Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2010 | 114 | 685–696 doi: 10.1111/j.1471-4159.2010.06773.x

Apolipoprotein E mRNA is transported to dendrites and may have a role in synaptic structural plasticity

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Abstract

Although the dendritic localization and translation of a subset of mRNAs plays a pivotal role in synaptic plasticity, the dendritic mRNAs and their functions have been only minimally characterized thus far. In this study, we isolated mRNAs from Staufen2-containing ribonucleoprotein complexes, which function as modules for the transport of mRNA to the dendrites, and then constructed a cDNA library. Apolipoprotein E gene (*APOE*) mRNA was isolated from the dendritic mRNA-specific cDNA library. The specific localization of *APOE* mRNA was evaluated via *in situ* hybridization. The specific regions involved in the dendritic transport of *APOE* mRNA were determined using a visualization system employing green fluorescent protein-tagged bacteriophage MS2 RNA-binding protein. As a result, the proximal N-terminal or C-terminal regions of the ApoE-coding sequences were determined to be sufficient for dendritic transport. The level of dendritic *APOE* mRNA was significantly increased by depolarization-induced neuronal activity, but was reduced in the cell body regions. We assessed the functions of neuronal ApoE. The reduction of ganglioside GM1 by cholesterol depletion was completely blocked by ApoE over-expression. In addition, ApoE over-expression increased the immunoreactivity of the post-synaptic density 95 kDa antibody in the dendrites. These findings indicate that neuronal ApoE may be relevant to lipid rafts or synaptic structural plasticity.

Keywords: apolipoprotein E, cholesterol, lipid rafts, local protein synthesis, post-synaptic density-95 kDa, synaptic plasticity.

J. Neurochem. (2010) **114**, 685–696.

The dendritic transport of a subset of mRNAs and their subsequent translation is of great importance to the molecular mechanisms of synaptic plasticity, which is known as a cellular mechanism of learning and memory. Dendritic protein synthesis is regulated in a sophisticated manner in response to external stimuli or neuronal activity (Martin and Zukin 2006; Pfeiffer and Huber 2006; Schuman *et al.* 2006; Sutton and Schuman 2006). However more information is necessary to accurately identity of mRNAs localized to dendrites and their functions, to say nothing of the mechanisms underlying the transport and translation of mRNA. Although several research groups have previously attempted to identify the dendritic mRNAs, there has been little overlap among those results (Sung *et al.* 2004; Poon *et al.* 2006; Zhong *et al.* 2006). This new approach could help in determining the identities and functions of dendritic mRNAs, and may provide clues as to the roles of dendritic protein synthesis in synaptic plasticity.

Apolipoprotein E (ApoE), one of the major apolipoproteins, is involved in the transport of cholesterol and the

Received December 24, 2009; revised manuscript received March 25, 2010; accepted April 20, 2010.

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Abbreviations used: 3′UTR, 3′untranslated region; ApoE, apolipoprotein E; BDNF, brain-derived neurotrophic factor; CTB, cholera toxin B subunit; CTL, control; Cy3, cyanine 3; dsRBD, double-stranded RNA binding domain; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; MβCD, methyl-β-cyclodextrin; PSD-95, post-synaptic density 95 kDa; PSG, subgenomic promoter; RNP, ribonucleoprotein.

redistribution of lipids (Mahley 1988). The apolipoprotein E gene (*APOE*) located at a single locus on chromosome 19, exists in three isoforms (ApoE2, ApoE3, and ApoE4) in humans (Das *et al.* 1985). Recent research has discriminated the roles and expression patterns of these isoforms in the nervous system. Only ApoE4, which is involved in late-onset familial and sporadic Alzheimer's disease as a genetic risk factor (Corder *et al.* 1993; Saunders and Roses 1993), inhibits synaptic plasticity (Levi *et al.* 2003, 2005) and induces apoptosis (Levi and Michaelson 2007). By way of contrast, ApoE2 and ApoE3 protect neurons against excitotoxic stress and neurodegeneration (Boschert *et al.* 1999; Buttini *et al.* 1999, 2002), promote neurite outgrowth (Nathan *et al.* 2002), or synaptogensis and memory (Levi *et al.* 2003). Although the main sources of ApoE in the CNS are glial cells such as astrocytes, the level of ApoE expression in the neurons has been reported to be substantial (Xu *et al.* 1999; Dekroon and Armati 2001). In addition, ApoE expression in the neurons has been reported in the human cortical and hippocampal neurons of Alzheimer's disease (Han *et al.* 1994a,b), in rat hippocampal neurons by transient cerebral ischemia (Kida *et al.* 1995), and in rat hippocampal neurons as the result of excitotoxic stress (Boschert *et al.* 1999), thereby suggesting its protective role. Interestingly, cholesterol, the levels of which are sustained and transported by ApoE in the neurons, also plays a role in synaptic plasticity (Koudinov and Koudinova 2001; Mauch *et al.* 2001; Frank *et al.* 2008). In these contexts, ApoE might be surmised to perform a function in synaptic plasticity, but the functions of neuronal ApoE remain poorly understood.

Lipid rafts are more ordered microdomains in the plasma membrane; they are composed of cholesterol and sphingolipids such as ganglioside GM1, and are resistant to detergent-mediated solubilization. Proteins on the lipid rafts can be modified via the linkage of glycophosphatiylinositol anchors, binding cholesterol, or palmitoylation and/or myristoylation (Simons and Ikonen 1997; Brown and London 1998). Lipid rafts have been shown to be enriched with a variety of signaling proteins such as kinases, ion channels or receptors that play important roles in the signaling pathway (Simons and Toomre 2000) and are found in neuronal membranes (Suzuki *et al.* 2001). The depletion of lipid rafts from neuronal membrane induces changes in the synaptic structures and also reduces the level of surface α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (Hering *et al.* 2003) and NMDA-dependent calcium influx (Frank *et al.* 2004) as well as preventing the neuregulin-induced blockade of long-term potentiation induction in the hippocampus (Ma *et al.* 2003), thereby strongly suggesting that lipid rafts are involved in the maintenance of synaptic structure and synaptic plasticity.

The principal objective of the present study was to isolate mRNA localized in neuronal dendrites and to characterize its function. Thus we isolated mRNAs form the Staufen2-containing ribonucleoprotein (RNP) complexes of cultured hippocampal neurons and constructed a dendritic mRNA-specific cDNA library using them. *APOE* mRNA was isolated from the cDNA library. The dendritic localization of *APOE* mRNA was assessed via *in situ* hybridization, and specific localization in the dendrites was evaluated via *in situ* hybridization and immunostaining using Staufen2 antibody. Interestingly, the proximal N-terminal or C-terminal regions of the *APOE* mRNA coding sequences proved to be sufficient for dendritic transport, respectively. The levels of *APOE* mRNA were increased in the dendrites as the result of depolarization-induced neuronal activity, but were reduced in cell body regions. In addition, ApoE expression, induced by the Sindbis virus, blocked a loss of ganglioside GM1, which was observed as the result of cholesterol depletion induced by treatment with methyl-β-cyclodextrin (MβCD). Green fluorescent protein-tagged ApoE (GFP-ApoE) were indeed localized in dendrites near synaptic regions. Consistent with these results, ApoE expression significantly increased the immunoreactivity of the anti-post-synaptic density 95 kDa (PSD-95) antibody, a post-synaptic scaffolding protein in the dendrites. These findings indicate that ApoE may play a role in lipid rafts or synaptic structural plasticity by the regulation of its levels via dendritic localization and translation.

Materials and methods

Neuronal culture

Dissociated hippocampal neuron cultures were prepared from postnatal 1-day-old rat pups, as previously described elsewhere (Jeong *et al.* 2007).

Isolation of mRNAs from Staufen2-containing RNP complexes and construction of cDNA library

Cultured hippocampal neurons were infected with the Sindbis virus encoding for myc-tagged Staufen2 (Jeong *et al.* 2007) and allowed to express for 12 h. The cultures were supplemented with 1 mL ice-cold lysis buffer [10 mmol/L Hepes (pH 7.4), 200 mmol/L NaCl, 30 mmol/L EDTA, and 0.5% TritonX-100] with 1× complete protease inhibitors (Roche, Mannheim, Germany) and 400 U/mL rRNAsin (Promega, Madison, WI, USA) (Brown *et al.* 2001), and immunoprecipitated with monoclonal anti-myc antibody (Clone E910, Sigma, St Louis, MO, USA) or mouse IgG (Sigma). The purified RNA was utilized in RT-PCR analysis for mRNA of the Ca^{2+}/calmodulin-dependent protein kinase II α-subunit (CAMKIIα) and used in cDNA synthesis with SMART^{TM} system (Clontech, Mountain View, CA, USA). The synthesized cDNA was inserted into pGEM-T easy vector (Promega) and subsequently used in sequencing analysis and Basic Local Alignment Search Tool for nucleotide (Blastn) search (see Figure S1 for more detailed methods and the control data). Unless stated otherwise, all reagents were purchased from Sigma-Aldrich.

In situ hybridization and immunostaining

Probe preparation and *in situ* hybridization were conducted as in the previous experiment of Jeong *et al.* (2007). For the quantification of the *in situ* results, the development time of the signal was empirically determined less than 20 h to avoid saturation of the *in situ* signal. The *in situ* images were acquired using a Nikon inverted microscope (ECLIPSE TE2000-U, Tokyo, Japan) equipped with a digital camera (DP72-BSW, Olympus, Tokyo, Japan), a cooled CCD (charge coupled device, Cascade 512B, Photometrics, Tucson, AZ, USA) or a confocal microscope (TCS-SP2 AOBS, Leica Microsystems, Heidelberg, Germany). For the depolarization experiment, 12-day cultured neurons were incubated with high-K^+ HEPES buffered saline (HBS, 60 mmol/L KCl) for 10 min and subjected to an additional 2 h 50 min of incubation in 5% CO_2, at 37°C, and then immediately subjected to *in situ* analysis. For brain-derived neurotrophic factor (BDNF) treatment, the cultured neurons were treated for 3 h with 50 ng/mL of BDNF (Promega). Immunostaining was conducted as described by Jeong *et al.* (2007). Monoclonal anti-PSD-95 antibody (Clone: 6G6-1C9, Affinity Bioreagents, Golden, CO, USA) or polyclonal anti-Synapsin I antibody (Chemicon International, Temecula, CA, USA) was used in staining (1 : 200), respectively. A cyanine 3 (Cy3)-conjugated anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was utilized as the secondary antibody for PSD-95 staining, a Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for Synapsin I staining.

DNA construction for Sindbis viral expression

For the Sinbis viral expression of ApoE, ApoE coding sequences were PCR amplified (ApoE-Mlu-S, ApoE-Sph-A) and inserted into the *Mlu*I/*Sph*I site of pSinRep5 (Invitrogen, Carlsbad, CA, USA), then amplified with the subgenomic promoter (PSG) of the pSinRep5 vector (PSG-Sph-S, ApoE-Sph-A). The PCR products were inserted into the *Sph*I site of pSinRep5-GFP and pSinRep5-monomeric red fluorescent protein (mRFP) (Jeong *et al.* 2007), resulting pSinRep5-GFP-PSG-ApoE and pSinRep5-mRFP-PSG-ApoE, respectively. For the expression of GFP-tagged ApoE (GFP-ApoE), ApoE coding sequences were amplified [ApoE-Bam-S, ApoE3′untranslated regions (UTR)-Sal-S] and inserted into the *Bgl*II/*Sal*I site of pEGFP-C1 (Clontech), then amplified with the GFP (GFP-Mlu-S, ApoE-Sph-A). And the PCR products were inserted into the *Mlu*I/*Sph*I site of pSinRep5. The Sindbis viruses were constructed in accordance with Invitrogen protocols. For Sindbis viral expression, viruses were added directly to media and expression was allowed for 6–12 h, according to the experimental design. All restriction enzymes employed in this research were purchased from New England Biolabs (Beverly, MA, USA).

mRNA transport assay using MS2-GFP reporting system

The constructs, involved in the visualization system employing GFP-tagged bacteriophage MS2 RNA-binding protein, were kindly provided by Dr. Kenneth Kosik (University of California, Santa Barbara, CA, USA). The full-length coding regions of *APOE* mRNA (ApoE-BamH-S, ApoE-Not-A), 40–420 bp regions (containing 1–127 amino acids; ApoE-BamH-S, ApoE420-Not-A), 40–229 bp regions (ApoE-BamH-S, ApoE229-Not-A), 230–420 bp regions (ApoE230-BamH-S, ApoE420-Not-A), 421–978 bp regions (containing 127–312 amino acids; ApoE421-BamH-S, ApoE-Not-A), 421–700 bp regions (ApoE421-BamH-S, ApoE700-Not-A), 701–978 bp regions of cDNA sequences (ApoE701-BamH-S, ApoE-Not-A), or 3′UTR (ApoE3′UTR-BamH-S, ApoE3′UTR-Not-A) were PCR amplified, subsequently digested with *BamH*I/*Not*I, and inserted into the *Bgl*II/*Not*I site of pRSV-MS2 bs vector, respectively (see Table S1 for primers and their sequences). Each plasmid or pRSV-MS2 bs-Ca^{2+}/calmodulin-dependent protein kinase II α-subunit 3′UTR vector (Rook *et al.* 2000) as a positive control was transfected to cultured hippocampal neurons with pCMV-GFP-MS2-nls vector via the Ca^{2+}-phosphate method (Goetze *et al.* 2004). After 36 h of incubation, the neurons were fixed and stained with anti-microtubule-associated protein 2 (MAP2) antibody (Chemicon International) or used directly for confocal microscopy and imaging analysis. Cy3-conjugated anti-mouse IgG was utilized as the secondary antibody for microtubule-associated protein 2 staining.

Cholesterol depletion and cholera toxin B subunit staining

Cultured hippocampal neurons were infected with Sindbis virus encoding for mRFP-PSG-ApoE or mRFP as a control virus. The cultures were then treated with 5 mmol/L MβCD (Sigma) for 20 min to deplete cholesterol, and subsequently fixed and stained with 1 μg/mL of FITC-conjugated cholera toxin B subunit (CTB, Sigma). Only the images of mRFP-positive neurons were acquired via confocal microscopy (TCS-SP2, Leica Microsystems). In order to assess the changes of lipid rafts on membranes as the result of ApoE over-expression, the levels of CTB bound to the membrane were measured in the cell body regions of mRFP-positive neurons.

Image analysis

According to the results of phase contrast microscopy and/or fluorescence imaging, typical hippocampal pyramidal neurons with similar morphologies were selected, and a single primary dendrite was scored for each analysis. For the quantification of PSD-95 or Synapsin I, the secondary and/or tertiary dendrites, which are similar in width, were selected and scored in the analyses. All images were processed with NIH image analysis software (ImageJ ver: 1.40g, Bethesda, MD, USA). All image acquisitions and analyses were conducted via blind experiments. Student's *t*-test was employed to determine the statistical differences between the two groups. One-way ANOVA was utilized to further compare the groups. *Post hoc* comparisons were conducted via Newman-Keuls multiple comparison tests.

Results

Dendritc mRNA candidates isolated from Staufen2-containing RNP complexes

In order to isolate mRNAs to be transported to dendrites or already localized in the dendrites, we constructed a cDNA library of mRNAs isolated from Staufen2-containing RNP complexes, which are involved in dendritic mRNA transport (Tang *et al.* 2001; Jeong *et al.* 2007). The estimated size of the cDNA library was approximately 4×10^4 colony forming unit (cfu). After a blue/white colony selection using β-galactosidase activity to remove self-ligated clones,

positive clones were randomly selected and subjected to sequencing analysis. The sequencing results were analyzed via a homology search using Basic Local Alignment Search Tool for nucleotide (Blastn). As a result, we identified 58 mRNA candidates which were already localized in the dendrites or are to be transported to the dendrites. According their molecular functions, mRNA candidates were classified into the following categories: ribosomal protein, channel, transcription/translation, growth factor, protein modification/signaling pathway, carrier, matrix/cytoskeleton associated, lipid metabolism, RNA binding/processing, proteosome-dependent degradation, and others (Table S2). Interestingly, our results showed little overlap with previous studies, with the exception of a few ribosomal proteins (Poon *et al.* 2006; Zhong *et al.* 2006).

APOE mRNA was localized in dendrites

In order to clarify the dendritic localization of the mRNA candidates, we selected four clones including apolipoprotein E (*APOE)* mRNA, and conducted *in situ* hybridization analyses. The *in situ* signals of *APOE* mRNA (Fig. 1a and b) and other candidates (see Figure S2) were detected in both the cell bodies and dendrites of the cultured hippocampal neurons. The mean *in situ* intensity of each region, relative to that of the sense controls, was increased significantly (Fig. 1c, 1–50 μm: 332.72 ± 59.36%, $n = 17$, $^{**}p > 0.01$; 51–100 μm: 199.0 ± 32.02%, $n = 17$, $^{*}p > 0.05$). ApoE is involved in the transport and homeostasis of cholesterol, which plays an important role in membrane dynamics; thus it might also be relevant to synaptic plasticity. Therefore, we elected to further analyze the localization of *APOE* mRNA in the dendrites. The localization of *APOE* mRNA was subsequently determined by assessing the relative localization with Staufen2-containing RNP complexes. As anticipated, following the expression of myc-tagged Staufen2, the immunoreactivity of anti-myc antibody was strongly colocalized with the *in situ* signals of *APOE* mRNAs in the dendrites. We noted that 37.9 ± 2.49% ($n = 8$) of *in situ* signals in the dendrites were colocalized with the immunoreactivities of anti-myc antibody (see Figure S3).

Coding regions of *APOE* mRNA were sufficient for dendritic transport

The specific sequences in the 3′untranslated regions (UTR) of some mRNAs are known to be involved in their transport to the neuronal dendrites (Andreassi and Riccio 2009).

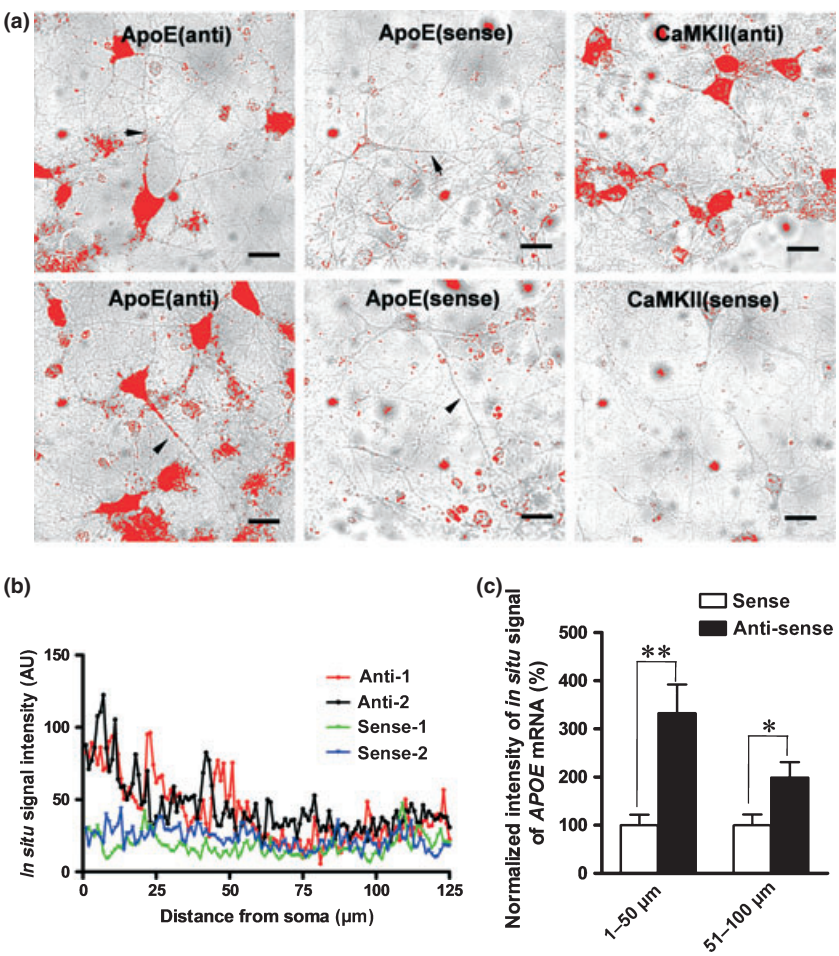
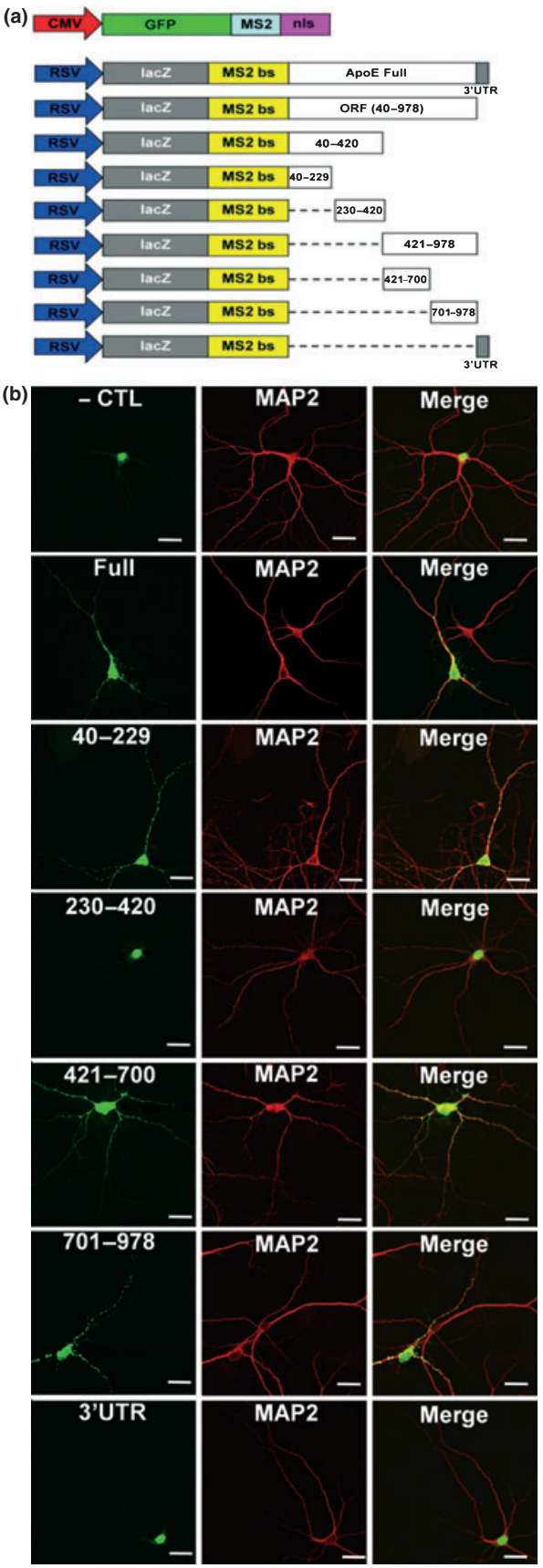


Fig. 1 *APOE* mRNA localizes in dendrites. The localization of *APOE* mRNA was evaluated via *in situ* hybridization. The cultured hippocampal neurons were hybridized with DIG (digoxigenin)-labeled sense or antisense ApoE and CaMKII α-subunit probes and incubated with alkaline phosphatase-coupled anti-DIG antibody. The *in situ* signals were developed via the addition of alkaline phosphatase substrates. All monochromatic images of *in situ* data were changed to the threshold images (identical threshold values in all images). (a) Representative *in situ* images (anti: antisense probe, sense: sense probe). (b) *In situ* signal intensity of the dendrites indicated by arrowheads in panel (a). (c) Bar graphic representation of the mean intensity of *in situ* signal. Each value indicates the mean intensities of *in situ* signals in indicated regions, relative to those of the sense control group (1–50 μm: 332.72 ± 59.36%, $n = 17$, $^{**}p > 0.01$; 51–100 μm: 199.0 ± 32.02%, $n = 17$, $^{*}p > 0.05$). Data are represented as the mean values ± SEM. Scale bar: 20 μm.



Indeed, compared with the 3′UTR length of different mRNAs, total length of the 3′UTR of *APOE* mRNA (109 bp) was considered to be relatively short to harbor some transporting elements. However, several reports has been shown that *cis*-acting elements for dendritic trafficking of mRNA was determined to less than 100 bp (Ainger *et al.* 1997; Muslimov *et al.* 1997; Carson *et al.* 1998; Mori *et al.* 2000; Tiruchinapalli *et al.* 2003). In an effort to determine the functions of the 3′UTRs of *APOE* mRNA, cultured neurons were transfected with pCMV-mRFP-3′UTR or mRFP vector, then subjected to *in situ* analysis for mRFP mRNA. Unexpectedly, the 3′UTR of *APOE* mRNA had no effect on its transport characteristics (see Figure S4). Thus, in the following experiments, the effects of the coding sequence in the dendritic transport of *APOE* mRNA were evaluated. In order to assess this, we took advantage of the visualization system of RNA transport using GFP and bacteriophage MS2 RNA-binding motifs, which this system has been widely used to detect mRNA regions directing to sub-cellular localization from *Drosophila* oocytes to mammalian neurons (Querido and Chartrand 2008). The bacteriophage MS2 coat protein binds the RNA stem-loop in the MS2 protein binding sites (MS2 bs, Fig. 2a). If an interest of mRNA is not transported to the dendrites or not presented, the MS2-GFP proteins bound to the MS2 bs are accumulated in the nucleus, because of a nuclear localization signal (nls). However, if an interest of mRNA is transported to the dendrites, MS2-GFP protein exported from the nucleus allowing the visualization using GFP (Querido and Chartrand 2008). Thus, we inserted a series of *APOE mRNA* regions to pRSV-MS2 bs vector. The neurons were incubated for 36 h after transfection and the fluorescence of GFP was monitored via confocal microscopy. As a result, not only full-length but also 40–229, 421–700 or 701–978 nucleotides of *APOE* mRNA-containing granules were transported to the dendrites. In accordance with our previous results (Figure S4), the 3′UTR of *APOE* mRNA-containing granules were not found in the dendrites. Thus, the proximal N-terminal (40–229 bp) or C-terminal regions (421–978 bp) were found to be sufficient for the transport of *APOE* mRNA to the dendrites (Figs 2 and S5).

Fig. 2 Coding regions but 3′UTR of *APOE* mRNA are sufficient for dendritic transport. The cultured hippocampal neurons were transfected with pCMV-GFP-MS2-nls (MS2: MS2 RNA-binding protein, nls: nuclear localization signal) and pRSV-MS2 bs-*APOE* mRNA region (MS2 bs: MS2-protein binding sites, eight copies of 12-bp hairpin) and additionally incubated for 36 h, and fixed. The cultures were stained with anti-MAP2 antibody. The dendritic transport of mRNAs was monitored by GFP signal in dendrites. (a) Plasmid constructs used in the visualization system of dendritic mRNA transport using a capsid assembly-deficient coat protein of MS2 bacteriophage and GFP. (b) Dendritic transport of each regions of *APOE* mRNA. Scale bar: 20 μm.

Neuronal activity increased the level of dendritic *APOE* mRNA

A variety of neuronal activities, including depolarization or neurotrophin treatment, increase the mRNA levels of BDNF and tyrosine kinase B (Tongiorgi *et al.* 1997; Righi *et al.* 2000), or zipcode binding protein1 and β-actin (Tiruchinapalli *et al.* 2003). Thus, in order to determine whether or not neuronal activity exerts an effect on the dendritic levels of *APOE* mRNA, cultured neurons were treated with high-K^+ HEPES buffered saline (60 mmol/L) for 10 min or with 50 μg/mL of BDNF for 3 h, then subjected to *in situ* analysis. Depolarization-induced neuronal activity significantly increased the levels of *APOE* mRNA in the distal dendrites (51–100 μm from cell body) [Fig. 3a and b, control (CTL): 24.61 ± 1.79, $n = 17$; +KCl: 29.54 ± 1.10, $n = 18$; $^*p < 0.05$, AU: arbitrary unit] but decreased it in the cell bodies (CTL: 134.5 ± 3.29, $n = 15$; +KCl: 121.1 ± 3.39, $n = 15$; $^{**}p > 0.01$, AU: arbitrary unit). This brief stimulation did not alter the levels of ApoE protein (data not shown). BDNF treatment exerted no detectable effects on the dendritic transport of *APOE* mRNA (Fig. 3c, distal dendrites, CTL 13.45 ± 0.85, $n = 20$; BDNF: 12.77 ± 0.89, $n = 22$, cell bodies: CTL: 162 ± 3.16, $n = 24$; BDNF: 170.8 ± 2.79, $n = 23$, AU: arbitrary unit).

ApoE expression blocked reduction of lipid rafts by cholesterol depletion

Cholesterol is known to be enriched in the lipid rafts on plasma membranes (Simons and Ikonen 1997; Brown and London 1998). In an effort to assess the change of lipid rafts on membranes following the expression of ApoE, cultured hippocampal neurons were infected with the two-cistron Sindbis virus encoding for mRFP-PSG-ApoE to avoid the functional hindrance by a tagging protein. We then assessed *APOE* mRNA and protein levels in the distal dendrites (51–100 μm from cell body) following over-expression by the Sindbis virus. Viral infection induced an approximate 2.5-fold increase in the levels of the ApoE protein as compared to the uninfected controls, and an approximate 3.8-fold increase in mRNA levels (Figure S6). In an effort to assess the functions of dendritic ApoE, we evaluated the effects of ApoE expression on the lipid rafts using this expression system. The most profound change in the rafts was evaluated via the FITC-conjugated CTB, which has a high degree of affinity with ganglioside GM1 (Lencer *et al.* 1999) and is widely used to measure membrane rafts (Bruses *et al.* 2001). MβCD treatment can effectively extract cholesterol from the neuronal membrane (Bruses *et al.* 2001; Hering *et al.* 2003; Frank *et al.* 2004). Neurons infected with the Sindbis virus encoding for mRFP-PSG-ApoE or mRFP were incubated for 6 h to induce expression, treated with 5 mmol/L MβCD, and stained with FITC-CTB. Interestingly, although the results were not statistically significant, ApoE expression itself reduced the reactivity of

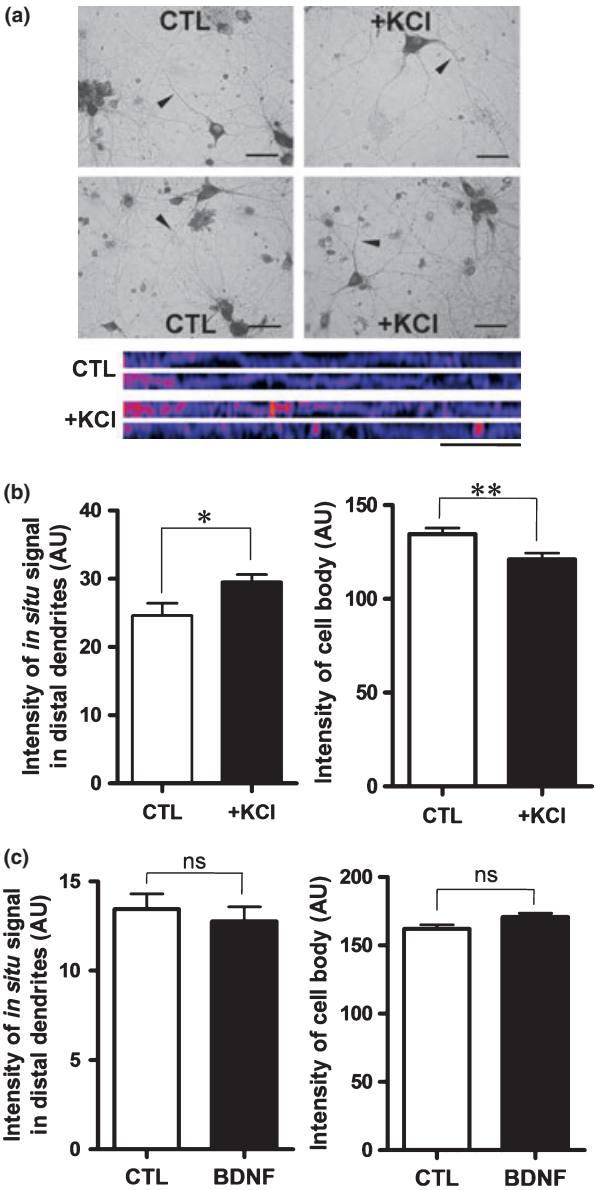


Fig. 3 Depolarization increases the dendritic levels of *APOE* mRNA. The cultured hippocampal neurons were briefly stimulated with high-K^+ HBS (60 mmol/L KCl, 10 min) and incubated for 2 h 50 min or treated with 50 ng/mL of BDNF for 3 h, then subjected to *in situ* analysis. The values of 51–100 μm long distal dendrites were scored in analysis. (a) Representative images of *in situ* analysis. The arrowheads indicates the dendrites used in image analysis. In lower panel, straightened images of dendrites were transited into fired ones for easy visualization, and presented. Scale bar: 20 μm. (b) Bar graphic representation of image analysis results. Each value indicates the mean *in situ* intensity of the indicated dendritic regions in arbitrary units (AU). Data are represented as the mean values ± SEM. Student's *t*-test was employed to determine the statistical difference between the groups ($^*p < 0.05$, $^{**}p < 0.01$). (c) BDNF treatment had no effects on dendritic levels of *APOE* mRNA. The cultured hippocampal neurons were treated with 50 ng/mL of BDNF for 3 h, and subjected to *in situ* analysis and image analysis (ns: not significant).

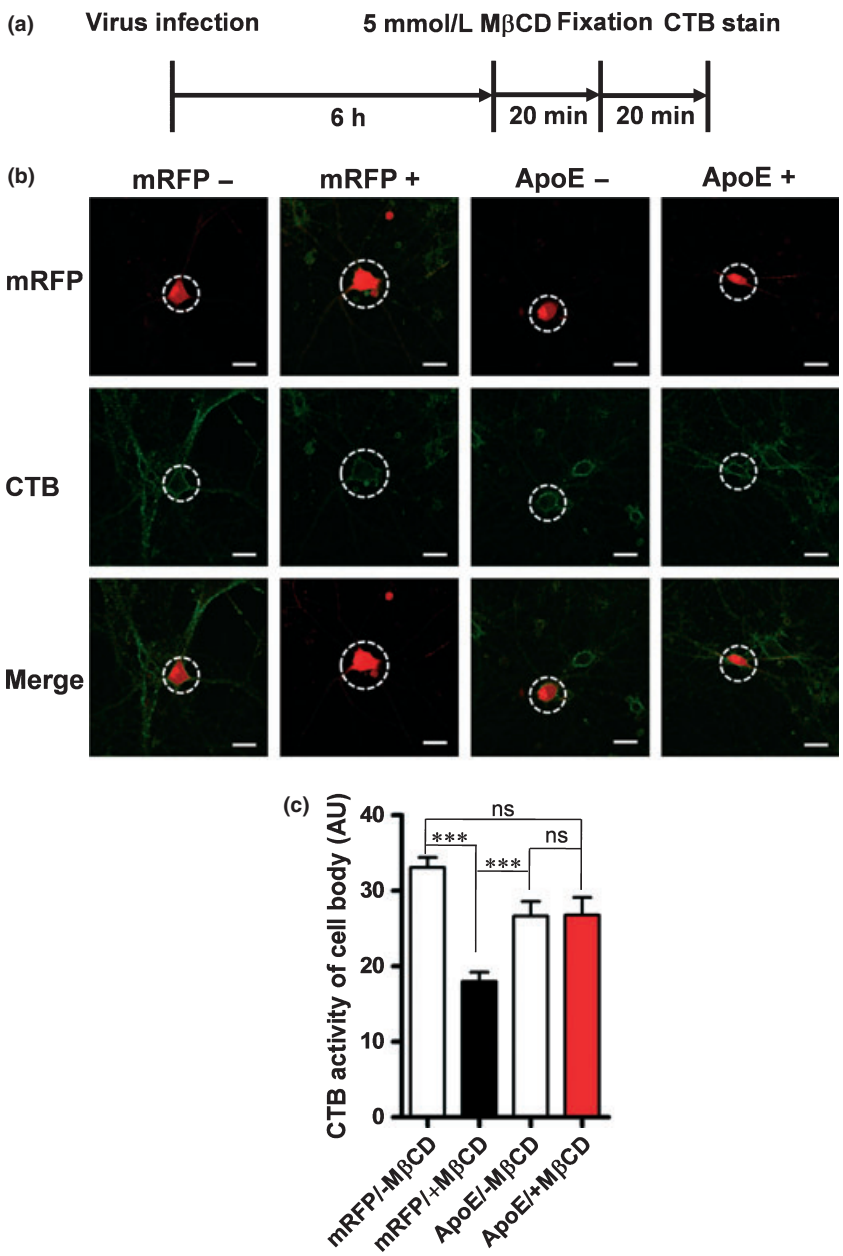


Fig. 4 ApoE expression blocks the reduction of lipid rafts by cholesterol depletion. The cultured hippocampal neurons were infected with the Sindbis virus encoding for mRFP-PSG-ApoE (ApoE) or mRFP, incubated for 6 h and treated with 5 mmol/L methyl-β-cyclodextrin (MβCD) for 20 min. Immediately the cultures were fixed and stained with 1 μg/mL FITC-conjugated cholera toxin B subunit (CTB) for 20 min. Stained images were acquired using a laser scanning confocal microscope. Each value indicates the mean fluorescent intensity of cell body membranes from mRFP-PSG-ApoE (ApoE) or mRFP expressed neurons in arbitrary units (AU). Data represent the mean values ± SEM. (a) Schematic diagram of experimental procedure. (b) Representative images of neurons (mRFP –: infected with mRFP virus and not treated with MβCD, mRFP +: infected and treated with MβCD, ApoE –: infected with mRFP-PSG-ApoE virus and not treated, ApoE +: infected and treated with MβCD). The white circles indicate the cell bodies of neurons which express mRFP or ApoE. Scale bar: 20 μm. (c) ApoE expression completely blocked the reduction of CTB activities induced by cholesterol depletion in cell body regions. One-way ANOVA and Newman–Keuls multiple comparison test were used to determine statistical difference among groups (***$p < 0.001$, ns: not significant).

CTB on the membranes by approximately 19%, but completely blocked reduction by MβCD treatment, thereby suggesting the protective effects of ApoE on the reduction of lipid rafts on membranes via cholesterol depletion (Fig. 4, mRFP/– MβCD: 33.07 ± 1.35, $n = 12$; mRFP/+ MβCD: 17.99 ± 1.21, $n = 25$; ApoE/– MβCD: 26.44 ± 1.94, $n = 11$; ApoE/+ MβCD: 26.78 ± 2.35, $n = 14$, AU: arbitrary unit, one-way ANOVA ***$p < 0.001$, ns: not significant).

ApoE expression increased PSD-95 immunoreactivity in synapses

In order to determine the effects of ApoE expression on synaptic structure and discriminate the effects of neuronal ApoE with those originated form glial cells, we constructed the Sindbis virus encoding GFP-tagged ApoE (GFP-ApoE). At first we examined whether neuronal ApoE proteins were localized to dendrites. Neurons infected with the Sindbis virus encoding GFP-ApoE were stained with anti-PSD-95 or anti-Synapsin I antibody, and examined the localization of GFP and PSD-95 or Synapsin I reactivity. GFP-tagged ApoE was localized in the dendrites near synaptic regions (see Figure S7). In the following experiments, neurons infected with the two-cistron Sindbis virus encoding for GFP-PSG-ApoE were stained with anti-PSD-95 antibody or anti-Synapsin I antibody, after which its reactivity was evaluated. ApoE expression significantly increased the immunoreactivity of PSD-95 antibody in the dendrites by approximately 26% [Fig. 5a and

b, GFP: 22.60 ± 1.80, $n = 19$ dendrites from nine neurons (total length: 2346 μm); ApoE: 28.47 ± 2.03, $n = 16$ dendrites from 10 neurons (total length: 1663 μm), $*p < 0.05$, AU: arbitrary unit], but significantly not particle density (Fig. 5c, GFP: 14.02 ± 0.95; ApoE: 15.96 ± 1.64, mean number of particles/10 μm dendrite, ns: not significant). However, our data did not definitively discriminate the roles of ApoE protein translated in dendrites, by local protein synthesis, from those translated in the cell bodies.

Discussion

In this study, *APOE* mRNA was isolated from the Staufen2-containing RNP complexes of rat cultured hippocampal neurons. The *APOE* mRNAs were detected in the dendritic regions and colocalized with Staufen2 protein. The proximal N-terminal or C-terminal regions of the coding sequences proved to be sufficient for their dendritic transport, but the 3′UTRs proved to be unnecessary. Interestingly, the dendritic level of *APOE* mRNAs was increased by depolarization-induced neuronal activity, but the levels in cell bodies were reduced significantly, thereby implying an active transport mechanism. According to our results, the reduction of lipid rafts by cholesterol depletion was reversed as the result of ApoE over-expression. In addition, neuronal ApoE was localized in the dendrites and ApoE over-expression increased the immunoreactivity of PSD-95 antibody in the dendrites.

A previous study in which the mRNAs were isolated from Staufen2-RNP complexes did not include *APOE* mRNA in the list of candidate mRNAs (Furic *et al.* 2008). This might be because a different cellular system was used for the expression of an epitope tagged-Staufen2 protein. Furic *et al.* took advantage of kidney cell originated human embryos (HEK293 cells) for the expression and isolation of hemagglutinin (HA)-tagged Staufen2-RNP complexes. On the other hand, we utilized cultured hippocampal neurons for this purpose. Consequently, there might be some differences in the mRNA profiles associated with Staufen2-containing

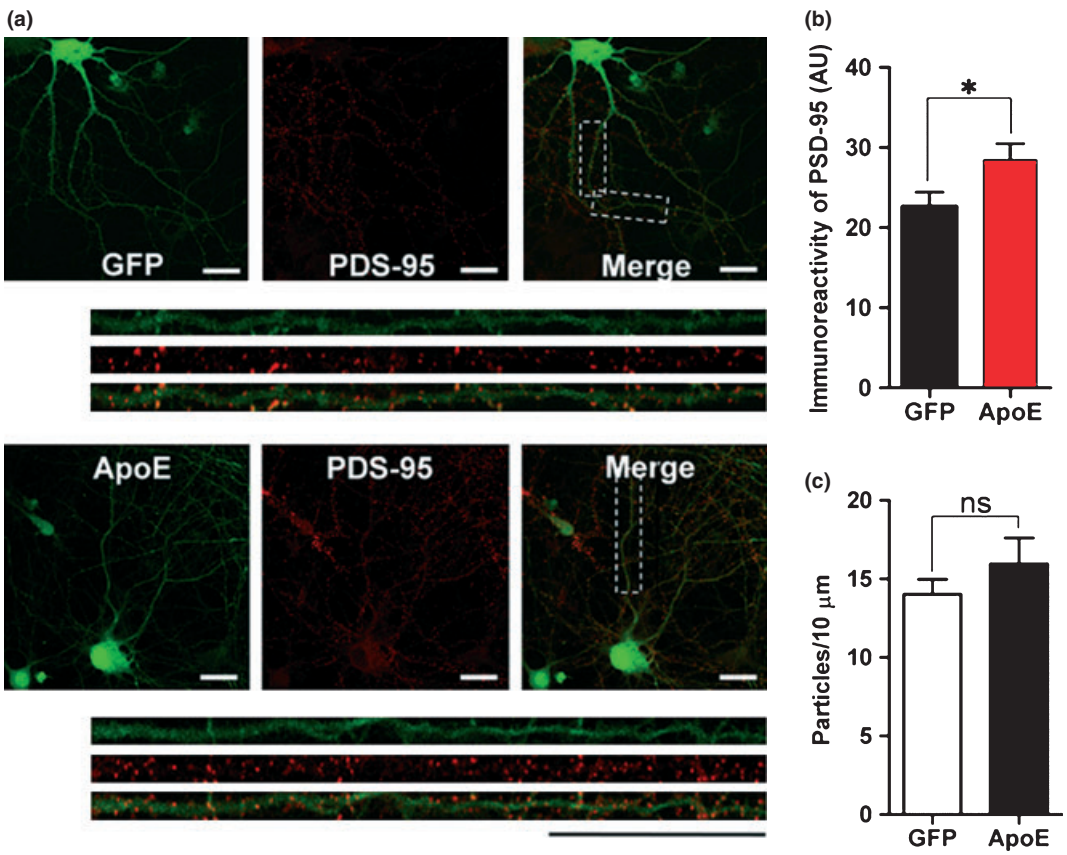


Fig. 5 ApoE expression increases the immunoreactivity of PSD-95 in dendrites. The cultured hippocampal neurons were infected with Sindbis virus encoding GFP-PSG-ApoE (ApoE) or GFP, and incubated 12 h and stained with anti-PSD-95 antibody. The secondary and/or tertiary dendrites were selected from the neurons showing a similar morphology and fluorescent intensities were scored in data analysis. (a) Representative images of neurons. Boxed regions indicate the dendrites used in image analysis. Lower panels show straightened images of dendrites indicated by the dotted-line boxes in upper images. Scale bar: 20 μm. (b), (c) Bar graphic representation of data analysis. Data represent the mean values ± SEM. Student's *t*-test was employed to determine the statistical difference between the groups ($*p < 0.05$, ns: not significant, AU: arbitrary unit).

RNP complexes between neurons and kidney cells. Little overlap was detected between this study and other studies, with the exception of a few ribosomal proteins between the dendritic mRNAs (Poon *et al.* 2006; Zhong *et al.* 2006); this underlines the broad diversity of dendritic mRNAs or different mRNA pools isolated via different methodologies. In addition, localization of mRNAs to Staufen2-containing RNP complexes is not sufficient for their dendritic targeting, and Staufen2-containing RNP complexes may also be present in the cell soma. Thus the mRNAs in our list need an additional validation such as *in situ* hybridization. Even this limitation, the approach utilized in this study may help in determining the identities and functions of dendritic mRNAs, and may also provide some clues as to the roles of dendritic protein synthesis in synaptic plasticity.

The transport of mRNA to dendrites and its translation therein, a process known as local protein synthesis, perform a critical role in synaptic plasticity (Martin and Zukin 2006; Pfeiffer and Huber 2006; Schuman *et al.* 2006; Sutton and Schuman 2006). The mRNAs are transported to the dendrites via the use of molecular motors such as kinesin, forming ribonucleoprotein complexes or RNA granules, and this transport is finely regulated by specific signaling pathways and neuronal activities (Hirokawa 2006). According to our results, the dendritic level of *APOE* mRNA was significantly increased in response to depolarization-induced neuronal activity (Fig. 3), and this was accompanied by a reduction in cell body regions. A brief stimulation protocol (60 mmol/L KCl, 10 min) induced no change in ApoE expression (data not shown). Indeed, these results are consistent with previous data showing that depolarization-induced neuronal activity increased the dendritic levels of Staufen2-containing RNP complexes (Kim and Kim 2006; Jeong *et al.* 2007). BDNF treatment exerted no detectable effects on the dendritic transport of *APOE* mRNA. This is also consistent with our previous data, in which BDNF was shown to exert no effects on the dendritic transport of Staufen2-containing RNP complexes (data not shown). This may be attributable to the weak intensity of the BDNF-induced signaling pathway, as compared with depolarization. There may be different regulatory mechanisms to two external stimuli in the dendritic transport of *APOE* mRNA. Collectively, these findings corroborate that *APOE* mRNA is transported via a Staufen2-mediated mechanism.

APOE mRNA was isolated from a dendrite-specific cDNA library (Figure S1, Table S2) and the localization of *APOE* mRNA was verified via *in situ* hybridization analysis (Figs 1 and S3). As described previously, dendritic mRNAs are transported via the formation of ribonucleoprotein complexes or RNA granules (Hirokawa 2006). Proteins harboring double-stranded RNA binding domain (dsRBD), such as Staufen and *Xenopus laevis* RNA-binding protein A, bind to the double-stranded RNA produced by stem-and-loops (St Johnston *et al.* 1991; Eckmann and Jantsch 1997). The third dsRBD of *Drosophila* Staufen binds to RNA stem-and-loops harboring more than eight uninterrupted base pairs, and optimally 12 uninterrupted base pairs (Ramos *et al.* 2000). This suggests that many mRNAs might function as targets for double-stranded RNA binding proteins such as Staufen. In this study, we did not isolate a specific region of *APOE* mRNA with regard to binding to Staufen2, but predicted that the secondary structure of *APOE* mRNA would evidence several potential bind sites of dsRBD (data not shown), suggesting its dendritic localization. In addition, as we did not assess the direct interaction of *APOE* mRNA with the Staufen2 protein, we are unable to dismiss the possibility that *APOE* mRNA might interact with other RNA-binding proteins in Staufen2-containing RNP complexes.

Although the astrocytes are the primary expression sites of ApoE, ApoE is also expressed in neurons (Xu *et al.* 1999; Dekroon and Armati 2001). This raises a question as to how the role of ApoE expressed in neurons differs, particularly in the dendrites? ApoE performs pivotal roles in maintenance of synaptic structures or in aging-related neurodegeneration (Masliah *et al.* 1995). The expression of ApoE is increased after injury to the CNS or PNS (Ignatius *et al.* 1986; Mahley 1988). In addition, the ApoE-containing lipoprotein induces synaptogenesis (Mauch *et al.* 2001) and axonal growth (Hayashi *et al.* 2004), and protects neurons against cell death induced by a receptor-mediated signaling pathway (Hayashi *et al.* 2007). Recent data indicate that ApoE plays a role in facilitating the proteolytic clearance of Aβ in the brain, thereby suggesting the possible use of ApoE as a therapeutic target in cases of Alzheimer's disease (Jiang *et al.* 2008). The depletion of lipid rafts from neuronal membranes induces a change in synaptic structures and function (Hering *et al.* 2003; Frank *et al.* 2004) and cholesterol levels in the nervous system affect on synaptic plasticity (Koudinov and Koudinova 2001; Mauch *et al.* 2001; Frank *et al.* 2008). In humans, ApoE proteins function differently in the nervous system according to their specific isoform. ApoE in rodents is structurally and functionally similar to human ApoE3 (Weisgraber 1994). Thus, we expected to see similar roles of ApoE3 among human isoforms. ApoE over-expression completely blocked the reduction of membrane lipid rafts as the result of cholesterol depletion (Fig. 4). Consequently, ApoE proteins were localized in the dendrites near synaptic regions (Figure S7) and ApoE over-expression increased the immunoreactivity of anti-PSD-95 antibody in the dendrites (Fig. 5). These findings suggest that ApoE might prevent the reduction of lipid rafts by sustaining cholesterol level in membrane, and might increase PSD-95 in post-synaptic regions by increasing raft-associated PSD-95. And these are consistent with previous reports suggesting that ApoE plays a role in the functional or structural aspects of neurons.

In this study, we exploited the Sindbis viral expression system to determine the role of dendritic ApoE protein or

ApoE protein translated from dendritically localized *APOE* mRNAs. The Sindbis virus preferentially infects neurons over non-neuronal cells-especially astrocytes, oligodendrocytes and microglia (Ehrengruber 2002). This supports the supposition that the function of ApoE originates mainly from that of neuronal ApoE. Although the infection of Sindbis virus encoding for ApoE significantly increased the mRNA levels of ApoE in both cell bodies and distal dendrites (51–100 μm from cell body), we were unable to exclude ApoE protein translocalized from the cell body. Thus, our data does not definitively discriminate the roles of ApoE protein translated in dendrites, by local protein synthesis, from those translated in the cell body. Were a new expression system developed by which the dendritic level of *APOE* mRNA could be specifically increased, we could answer this question more clearly. Unfortunately, because of issues with the resolution of the confocal microscopic images obtained by FITC-CTB staining, the alterations of lipid rafts by ApoE expression were examined in cell body regions, rather than dendritic regions (Fig. 4). Thus, the interpretation of our results is restricted to the effects of neuronal ApoE on lipid rafts. If this technical problem can be resolved by the development of novel staining or detection methods, the role of dendritic ApoE can be better understood.

In this study, we isolated *APOE* mRNA from an mRNA pool localized to the dendrites and assessed its dendritic transport characteristics. We subsequently assessed post-synaptic structures via ApoE over-expression in cultured hippocampal neurons. According to our results, neuronal ApoE completely blocked the reduction of membrane lipid rafts because of cholesterol depletion, was localized in the dendrites near synaptic regions, and enhanced the immunoreactivity of anti-PSD-95 antibody in the dendrites. Collectively, these data indicate that neuronal ApoE may play a role in synaptic plasticity, stabilizing synaptic structure by regulating the levels of cholesterol in the membranes.

Acknowledgements

This work was supported by the Korea Research Foundation Grant (KRF-2007-521-C00242) and by Basic Science Research Program (2009-0063679) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology.

Supporting Information

Additional Supporting information may be found in the online version of this article:

Figure S1. Experimental Scheme for isolation of Staufen2-containing RNP complexes and construction of cDNA library.

Figure S2. mRNA candidates isolated from Staufen2-containing RNP complexes are localized in dendrites.

Figure S3. *APOE* mRNA colocalizes with Staufen2-containing RNP complexes.

Figure S4. 3′UTR of APOE mRNA are not required for dendritic transport.

Figure S5. N-terminal or C-terminal regions of ApoE coding sequences are sufficient for dendritic transport.

Figure S6. Infection of Sindbis virus encoding for ApoE increases ApoE protein and mRNA levels in dendrites.

Figure S7. Neuronal ApoE proteins are localized in synapses.

Table S1. Primers and sequences used in subcloning.

Table S2. mRNAs isolated from Staufen2-containing RNP complexes.

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