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EXPRESSION AND REGULATION OF APOLIPOPROTEIN E RECEPTORS IN THE CELLS OF THE CENTRAL NERVOUS SYSTEM IN CULTURE: A REVIEW

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ABSTRACT

The importance of apolipoprotein E (apoE) in the central nervous system (CNS) became increasingly clear since the descovery that apoE ϵ4 allele is a major risk factor for Alzheimer's disease. ApoE is one of the major apolipoproteins that acts as a ligand for the cellular uptake of lipoproteins via apoE receptors, members of low-density lipoprotein receptor (LDLR) family, in the CNS. Recently, LDLR family has been shown to have new functions that modulate intracellular signalling and affect neuronal and glial functions, survival and regeneration. However, the pattern of expression of apoE receptors in the CNS has not been fully clarified yet. The LDLR, very low density lipoprotein receptor (VLDLR), LDLR-related protein (LRP), and apolipoprotein E receptor 2 (apoER2) are known to bind to and internalize apoE-containing lipoproteins. Here we summarize the expression of apoE receptors in the CNS and demonstrate additional our original data on cell type specific expression and regulation of those receptors in the CNS, using *in situ* hybridization and RT-PCR. The cells used in our study were highly enriched cultures of neurons, astrocytes, microglia and oligodendrocytes isolated from rat brain and neuroblastoma cell line, Neuro2a. All of these four types of receptors were shown to be expressed in neurons, astrocytes, microglia and oligodendrocytes, while LDLR and LRP were expressed in Neuro2a cells. We further examined the regulation of the expression of these receptors by altering the cholesterol content of the cells, and found that only the LDLR expression was downregulated following internalization of lipoprotein cholesterol and upregulated by cholesterol deprivation, in neuronal and astroglial cells. These data together with previous studies suggest that LDLR, VLDL, LRP, and apoER2 may be involved in apoE-mediated lipid uptake and/or intracellualr signalling in the cells of the CNS cells, i.e., neurons, astrocytes, microglia, and oligodendrocytes.

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INTRODUCTION

Apolipoprotein E (apoE) is a major apolipoprotein in the central nervous system (CNS), plays important roles in the maintenance of lipid homeostasis, and is involved in the development, remodeling, and regeneration of the nervous system (1-3). Ever since the discovery that inheritance of the apoE ϵ4-allele gene is associated with Alzheimer's disease (AD) (4-7), considerable attention has been paid to knowing the roles of apoE in the CNS. Although, great number of studies have been performed to elucidate apoE isoform-specific actions, the molecular mechanisms by which apoE is involved in the development of AD have not been fully ascertained yet. We have addressed these issues from the point of view of the role of apoE in cholesterol metabolism, and found a cholesterol-related apoE isoform-specific activity in neurons (8-10). In addition, different from nonneuronal cells, our previous finding also highlighted the importance of cholesterol for maintenance of neuronal cell viability (11), suggesting that the role of apoE in CNS cells may be cell-type specific.

Since apoE is a ligand for several receptors and apoE-containing lipoproteins bind to these receptors to be internalized into the cells, determination of cell-type specific expression of apoE receptors on CNS cells provides us the fundamental knowledge for investigating the role of apoE in lipids metabolism in the CNS. The apoE receptors identified to date are low-density lipoprotein receptor (LDLR) (12), LDL-related protein (LRP) (13), very low density lipoprotein receptor (VLDLR) (14), gp330 (15) and apolipoprotein E receptor 2 (apoER2) (16), which are members of LDL receptor family and which bind to and internalize apoE-containing lipoproteins. Among these, LDLR, VLDL, LRP and apoER2 have been shown to be expressed in mammalian neurons, and LDLR, VLDL and LRP in astrocytes (17); however, their cell type-specific expression in glial cells has not been fully clarified. Further, recent studies have shown that all members of LDL receptor family bind to

apoE on their extracellular domains and modulate intracellular signaling that affects neuronal and glial functions, survival and regeneration (18), indicating that apoE receptors have new functions other than endocytic receptor for lipid uptake. Thus determination of the expression of apoE receptors on glial cells also provide us the important knowledge for studying the biological roles of apoE other than that in lipid metabolism. To determine the cell type-specific expression of apoE receptors such as LDLR, VLDLR, LRP and apoER2 in the cells of the CNS including neurons, astrocytes, oligodendroglia and microglia, we established cultures rich in each of the cell types. In addition, we also examined the effect of cholesterol on the regulation of expression of these apoE receptors in cultured neurons and astrocytes. We found that all of these receptors are expressed in all the cell types as demonstrated by RT-PCR and *in situ* hybridization. In addition, as has been reported for nonneuronal cells, only LDLR was regulated by the cholesterol content as demonstrated by RT-PCR and Western blot in neuron- and astrocyte-rich cultures.

RESULTS

Cultures highly rich in Neurons, Astrocytes, Microglia and Oligodendrocytes Isolated from Rat Brain

To confirm the purity of the cultures rich in neurons, astrocytes, microglia and oligodendrocytes, each of the cultures was processed for double immunolabeling with cell-type-specific markers. The purity of each of the cultures was shown in Figure 1. Figures 1a and b show that more than 99% of the cells in the neuron-rich cultures were demonstrated to be tau-1 positive. Figures 1c and d show that more than 96% of the cells in the astrocyte-rich cultures were GFAP positive. Figures 1e and f show that 99% of the cells in the microglia-rich cultures were ED1 positive, and Figures 1g ad h show that more than 95% of the cells in the oligodendrocyte-rich cultures expressed the O1 phenotype. These results indicate that the cultures were highly rich in neurons, astrocytes, microglia and oligodendrocytes, respectively.

The Expression of apoE Receptors in Neurons, Astrocytes, Microglia and Oligodendrocytes in Culture

To examine the expression of apoE receptors in neuron, astrocyte, microglia and oligodendrocyte cultures prepared from embryonic rat cerebral cortices, RT-PCR analysis was performed. A 30-cycle RT-PCR analysis was carried out on total RNA from primary neuron-, astrocyte-, microglia- and oligodendrocyte-rich cultures. The predicted 292-bp amplification product of LRP mRNA, 280-bp LDLR mRNA, 288-bp VLDLR mRNA and 250-bp apoER2 mRNA were detected by ethidium bromide staining. Figure 2 shows that RT-PCR products of LRP, LDLR, VLDLR and apoER2 mRNA were detected in all cell types, while only LDLR and LRP mRNA were detected in Neuro2a cells, indicating that the expression pattern of apoE receptors is cell-type specific. The mRNA for the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference cellular transcript.

Cell-type Specific Expression of the Putative Receptors of apoE by in situ Hybridization

To confirm the RT-PCR data, apoE receptor expression in neuron-, astrocyte-, microglia- and oligodendrocyte-rich cultures was examined by *in situ* hybridization using four kinds of cDNA probes specific for each apoE receptor. The apoE receptors examined were LRP, LDLR, VLDLR and apoER2. As shown in Figure 3, strong expression of all four kinds of apoE receptors was detected in neurons, astrocytes, microglia and oligodendrocytes, supporting the RT-PCR data. The sense probes for LDLR, VLDL, apoER2 (data not shown) and LRP did not give any specific signal in each sister culture.

Regulation of the Level of Expression of ApoE Receptor mRNA by the Cellular Cholesterol Content in Rat Neuronal and Astrocyte Cultures

A comparison of the expression levels of LDLR, LRP, VLDLR and apoER2 mRNA in cultures subject to treatments which altered the cholesterol content of the cells was performed using RT-PCR analysis. A 25-cycle PCR was performed and the ratio of LDLR, LRP, VLDLR and apoER2 mRNAs in neurons and astrocytes was determined by direct comparison of the levels of cDNA generated at a given cycle within a linear range of amplification. We demonstrated that, for 1 μg of RNA subjected to reverse transcription, a linear range of PCR amplification of LDLR, LRP, VLDL and apoER2 cDNA was obtained between 20 to 25 cycles in this system (data not shown). The basal levels of the expression of apoE receptor mRNA in neurons and astrocytes cultured in cholesterol-containing medium are shown in Figures 4a and b, lane 1. As Figures 4a and b, lane 2 and 3, demonstrate, the expression levels of apoE receptor mRNA remained unchanged when the cells were incubated with LD-FBS and β-VLDL. In contrast, the expression of LDLR mRNA in neurons and astrocytes was increased after 12 and 24 hr of incubation with 5% LD-FBS alone. However, the expression levels of other apoE receptors remained unchanged. In addition, we examined the effect of a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, compactin, on the expression of the apoE receptors. Neuronal and astrocytic cultures were exposed to 5% LD-FBS plus compactin (500 nM) in DMEM. The expression of LDLR mRNA was increased in the cells treated with 5% LD-FBS plus compactin compared with that with 5% LD-FBS alone in both types of cultures. However, there was no apparent effect on LRP, VLDLR or apoER2 mRNA expression, as shown in Figures 4a and b.

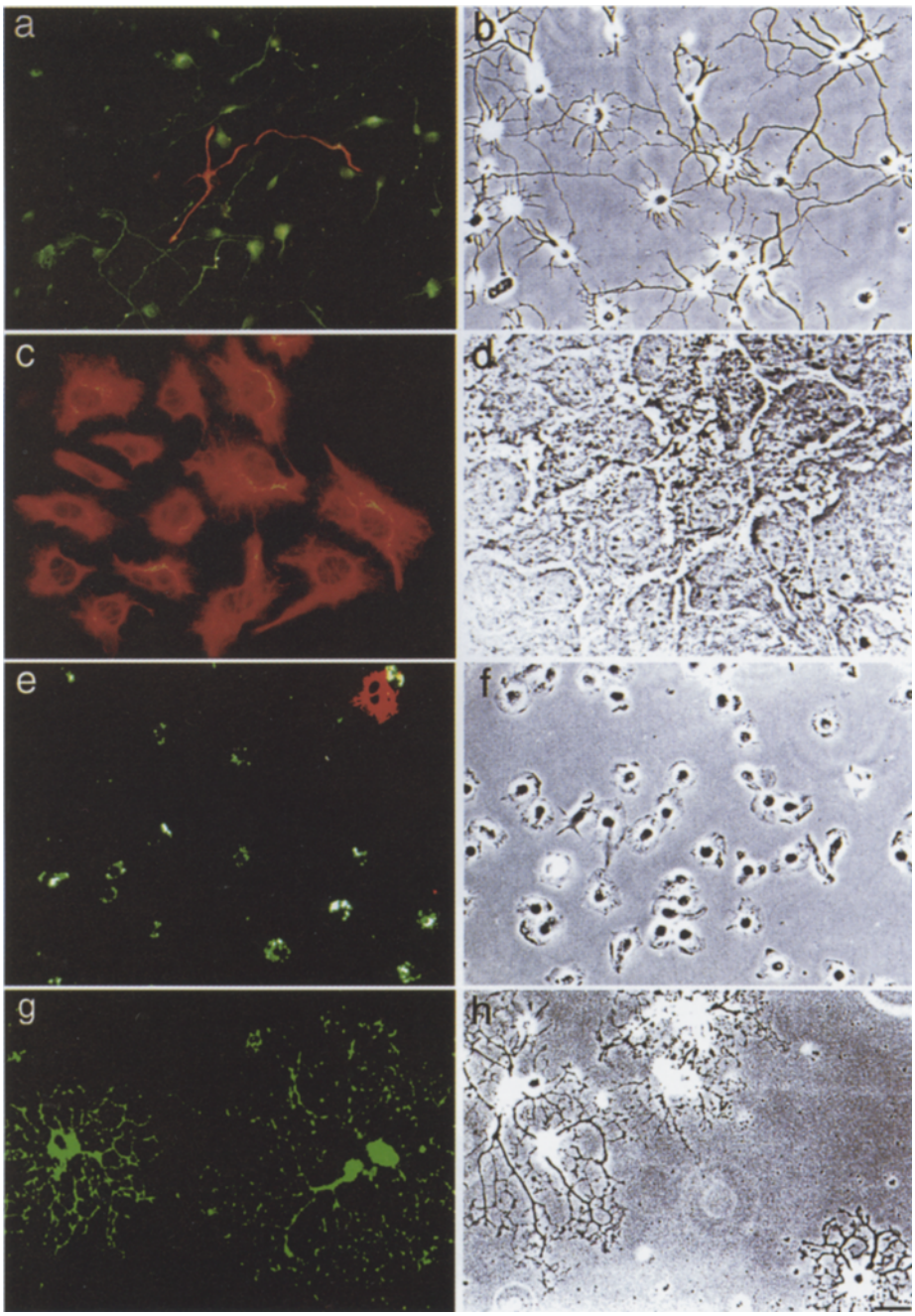

Figure 1. Rat cortical cell cultures, highly rich in neurons, astrocytes, microglia and oligodendrocytes.
Neuron-, astrocyte- microglia- and oligodendrocyte-rich populations were isolated from embryonal rat brains as described under **Materials and Methods**. They were plated on coverslips and processed for immunolabeling. (a) Neuronal cells double-immunostained with anti- tau-1 antibody (visualized by FITC), and anti-GFAP antibody (visualized by rhodamine); (c) Astrocytes immunoreactive for GFAP; (e) Microglial cells double-immmunostained with anti-ED1 antibody (visualized by FITC), and anti-GFAP antibody (visualized by rhodamine); (g) Oligodendrocytes immunoreactive for O1. Phase-contrast micrographs of neuron-, astrocyte-, microglia- and oligodendrocyte-rich cells were shown in (b), (d), (f) and (h). Bar=30 µm.

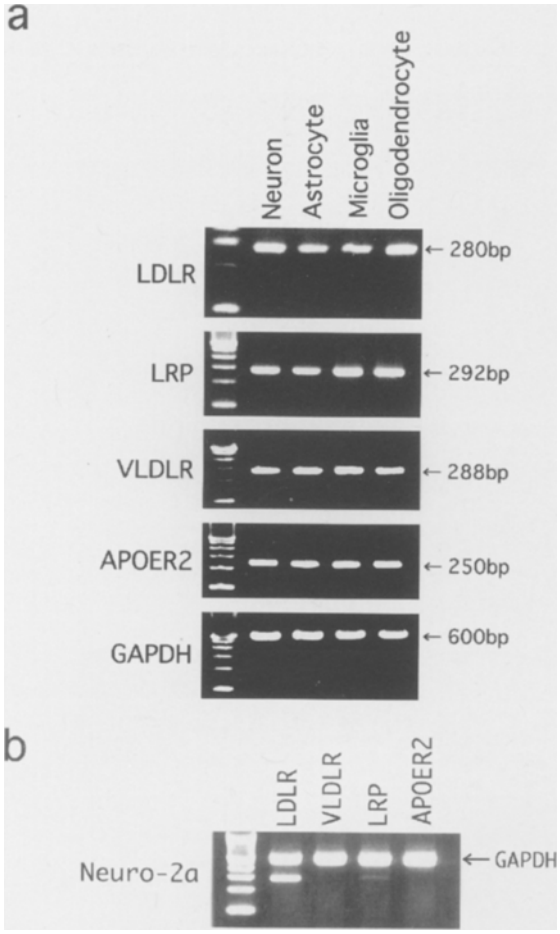

Figure 2. The expression of apoE receptors in neurons, astrocytes, microglia and oligodendrocytes and Neuro2a cells.

Neuron-, astrocyte- microglia- and oligodendrocyte-rich populations were isolated from primary cultures prepared from embryonal rat brain as described under the "Materials and Methods". Neuro2a cells were grown in DMEM containing 10% FBS. Total RNA was isolated form cell-type-specific-rich cultures and Neuro2a cells. The RNA samples were analyzed by RT-PCR using specific primers for LRP, LDLR, VLDLR, apoER2 and GAPDH. The PCR products were electrophoresed on a 3.5% agarose gel and visualized by ethidium bromide staining. MW, molecular weight markers (100-bp DNA ladder).

Determination of Cholesterol Content of Astrocyte- and Neuron-rich Cultures Treated with the Reagents.

To confirm that the upregulation of LDLR was due to cholesterol deficiency, we next determined the cholesterol content in the sister cultures. The cultures rich in astrocyte and neurons were treated with 5% FBS, 5% LD-FBS plus ß-VLDL, 5% LD-FBS, and 5% LD-FBS plus compactin (500 nM) for 24 h. As shown in Table 1, treatment with 5% LD-FBS and 5% LD-FBS plus compactin (500 nM), which upregulated the expression of LDLR, significantly reduced the cholesterol content compared with that with 5% FBS and 5% LD-FBS plus ß-VLDL in both cell types of cultures.

Table 1: Determination of Cholesterol Content of Astrocyte- and Neuron-rich Cultures Treated with the Reagents.

	Cholesterol (µg/mg protein)	
Treatment	*Astrocytes*	*Neurons*
5% FBS	228±13	147± 4
5% LD-FBS + ßVLDL (90 µg cholesterol/ml)	286±16	173±19
5% LD-FBS	124±12*	89± 5***
5% LD-FBS+ Compactin (300nM)	89±19*	56± 5**

Astrocyte- and neuron-rich cultures plated onto 12-well were treated with reagents shown above for 24 h. Cholesterol content of those cultures were then determined as described under **Materials and Methods**. Statistics were performed on six independent paired samples and analyzed by ANOVA. *P<0.001 vs. 5% FBS and 5% LD-FBS + ß-VLDL (90 µg cholesterol/ml), and **P<0.002 vs. 5% FBS and 5% LD-FBS + ß-VLDL.

Regulation of the Level of LDLR by the Cellular Cholesterol Content in Rat Astrocyte Cultures.

A comparison of the LDLR levels in astrocyte cultures subject to treatments, which altered the cholesterol content of the cells, was performed using Western blot analysis. Cellular lysates of astrocytes treated or untreated for different times were electrophoresed, blotted to a nitrocellulose membrane and incubated with a monoclonal antibody against LDLR. The basal levels of the expression of LDLR in astrocyte cultured in cholesterol-containing medium are shown in Figure 5, lane1. Treatment of astrocyte cultures with 5% LD-FBS or 5% LD-FBS plus compactin increased the levels of LDLR, while treatment of astrocyte cultures with 5% LD-FBS plus é-VLDL decreased the levels of LDLR after 12 and 24 hr of incubation. These changes were consistent with the results obtained by RT-PCR analysis, which are shown in Figure 4.

DISCUSSION

Our experiments show that the apoE receptors, LDLR, VLDLR, LRP and apoER2, are expressed in cultured neurons, astrocytes, microglia and oligodendrocytes isolated from rat cerebral cortices. Our present study show for the first time that only the expression of LDLR is regulated by altering the intracellular cholesterol content in neurons and astrocytes. In addition, only LDLR and LRP are expressed in Neuro2a, indicating that the pattern of the expression of these receptors is cell type-specific. Previous studies have shown that LDLR, VLDLR, LRP and apoER2 are expressed in neurons (16, 19-24), LDLR, VLDL and LRP in astrocytes (19, 21-24), LDLR in oligodendrocytes (22), and LDLR and VLDL in microglia (25, 26). The results of our present study not only confirm these results of previous studies, but also provide new evidence: astrocytes also express apoER2, oligodendrocytes express VLDLR, LRP and apoER2, and microglia express LRP and apoER2. Our observations do not permit us to evaluate the receptor(s) through which apoE-containing lipoproteins are internalized into the cells. There are some

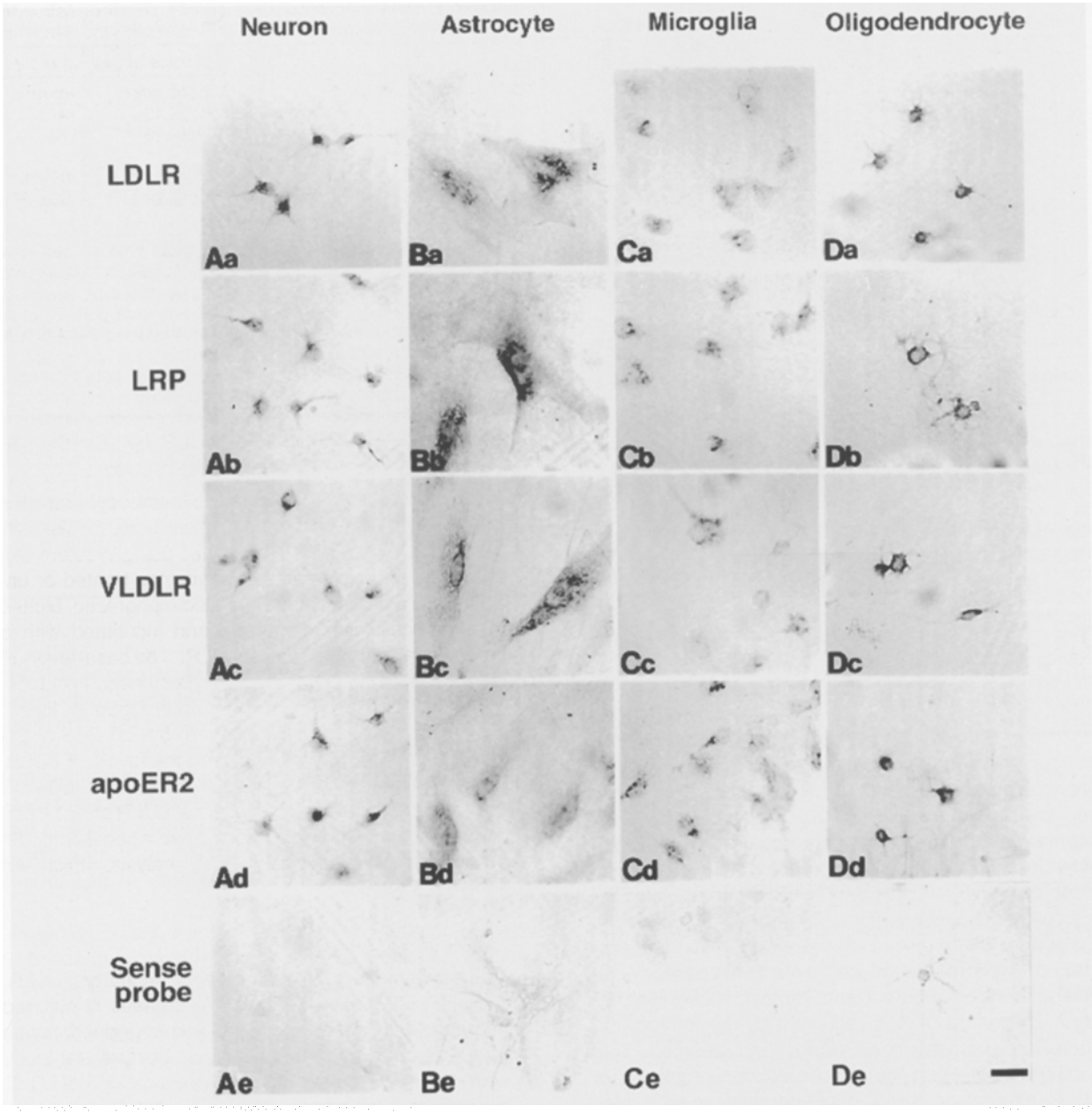

Figure 3. Cell-type Specific Expression of the Putative Receptors of apoE by *in situ* Hybridization
Cultures highly rich in neuron, astrocyte, microglia and oligodendrocyte populations plated on glass coverslips were processed for *in situ* hybridization with antisense LDLR, LRP, VLDLR, apoER2, and sense LRP probes. Aa-Ae, neuronal cells; Ba-Be, astrocytes; Ca-Ce, microglia; Da-De, oligodendrocytes. Aa-Da, LDLR antisense probe; Ab-Db, LRP antisense probe; Ac-Dc, VLDLR antisense probe; Ad-Dd, apoER2 antisense probe. Ae-De, LRP sense probe. Hybridization signals were visualized with NBT and BCIP. Bar =30 μm.

studies describing that apoE-containing lipoproteins are internalized through LRP in neurons using a 39-kDa protein referred to as receptor-associated protein (RAP), which is known to regulate ligand-binding to LRP (27-29). However, RAP has been also shown to bind to LDLR and VLDLR (30, 31). In addition, neither LDLR nor LRP has been shown to be responsible for the internalization of apoE-containing lipoproteins in neurons (32). Thus, the receptor(s) involved in apoE-mediated lipoprotein transfer in CNS cells have not been well defined yet. Further experiments are needed to identify the receptor(s) through which apoE-containing lipoproteins bind to and are internalized into cells of the CNS, including neurons, astrocytes, microglia and oligodendrocytes.

It is well known that when cells are deprived of sterols, proteolysis process for sterol regulatory element binding proteins (SREBPs) is activated and active portions of the SREBPs from cell membranes translocate to the nucleus, where they activate transcription of LDLR, HMG-CoA reductase and genes encoding many other enzymes in the cholesterol biosynthetic pathway (33-

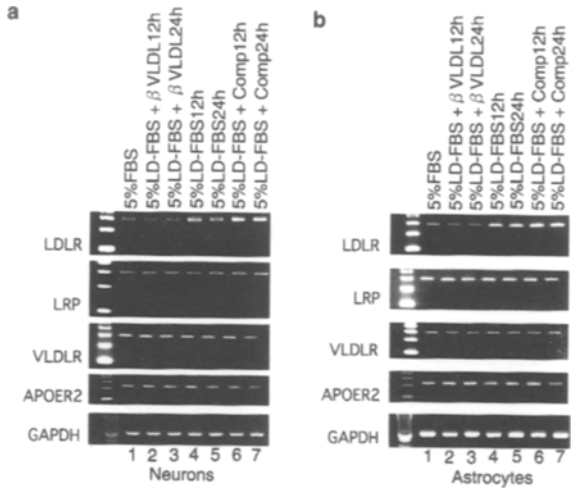

Figure 4. Regulation of the apoE receptor mRNA by cholesterol in rat neuronal and astrocyte cultures.
Both neuronal and astrocyte cultures were prepared from E17 rat brains according to the methods described under the **Materials and Methods**. Each cells plated onto 6-well plates was incubated in 5% FBS DMEM, 5% LD-FBS, 5% LD-FBS plus ß-VLDL (90 µg cholesterol/ml), or 5% LD-FBS with compactin (500 nM), for 12h and 24h. Total RNA was prepared and the same sample was analyzed by RT-PCR using LRP-, LDLR-, VLDLR-, apoER2- and GAPDH-specific primers. The PCR products were electrophoresed on a 3.5% agarose gel and visualized by ethidium bromide staining. MW, molecular weight markers (100-bp DNA ladder).

35). In the light of the above and together with the previous report describing LDLR-expressing astrocytes and the downregulation of LDLR expression following internalization of lipoprotein cholesterol (36), it is reasonable to ask whether the expression of the other apoE receptors, LRP, VLDLR and apoER2, is regulated by the cellular cholesterol content in our culture systems. Consistent with previous studies which have been performed using nonneural cells, our present study, as demonstrated in Figure 4 and Table 1, shows that the gene encoding LDLR is regulated by the intracellular cholesterol content in astrocytes and neurons. This finding indicate that the sterol-mediated regulation of LDLR expression seen in proliferating cells is also present in neurons and astrocytes. However, the expression of other apoE receptors, including VLDLR, LRP and apoER2 is not regulated by internalization of lipoprotein cholesterol or suppression of cholesterol biosynthesis by compactin. In this study, we used ß-VLDL as a cholesterol carrier which is known to be rich in cholesterol and apoE. As for the regulation of LRP expression, our results may be explained by the fact that the promoter for the LRP gene contains no sterol regulatory element (37). It is shown that LRP binds to and internalizes apoE-containing lipoproteins but also has additional ligands unrelated to lipid metabolism (41). This is supported by the fact that LRP has been shown to be upregulated by NGF in neurons (38), and downregulated by lipopolysaccharides and interferon-γ in macrophages (39). In addition, the expression of

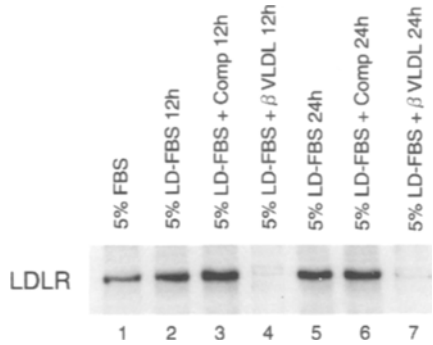

Figure 5. Regulation of the LDLR protein by cholesterol in rat astrocyte cultures.
Western blot analysis of the LDLR in cell extracts of primary cultured rat astrocytes. Astrocyte cultures were prepared from E17 rat brains according to the methods described under the "Materials and Methods". Astrocytes plated onto 6-well plates were incubated in 5% FBS DMEM, 5% LD-FBS, 5% LD-FBS plus b-VLDL (90mg cholesterol/ml), or 5% LD-FBS with compactin (500nM), for 12h and 24h. Samples corresponding to 10mg protein from the respective sources were resolved by SDS-PAGE in 4-20% gradient gel, blotted to a membrane, and immunostained using anti-LDLR antibody.

VLDLR is regulated by thyroid hormonal manipulation (40). Interestingly, recent studies have shown that lipoprotein receptors have been shown to mediate the endocytic uptake of retinoids and steroids, and interact with intracellular signaling pathways (18). Thus, although these receptors are assumed to play an important role in apoE-mediated lipid uptake, our finding that the expression of LRP, VLDL and apoER2 is not regulated by intracellular cholesterol content, suggests that the VLDLR and apoER2, like LRP, may have physiological functions that are not directly related to lipid transport.

In summary, we have shown that the apoE receptors, LDLR, VLDLR, LRP and apoER2, are expressed in neurons, astrocytes, microglia and oligodendrocytes in cultures prepared form rat cerebral cortices. Only the expression of LDLR is regulated by the cellular cholesterol content in neuronal and astrocytic cultures.

MATERIALS AND METHODS

Experimental Procedures

Cell Culture

Highly neuron-rich cultures were prepared from embryonic day 17 (E17) rat brain according to a previously described method (8). In brief, the cerebral cortices isolated from E17 Sprague-Dawley rats were dissected into small cubes, incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin and 20 mg/ml DNase I for 20 min at 37°C, and dissociated into single cells by gentle pipetting. The dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and plated onto poly-D-Lysine precoated 6-well dishes at a cell density of 2 x $10^6/cm^2$. Six hours following plating, the medium was

replaced with serum-free N2 medium (DMEM/F12 containing growth supplements). After 1-3 days *in vitro*, neuron-rich cultures were harvested.

For microglia- and astrocyte-rich cultures, mixed glial cells were prepared according to the methods described previously (42). In brief, dissociated cells were prepared from E17-18 rat cerebral cortices as described above and seeded in 75-cm^2 flasks at a cell density of 1 x 10^7. After 2 weeks *in vitro*, the mixed glial cultures were treated with 12 mM lidocaine for 10 min, and the cells floating in the medium were collected as a source of microglia. The cells were pelleted and washed with the culture medium (DMEM/F12 supplemented with 5% FBS, 5 µg/ml insulin and 100 µg/ml transferrin). The cells resuspended in the culture medium were plated on appropriate plates for each experiment. For astrocyte-rich cultures, mixed glial cultures maintained for 2-4 weeks after preparation of the primary culture were used. The cells on the astrocytic monolayer were removed by shaking the flasks vigorously at 200 rpm for 17 hr. The medium was removed and the remaining monolayer cells were trypsinized (0.1%) and reseeded in 75-cm^2 flasks or on glass coverslips.

Oligodendrocyte-rich cultures were prepared according to the previously described method (43) with some modifications. In brief, the dissociated cells from E17 rat cerebral cortices were prepared according to the same method as described above. The cells were seeded on poly-D-Lysine-coated culture dishes (1.4 x 10^7 cells/60-mm dish) in DMEM containing 10% FBS. After 7 and 14 days *in vitro*, the cultures were passaged and seeded in tissue culture dishes at a density of 2 x 10^6 cell/60-mm dish in DMEM containing 10% FBS. Two days following the replating, the medium was exchanged for a serum-free defined medium consisting of DMEM supplemented with glucose (5 mg/ml), insulin (5 µg/ml), sodium selenite (40 ng/ml), transferrin (100 µg/ml), progesterone (0.06 ng/ml), putrescine (16 µg/ml), thyroxine (40 ng/ml), triiodothyronine (30 ng/ml), and bFGF (2ng/ml). The cultures were maintained for 5 days in the serum-free medium, and then the cells were reseeded at a density of 1.7 x 10^6/60-mm dish. These procedures were repeated a total of 3 times, and the cells were cultured for more than 1 month. Each kind of cell plated at a density of 1 x 10^6 cells per dish was used for RT-PCR analysis, and the cells plated on poly-D-Lysine-coated glass coverslips at a density of 2 x 10^5 cells/cm^2 were processed for immunocytochemistry or *in situ* hybridization.

Immunocytochemistry

To confirm the purity of the cell preparation, the cells cultures were processed for immunolabelling with cell-type-specific markers. The cell-type-specific markers employed in this study were anti-tau-1 monoclonal antibody (Boehringer Mannheim, Germany) for neurons, anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (DAKO, Glostrup, Denmark) for astrocytes, anti-ED1 antibody (Serotec Ltd,) for microglia and O1 (44) for oligodendrocytes. For double immunostaining of the neuron-, astrocyte- and microglia-enriched cultures, the cells on poly-D-lysine glass coverslips at a density of 2 x 10^5 cells per cm^2 were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min at room temperature (RT) and 0.2% Triton X-100 in PBS for 5 min. Before applying the primary antibodies, the cells were incubated with PBS containing normal goat serum for 1 hour in order to block any potential interaction between Fc receptors and Fc fragments. The cells were incubated overnight with a mixture of two appropriate cell-type-specific markers at 4°C. A combination of mouse anti-tau1 monoclonal antibody (1:200) and rabbit anti-GFAP polyclonal antibody (1:500) was used to determine the percentage of neurons in the neuron-enriched cultures, and that of mouse anti-ED1 monoclonal antibody (1:100) and rabbit anti-GFAP polyclonal antibody (1:500) was used for determining of the percentage of astrocytes in astrocyte-enriched cultures. To determine the ratio of microglial cells in microglia-enriched cultures, a combination of mouse anti-ED1 monoclonal antibody (1:100) and rabbit anti-GFAP polyclonal antibody (1:500) was used. The cultures were followed by incubation with a mixture of rhodamine-conjugated anti-rabbit IgG (1:50; Single Oak Drive, Temecula, CA) and FITC-conjugated anti-mouse IgG (1:20 Calle Negocio, San Clemente, CA) at RT for 40 min. After three washes in PBS, coverslips mounted on slides with Vectashield were examined under an Olympus Universal microscope equipped with fluorescein and rhodamine optics. For oligodendrocyte-rich cultures, cells on coverslips at a density of 2 x 10^5/cm^2 were incubated with medium containing mouse anti-O1 monoclonal antibody (1:1) at RT for 30 min. The cells were then washed in PBS and fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PH 7.4) for 15 min at RT, followed by several washes in PBS, and then incubated with anti-GFAP polyclonal antibody (1:500) at RT for 1h. For negative controls, primary antibodies were omitted from the reaction. Over 300 cells were counted for each staining condition and the results were expressed as a percentage of the number of immunoreactive cells per total number of cells.

RT-PCR Analysis

Total RNA was extended with TRI ZOL reagent (GIBCO-BRL) according to the supplier's instructions, and 1 µg from each sample was subjected to DNase treatment and processed for first-strand cDNA synthesis using the Reverse Transcription system Kit (Promega). An aliquot (1ml) of each cDNA product was amplified by PCR using the specific sense and antisense primers designed from the cDNA sequence for LDLR (GenBank X13722), LRP (GenBank X67469), VLDLR (GenBank L35767), and apoER2 (GenBank D50678) genes. The sense and antisense primers were as follows: LDLR 5'-primer, CCTGCCAAGATCAAGAAAGG and 3'-primer, GGTTGGCACTGA AAATGGGCT; LRP 5'-primer, TGAGAATGACCAGTACGGGA and 3'-primer,

CAAAGTAGATGAAGCCGGTC; VLDLR 5'-primer, TGGAATTACCCTCG ACCTTG and 3'-primer, CTGGACGAGTTCATGGTAGA; and apoER2 5'-primer, TGTCCTGATGGCTCCGATGA and 3'-primer TCACCACAGTCGTCATCGCC. Glycerylaldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reaction standard. Each amplification cycle comprised 25 or 30 cycles, each consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C in each experiment. The amplified fragments were examined by electrophoresis on a 3.5% agarose gel (Nasieve 3:1 agarose Rockland), stained with 1 mg/ml ethidium bromide. The mRNA for the constitutive GAPDH was examined as the reference cellular transcript.

RNA Probes and in situ Hybridization

For LRP, the antisense and sense probes of LRP were generously provided by Dr. Kohsaka (21). A 604-bp fragment of LRP cDNA was inserted into pGEM-1 at the BamHI/SmaI site. For apoER2, a 770-bp fragment of apoER2 cDNA (nucleotide numbers 491-1261), which was kindly provided by Dr. Yamamoto (16), was inserted into pBluescript KSII+(Stratagene, CA) at the HindIII/EcoRI site. For LDLR and VLDLR, a 1003-bp fragment of LDLR cDNA (nucleotide numbers 1092-2095 GenBank X13722) and a 1066-bp fragment of VLDLR cDNA (nucleotide numbers 1690-2756 GenBank L35767) were inserted into pBluescript KSII+ (Stratagene, CA) at the HindIII/EcoRI site. After linearizing the template plasmid by digestion with an appropriate restriction enzyme, T3, T7 or SP6 RNA polymerase was used to perform transcription *in vitro*. Digoxigenin-11-UTP-labeled single-stranded RNA was prepared using a DIG RNA labeling kit (Boehringer Mannheim, Germany). The RNA was heated with 40 mM $NaHCO_3$, 60 mM Na_2CO_3, pH 10.2 for 50 min at 60°C to generate shorter fragments with an average length of 150 bases. After ethanol precipitation, the products were dissolved in 50ml of deionized formamide, and then stored at -80°C until use. *In situ* hybridization was performed as described previously (45).

Lipoproteins

ß-VLDL (d<1.006 g/ml) was prepared from the plasma of male New Zealand white rabbits according to the methods previously described (8), and used as the cholesterol source. The ratio of total cholesterol to protein ranged from 10 to 13 in the three preparations used in this study.

Cellular Cholesterol Measurement

Astrocyte- and neuron-rich cultures were cultured in DMEM containing 5% FBS, 5% lipoprotein-deficient (LD)-FBS, 5% LD-FBS plus ß-VLDL, or 5% LD-FBS plus compactin for 24 h. The cultures were then washed with PBS for two times and dried at room temperature. Cellular lipids were extracted with hexane:isopropanol (3:2 v/v) and were analyzed with cholesterol determinant kit (Kyowa Ltd., Tokyo, Japan).

Western Blot Analysis

Treated and untreated cells were lysed in 1% SDS, 100mM Tris-HCl PH 8.5 containing 1mM EGTA, a mixture of proteases, Complete™ (Boehringer Mannheim, Germany), and centrifuged at 10,000 x g for 5 min. The protein content of each clear supernatant was equalified using BCA protein assay kit and was subjected to 4-20% gradient Tris/tricine SDS-PAGE (Dia-ichi pure chemical Co. Ltd., Tokyo, Japan). The separated proteins were transferred to a polyvinylidene diffuoride membrane (Millipore, Bedford, MA). The blot was blocked with 100% Block Ace (Dainippon pharmacetical Co. Ltd., Osaka, Japan) for 1h, and incubated with primary antibody for overnight at 4°C. The first antibody used was a monoclonal antibody against LDL (1:100) (Calbiochem, La Jolla, CA). The blot was washed four times with PBS over a period of 60 min in PBS-T (PBS containing 0.05% Tween 20), and then incubated with secondary antibody (horseradish peroxidase-conjugated anti-mouse antibody, diluted 1:5000) for 1h. Between the steps, the blot was washed four times with PBS-T over 15 min. The bound antibody wasdetected using the ECL (Amersham Pharmacia Biotechnology, England).

Treatment

To examine the effects of inhibition of cholesterol synthesis on apoE receptors expression in neuron- and astrocyte-rich cultures, the cultures were incubated with 5% FBS, 5% LD-FBS, 5% LD-FBS plus ß-VLDL (90 µg cholesterol/ml), or 5% LD-FBS with 500 nM compactin in DMEM. The cultures were harvested and the total RNA was extracted for RT-PCR analysis 12 and 24 hr after the beginning of the treatment.

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