Neuroscience 122 (2003) 291–303

apoE ISOFORMS AFFECT NEURONAL *N*-METHYL-D-ASPARTATE CALCIUM RESPONSES AND TOXICITY VIA RECEPTOR-MEDIATED PROCESSES

Z. QIU,[a] K. A. CRUTCHER,[b] B. T. HYMAN[a] AND G. W. REBECK[c]*

[a]*Department of Neurology, Massachusetts General Hospital, 114 16th Street, Charlestown, MA 02129, USA*

[b]*Department of Neurosurgery, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA*

[c]*Department of Neuroscience, Georgetown University, 3970 Reservoir Road NW, Washington, DC 20057-1464, USA*

Abstract—Apolipoprotein E (apoE) alters the pathophysiology of Alzheimer's disease, but its mechanism is not fully understood. We examined the effects of recombinant human apoE3 and apoE4 on the neuronal calcium response to *N*-methyl-D-aspartate (NMDA), and compared them to their toxicity. ApoE4 (100 nM) significantly increased the resting calcium (by 70%) and the calcium response to NMDA (by 185%), whereas similar changes were not obtained in apoE3-treated neurons. ApoE4, but not apoE3, also significantly increased neurotoxicity, as evidenced by enhanced lactate dehydrogenase release (by 53%) and reduced 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide levels (by 32%). ApoE4-induced changes in the calcium response to NMDA and associated neurotoxicity were blocked by coincubation with MK-801. Both the receptor-associated protein, which inhibits interaction of apoE with members of the LDL receptor family, including the low-density lipoprotein receptor-related protein (LRP), and activated α2-macroglobulin, another LRP ligand, prevented apoE4-induced enhancement of the calcium response to NMDA, resting calcium levels, and neurotoxicity. A tandem apoE peptide (100 nM) containing only the receptor binding region residues also eliminated the enhanced calcium signaling and neurotoxicity by apoE4.

Taken together, our data demonstrate that differential effects of apoE3 and apoE4 on the calcium signaling in neurons correlate with their effect on neurotoxicity, which are secondary to receptor binding.

Key words: Alzheimer's disease, hippocampus, fluo-3, α2-macroglobulin, calcium, lipoprotein.

Genetic studies have found an increased risk of Alzheimer's disease associated with inheritance of the apolipoprotein E (apoE) ε4 allele compared with the ε2 and ε3 alleles. Differential effects of apoE3 and apoE4 or related peptides *in vitro* have been documented on neurite outgrowth (Crutcher et al., 1994; Holtzman et al., 1995; Narita et al., 1997), formation of SDS-stable complexes with Aβ (LaDu et al., 1995), facilitation of transcription of cAMP-response element-binding protein (CREB)-dependent genes (Ohkubo et al., 2001), cholesterol efflux (Michikawa and Yanagisawa, 1999b), cytoskeletal assembly (Huang et al., 2001), and neurotoxicity (Marques et al., 1996; Jordan et al., 1998; Tolar et al., 1999). Many neuronal effects of apoE isoforms are mediated by the apoE receptor, the low-density lipoprotein receptor-related protein (LRP). In general, apoE3 acts as a neurotrophic or neuroprotective factor (Hashimoto et al., 2000), while apoE4 is associated with neurotoxicity (Tolar et al., 1999), and both effects are associated with the function of LRP.

LRP is a 600 kDa multifunctional cell surface receptor (a member of the LDL receptor family), containing multiple ligand sites and a high affinity calcium-binding site, which is important for receptor conformation and ligand recognition (Strickland et al., 1991). LRP directs ligands, including apoE, activated α2-macroglobulin (α2M*), and lactoferrin to degradation. LRP is expressed in the CNS (Rebeck et al., 1993), and also in primary neurons (Holtzman et al., 1995). Little is known about the differential effects of physiological concentrations of apoE isoforms (5–10 μg/ml in CSF) on intracellular calcium signaling. Calcium plays an important role in both physiological and pathological processes. Physiologically, calcium is an important intracellular second messenger and controls a variety of neuronal functions. However, excessive intracellular calcium is associated with neurotoxicity (Gibbons et al., 1993; Lipton and Rosenberg, 1994), and hyperphosphorylation of tau (Yu and Fraser, 2001). Therefore, apoE induced changes in calcium signaling could profoundly affect neuronal function in the CNS. We focused on the chronic effects of apoE because the presence of apoE on plaques in the Alzheimer's disease (AD) brain could expose surrounding neurons to plaque-associated protein for the life of the plaque.

In the current study, apoE isoforms' effects were examined in cultured hippocampal neurons, which express *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid, and kainate subtypes of glutamate receptors. Glutamate is the main excitatory transmitter in the CNS and is known to play an important role in neuronal degeneration (Choi, 1992; Olney et al., 1997). Calcium signaling elicited by activation of NMDA receptors was assessed. ApoE4, but not apoE3, increased

*Corresponding author. Tel: +1-202-687-1534; fax: +1-202-687-0617.
E-mail address: gwr2@georgetown.edu (G. W. Rebeck).

Abbreviations: AD, Alzheimer's disease; apoE, apolipoprotein E; CICR, calcium-induced calcium release; CREB, cAMP-response element-binding protein; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; LRP, low-density lipoprotein receptor-related protein; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; RAP, receptor-associated protein; VSCC, voltage sensitive calcium channels; α2M*, activated α2-macroglobulin.

0306-4522/03$30.00+0.00
doi:10.1016/j.neuroscience.2003.08.017

neurotoxicity as a consequence of the alteration of intracellular calcium homeostasis, and enhancement of intracellular calcium signals produced by NMDA receptor activation. These effects were blocked by the receptor-associated protein (RAP), an antagonist of the LDL receptor family, α2M*, an LRP ligand, and a tandem apoE peptide containing the apoE receptor binding region. Thus the differential effects of apoE isoforms are associated with neurotoxicity, mediated by LRP.

EXPERIMENTAL PROCEDURES

Cell culture

Hippocampal neurons from 19-day-old embryonic Sprague–Dawley rats were isolated by a standard enzyme treatment protocol (Przewlocki et al., 1999; Qiu et al., 2002). Briefly, hippocampi were dissociated in calcium-free saline and plated on poly-D-lysine (Sigma Chemical Co., St. Louis, MO, USA) coated tissue culture dishes at a density of 1.5×10^6 cells/ml. The neurons were grown in MEM plus 5% horse serum and 5% fetal bovine serum supplemented with 30 mM glucose, and 25 μM penicillin–streptomycin. Medium with 10% horse serum was replaced every 3 days. Treatment with 5-fluoro-2′-deoxyuridine (20 μg/ml) on the third day after plating minimized non-neuronal cell proliferation. The cultures survive for about 20 days in a standard CO_2 incubator.

Treatment with apoE receptor ligands

Receptor ligands, including apoE3, apoE4, α2M*, and the tandem apoE peptide, were added to primary neuronal cultures 6 or 8 days after plating, at which time the culture media was replaced with medium containing 5% horse serum. Control cultures consisted of sister cultures, which were untreated with receptor ligands. The neurons were observed throughout the first 10 days in culture. The ligand-containing medium was removed before calcium measurements were made. RAP was used as an LRP antagonist. RAP, α2M*, and the tandem apoE were applied to the cultures for 24–48 h (defined here as a "chronic" *in vitro* treatment) in order to determine whether they would alter the apoE4 effects on calcium signaling and neurotoxicity. Control cultures (sister cultures) were not treated with receptor ligands. The medium was replaced by physiological saline before calcium measurements were made. The composition of the physiological saline was (in mM): 140 NaCl, 3.5 KCl, 0.4 KH_2PO_4, 0.33 Na_2HPO_4, 2 $MgSO_4$, 2.2 $CaCl_2$, 10 glucose and 10 HEPES–NaOH, pH 7.3.

Intracellular calcium measurement

Intracellular calcium was determined for individual cells using standard microscopic fluo-3 digital imaging (Minta et al., 1989; Qiu et al., 2002). Briefly, hippocampal neurons were loaded with 1 μM fluo-3/AM, and live video imagines of selected microscopic fields were recorded with a photomultiplier (Hamamatsu Photonics, Hamamatsu City, Japan) and digitized by computer with Bio-Rad imaging time course software (Imaging Research Inc., St. Catherines, Ontario, Canada). The somata of approximately five to 10 cells in each microscopic field were individually measured. Intracellular calcium levels were estimated by converting fluorescent intensity to intracellular calcium concentration using the following formula: $[Ca^{2+}]i=K_d(F-F_{min})/(F_{max}-F)$. Calibration was done *in vitro* using fluo-3 salt (100 μM) in solutions of known calcium concentration (Molecular Probes, Eugene, OR, USA), and *in vivo* under saturating calcium concentrations facilitated by introducing extracellular calcium into cells with the calcium ionophore A23187 (Molecular Probes). The calcium calibration *in vivo* was consistent with calcium calibration *in vitro*, and the *in vitro* calibrations were applied in the current study. All experiments were performed at room temperature (approximately 23 °C).

Drug application

In calcium studies, cells were stimulated with micropressure application of either NMDA (100 μM) or selective agonists for the NMDA receptor subtypes of glutamate receptors. The agonists were dissolved in bath saline and applied by brief (1 sec) micropressure pulse from drug pipettes (1–3 μ tip) placed under visual control near target neurons (Qiu et al., 1995). A dye was included in the treatment solutions to monitor neuronal exposure, demonstrating that the agonist was rapidly distributed over an area sufficient to expose the target neurons. For NMDA stimulation the cell bath and agonist solutions were magnesium-free physiological saline containing 5 μM glycine. In some experiments the neurons were exposed to drugs by bath exchange, such as MK-801.

Chemicals

Recombinant human apoE3 and apoE4 from Panvera (Madison, WI, USA) were used in this study, and we found that the apoE isoforms from CalBiochem (San Diego, CA, USA) produced similar results in the calcium studies. Recombinant human LRP RAP was prepared from a glutathione S-transferase fusion protein as described (Williams et al., 1992). Recombinant human α2 M (Sigma Chemical Co.) was activated in methylamine at 10 mg/ml as stock solution and stored at −20 °C for no more than 2–3 weeks. NMDA was obtained from Tocris Neuramin, UK, and stored as stock solutions at concentrations of 50 mM. MK-801 (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM as a stock solution. The final concentration of DMSO is not more than 0.05% in the cell bath solution. In control experiments DMSO had no effects by itself. Synthetic tandem apoE peptide E(141–149)2, consisted of a double sequence of apoE amino acids 141 through 149 (Tolar et al., 1997).

Neurotoxicity assay

Neuronal toxicity was assessed using a standard lactate dehydrogenase assay (LDH kit; Boehringer Mannheim; Koh and Choi, 1987; Qiu et al., 1998) and by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide; Sigma Chemical Co.; Ankarcrona et al., 1995). For studies involving LRP ligands the primary hippocampal neurons were studied using the same paradigm mentioned above. For studies involving NMDA toxicity, delayed toxicity was measured (Koh and Choi, 1987). The hippocampal neurons were challenged with NMDA for 5 min, and the NMDA solution was replaced with the same low serum media as mentioned above and the cultures returned to the incubator. On the next day (18–24 h), toxicity was assessed by measurement of LDH release and by the MTT assay. For both the MTT and LDH assays, results from each culture set were normalized to the appropriate control values for the culture set and then the results were combined.

Data analysis

For calcium studies, intracellular calcium responses were quantified by measurement of the peak amplitude. Resting calcium levels were subtracted from all peak amplitude values on an individual cell basis (Qiu et al., 2002). Each protocol consists of two or three culture sets of hippocampal neurons, in which five to 15 hippocampal neuronal somata in each field were measured. In both calcium studies and neurotoxicity assays, data from several cultures were pooled for statistical analyses. Values are expressed as mean±S.E.M. Two-way analysis of variance followed

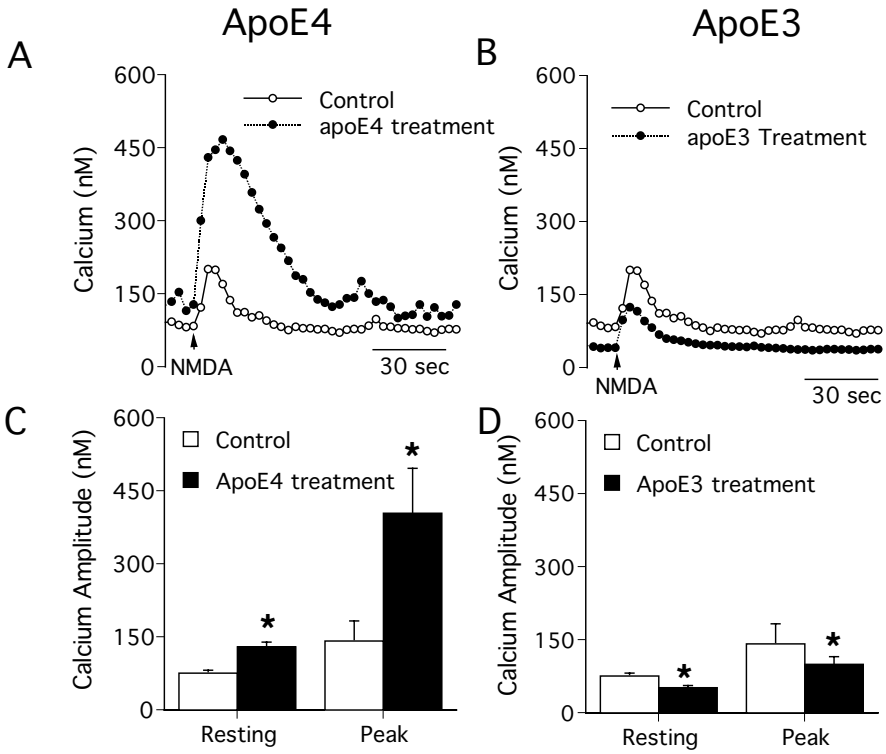

Fig. 1. Differential effects of apoE3 and apoE4 on intracellular calcium signals evoked in cultured hippocampal neurons by NMDA stimulation. Panels A and B show representative recordings of intracellular calcium signals evoked by brief (1 s) application of NMDA (at the arrow) from a micropipette in both control and apoE treated neurons. Estimated intracellular calcium are plotted vs. time for representative cells under control condition (open circles in panels A and B) and either apoE4 (panel A) or apoE3 (panel B) treated conditions (filled circle in panels A and B). Panels C and D show mean values (mean±S.E.M.) for the population of neurons studied for control neurons (open bars) and either apoE4- (panel C) or apoE3- (panel D) treated neurons (filled bars). Averaged data include resting calcium levels and peak calcium amplitude, in which the resting levels have been subtracted. Results are pooled from two to three sets of cultures, and each culture includes two or three fields, containing seven to 12 cells per field. * $P<0.05$.

by the Fisher post hoc test for multiple comparisons determined statistical significance. $P<0.05$ was considered statistically significant.

RESULTS

Differential effects of apoE3 and apoE4 on calcium signaling correlate with their effects on neurotoxicity

In the current study the effects of chronic exposures (1–2 days) of apoE isofoms on neuronal intracellular calcium signaling have been investigated *in vitro*. Fig. 1 shows the effects of 100 nM apoE3 and apoE4 on calcium signaling evoked in cultured hippocampal neurons by NMDA stimulation. The typical intracellular calcium recordings from the control or apoE treated neurons at 8 DIV are shown in Fig. 1A for apoE4 and Fig. 1B for apoE3. NMDA was applied as indicated by an arrow. NMDA stimulation produced an increase in intracellular calcium, which was characterized by an initial peak and slow recovery phase in both control and apoE-treated cultures. ApoE4 treatment dramatically increased the calcium response to NMDA stimulation in the cultured hippocampal neurons, in which the peak calcium amplitudes were enhanced by 185%, and the resting calcium levels were increased by 70% (Fig. 1C). In contrast to apoE4, apoE3 treatment significantly reduced the peak amplitude of calcium response to NMDA, and decreased resting calcium levels as shown in panel D. No intracellular calcium changes were obtained immediately after challenge of the neurons with recombinant human apoE4 alone ("acute" treatments, data not shown). Thus, chronic treatment of neurons with apoE3 and apoE4 had differential effects on intracellular calcium signaling.

Excessive calcium influx is a component of some neuronal loss. The profound increases in intracellular calcium levels and calcium response to NMDA in apoE4-treated neurons indicated that apoE4 might potentially induce calcium overload, leading to neuronal damage. To determine if the effects of apoE on calcium signaling correlated with their effects on neurotoxicity, we examined the effects of apoE3 and apoE4 using the LDH and MTT assays. Fig. 2 shows the average effects of chronic apoE4 and apoE3 treatments on neurotoxicity in cultured hippocampal neurons. ApoE4 (100 nM) significantly increased toxicity by enhancing LDH release by 52% and reducing the MTT levels by 32% (panel A), whereas similar effects were not obtained by apoE3 (100 nM) treatment (panel B). These results show that differential neurotoxicities of apoE3 and

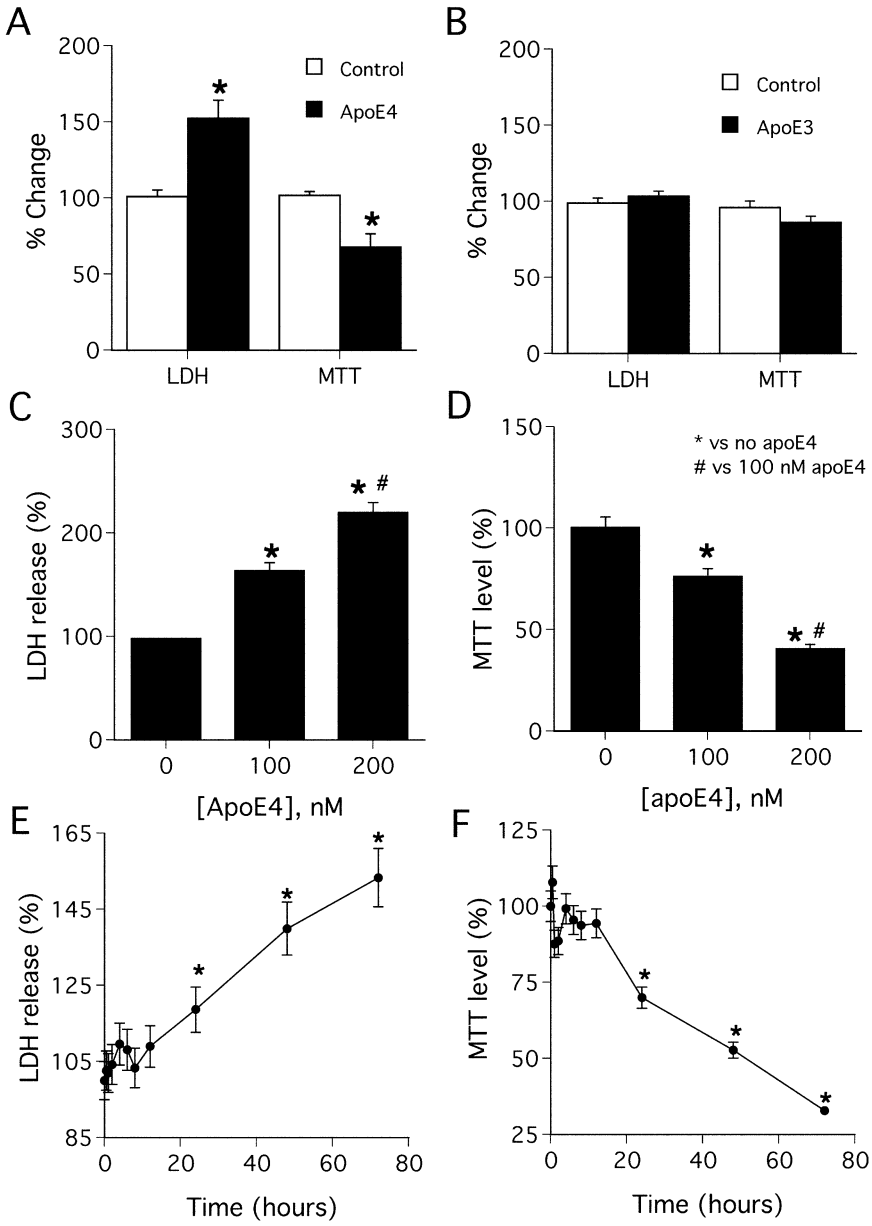

Fig. 2. Differential neurotoxic effects induced by apoE3 and apoE4 in hippocampal neuronal cultures. The neurotoxicity was estimated using standard MTT and LDH assays in apoE-treated hippocampal neuronal culture. Mean values (mean±S.E.M.) are recorded for MTT and LDH in control (open bars), 100 nM apoE4- (filled bars, panel A), and 100 nM apoE3- (filled bars, panel B) treated cultures (normalized to control values). Dose-dependent changes of LDH (panel C) and MTT (panel D) are recorded after treatment of neurons with apoE4 from 100 nM to 200 nM for 48 h. Time-dependent changes of 100 nM apoE4 on neurotoxicity are summarized in panel E and F. Data are analyzed from eight to 12 sets of experiments. Significant differences ($P<0.05$) between control and apoE-treated neurons are indicated by asterisks (*).

apoE4 correlate with their effects on the calcium response to NMDA, and suggest that intracellular calcium signaling plays an important role in the isoform-specific effects of apoE.

The concentration of apoE in CSF is in the range of 100–200 nM (Riemenschneider et al., 2002). It is likely that the neuronal impairment varies depending on the intensity of insult around the neurons, such as the dose and the duration of apoE4 exposure. Thus, it was of interest to determine if apoE4 was toxic at physiologically relevant doses and in a dose- and time-dependent manner. Fig. 2C and 2D show the summarized effects of apoE4 at the concentrations of 100 nM and 200 nM on toxicity using LDH assay and MTT assay. We found that apoE4's effects were dose-dependent, and 100 nM apoE4 did not cause the maximum neurotoxicity. Further study showed that significant neurotoxicity was not detected until 24 h after apoE4 treatment, and continued to increase up to 72 h after treatment (Fig. 2E and 2F). Thus, apoE4 contributed to cytotoxicity in a dose-dependent and time-dependent manner.

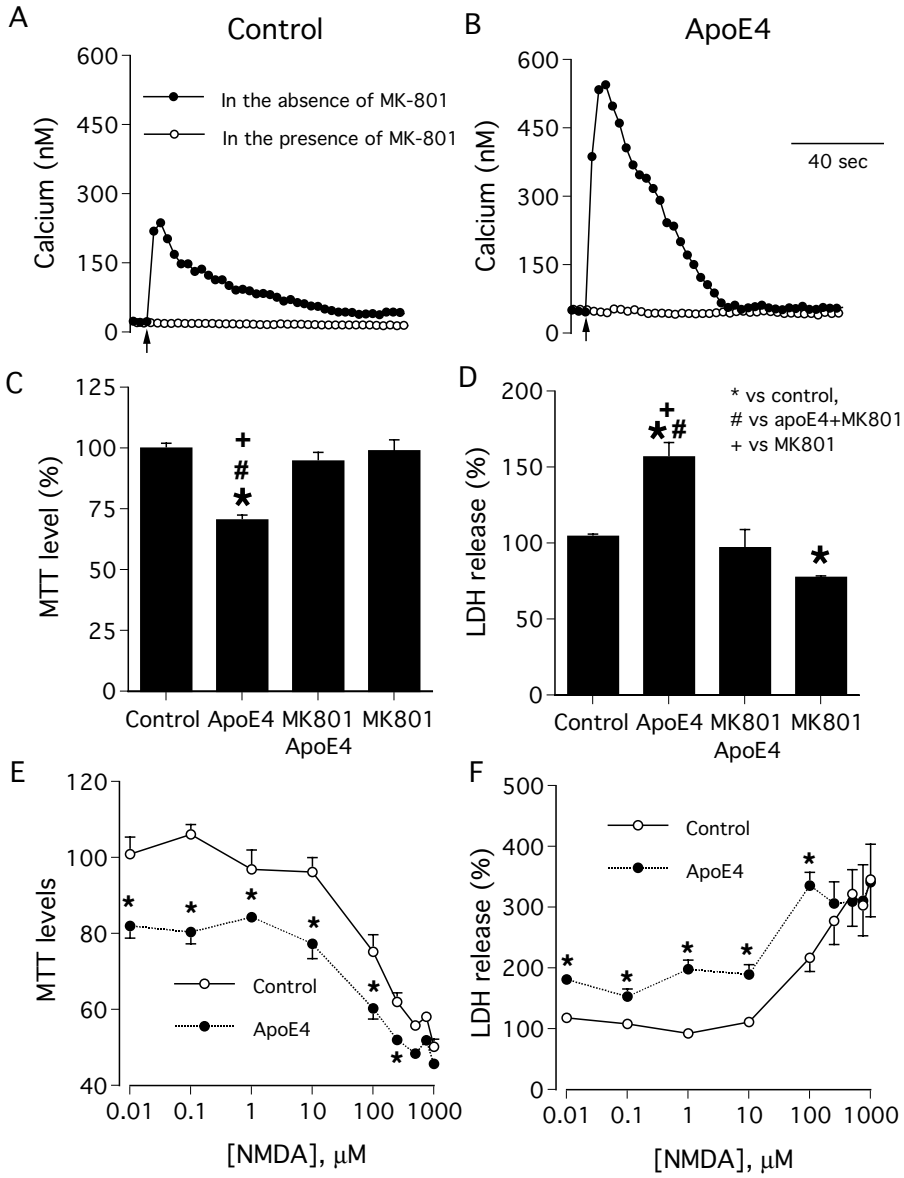

Fig. 3. MK-801 abolishes apoE4-induced increases in intracellular calcium and neurotoxicity. The effect of MK-801 (10 μM), an NMDA receptor noncompetitive antagonist, was tested to determine if activation of the NMDA receptor was involved in the intracellular calcium signaling to NMDA and neurotoxicity in apoE4-treated neurons. Panels A and B show representative recordings of intracellular calcium signals evoked by brief (1 s) application of NMDA at the concentration of 100 μM (at the arrow) from a micropipette in both control (panel A) and apoE4-treated neurons (panel B) in the absence of MK-801 (filled circles in panels A and B) and in the presence of MK-801 (open circles in panels A and B). Panels C and D show mean values (mean±S.E.M.) for the population of neurons studied for control, apoE4, MK-801, and apoE4/MK-801-treated neurons. Averaged data show the neurotoxicity detected by the MTT assay (panel C) and the LDH assay (panel D), which are normalized to control values. To determine if apoE4 altered the dose-response relationships for the neurotoxicity, hippocampal neurons were challenged with NMDA at concentrations ranging from 10 nM to 1 mM. Neurotoxicity was determined using the MTT assay (panel E) and the LDH assay (panel F). Control (open circles) and apoE4-treated neurons (filled circles) were exposed to the indicated concentrations of NMDA for 5 min as mentioned in the methods. Data are analyzed from six to eight experiments. Significant differences ($P<0.05$) between control and apoE-treated neurons are indicated by asterisks (*).

NMDA receptor-mediated alteration of calcium signaling and apoE4 toxicity

Glutamate receptor-mediated excitotoxicity results from increased calcium influx leading to toxic intracellular calcium overload, with the NMDA receptor subtype primarily associated with calcium flux. The effect of MK-801, a NMDA receptor antagonist, was tested in order to determine if activation of NMDA receptor was involved in the neuronal damage in apoE4-treated neurons. Fig. 3 shows the effects of MK-801 on the intracellular calcium response to NMDA and neurotoxicity in apoE-treated neurons. Figs. 3A and 3B are representative intracellular calcium recordings from the control and apoE-treated neurons in the absence

and presence of 10 μM MK-801. MK801 eliminated the NMDA-induced intracellular calcium signaling in both control and apoE4 treated neurons. Furthermore, MK801 abolished the substantial increase in neuronal death by apoE4 in both assays (Figs. 3C and 3D). MK801 alone did not alter MTT levels (Fig. 3C), but did slightly decrease LDH release (Fig. 3D), which could be due to the MK-801 blocking effects of endogenous glutamate on primary neurons. These results demonstrate that the alteration in intracellular calcium response to NMDA and neurotoxicity by apoE4 were specifically induced by NMDA receptor activation.

In the calcium signaling studies outlined above, NMDA was tested at a standard dose of 100 μM. *In vivo*, it is likely that the amplitude of the synaptic response to NMDA varies depending on the intensity of the synaptic input. Thus, it was important to determine if apoE4 altered the dose-response relationships for the neurotoxicity subsequent to the intracellular calcium signal to NMDA. NMDA concentrations from 10 nM to 1 mM were tested. Control and apoE4-treated neurons were exposed to the indicated concentrations of NMDA for 5 min as mentioned in the Experimental Procedures. Neurotoxicity was determined using the MTT assay (Fig. 3E) and LDH assay (Fig. 3F). In control neurons, neuronal damage was enhanced with increasing NMDA dose. ApoE4 treatment enhanced toxicity at most doses of NMDA (0.01 μM to 100 μM), but did not cause neurotoxicity at dramatically lower concentrations of NMDA (Fig. 3E and 3F). Take together, our data indicate that interactions between NMDA and apoE4 can occur that lead to enhanced cell damage, and this enhanced damage is likely through the activation of NMDA receptor-mediated calcium influx, since MK-801 could abolish the toxicity (Fig. 3A, 3B and 3C). However, apoE4 did not dramatically increase the sensitivity of neurons to NMDA, suggesting that the effects of apoE4 on NMDA receptor is likely through the alteration of the calcium permeability rather than the density of NMDA receptors on the neuronal surface.

ApoE isoforms alter calcium signaling and neurotoxicity via an apoE receptor

LRP is strongly expressed on neurons *in vivo* and *in vitro* (Rebeck et al., 1993; Holtzman et al., 1995). RAP is a protein that facilitates the proper folding and trafficking of LRP and other apoE receptors within the early secretory pathway (Zheng et al., 1994), and antagonizes the binding of all known ligands to these receptors *in vitro* (Battey et al., 1994). To test if the tremendous increase in calcium responses to NMDA by apoE4 was due to a receptor-mediated pathway, we co-incubated cultures with RAP (500 nM). The effects of RAP on apoE4-induced changes in calcium signaling and neuronal toxicity are summarized in Fig. 4, which shows representative recordings (Fig. 4A), average resting calcium (Fig. 4B), and peak calcium amplitude in response to NMDA stimulation (Fig. 4C). RAP reduced the apoE4-induced enhancement in resting calcium levels, but did not eliminate the increases in resting calcium levels by apoE4 treatment (Fig. 4B), suggesting that an apoE receptor-mediated effect and a non-apoE receptor-mediated effect were involved. However, the apoE4-induced substantial increases in calcium response to NMDA were abolished by co-incubation with RAP. RAP alone did not alter the resting or the peak calcium responses to NMDA (data not shown). RAP also diminished the small but significant alterations of the intracellular calcium signaling induced by apoE3 (Fig. 1B and 1D; data not shown). Importantly, RAP also prevented apoE4-induced neurotoxicity as detected in both MTT and LDH assays (Fig. 4D). ApoE3 treatment and RAP alone did not cause neurotoxicity as determined by levels of MTT and LDH (Fig. 4E). Our data indicate that apoE4 alters calcium signaling via an apoE receptor, and that the receptor-mediated alterations of intracellular calcium levels are likely involved in altered neuronal viability. Additional experiments were carried out to identify the mechanisms mediating the effects of apoE isoforms via apoE receptors on NMDA-stimulated responses.

α2M* prevents apoE4-induced alterations in calcium signaling and neurotoxicity

LRP binds multiple ligands. One approach to identify the mechanisms mediating the effects of apoE isoforms via LRP on NMDA-stimulated responses is to determine whether the effects of apoE4 can be reversed by other LRP ligands. Among the diverse ligands for LRP, α2M* is of particular interest due to its reported association with AD (Rebeck et al., 1995; Qiu et al., 1999, 2001; Blacker et al., 1998). We co-incubated cultures with α2M* at the concentration of 500 nM, which is known to reduce NMDA calcium signaling (Qiu et al., 2002). The effects of α2M* on apoE4-induced alterations in calcium signaling and neurotoxicity are shown in Fig. 5 (representative recordings in Fig. 5A, average resting calcium in Fig. 5B, and peak calcium amplitudes in response to NMDA stimulation in Fig. 5C). α2M* eliminated the apoE4-induced increases in the calcium response to NMDA stimulation in the cultured neurons. The average data show that chronic treatment with α2M* prevented the enhancement of resting calcium levels and calcium response to NMDA by apoE4. These results suggest that LRP is involved in the apoE4-induced intracellular calcium homeostasis and intracellular calcium signaling to NMDA. Chronic treatment of neurons with α2M* also prevented apoE4-induced neurotoxicity as shown by changes in MTT and LDH assays (Fig. 5D and 5E). α2M* completely antagonized apoE4-induced reduction of cell survival detected in the MTT assay, and partially prevented cell damage detected in the LDH assay. α2M* alone showed a non-significant trend to improve neuronal survival (panel D).

Tandem apoE peptide also eliminates the intracellular calcium signaling and neurotoxicity of apoE4

Our results suggested that apoE isoforms exert their differential effects on calcium signaling and neuronal toxicity by an apoE receptor such as LRP. To clarify the role of this receptor in the effects of apoE on calcium signaling and

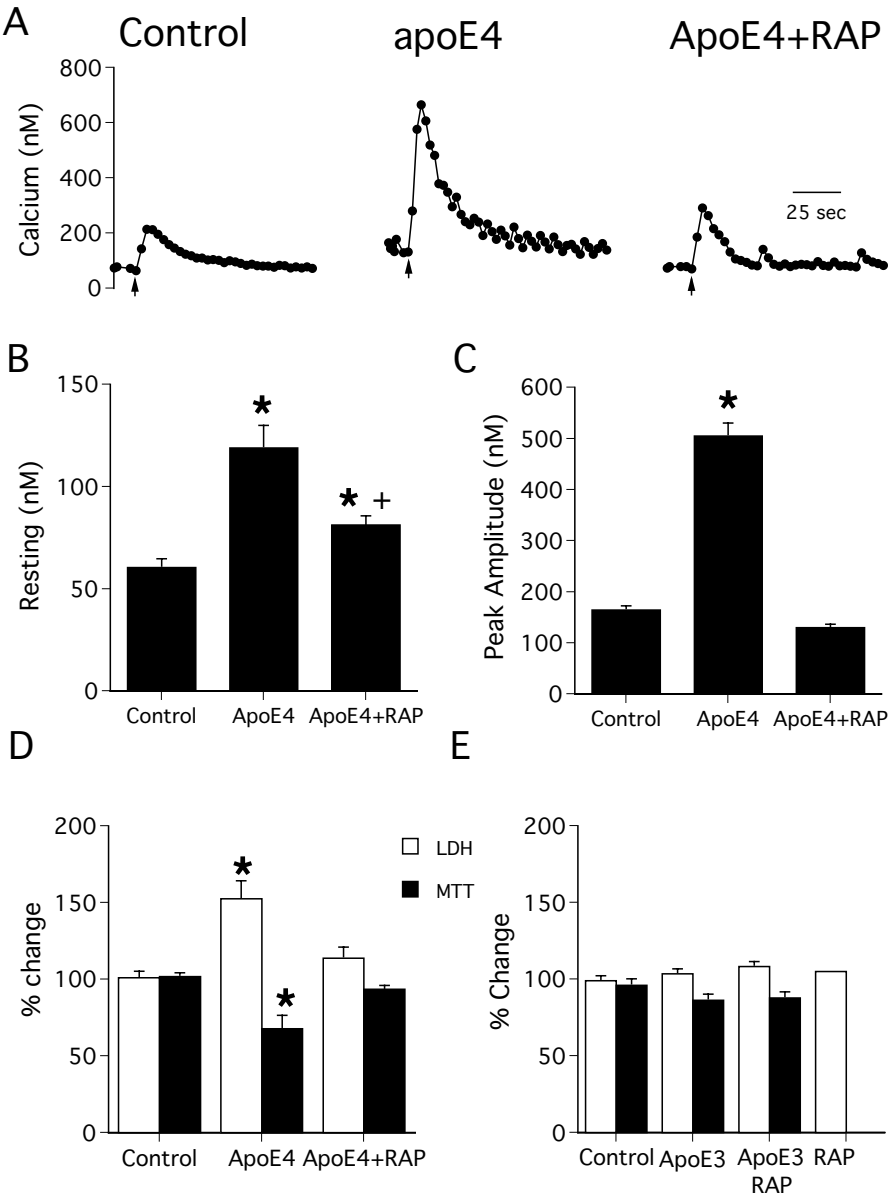

Fig. 4. ApoE4 alters calcium signaling and neurotoxicity via receptor-mediated processes. Neurons were incubated with apoE4 with or without RAP at a concentration of 500 nM for 48 h. The representative recordings (panel A) and averaged data (mean±S.E.M.) in the resting calcium (panel B) and calcium response to NMDA (panel C) show the effects of RAP on apoE4-induced alteration of calcium signaling. The intracellular calcium signals were evoked by brief (1 s) application of NMDA (at the arrow) from a micropipette in control, apoE-, and apoE/RAP- treated neurons. Estimated intracellular calcium is plotted vs. time for representative cells under each condition. Averaged data include resting calcium levels and peak calcium amplitude, in which the resting levels have been subtracted. Results are pooled from two to three sets of cultures, and each culture includes two or three fields, containing seven to 12 cells per field. Asterisks (*) indicate significant differences ($P<0.05$) from control. Crosses (+) in panel B indicate significant differences ($P<0.05$) from apoE4. Panels D and E show mean values (mean±S.E.M.) for the population of neurons of apoE4-, and apoE3-treated groups. Averaged data show the neurotoxicity detected by the MTT assay (filled squares) and the LDH assay (open squares), which are normalized to control values. Data are analyzed from six to eight experiments. Significant differences ($P<0.05$) from control are indicated by asterisks (*).

toxicity, we used a tandem apoE peptide containing only the receptor binding domain, which is identical in both apoE3 and apoE4. The synthetic tandem apoE peptide consists of a double sequence of apoE amino acids 141 through 149 [$E(141–149)_2$]. To characterize the function of the tandem apoE peptide, we first tested its effects on the viability of hippocampal neurons (Fig. 6A), and secondly, we tested its effects on the intracellular calcium response to NMDA (Fig. 6C and 6D). It has been reported that this peptide substantially increases neurotoxicity when used at micromolar levels (Crutcher et al., 1994; Tolar et al., 1997). However, we found that the tandem apoE peptide protected neurons in a dose-dependent manner when used at concentrations from 100 nM to 700 nM (Fig. 6A), evidenced as decreased LDH release by 20–25% without significant changes in MTT levels. The neuroprotective

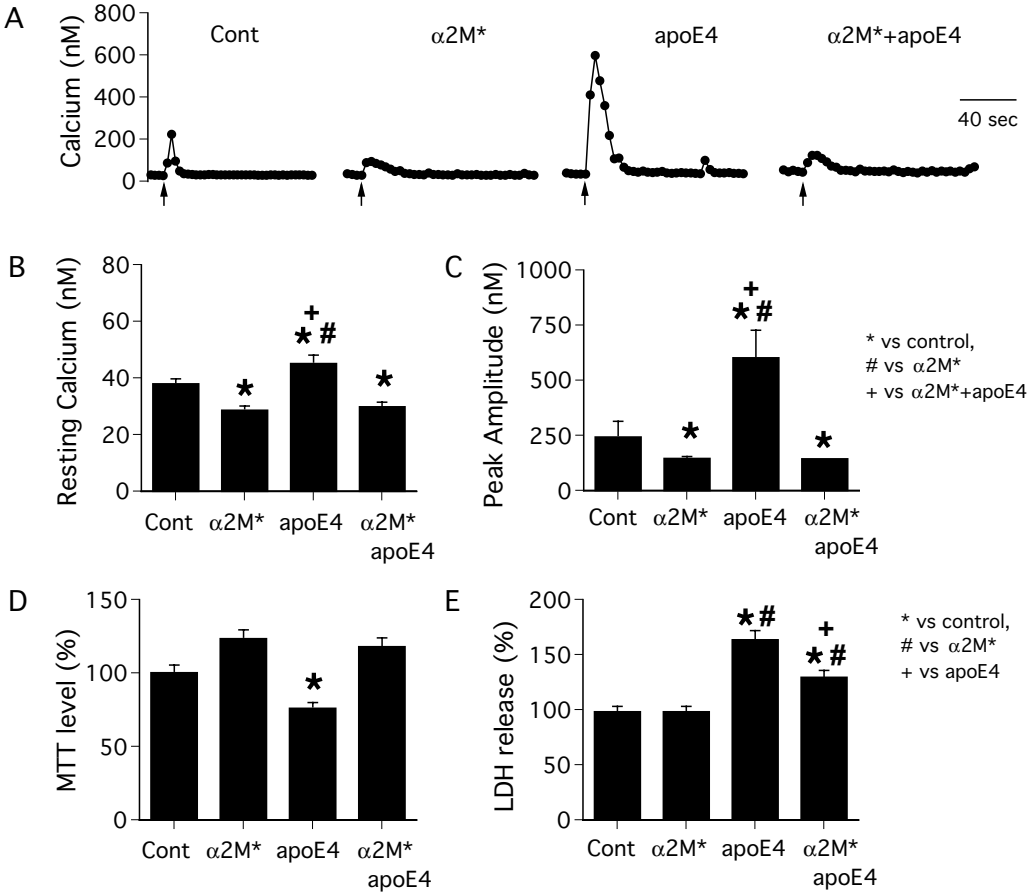

Fig. 5. α2M* prevents apoE4-induced alterations in calcium signaling and neurotoxicity. We co-incubated cultures with α2M* at a concentration of 50 nM, which is known to reduce calcium signaling to NMDA. The typical intracellular calcium recordings (panel A) represent calcium signaling from control, α2M*-, apoE4-, and apoE4/α2M*-treated neurons. NMDA was applied at the point indicated by an arrow. Averaged data include resting calcium levels (panel B) and peak calcium amplitude (panel C), in which the resting levels have been subtracted, in control, α2M*-, apoE4-, and apoE4/α2M*-treated neurons. Results are pooled from two to three sets of cultures, and each culture includes two or three fields, containing seven to 12 cells per field. Asterisks (*) indicate significant differences ($P<0.05$) from control. Numbers (#) indicate significant differences ($P<0.05$) from α2M*. Crosses (+) indicate significant differences ($P<0.05$) from apoE4/α2M*. Panels D and E show mean percent changes (mean±S.E.M.) of MTT and LDH assays after treatment with apoE4, α2M* and apoE4. Data are analyzed from six to eight experiments. Asterisks (*) indicate significant differences ($P<0.05$) from control. Numbers (#) indicate significant differences ($P<0.05$) from α2M*. Crosses (+) indicate significant differences ($P<0.05$) from apoE4/α2M*.

effects of tandem apoE peptide occurred in a RAP-blockable manner as shown in Fig. 6B. No obvious neurotoxicity was obtained from hippocampal neurons treated with tandem apoE peptide, although there was an increase in LDH release at the highest concentration tested (1 μM). Similar to α2M* and apoE3, the tandem apoE peptide substantially decreased the intracellular calcium response to NMDA in hippocampal neurons without significantly altering resting calcium levels (Fig. 6C and 6D). These data suggest that the neuroprotection of the tandem apoE peptide is also associated with its effects on the intracellular calcium response to NMDA. This peptide may be a convenient reagent for investigating the connection between LRP and the NMDA receptor.

Since apoE4 and this tandem apoE peptide both bind to apoE receptors with dramatically different cellular effects, we tested whether the tandem apoE peptide could inhibit the toxic effects of apoE4. The tandem apoE peptide blocked apoE4-induced increases in the calcium response to NMDA stimulation of the cultured hippocampal neurons (Fig. 7A). However, apoE peptide was not able to eliminate the enhancement of resting calcium levels by apoE4 (Fig. 7A). Tandem apoE peptide treatment also significantly antagonized apoE4-induced reduction of cell survival and this effect was associated with decreased MTT levels (Fig. 7B) and increased levels of LDH (Fig. 7C). These data further demonstrate that apoE4 exerts its effects on the calcium signaling through receptor binding and that such calcium overload effects correlate with its neurotoxicity. Interestingly, neither the tandem apoE peptide (Fig. 7D) nor RAP (Fig. 4B) prevents the apoE4 increase in resting calcium, suggesting that apoE4 may have cellular effects independent of its interaction with members of the LDL receptor family.

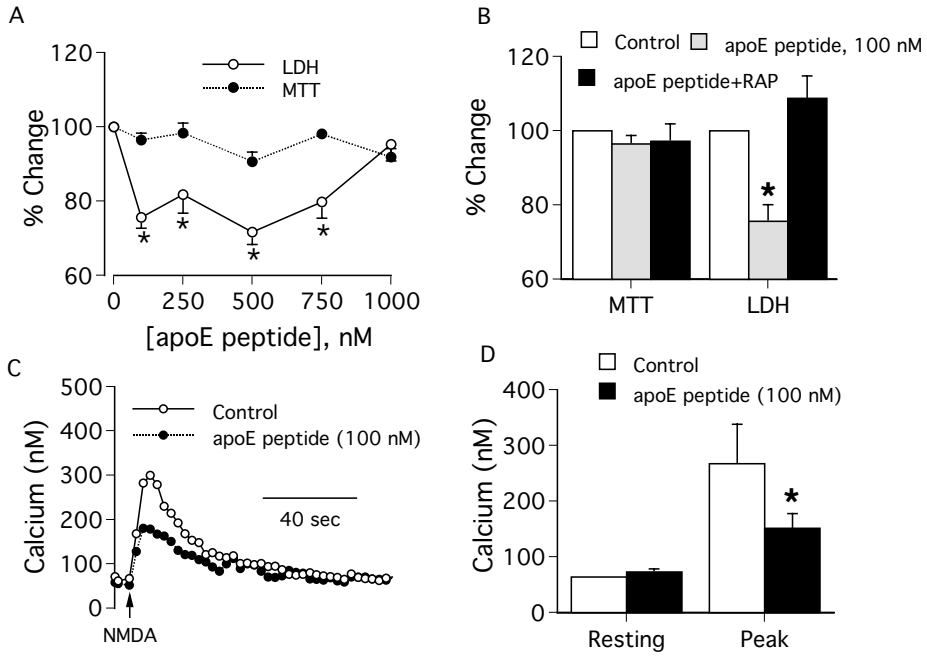

Fig. 6. Characterization of the function of tandem apoE peptide. Panel A shows the dose-response effects of tandem apoE peptide on neuronal survival. To determine if apoE peptide altered the neuronal viability, hippocampal neurons were challenged with apoE peptide at the concentrations from 100 nM to 1 μM. Neurotoxicity was determined using the MTT (filled circles) and LDH assays (open circles). Control neurons were exposed to the indicated concentrations of apoE peptide for 24–48 h. Data are analyzed from six to eight experiments. Significant differences ($P<0.05$) are indicated by asterisks (*). We co-cultured the hippocampal neurons with RAP at 500 nM, and tested the effects of apoE peptide at a concentration of 100 nM in panel B. RAP at the concentration of 500 nM blocked the neuroprotective effects of 100 nM apoE peptide by decreasing LDH release. Panels C and D show that the neuroprotection of tandem apoE peptide is associated with its effects on calcium signaling. The typical intracellular calcium recordings (panel C) represent calcium signaling from control (open circles) and apoE peptide (filled circles)-treated neurons. NMDA was applied as indicated by an arrow. Averaged data (panel D) include resting calcium levels and peak calcium amplitude, in which the resting levels have been subtracted in control (open bars) and apoE peptide- (filled bars) treated neurons. Results are pooled from two to three sets of cultures, and each culture includes two or three fields, containing seven to 12 cells per field. Asterisks (*) indicate significant differences ($P<0.05$) from control.

DISCUSSION

ApoE has numerous effects on CNS cells, and many of these effects are altered between apoE isoforms. In the present study, we found that chronic exposure of cultured rat hippocampal neurons to apoE3 or apoE4 changed intracellular calcium homeostasis and calcium signaling to NMDA, which correlated with neuronal toxicity. Activation of NMDA receptors was specifically involved in the effects of apoE4 on intracellular calcium signaling and subsequent neurotoxicity. We found that RAP prevented apoE-induced alteration of calcium responses to NMDA and eliminated apoE4-induced neurotoxicity, suggesting that an apoE receptor is involved in these effects of apoE. Simultaneous exposure of neurons to other LRP ligands, α2M* or tandem apoE peptide, abolished the apoE4-induced intracellular calcium response to NMDA and associated neurotoxicity. These differential effects of apoE isoforms demonstrate the potential for apoE receptors to alter important aspects of neuronal function in the brain.

Neurons obtained from the rat hippocampus chronically treated with recombinant human apoE showed distinctive isoform-specific changes in intracellular calcium signaling (Fig. 1) and neuronal toxicity as assessed by both LDH release and MTT levels (Fig. 2), thus providing an accessible *in vitro* model to investigate the neuropathophysiology of apoE. The 48 h treatment of apoE at a concentration of 100 nM produced a sensitive, reliable, and consistent change in both calcium signals and neuronal damage (Fig. 2). The time-dependent neurotoxic effects by apoE isoforms are consistent with the apoE4 toxicity measured by Trypan Blue exclusion (Hashimoto et al., 2000) and by vital dye (Marques et al., 1997; Tolar et al., 1999). Our analysis of the differential effects of apoE isoforms on hippocampal neurons in culture may apply to *in vivo* conditions for several reasons. First, the concentration of apoE in the CSF is between 100 nM and 200 nM (Riemenschneider et al., 2002). Second, 48 h treatment provided enough time for the recombinant apoE to interact with lipoproteins or other cofactors in the media. Third, apoE4 caused intracellular calcium signaling via a receptor-mediated process. Finally, the chronic treatment paradigm may imitate the deteriorative process of AD in which apoE may be present on amyloid deposits for a long period of time. These findings suggest that neuronal signaling may differ in brains of humans or transgenic mice of APOE ε4 genotype. These situations would reflect a more chronic exposure of cells to apoE isoforms than in our *in vitro* study; future studies of NMDA receptor signaling in APOE transgenic mice could address these issues *in vivo*.

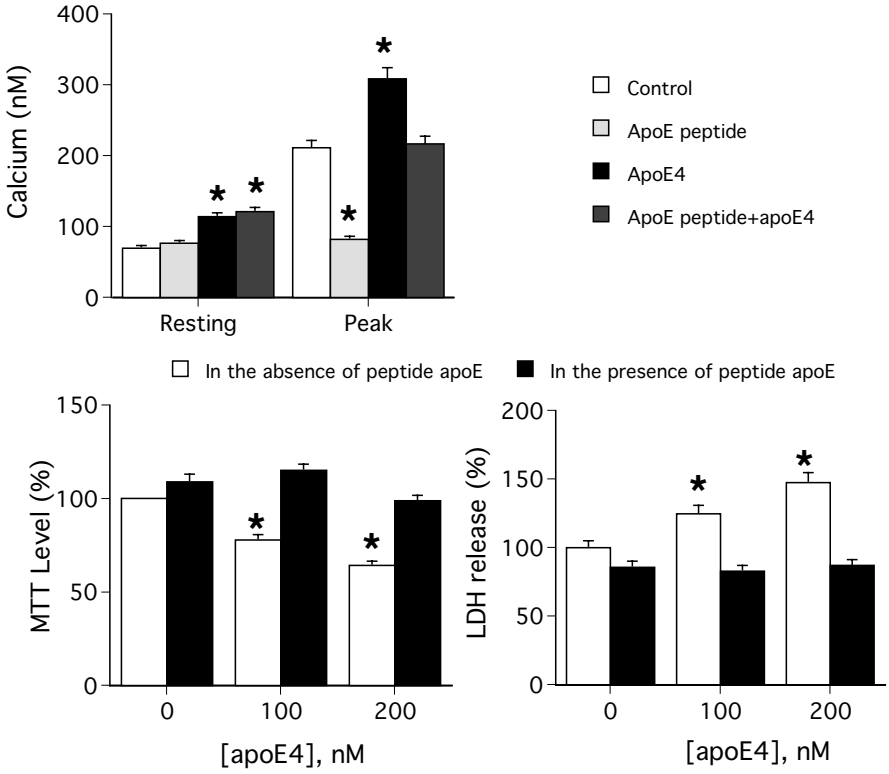

Fig. 7. Tandem apoE peptide blocks the enhanced intracellular calcium response to NMDA and neurotoxicity by apoE4. To differentiate the function of apoE's receptor binding site, we co-cultured the hippocampal neurons with apoE4. Averaged data include resting calcium levels and peak calcium amplitude (panel A), in which the resting levels have been subtracted, in control, apoE peptide-, apoE4-, and apoE4/apoE peptide-treated neurons. Results are pooled from two to three sets of cultures, and each culture includes two or three fields, containing seven to 12 cells per field. Asterisks (*) indicate significant differences ($P<0.05$) from control. The tandem apoE peptide blocked apoE4-induced dramatic increases in the calcium response to NMDA stimulation in the cultured hippocampal neurons as shown in panel A. However, apoE peptide was not able to eliminate the enhancement of resting calcium levels by apoE4. Panels B and C show mean values (mean±S.E.M.) of chronic treatment with apoE4, apoE peptide and apoE4/apoE peptide on the neurotoxic effects in cultured hippocampal neurons. The effects of apoE peptide on apoE4-induced neurotoxicity were assessed by using the MTT assay (panel B), and the LDH assay (panel C), which are normalized to control values. Data are analyzed from six to eight experiments. Asterisks (*) indicate significant differences ($P<0.05$) from control.

ApoE exists in different conformations depending on whether it is lipid-free, or present on lipoproteins of different densities (Weisgraber, 1994). The recombinant human apoE proteins (lipid free) used here may bind to the lipoproteins in the serum or conditioned media, suggesting that apoE may exist in several conformations under our experimental conditions. In normal CNS, apoE is secreted from astrocytes and microglia in association with a very high density lipoprotein, which likely accumulate lipids to become the larger lipoproteins found in the CSF. In AD CNS, apoE is also present on Aβ deposits, and the conformation of apoE on an Aβ deposit is unknown. No immediate effects of recombinant human apoE4 on intracellular calcium signaling were observed even when it was preincubated with lipoproteins, HDL and β-VLDL (data not shown), indicating that apoE isoforms interacting with lipoprotein requires either a certain time or some cofactors secreted from the cells. ApoE could also be accumulating lipid from cells via cholesterol efflux (Michikawa et al., 2000). Another possibility is that the proteolysis of apoE may be important for determining neurotoxic effects (Tolar et al., 1999; Zhang et al., 2001), which needs to be further investigated.

We have shown that apoE altered intracellular calcium signaling and neuronal toxicity in hippocampal neurons in an isoform-specific manner. The mechanism of the apoE isoform-specific effects on neurotoxicity might be associated with these processes: their effects on lipid metabolism, receptor-mediated signaling, or receptor structure. First, apoE isoforms differ in binding to lipoprotein, with apoE4 preferentially bound to VLDL over HDL (Mahley, 1988), although the latter is the major lipoprotein type in CNS (Roheim et al., 1979; Pitas et al., 1987). The receptor-mediated effects of apoE may depend on lipoprotein binding in the culture media, with apoE3 and apoE4 interacting with different lipoprotein subpopulations (Michikawa and Yanagisawa, 1999a; Michikawa et al., 2000). Second, the receptor-mediated downstream signaling could depend only on effects of apoE4 that differed from the other ligands that we tested, i.e. apoE3, the tandem apoE peptide, and α2M*. Defining this effect will require an understanding of whether receptors such as LRP and the NMDA receptor interact directly or whether other adaptor proteins are involved. Our results are consistent with the finding that apoE4 affects transcriptional activity of CREB protein

via LRP (Ohkubo et al., 2001) due to the alteration of intracellular calcium signaling. Third, apoE3 (but not apoE4) can form dimers thus potentially binding two LRP molecules. Similarly, α2M* also can form tetramers, and both apoE3 and α2M* generate similar effects on intracellular calcium signaling (Fig. 1B and Fig. 5A).

We tested whether the effects of apoE4 on calcium signaling and neurotoxicity could be blocked by other LRP binding molecules. We found that α2M* and a tandem apoE peptide could block the effects of apoE4, but that apoE3 could not (Fig. 7). Others have reported that apoE3 produces similar protective effects as α2M* on neuronal viability when co-cultured with apoE4 (Hashimoto et al., 2000). We hypothesize that the lipoprotein binding region of apoE, which is altered structurally in apoE4 (Weisgraber, 1994), might play an important role in the differential effects of apoE isoforms. Our studies suggest that the isoform-specific effects of apoE on cultured hippocampal neurons require LRP binding (Fig. 4), but also may be involved an LRP independent pathway. In addition, LRP and apoER2 (Stockinger et al., 1998) are the only known brain receptors for α2M*, and apoER2 is also expressed strongly on mature neurons (Christie et al., 1996; Clatworthy et al., 1998). Our data do not rule out a role for apoER2 in the effects of the apoE tandem peptide or apoE4.

We previously reported that α2M* decreased calcium influx after NMDA receptor activation (Qiu et al., 2002). This decrease involved reducing calcium entry through NMDA receptors, which subsequently reduced flux through voltage sensitive calcium channels (VSCCs) and calcium-induced calcium release (CICR) from intracellular calcium stores. Enhanced calcium influx via NMDA receptor-gated channels and a larger membrane depolarization subsequent to the NMDA receptor activation in apoE4-treated neurons could be the trigger for the enhanced calcium release from stores. Previous studies have demonstrated apoE effects on several calcium pathways, including NMDA receptors (Tolar et al., 1999), VSCCs (Ohkubo et al., 2001), and CICR (Ohkubo et al., 2001), although not all studies agree (Muller et al., 1998; Veinbergs et al., 2002). It is clear that the potential contribution to the calcium response elicited by apoE4 could be selectively blocked by the NMDA receptor antagonist, MK-801 (Fig. 3B). We found that apoE4 did not affect the calcium response to kainate, another ion-gated glutamate receptor subtype, in the cultured primary neurons (data not shown). The mechanism connecting LRP to the NMDA receptor remains unknown. *In vitro* experiments demonstrate binding of several cytoplasm proteins to LRP, including the disabled-1, and the scaffold protein, postsynaptic density-95 (Sheng, 1997; Gotthardt et al., 2000). In particular, postsynaptic density-95 is of interest because of its binding to the NMDA receptor. These proteins may help in signaling transduction from LRP to the NMDA receptor (Christopherson et al., 1999; Lei et al., 2001). Other studies found that cytoplasmic residues of LRP can be phosphorylated (Bu et al., 1994), potentially affecting the association of LRP with those cytoplasmic proteins. The molecular connection between LRP and NMDA receptors will be pursued in the future. Our studies suggest a pathological role for apoE4 in the CNS and indicate that NMDA receptor-mediated functions may play a critical role in the neuropathological changes observed in AD associated with apoE4.

Several groups have shown that apoE4 is neurotoxic both *in vivo* (Buttini et al., 1999; Veinbergs et al., 2002) and *in vitro* (Marques et al., 1997; Tolar et al., 1999; Hashimoto et al., 2000), although there is disagreement about whether LRP is involved (Jordan et al., 1998; Moulder et al., 1999; Hagiwara et al., 2000). In this study, several pieces of data strongly support a role for LRP in apoE4's effect on calcium signaling and neurotoxicity *in vitro*: 1) the increase in intracellular calcium response to NMDA and subsequent neuronal death after treatment with apoE4 could be completely eliminated by RAP (Fig. 4); 2) α2M*, an LRP ligand, eliminated the alterations in calcium signaling and neurotoxicity induced by apoE4 (Fig. 5); 3) the tandem apoE peptide, containing only the receptor binding region residues, eliminated the enhanced calcium signaling and neuronal neurotoxicity by apoE4. This peptide has been reported to be toxic to neurons at higher concentrations under some culture condition, which is dependent (Crutcher et al., 1994; Tolar et al., 1999) or independent of LRP (Hagiwara et al., 2000). Recently, a protective effect on NMDA excitotoxicity was obtained from a similar apoE peptide in primary rat neuronal–glial cell cultures (Aono et al., 2003). It seems that many LRP ligands (Du et al., 1998; Qiu et al., 1999, 2001), including apoE3, exert neurotrophic effects in the CNS, while only apoE4 increases the intracellular calcium response to NMDA and is toxic to neurons. Recent work found that neurotrophic signals rely on synaptic NMDA receptors and neurotoxic signals involve extrasynaptic NMDA receptors (Hardingham et al., 2002). We hypothesize that interactions of apoE3 and apoE4 with LRP may differentially alter the distribution of NMDA receptors. We have previously found that culture conditions greatly affect the calcium responses of neurons to LRP binding (Bacskai et al., 2000; Qiu et al., 2002). *In vivo*, neuronal responses to LRP ligands may likewise be heterogeneous, particular in neurodegeneration.

A number of human magnetic resonance imaging studies suggest that APOE-e4 is associated with hippocampal atrophy, even in the absence of a diagnosis of AD (Tohgi et al., 1997; den Heijer et al., 2002; Kim et al., 2002; Mori et al., 2002). In addition, positron emission tomography studies suggest that cerebral glucose metabolism is impaired in APOE-e4 individuals (Mielke et al., 1998; Small et al., 2000; Reiman et al., 2001). The neurotoxic properties of the apoE4 protein, such as those demonstrated here on hippocampal neurons *in vitro*, may account for the results of these various imaging studies *in vivo*.

Acknowledgements—Supported by AG14473 (GWR) and AG12406 (BTH).

REFERENCES

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P (1995) Glutamate-induced neuronal death: a

succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15:961–973.

Aono M, Bennett ER, Kim KS, Lynch JR, Myers J, Pearlstein RD, Warner DS, Laskowitz DT (2003) Protective effect of apolipoprotein E-mimetic peptides on *N*-methyl-D-aspartate excitotoxicity in primary rat neuronal-glial cell cultures. Neuroscience 116:437–445.

Bacskai BJ, Xia MQ, Strickland DK, Rebeck GW, Hyman BT (2000) The endocytic receptor protein LRP also mediates neuronal calcium signaling via *N*-methyl-D-aspartate receptors. Proc Natl Acad Sci USA 97:11551–11556.

Battey FD, Gafvels ME, FitzGerald DJ, Argraves WS, Chappell DA, Strauss JF III, Strickland DK (1994) The 39 kDa receptor-associated protein regulates ligand binding by the very low density lipoprotein receptor. J Biol Chem 269:23268–23273.

Blacker D, Wilcox MA, Laird NM, Rodes L, Horvath SM, Go RCP, Perry R, Watson B, Bassett SS, McInnis MG, Albert MS, Hyman BT, Tanzi RE (1998) Alpha-2 macroglobulin is genetically associated with Alzheimer disease. Nat Genet 19:357–360.

Bu G, Maksymovitch EA, Nerbonne JM, Schwartz AL (1994) Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian central neurons. J Biol Chem 269: 18521–18528.

Buttini M, Orth M, Bellosta O (1999) Expression of human apolipoprotein E3 or E4 in the brains of apoe $-/-$ mice: isoform specific effects on neurodegeneration. J Neurosci 19:4867–4880.

Choi DW (1992) Excitotoxic cell death. J Neurobiol 23:1261–1276.

Christie RH, Chung H, Rebeck GW, Strickland D, Hyman BT (1996) Expression of the very low density lipoprotein receptor (VLDL-r), an apolipoprotein E receptor, in the central nervous system and in Alzheimer disease. J Neuropath Exp Neurol 55:491–498.

Christopherson KS, Hillier BJ, Lim WA, Bredt DS (1999) PSD-95 assembles a ternary complex with the *N*-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. J Biol Chem 274:27467–27473.

Clatworthy AE, Stockinger W, Christie RH, Schneider WJ, Nimpf J, Hyman BT, Rebeck GW (1998) Expression and alternate splicing of apoE receptor 2 in brain. Neuroscience 90:903–910.

Crutcher KA, Clay MA, Scott SA, Tian X, Tolar M, Harmony JA (1994) Neurite degeneration elicited by apolipoprotein E peptides. Exp Neurol 130:120–126.

den Heijer T, Oudkerk M, Launer LJ, van Duijn CM, Hofman A, Breteler MM (2002) Hippocampal, amygdalar, and global brain atrophy in different apolipoprotein E genotypes. Neurology 59:746–748.

Du Y, Bales KR, Dodel RC, Liu X, Glinn MA, Horn JW, Little SP, Paul SM (1998) α2-Macroglobulin attenuates β-amyloid peptide 1–40 fibril formation and associated neurotoxicity of cultured fetal rat cortical neurons. J Neurochem 70:1182–1188.

Gibbons SJ, Brorson JR, Bleakman D, Chard PS, Miller RJ (1993) Calcium influx and neurodegeneration. Ann NY Acad Sci 679:22–23.

Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J (2000) Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. J Biol Chem 275:25616–25624.

Hagiwara A, Hashimoto Y, Niikura T, Ito Y, Terashita K, Kita Y, Nishimoto I, Umezawa K (2000) Neuronal cell apoptosis by a receptor-binding domain peptide of ApoE4, not through low-density lipoprotein receptor-related protein. Biochem Biophys Res Commun 278:633–639.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405–414.

Hashimoto Y, Jiang H, Niikura T, Ito Y, Hagiwara A, Umezawa K, Abe Y, Murayama Y, Nishimoto I (2000) Neuronal apoptosis by apolipoprotein E4 through low-density lipoprotein receptor-related protein and heterotrimeric GTPases. J Neurosci 20:8401–8409.

Holtzman DM, Pitas RE, Kilbridge J, Nathan B, Mahley RW, Bu G, Schwartz AL (1995) Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line. Proc Natl Acad Sci USA 92:9480–9484.

Huang Y, Liu X Q, Wyss-Coray T, Brecht WJ, Sanan DA, Mahley RW (2001) Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. Proc Natl Acad Sci USA 98:8838–8843.

Jordan J, Galindo MF, Miller RJ, Reardon CA, Getz GS, LaDu MJ (1998) Isoform-specific effect of apolipoprotein E on cell survival and β-amyloid-induced toxicity in rat hippocampal pyramidal neuronal cultures. J Neurosci 18:195–204.

Kim DH, Payne ME, Levy RM, MacFall J, Steffens DC (2002) APOE genotype and hippocampal volume change in geriatric depression. Biol Psychiatry 51:426–429.

Koh JY, Choi DW (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods 20:83–90.

LaDu MJ, Pederson TM, Frail DE, Reardon CA, Getz G, Falduto MT (1995) Purification of apolipoprotein E attenuates isoform-specific binding to β-amyloid. J Biol Chem 270:9039–9042.

Lei S, Czerwinska EWC, Walsh MP, MacDonald JF (2001) Regulation of NMDA receptor activity by F-actin and myosin light chain kinase. J Neurosci Res 21:8464–8472.

Lipton SA, Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. New Engl J Med 330: 613–622.

Mahley RW (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240:622–630.

Marques MA, Tolar M, Crutcher KA (1997) Apolipoprotein E exhibits isoform-specific neurotoxicity. Alzheimers Res 3:1–6.

Marques MA, Tolar M, Harmony JA, Crutcher KA (1996) A thrombin cleavage fragment of apolipoprotein E exhibits isoform-specific neurotoxicity. Neuroreport 7:2529–2532.

Michikawa M, Yanagisawa K (1999a) Inhibition of cholesterol production but not of nonsterol isoprenoid products induces neuronal cell death. J Neurochem 72:2278–2285.

Michikawa M, Yanagisawa K (1999b) Apolipoprotein E4 isoform-specific actions on neuronal cells in culture. Mech Ageing Dev 107: 233–243.

Michikawa M, Fan QW, Isobe I, Yanagisawa K (2000) Apolipoprotein E exhibits isoform-specific promotion of lipid efflux from astrocytes and neurons in culture. J Neurochem 74:1008–1016.

Mielke R, Zerres K, Uhlhaas S, Kessler J, Heiss WD (1998) Apolipoprotein E polymorphism influences the cerebral metabolic pattern in Alzheimer's disease. Neurosci Lett 254:49–52.

Minta A, Kao JPY, Tsien RY (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. J Biol Chem 264:8171–8178.

Mori E, Lee K, Yasuda M, Hashimoto M, Kazui H, Hirono N, Matsui M (2002) Accelerated hippocampal atrophy in Alzheimer's disease with apolipoprotein E epsilon4 allele. Ann Neurol 51:209–214.

Moulder KL, Narita M, Chang LK, Bu G, Johnson EMJ (1999) Analysis of a novel mechanism of neuronal toxicity produced by an apolipoprotein E-derived peptide. J Neurochem 72:1069–1080.

Muller W, Meske V, Berlin K, Scharnagl H, Marz W, Ohm TG (1998) Apolipoprotein E isoforms increase intracellular Ca^{2+} differentially through a omega-agatoxin IVa-sensitive Ca^{2+}-channel. Brain Pathol 8:641–653.

Narita M, Bu G, Holtzman DM, Schwartz AL (1997) The low-density lipoprotein receptor-related protein, a multifunctional apolipoprotein E receptor, modulates hippocampal neurite development. J Neurochem 68:587–595.

Ohkubo N, Mitsuda N, Tamatani M, Yamaguchi A, Lee YD, Ogihara T, Vitek MP, Tohyama M (2001) Apolipoprotein E4 stimulates cAMP response element-binding protein transcriptional activity through the extracellular signal-regulated kinase pathway. J Biol Chem 276:3046–3053.

Olney JW, Wozniak DF, Farber NB (1997) Excitotoxic neurodegeneration in Alzheimer disease: new hypothesis and new therapeutic strategies. Arch Neurol 54:1234–1240.

Pitas RE, Boyles JK, Lee SH, Hui D, Weisgraber KH (1987) Lipoproteins and their receptors in the central nervous system. J Biol Chem 262:14352–14360.

Przewlocki R, Parsons KL, Sweeney DD, Trotter C, Netzeband JG, Siggins GR, Gruol DL (1999) Opioid enhancement of calcium oscillations and burst events involving NMDA receptors and L-type calcium channels in cultured hippocampal neurons. J Neurosci 19:9705–9715.

Qiu Z, Parsons KL, Gruol DL (1995) Interleukin-6 selectively enhances the intracellular calcium response to NMDA in developing CNS neurons. J Neurosci 15:6688–6699.

Qiu Z, Sweeney DD, Netzeband JG, Gruol D (1998) Chronic interleukin-6 alters NMDA receptor mediated membrane responses and enhances neurotoxicity in developing CNS neurons. J Neurosci 18:10445–10456.

Qiu Z, Strickland DK, Hyman BT, Rebeck GW (1999) α2-Macroglobulin enhances the clearance of endogenous soluble β-amyloid peptide via low-density lipoprotein receptor-related protein in cortical neurons. J Neurochem 73:1393–1398.

Qiu Z, Strickland DK, Hyman BT, Rebeck GW (2001) Elevation of LDL receptor-related protein levels via ligands interactions in Alzheimer's disease and *in vitro*. J Neuropathol Exp Neurol 60:430–440.

Qiu Z, Strickland DK, Hyman BT, Rebeck GW (2002) Alpha 2-macroglobulin exposure reduces calcium responses to NMDA via LDL receptor-related protein in cultured hippocampal neurons. J Biol Chem 277:14458–14466.

Rebeck GW, Reiter JS, Strickland DK, Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. Neuron 11:575–580.

Rebeck GW, Harr SD, Strickland DK, Hyman BT (1995) Multiple, diverse senile plaque-associated proteins are ligands of an apolipoprotein E receptor, the α2-macroglobulin receptor/low-density-lipoprotein receptor-related protein. Ann Neurol 37:211–217.

Reiman EM, Caselli RJ, Chen K, Alexander GE, Bandy D, Frost J (2001) Declining brain activity in cognitively normal apolipoprotein E epsilon 4 heterozygotes: a foundation for using positron emission tomography to efficiently test treatments to prevent Alzheimer's disease. Proc Natl Acad Sci USA 98:3334–3339.

Riemenschneider M, Schmolke M, Lautenschlager N, Vanderstichele H, Vanmechelen E, Guder WG, Kurz A (2002) Association of CSF apolipoprotein E, Abeta42 and cognition in Alzheimer's disease. Neurobiol Aging 23:205–211.

Roheim PS, Carey M, Forte T, Vega GL (1979) Apolipoproteins in human cerebrospinal fluid. Proc Natl Acad Sci USA 76:4646–4649.

Sheng M (1997) Excitatory synapses: glutamate receptors put in their place. Nature 386:221–223.

Small GW, Ercoli LM, Silverman DH, Huang SC, Komo S, Bookheimer SY, Lavretsky H, Miller K, Siddarth P, Rasgon NL, Mazziotta JC, Saxena S, Wu HM, Mega MS, Cummings JL, Saunders AM, Pericak-Vance MA, Roses AD, Barrio JR, Phelps ME (2000) Cerebral metabolic and cognitive decline in persons at genetic risk for Alzheimer's disease. Proc Natl Acad Sci USA 97:6037–6042.

Stockinger W, Hengstschlager-Ottnad E, Novak S, Matus A, Huttinger M, Bauer J, Lassmann H, Schneider WJ, Nimpf J (1998) The low density lipoprotein receptor gene family: differential expression of two alpha2-macroglobulin receptors in the brain. J Biol Chem 273: 32213–32221.

Strickland DS, Ashcom JD, Williams S, Battey F, Behre E, McTigue K, Battey JF, Argraves WS (1991) Primary structure of α2-macroglobulin receptor-related protein. J Biol Chem 266:13364–13369.

Tohgi H, Takahashi S, Kato E, Homma A, Niina R, Sasaki K, Yonezawa H, Sasaki M (1997) Reduced size of right hippocampus in 39- to 80-year-old normal subjects carrying the apolipoprotein E epsilon4 allele. Neurosci Lett 236:21–24.

Tolar M, Marques MA, Harmony JA, Crutcher KA (1997) Neurotoxicity of the 22 kDa thrombin-cleavage fragment of apolipoprotein E and related synthetic peptides is receptor-mediated. J Neurosci 17: 5678–5686.

Tolar M, Keller JN, Chan S, Mattson MP, Marques MA, Crutcher KA (1999) Truncated apolipoprotein E (ApoE) causes increased intracellular calcium and may mediate ApoE neurotoxicity. J Neurosci 19:7100–7110.

Veinbergs I, Eversonm A, Sagara Y, Masliah E (2002) Neurotoxic effects of apolipoprotein E4 are mediated via dysregulation of calcium homeostasis. J Neurosci Res 67:379–387.

Weisgraber KH (1994) Apolipoprotein E: structure-function relationships. Adv Prot Chem 45:249–302.

Williams SE, Ashcom JD, Argraves WS, Strickland DK (1992) A novel mechanism for controlling the activity of α2-macroglobulin receptor/low density lipoprotein receptor-related protein. J Biol Chem 267: 9035–9040.

Yu WH, Fraser PE (2001) S100beta interaction with tau is promoted by zinc and inhibited by hyperphosphorylation in Alzheimer's disease. J Neurosci 21:2240–2246.

Zhang D, McQuade J-A, Shockley K, Levin L, Marques M, Crutcher K (2001) Proteolysis of apolipoprotein E and Alzheimer's disease pathology. Alzheimers Rep 4:67–80.

Zheng G, Bachinsky DR, Stamenkovic I, Strickland DK, Brown D, Andres G, McCluskey RT (1994) Organ distribution in rats of two members of the low-density lipoprotein receptor gene family, gp330 and LRP/α2MR, and the receptor-associated protein (RAP). J Histochem Cytochem 42:531–542.

(Accepted 14 August 2003)