentiation in cells and directs them to remain in an undifferentiated state. Thus, activation of Notch signaling inhibits NSC differenti-

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ation, whereas the suppression of Notch signaling promotes stem cell neurogenesis [6, 7]. Numb signaling plays multiple roles for the generation of neural lineages through tuning development cell fates. Numb signaling is widely regarded as an inhibitor of Notch signaling, which suppresses neurogenesis [5]. However, the mechanism of this inhibition is not well understood, and suggestions are that it may involve interaction with α -Adaptin, a component of the AP-2 complex of the endocytic pathway machinery [8, 9]. Given that the fate of NSCs is governed by a transcriptional network pathway in the developing mouse brain [10] and that the mitogen-activated protein kinase (MAPK) and its downstream nuclear targets (important mediators of signal transduction that play a key role in the regulation of many cellular processes in the central nervous system [CNS]) are activated during various physiological and pathological events [11, 12], interest in the role of the activation of p38 MAPK signaling in differentiation abounds. The p38 MAPK and c-Jun N-terminal protein kinase signaling are known to regulate differentiation and apoptosis in immortalized neuronal stem cells [13] as well as Gap junctional intercellular communications, and these may play an important role during neuronal stem cell differentiation [14]. The extracellular signal-regulated kinase 1 (ERK1)/ERK2 and p38 MAPK signaling pathway may be closely related functionally to regulate gap junction in rat NSCs [14]. Interestingly, MAPK signaling may also be involved in neurogenesis of NSCs in the determination of either cell survival or apoptosis [15, 16].

Niemann-Pick type C1 (NPC1) is an autosomal-recessive, lysosomal lipid storage disease characterized by defective trafficking of intracellular cholesterol and lysosomal accumulation of unesterified cholesterol gangliosides and other lipids [17–20]. Loftus et al. [21] reported that NPC1 mice are asymptomatic at birth, with the earliest definitive symptoms of the disease apparent by 4 to 6 weeks of age and death ensuing by 10 to 15 weeks of age. These are characterized by a fatal buildup of unesterified cholesterol and sphingolipids in late endocytic organelles, leading to demyelination and progressive neurodegeneration [22, 23]. Moreover, abnormal cholesterol metabolism due to the *NPC1*^{-/-} may be responsible for activation of the MAPK signaling pathway and site-specific phosphorylation of tau in vivo, leading to tauopathy in NPCs [24].

There is an increasing interest in the importance of glial cells for neuronal survival. Astrocytes that ensheath the synapses within the CNS are known to play an important role in synapse formation [25, 26], but they are also suggested to contribute to neurodegeneration in the NPC1 mouse [27]. NSCs have potential clinical applications to overcome neurodegenerative diseases, such as Parkinson's disease and Huntington's disease [28, 29]. However, whether deficiency of certain genes may affect self-renewal and differentiation in NSC fate remains unclear. This study investigated whether gene mutation can affect self-renewal of NSCs in NPC knockout mice that are mutated in NPC1 protein-mutated mice and conclusively showed that NSCs isolated from NPC^{-/-} mice were unable to self-renew and altered differentiation by the activation of p38 MAPK signaling.

MATERIALS AND METHODS

Mice

A breeding pair of BALB/c NPC1^{N^{HH}} mice, heterozygous for NPC1 (NPC1^{-/+}), were purchased from Jackson Laboratory (Bar Harbor, ME, <http://www.jax.org>). For genotyping of cultured NSCs prepared from fetal mice, DNA was isolated from tail tips of each mouse and polymerase chain reaction was performed as described in Loftus et al. [21].

Neurosphere Cell Culture

For neurospheres, fetal cerebral cells from NPC transgenic mice were collected at day E16. After trypsin dissociation, primary cells were seeded at the concentration of 1 cell per microliter and expanded in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco, Carlsbad, CA, <http://www.invitrogen.com>) supplemented with epidermal growth factor (EGF) (10 ng/ml) (Roche Applied Science, Mannheim, Germany, <http://www.roche-applied-science.com/>) and basic fibroblast growth factor (bFGF) (20 ng/ml) (Roche Applied Science), 2% B27 supplement (Gibco), and penicillin-streptomycin-neomycin antibiotic mixture (100 mg/ml, Gibco). The ratio of the number of spheres formed after 7 days (formation of secondary spheres) and 21 days (formation of tertiary spheres) in vitro to the number of cells plated is the NSC frequency. Subsequent passaging of primary spheres was performed by mechanically dissociating collected spheres (centrifuged for 5 minutes at 100g) into a single-cell suspension and replating in basal media containing EGF and bFGF. After NSCs were treated with MAPK inhibitors PD 980590 and SB202102 (Tocris, Avonmouth, U.K., <http://www.tocris.com/>), they were differentiated by transfer to chamber slides coated with poly-D-lysine (Nunc, Roskilde, Denmark, <http://www.nuncbrand.com>) in basal medium with 1% fetal bovine serum (Gibco) for 5 to 6 days and then assessed by immunocytochemistry for astrocytes.

Immunocytochemistry

Neurospheres were fixed immediately in 4% paraformaldehyde and preincubated for 1 hour with normal goat serum (10%) (Zymed Laboratories Inc, San Francisco, <http://www.zymed.com>). The neurospheres were performed as described previously, using known marker for astrocytes (anti-glial fibrillary acidic protein [anti-GFAP], 1:200, Chemicon, Temecula, CA, <http://www.chemicon.com>), and were incubated with peroxidase-conjugated secondary antibody (anti-mouse TRITC, 1:200, Zymed Laboratories Inc). Images were captured on a Nikon Diaphot microscope using a Progress camera.

Western Blot Analysis

Neurosphere-derived cells were lysed with a buffer (150 Mm NaCl, 20 Mm Tris-HCl, 1 mM EDTA) containing protein inhibitors (1 μ g/ml aprotinin, 1 μ M leupeptin, 1 mM PMSF) and protease inhibitors (1 mM NaO₂, 1 mM NaF). Collected proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose, hybridized with antibody to GFAP (1:1,000, Chemicon), MKK3 (1:1,000, Cell Signaling Technology, Beverly, MA, <http://www.cellsignal.com>), pMKK3/6 (1:1,000, Cell Signaling Technology), and p38 (1:1,000, Cell Signaling Tech-

nology), phospho-p38 (1:1,000, Promega, Madison, WI, <http://www.promega.com>), phospho-ERK1/2 (1:1,000, Promega), ERK1/2 (1:1,000, Zymed Laboratories Inc), and B-actin (1:5,000, Sigma), and detected by chemiluminescence.

Statistical Analysis

Statistical analysis was performed using analysis of variance followed by Duncan's multiple-range test.

RESULTS

Multipotent Neurospheres from $NPC^{-/-}$ Mice Significantly Showed Lack of Self-Renewal Ability

NSC self-renewal in the presence of a deficiency of NPC1 gene was assessed in isolated cells from brain cerebral cortices of $NPC1^{+/+}$, $NPC1^{+/-}$, and $NPC1^{-/-}$ mice at embryonic day 16 (E16). The genotype was determined by tail DNA analysis (Fig. 1). The cells were plated as single cells at a density of 5×10^4 cells/ml in 24 wells with DMEM/F12 medium containing bFGF and EGF. After incubation for 7 days, cerebral cells from $NPC1^{+/+}$, $NPC1^{+/-}$, and $NPC1^{-/-}$ mice generated multipotent neurospheres, but neurospheres from $NPC1^{+/-}$ and $NPC1^{-/-}$ mice were formed at significantly lower numbers and smaller diameters ($p < .05$) than those from $NPC1^{+/+}$ mice (Figs. 2A–2C).

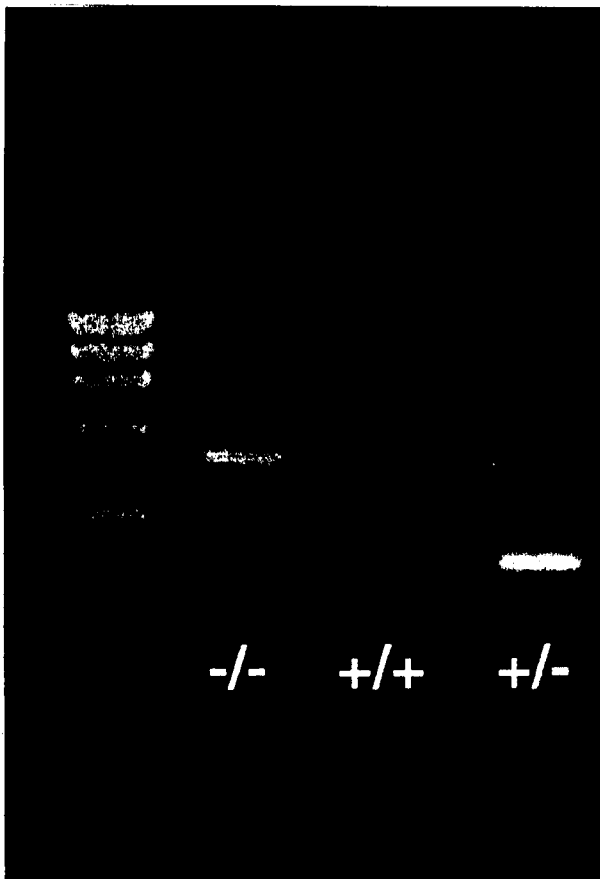


Figure 1. Genotyping analysis with tail-DNA by polymerase chain reaction as described in Materials and Methods. $NPC1^{-/-}$ is presented in 475 bp, $NPC1^{+/+}$ in 173 bp, and $NPC1^{+/-}$ in both 475 bp and 173 bp. Abbreviation: NPC1, Niemann-Pick type C.

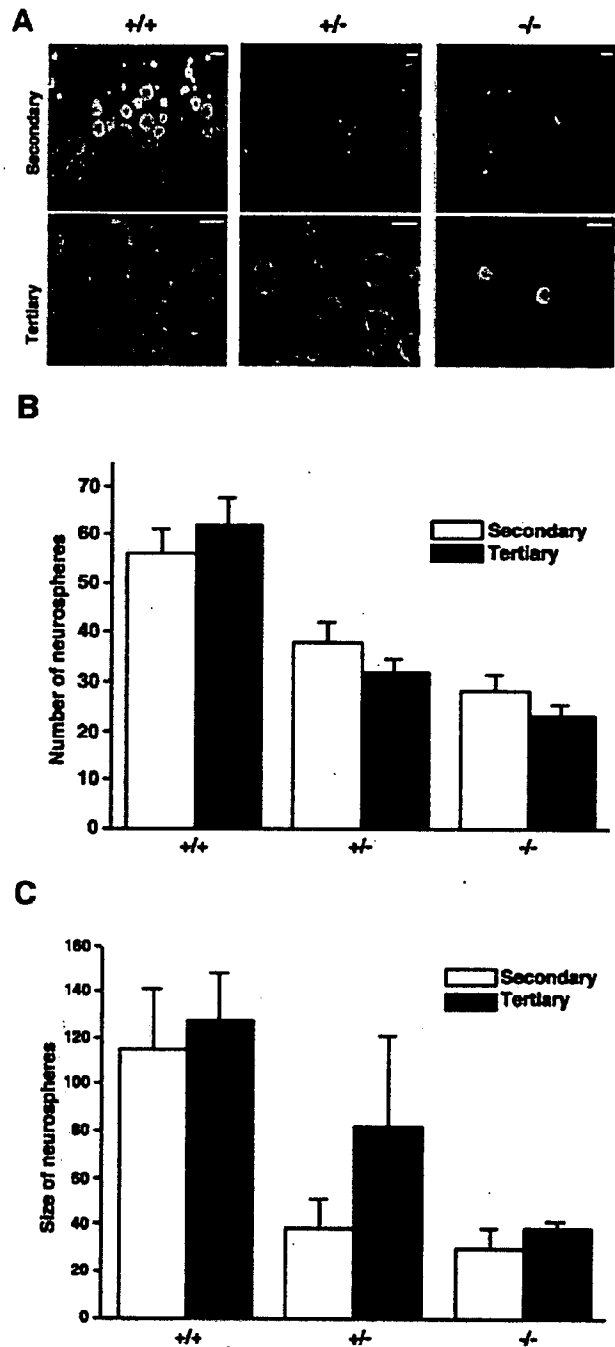


Figure 2. Neural stem cells (NSCs) require Niemann-Pick type C (NPC) to self-renew normally. Images show typical neurospheres that formed after 7 days from E16 fetal mice brain. (A): Generation of $NPC1^{-/-}$ E16 cerebral cells; the cells that formed multipotent neurospheres were significantly reduced relative to wild-type cells. (B, C): Self-renewal capacity is expressed as the number and diameter of secondary and tertiary neurospheres generated per primary neurospheres on subcloning.

NPC1 Deficiency–Mediated Activation of the MAPK Impaired the Generation of Neurospheres from $NPC1^{-/-}$

Because several different MAPK-dependent pathways mediate the cell differentiation of astroglia, we extracted and examined

several MAPK pathway-related proteins from NSCs from *NPC1*^{+/+}, *NPC1*^{+/-}, and *NPC1*^{-/-} mice. The levels of MKK3, p38, pMKK3/6, and pp38 in the NSCs from *NPC1*^{+/-} and *NPC1*^{-/-} were significantly higher than those in the *NPC1*^{+/+} (Fig. 3A). Moreover, the levels of ERK1/2 and phosphorylated ERK1/2 were also increased in NSCs from *NPC1*^{-/-} compared with those in the cells from *NPC1*^{+/+} (Fig. 3C).

Self-Renewal Ability of NSCs Was Improved by p38 MAPK Inhibitor in *NPC1*^{-/-} Mice-Derived Neurospheres

To determine if the higher levels of MAPK were impairing the self-renewal, proliferation, and differentiation of the neurospheres from *NPC1*^{-/-} mice, NSCs were treated with a MAPK kinase (MEK) inhibitor (2 μM PD98059) or a p38 MAPK inhibitor (2 μM SB202190), respectively. When cells were treated with SB202190, they gave rise to larger secondary and tertiary neurospheres and significantly greater numbers of neurospheres compared with the untreated neurospheres from *NPC1*^{-/-} mice. Treatment with the MEK inhibitor PD98059 also could increase sizes of neurospheres from *NPC1*^{-/-} mice. However, there was no significant difference in the number of neurospheres between the PD98059-treated group and control group (Fig. 4A). Quantitative results of these treatments are shown in Figures 4B and 4C.

The effect of PD98059 and SB202190 treatment on the expression levels of the proteins in the NSCs from *NPC1*^{-/-} mice was assessed. As shown in Figure 5, protein expression of GFAP did not change after either treatment; however, the activation of the phosphorylated MAPK-related proteins, including

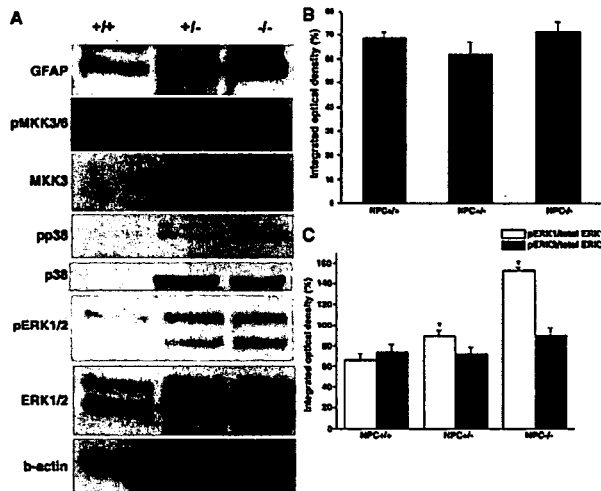


Figure 3. Western blotting results from Niemann-Pick type C (NPC) neurosphere-derived cell. (A): Expression of protein level with various antibodies. GFAP, pMKK3/6, MKK3, pp38, p38, pERK1/2, and ERK1/2 in wild-type. *NPC1*^{+/-}, and *NPC1*^{-/-} mice (protein, 25 μg/well). Values are the mean ± standard deviation for three to five independent experiments. (B, C): Protein level was determined by densitometry programs, Image J analysis software. Values are the mean ± standard deviation for three to five independent experiments. (B): Protein expression of GFAP/β-actin. (C): Open bar represented expression of pERK1/ERK1, and closed bar represented expression of pERK2/ERK2.

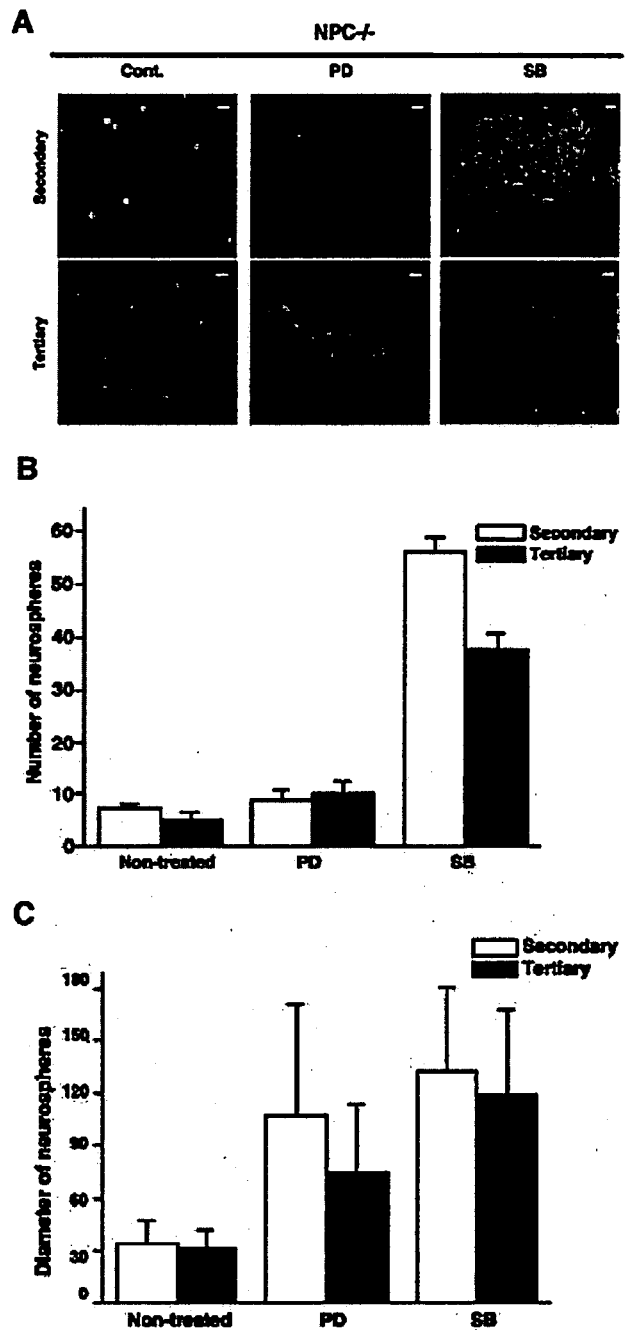


Figure 4. MAPK inhibitors increase neurosphere formation from derived cells in *NPC1*^{-/-}. (A): Cerebral cells from E16 *NPC1*^{-/-} mice were dissociated and cultured to generate neurospheres in the presence of MAPK kinase inhibitor (2 μM PD98059) and p38 MAPK inhibitor (2 μM SB202190). (B, C): Self-renewal capacity is expressed as the number and diameter of secondary and tertiary neurospheres generated per primary neurosphere on subcloning. Abbreviations: MAPK, mitogen-activated protein kinase; NPC1, Niemann-Pick type C.

pMKK3/6, pp38 in *NPC1*^{-/-} cells treated with SB202190, was significantly inhibited. Even though the phosphorylated pERK was highly activated in *NPC1*^{+/-} and NSCs from *NPC1*^{-/-} compared with *NPC1*^{+/+}, this was not the case after treatment with PD98059 in NSCs from *NPC1*^{-/-}.

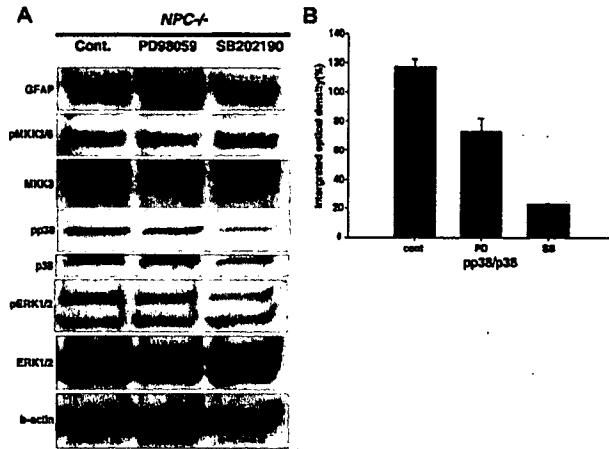


Figure 5. (A): Expression of GFAP, pMKK3/6, pp38, and pERK1/2 was reduced in the neurosphere-derived cells treated with MAPK inhibitors PD98059 (MAPK kinase inhibitor) and SB202190 (P38 inhibitor). Western blotting was done on neurosphere-derived cells (25 μg of protein) from each mouse. (B): Protein level of pp38/p38 was determined by densitometry programs. Image J analysis software. Values are the mean ± standard deviation for three to five independent experiments. Abbreviations: MAPK, mitogen-activated protein kinase; NPC1, Niemann-Pick type C.

NSCs from NPC1^{+/+} and NPC1^{-/-} Mice Give Rise to Different Astrocytes

To examine differentiation ability into astrocytes from neurospheres derived from NPC^{+/+} or NPC^{-/-} mice, differentiation of neurospheres was induced by adding 1% fetal bovine serum to culture medium in the absence of bFGF and EGF. As shown in Figure 6, the NSCs from NPC^{+/+} and NPC^{-/-} mice showed a different pattern of immunoreactivity against GFAP, a marker of astrocytes. Astrocytes differentiated from NSCs of NPC^{+/+} showed typical polygonal morphology. The astrocytes derived from NSCs of NPC^{-/-} had larger diameters,

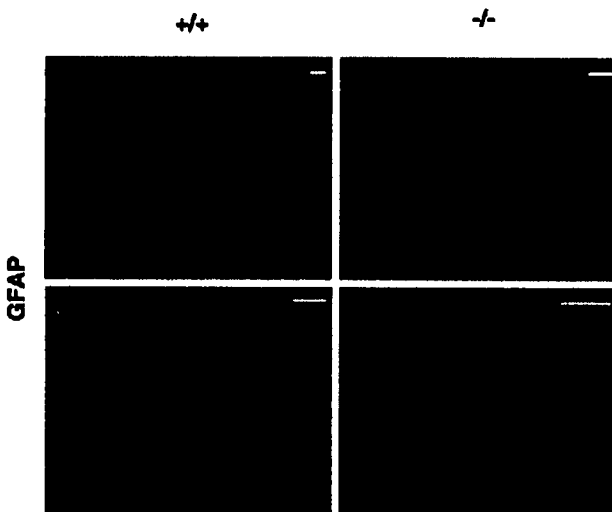


Figure 6. Generated neurospheres were differentiated by 1% fetal bovine serum; after 7 days, the spheres were differentiated into astrocytes (GFAP) between wild-type (+/+) and knockout (-/-). Scale bars = 100 μm.

appeared clumpy, and had rounded appearance, results that are consistent with those of literature reports [19, 30].

DISCUSSION

Self-renewal of NSCs is important to progression into multipotent lineages and maintenance of adult tissue. The polycomb family transcriptional repressor *Bmi-1* has been suggested to be a requirement for self-renewal and multiple tissues, including CNS and peripheral nervous system in NSCs [31]. Deficiency of *Bmi-1* leads to progressive postnatal growth retardation and neurological defects and retardation [31]. However, studies have not been directed at the identification of how the implications of deficiency of certain genes can affect NSCs of self-renewal and differentiation in neurodegenerative diseases. Here, we investigated self-renewal and differentiations of NSCs using one of the neurodegenerative diseases, NPC1 animal model.

To determine whether NPC1 gene deficiency can cause self-renewal of NSCs, NSCs from mice brain at E16 were isolated and characterized. It became clear that the NSCs from NPC^{-/-} mice lacked the ability to self-renew compared with those from the NPC^{+/+} mice. Based on previous results [13], it was hypothesized that low self-renewal ability of NSCs from NPC^{-/-} may be caused by MAPK proteins. MAPK-related proteins, MKK3/6, p38, and ERK1/2 were highly phosphorylated in NSCs from NPC^{-/-}. This was consistent with the observation by Sawamura et al. [24] that hyperphosphorylated tau and enhanced MAPK activity occur in the brains of NPC mice [24]. Further insight into the mechanism of MAPK-mediated self-renewal of NSCs was sought by studies involving the MAPK inhibitors SB 202190 for p38 MAPK and PD 98059 for pERK kinase in the NSCs from NPC^{-/-} mice. The self-renewal

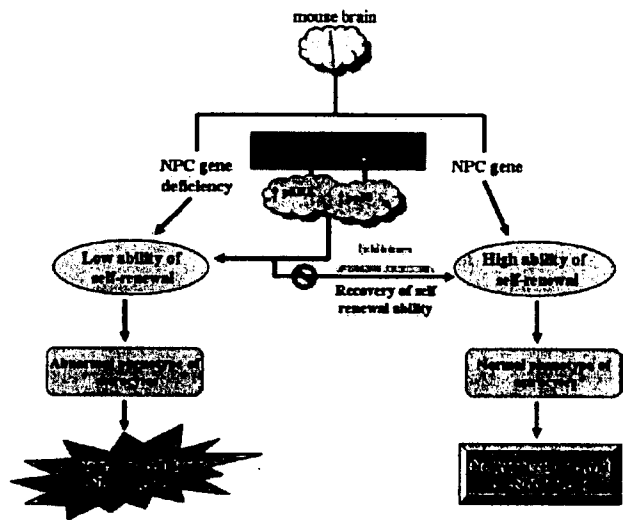


Figure 7. Schematic diagram for expression and function of NPC1 gene is mediated by MAPK in regulation of neuronal stem cell self-renewal and differentiation. Neuronal stem cells were isolated from brain of wild-type and NPC^{-/-} mice; NPC1 gene affects self-renewal ability and differentiation of neuronal stem cell not through the MAPK (pERK, pp38) depending on NPC1 gene presence. MAPK inhibitors PD 98059 (MAPK kinase inhibitor) and SB202190 (p38 inhibitor) could induce improvement of self-renewal ability in neuronal stem cells from NPC^{-/-}. Abbreviations: MAPK, mitogen-activated protein kinase; NPC1, Niemann-Pick type C.