Inheritance of the apoE4 allele (e4) increases the risk of developing Alzheimer’s disease; however, the mechanisms underlying this association remain elusive. Recent data suggest that inheritance of e4 may lead to reduced apoE protein levels in the CNS. We therefore examined apoE protein levels in the brains, CSF and plasma of e2/2, e3/3, and e4/4 targeted replacement mice. These apoE mice showed a genotype-dependent decrease in apoE levels; e2/2 > e3/3 > e4/4. Next, we sought to examine the relative contributions of apoE4 and apoE3 in the e3/4 mouse brains. ApoE4 represented 30–40% of the total apoE. Moreover, the absolute amount of apoE3 per allele was similar between e3/3 and e3/4 mice, implying that the reduced levels of total apoE in e3/4 mice can be explained by the reduction in apoE4 levels. In culture medium from e3/4 human astrocytoma or e3/3, e4/4 and e3/4 primary astrocytes, apoE4 levels were consistently lower than apoE3. Secreted cholesterol levels were also lower from e4/4 astrocytes. Pulse-chase experiments showed an enhanced degradation and reduced half-life of newly synthesized apoE4 compared with apoE3. Together, these data suggest that astrocytes preferentially degrade apoE4, leading to reduced apoE4 secretion and ultimately to reduced brain apoE levels. Moreover, the genotype-dependent decrease in CNS apoE levels, mirror the relative risk of developing AD, and suggest that low levels of total apoE exhibited by e4 carriers may directly contribute to the disease progression, perhaps by reducing the capacity of apoE to promote synaptic repair and/or Aβ clearance.

**Key words:** apolipoprotein E; astrocyte; degradation; Alzheimer’s disease; immunoassay; liver X receptor

**Introduction**

Apolipoprotein E (apoE) is a well characterized 34 kDa protein that assists in the regulation of lipid metabolism (Weisgraber and Mahley, 1996). ApoE is expressed throughout the brain, and is produced by astrocytes that secrete apoE as part of a cholesterol-rich lipoprotein particle (Boyles et al., 1985). ApoE in the brain is derived from local synthesis with little contribution from the periphery (Fernández-Miranda et al., 1997). In humans, apoE has three major protein isoforms: apoE2 (cys112, cys158), apoE3 (cys112, arg158), and apoE4 (arg112, arg158). The association of allele four of apoE (e4) as a genetic risk factor for Alzheimer’s disease (AD) has been well established, accounting for between 50–60% of the genetic variation in the disease (Corder et al., 1993; Raber et al., 2004). Patients expressing the e4 allele have an earlier age at onset, a greater amyloid burden and reduced capacity for synaptic plasticity (Corder et al., 1993, 2004; Ji et al., 2003; Lai et al., 2006). In contrast, inheritance of e2 has been associated with a decreased risk for developing AD. e4 also appears to be a risk factor for poor outcome after head trauma (Friedman et al., 1999), cerebral hemorrhage (O’Donnell et al., 2000), and stroke (Schneider et al., 2005), as well as influencing the age of onset of other neurodegenerative diseases such as Parkinson’s disease (Pankratz et al., 2006), multiple sclerosis (Fazekas et al., 2001; Enzinger et al., 2004) and amyotrophic lateral sclerosis (Moulard et al., 1996). Although not all of these associations have been consistently replicated (Siddique et al., 1998; Jasinska-Myga et al., 2007; Guerrero et al., 2008). However, together, these data do suggest that apoE plays a role in the pathophysiology of a wide range of neurological conditions, not only AD.

Despite knowing for over a decade that apoE4 is a risk factor for multiple neurodegenerative diseases, the underlying molecular mechanisms attributing to the risk-factor activity of apoE4 remains unclear. ApoE4 has two major structural characteristics that distinguish it from apoE3 or apoE2 (Hatters et al., 2006). First, apoE4 preferentially forms a “molten globule state” that ultimately reduces the *in vitro* stability of apoE4 relative to the other isoforms. Second, apoE4 forms a unique salt bridge interaction between Arg-61 in the N terminal and Glu-255 in the carboxy-terminal domain. This domain interaction results in apoE4 preferentially binding to lower density lipoprotein particles and enhanced clearance of apoE4 from the periphery (Raffai et al., 2001). Recently, Ramaswamy et al. (2005) published data to suggest that engineering an apoE4-like, Arg-61 domain interaction into murine apoE also affects CNS apoE biology and leads to low brain levels of apoE. Here, we confirm and expand on the findings of Ramaswamy et al. (2005); we demonstrate that astrocytes preferentially degrade apoE4, leading to reduced apoE4 se-
creation and reduced brain apoE levels. Moreover, the genotype-dependent decrease in CNS apoE levels mirror the relative risk of developing AD and suggest that low levels of total apoE exhibited by e4 carriers may directly contribute to the disease progression.

Materials and Methods

Animals. Twelve- to twenty-week-old male e2/2, e3/3, e3/4 and e4/4 targeted replacement mice were purchased from Taconic. The mice were individually housed for at least 2 weeks before any testing and allowed ad libitum access to food and water. Animal experiments were approved by the Institutional Animal Care and Use Committee of Wyeth Research.

The evening before the experiment the animals were fasted. The following day the mice were anesthetized using isoflurane, plasma and CSF harvested, saline perfused and the brains removed. CSF was collected as previously described (DeMattos et al., 2002). Each brain was hemisected and frontal cortex and/or hippocampus dissected and weighed. Tissues were homogenized at a ratio of 10 ml/g wet weight brain in ice-cold detergent-rich lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% IGEPA CA-630, 0.5% sodium deoxycholate, and Complete protease inhibitors; Roche) using a 10–20 s burst in Polytron homogenizer. Such a detergent-rich lysis buffer has been shown to inhibit apoE oligomerization and fully release the lipid-bound apoE epitopes which, if not exposed, may hamper accurate detection of apoE levels by immunobassay (Kru and Cole, 1996). Ramaswamy et al., 2005). The homogenates were centrifuged at 14,000 rpm for 10 min at 4°C in an Eppendorf 5417-R refrigerated Microfuge. The supernatant was collected and analyzed for apoE levels.

Immunoassays. Human apoE2, apoE3 and apoE4 standards were purchased from Calbiochem. Total apoE and apoE4 in cell culture media or brain homogenates were measured by a double-sandwich Mesoscale diagnostics (MSD)-based immunoassay (Riddell et al., 2007). Briefly, 96- well streptavidin-coated MSD plates were washed three times in Tris-buffered saline containing 0.1% Tween 20 (TBST). Ten microliters of tissue culture supernatant, diluted brain homogenates or human apoE standards were loaded into wells together with 90 μl of 1:5000 biotinylated goat anti-human apoE (Biodesign; K741808) in MSD blocker A. Plates were incubated overnight at 4°C while shaking. The following day, the plates were then washed three times in TBST and then incubated with 100 μl of detection antibody mastermix (1:5000 anti-human pan-apoE monoclonal antibody (Millipore Bioscience Research Reagents; Mab1062) and 1:10000 anti-mouse MSD-Tag in MSD blocker A) for 2 h.

For the apoE4 selective immunoassay the anti-human pan-apoE antibody was replaced with 1:5000 anti-human apoE4 monoclonal antibody (MBL International; M067-3). The plates were then washed three times in TBST and 150 μl of MSD read buffer-T was added to each well. Plates were read in a Sector Imager 6000. To ensure that the apoE measured in the brain homogenates remained in the linear range of the immunoassay, the conditioned media were collected for analysis and cell viability measured using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-H2-tetrazolium] assay (Promega). In some experiments, apoE was detected from media that was concentrated 10-fold in Vivaspin concentrators (10,000 molecular weight cut-off; Pierce) or from apoE-containing lipoprotein particles (density <1.21 g/ml) that were isolated from media by density ultracentrifugation (LaDu et al., 1998).

Gene sequencing and DNA sequencing. Genomic DNA was extracted from cells for PCR restriction fragment length polymorphism (PCR-RFLP) genotyping, as described previously (Hixson and Vernier, 1990). Briefly, a 244-bp apoE fragment was amplified by PCR (35 cycles; 97°C for 1 min, 63°C for 1 min, and 72°C for 1 min) with the primer pair: 5’-GATCACAGGTTCCATACACAGCGAGAG-3’ and 5’-GATCCGGCCGCACAGCTTCCTCATG-3’. The amplified fragment was digested using HhaI and the products were visualized on a 20% Tris-buffereDTA polyacrylamide gels (Invitrogen). Each genotype gives a specific combination of HhaI fragment sizes: e2/2, 91 and 83 bp; e3/3, 91 and 48 bp; e4/4, 72 and 48 bp and a mixed genotype: e2/3, 91, 83, and 48 bp; e3/4, 91, 72 and 48 bp; e2/4, 91, 83, 72 and 48 bp (Hixson and Vernier, 1990). For automatic DNA sequencing of PCR products, the sense primer was used on the 244-bp product purified from a 1.5% agarose gel.

Primary astrocyte cultures. Mixed glia cultures were prepared using a protocol adapted from Marriott et al. (1995). Neonatal (postnatal days 1–3) e3/3, e3/4 or e4/4 targeted replacement mice (Taconic) were decapitated and cortices were dissected and placed into 4°C HBBS. Cortical tissue was cut coarsely and incubated in HBBS containing 0.025% trypsin (100 units/ml) and DNase 1 (200 units/ml) for 10 min at 37°C. The solution was replaced by HBBS containing BSA and DNase and triturated 20 times. After allowing for debris to settle the supernatant was passed through a 40 μm cell strainer (BD Biosciences) and the process was repeated twice more. The supernatant was centrifuged at 1000 rpm for 5 min and the pellet was resuspended in 10 ml of DMEM containing 50 units of penicillin, 50 mg/ml streptomycin and 10% heat-inactivated fetal bovine serum (Invitrogen). Suspended cells were divided into T75 flasks, grown for 10 d in vitro and purified by overnight shaking (120 rpm). Remaining adherent cells, containing ~90% glial fibrillary acidic protein-positive astrocytes, were plated 48 h before experiments. At 80–90% confluency, cells were washed with HBBS and incubated in fresh serum-free medium (neurobasal medium containing 25 mM KCl, 2 mM glutamine, 100 μM penicillin, 100 μg/ml streptomycin with B27 supplementation) for 24 h. At the end of the incubation, the conditioned media were collected for analysis. Protein concentration of the lysate was measured using Micro BCA Protein Assay Kit (Pierce). Total cholesterol levels were measured using the Amplex Red cholesterol assay kit (Invitrogen). Total apoE and apoE4 levels were measured in conditioned media using the protocol described above.

Pulse-chase experiments and metabolic labeling of cell proteins with [35S]-methionine. Pulse-chase studies were performed as described previously (Kockx et al., 2004). Primary astrocytes were prepared from e3/3 and e4/4 homozygous mice were cultured for 24 h in serum-free media,
Frontal cortex and hippocampus from 20-week-old apoE-targeted replacement mice were detergent extracted and total apoE was measured using the pan-apoE immunoassay as outlined in Materials and Methods (Fig. 2). Both total apoE and apoE4 assays detected equivalent levels of apoE protein in e4/4 lysates (total apoE, 57.9 ± 5.8 ng/mg protein vs apoE4 61.3 ± 3.9 ng/mg protein, n = 14, p = 0.63).

Using these assays we sought to determine the absolute levels of apoE protein in the CNS. We choose not to examine apoE in over-expressing transgenic mouse models but rather examined apoE protein levels in the brains of 20 week old humanized e2/2, e3/3 and e4/4 targeted replacement mice, where the apoE expression is under the control of endogenous promoters (Knouff et al., 1999). Importantly, these mice showed a genotype-dependent decrease in cortical and hippocampal apoE levels, e2/2 > e3/3 > e4/4 (cortex, p < 0.0001; hippocampus, p < 0.0001, one-way ANOVA) (Fig. 2a), with e3/3 showing a 24.0 ± 4.1% and e4/4 showing a 39.4 ± 3.3% decrease in total cortical apoE relative to e2/2, whereas in the hippocampus e3/3 had a 12.0 ± 3.9% and e4/4 a 26.2 ± 3.8% reduction relative to e2/2. Interestingly, apoE levels were consistently 1.5–2-fold higher in the hippocampus compared with the frontal cortex, regardless of genotype. To
discount the possibility of a differential detection of the apoE isoforms from the brain lysates by immunoassay, we also measured apoE levels in the cortex and hippocampus using Western blotting. Using this technique we also observed a genotype-dependent decrease in cortical and hippocampal apoE levels, e2/2 > e3/3 > e4/4 (Fig. 2b). Next, we assessed the levels of apoE mRNA in the cortex and hippocampus. As noted for the Arg61 mouse (Ramaswamy et al., 2005), the levels of apoE mRNA were not significantly different across all three genotypes (Fig. 2c) implying that the altered levels of apoE are attributed to a post-translational mechanism. Next, we measured plasma levels of apoE across all three genotypes and, as reported in the human population (Eto et al., 1986), we observed a genotype-dependent decrease in plasma apoE levels (Fig. 2d). However, this time the e2/2 levels were 10 to 20-fold higher than that of e3/3 and e4/4 (e2/2, 758.5 ± 78.7 μg/ml vs e3/3, 74.0 μg/ml ± 11.3 vs e4/4, 37.1 ± 2.7 μg/ml). Finally, we measured CSF levels of apoE across all three genotypes and again observed a genotype-dependent decrease in apoE levels (e2/2, 3.5 ± 0.6 vs e3/3, 1.5 ± 0.3 vs e4/4, 1.1 ± 0.3 μg/ml); however, because of the high degree of variability in CSF apoE levels within each genotype, the differences between e3/3 and e4/4 did not reach statistical significance (Fig. 2e).

Next, we sought to determine the relative contribution of apoE4 to total apoE levels in 12-week-old e3/4 heterozygous mice. Consistent with the genotypic decrease in 20-week-old animals, we observed a genotype-dependent decrease in cortical and hippocampal apoE levels, e3/3 > e3/4 > e4/4 in the 12-week-old animals (cortex, \( p = 0.015 \); hippocampus, \( p = 0.0015 \), one-way ANOVA) (Fig. 3a). Moreover, using the apoE4 selective assay, we were able to determine the relative contribution of apoE4 to apoE3 in brain, CSF and plasma of e3/4 animals. In all compartments, including CSF, and in contrast to the expected equal contribution of each apoE isoform to the total apoE pool, apoE4 represented only between 30–40% of the total apoE (Fig. 3b). Moreover, in frontal cortex and hippocampus, the absolute amount of apoE3 per allele is similar between e3/3 and e3/4 mice, as is the amount of apoE4 per allele in e3/4 and e3/4 mice (Fig. 3c,d). These data imply that the reduced levels of total apoE in e3/4 mice can be fully explained by the reduction in apoE4 levels.

To determine the molecular mechanism by which e4 allele affects apoE protein levels, we sought to characterize apoE4 levels from human astrocytoma cell lines and primary astrocytes cultured from the apoE targeted replacement mice. Initially, we apoE genotyped a range of human astrocytoma cell lines with the hope of identifying apoE4 expressing cells. We subsequently identified two astrocytomas that expressed the apoE4 isoform; CCF-STTG1 genotyped as e3/4 and U118 genotyped as e2/4 (Fig. 4a). The genotype was confirmed by DNA sequencing (Fig. 4b) and by Western blotting of astrocytoma conditioned culture media using an apoE4 selective antibody (Fig. 4c). We next attempted to measure the levels of total apoE and apoE4 secreted by these cells. Although we could detect basal total apoE secretion from the CCF-STTG1 cells, apoE4 protein levels were below the sensitivity of our assay. To improve our assay signal, we sought to isolate and concentrate the astrocyte secreted apoE-containing lipoprotein particles using flotation ultracentrifugation (LaDu et al., 1998). Using this technique we could robustly measure both total apoE and apoE4 from the purified astrocytic lipoproteins. We found that although the CCF-STTG1-derived lipoproteins contained 67.9 ± 7.3 ng/ml total apoE, only 11.23 ± 2.7 ng/ml was attributed to apoE4 protein. Thus, apoE4 represented only 16.6 ± 4.1% of the total apoE secreted from CCF-STTG1 astrocytes (Fig. 5a). To discount the possibility of differential purification of apoE3 relative to apoE4, we sought to verify this result using a variety of apoE enrichment techniques; first, we directly concentrated the CCF-STTG1 conditioned media 10-fold, using filtration centrifugation, without lipoprotein isolation and again apoE4 represented only 23.5 ± 2.3% (\( n = 3, p < 0.0001 \)) of total apoE. Second, to fully remove any form of concentration protocol from our experimental design, we treated CCF-STTG1 cells with the liver X receptor (LXR) agonist, TO901317 (Quinet et al., 2004). TO901317 has recently been shown to potently induce apoE expression from astrocytes, including CCF-STTG1 cells (Liang et al., 2004). Similar to what has previously been demonstrated, we found that TO901317 potently induced total apoE expression from the cells (Fig. 5b). At all doses tested apoE4 represented <25% of the total apoE secreted from CCF-STTG1 cells treated with TO901317. We next examined the relative apoE4 secretion levels from the U118, e2/4 expressing cells. In these cells, secreted apoE4 levels only represented 21.4 ± 1.6% of the total apoE pool (Fig. 5a).

One potential explanation for the reduced levels of apoE4 in...
the conditioned media of CCF-STTG1 cells is enhanced cellular retention of apoE4. To examine this possibility we measured apoE levels from CCF-STTG1 cell lysates. Total apoE levels in CCF-STTG1 cell lysates were only 17.9 ± 1.3 ng/mg protein compared with 1377 ± 141 ng/mg protein secreted into the media over a 24 h period. As a result of the low levels of cell-associated apoE, apoE4 could not be accurately detected in the cell lysate. Because cell-associated apoE represented 1.3 ± 0.1% of total apoE secreted into the media, it is highly unlikely that preferential retention of apoE4 accounts for the differences in secreted apoE levels. Moreover, treating CCF-STTG1 cells with heparinase to release the cell surface bound apoE (Hara et al. 2003) increased the total amount of both apoE3 and apoE4 secreted into the media over a 24 h period, but it did not significantly alter the ratio of apoE4 to apoE3 (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

Next, we examined the apoE secretion profiles from e3/3, e3/4 and e4/4 primary astrocytic cultures. Astrocytes from e4/4 mice secreted 28 ± 8.4% less apoE into the culture medium than e3/3 cultures (n = 6, p < 0.05). Moreover, when we examined the relative contribution of apoE4 to total apoE levels secreted from primary astrocytes obtained from human e3/4 targeted replacement mice, and consistent with the human astrocytes and in vivo data, apoE4 only represented 31.1 ± 1.8% of the total apoE pool (n = 3, p < 0.001). Finally, there was a genotype-dependent decrease in cholesterol secreted into the medium, e3/3 > e3/4 > e4/4 (n = 3–9 per group, p < 0.001; one-way ANOVA), with e3/3 showing a 18.2 ± 6.2% and e4/4 showing a 52.0 ± 10.7% decrease in cholesterol relative to e3/3.

It has previously been shown that apoE can undergo significant intracellular degradation and thus escape secretion in macrophages, hepatocytes and CHO cells (Ye et al., 1993; Deng et al., 1995). To investigate this possibility as the basis for the isofrom-related differences in apoE secretion in astrocytes, the kinetics of apoE secretion and degradation in e3/3 and e4/4 primary astrocytes was measured by means of [35S]-pulse-chase experiments. Analysis of residual cell 35S-labeled apoE during chase incubations demonstrated that [35S]-apoE4 disappeared more rapidly from the astrocytes than [35S]-apoE3 (p = 0.0083, two-way ANOVA) (Fig. 6a). Cell-associated apoE4 had a half-life of 49 min compared with 96 min for apoE3. Net degradation of apoE was significantly higher in e4/4 cultures (p = 0.0045, two-way ANOVA) (Fig. 6c), with 43.1 ± 4.4% of apoE4 degraded after a 120 min chase, compared with 21.6 ± 7.9% for apoE3. This increased degradation of apoE4 corresponded with a decreased secretion of apoE4 after a 300 min chase (p < 0.05, two-way ANOVA) (Fig. 6b). As previously noted for macrophages (Kockx et al., 2004), newly synthesized astrocytic apoE appears to be present in two distinct pools, a stable pool of cell-associated apoE (~30%), that is neither secreted nor degraded over the duration of the experiment, and a mobile pool of apoE (~70%) that is targeted for either secretion or intracellular degradation. Inter-

Figure 4. Identification of human astrocytomas heterozygous for the e4 allele. a. ApoE genotyping of human astrocytoma cell lines (CCF-STTG, U87 and U118) was performed by amplifying a 244-bp PCR product from exon IV and restriction isotyping with HhaI (Hisson and Vernier, 1990). Each genotype gives a specific combination of HhaI fragment sizes: e2/2, 91 and 83 bp; e3/3, 91 and 48 bp; e4/4, 72 and 48 bp and a mixed genotype: e2/3, 91, 83, and 48 bp; e3/4, 91, 72 and 48 bp; e2/4, 91, 83, 72 and 48 bp. CCF-STTG1 cells were genotyped as e3/4, whereas U118 cells were e2/4. b. DNA sequencing of the 244 bp PCR product from CCF-STTG1 cells verified that they exhibited a mixed e3/4 genotype at codon 112. c. Conditioned media from CCF-STTG1 cells was analyzed by Western blotting using an apoE4 selective antibody (as outlined in Materials and Methods). Purified recombinant apoE3 and apoE4 were used as standards.

Figure 5. Reduced apoE4 levels in conditioned media from heterozygous human astrocyto-
mas. a. CCF-STTG1 cells were cultured in serum-free media for 48 h. The media was removed, apoE-containing lipoproteins isolated by flotation ultracentrifugation (density <1.21 g/ml) and assayed for total apoE and apoE4 protein levels using the MSD immunoassays as outlined in Materials and Methods (n = 3, mean ± SEM, ***p < 0.001). Because U118 have very low basal levels of apoE secretion, U118 cells (e2/4) were cultured in serum-free media containing 1 μg T0901317 for 48 h. The media was removed, concentrated 10-fold and assayed for total apoE and apoE4 protein levels using the MSD immunoassays as outlined in Materials and Methods (n = 3, mean ± SEM, ***p < 0.001). b. Dose-dependent upregulation of apoE3 and apoE4 by LXR agonist T0901317 in CCF-STTG1 cells. Cells were treated with T0901317 at the indicated concentrations for 48 h. Conditioned media were subjected to total apoE and apoE4 selective immunoassays as described in Materials and Methods. apoE3 levels were calculated by subtracting apoE4 from total apoE levels.
estingly, although apoE4 is degraded faster than apoE3, the secretion rate of apoE4 appears to be maintained at apoE3 levels for the first 120 min of the chase, after which the secretion of apoE4 drops off relative to apoE3 (Fig. 6b). It is of note that at the 120 min time point, the mobile pool of apoE4 appears to be completely exhausted, whereas apoE3 cells still have capacity in their mobile pool for either secretion or degradation (Fig. 6a). These data suggest that despite the enhanced rate of degradation, the apoE4 astrocytes maintain normal rates of apoE secretion until all available apoE is depleted. Ultimately, at the end of the 300 min chase period, 27.8 ± 2.7% of newly synthesized apoE3 has been secreted into the media and 41.0 ± 4.1% has been degraded, compared with 18.9 ± 2.5% of apoE4 being secreted and 51.7 ± 4.0% degraded (Fig. 6d). These data correspond very well with the 28% reduction of total apoE detected in the media of E4/4 astrocytes by immunomassay.

Recent evidence suggests that apoE4 is preferentially susceptible to proteolytic degradation into cytotoxic fragments (Huang et al., 2001), we therefore wished to determine whether the enhanced degradation of apoE4 led to an accumulation of the cytotoxic fragments in the astrocytes and brains of e4/4 mice. Although a low level of both 22 and 10 kDa fragments were detected in the pulse-chase experiments, these fragments degraded at the same rate as full-length apoE and did not accumulate in either e3/3 or e4/4 cells (data not shown). Moreover, using Western blotting, and despite heavily over-exposing our blots, we could find no evidence of the accumulation of apoE4 selective degradation products in the hippocampus of the e4/4 mice (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

Discussion

In humans and animal models, apoE genotype is correlated with apoE protein levels in plasma, with e2 showing the highest protein levels and e4 the lowest (Eto et al., 1986; Gregg et al., 1986; Raffai et al., 2001) (Fig. 2). In plasma, apoE4 preferentially binds to large, triglyceride-rich, very low-density lipoproteins and is more rapidly catabolized by the liver, thus leading to low steady state plasma levels. The effect of the e4 allele on the locally synthesized CNS apoE protein levels is less clear with multiple conflicting reports showing either reduced levels (Bertrand et al., 1995; Beffert et al., 1999; Glöckner et al., 2002; Poirier, 2005; Ramaswamy et al., 2005), no change (Sullivan et al., 2004; Fryer et al., 2005) or increases (Fukumoto et al., 2003) in apoE from both AD patient CNS and animal models. In this study, we have extensively analyzed the effect of the e4 allele on apoE levels both in vitro and in vivo. To avoid artificial measurements of apoE4 relative to apoE2 and apoE3, we used multiple methods of apoE enrichment (lipoprotein isolation, filtration concentration and LXR agonist induction) and detection (immunomassay and Western blot). We also detergent extracted plasma, CSF and brain samples before quantification, thus precluding well known issues of apolipoprotein detection in aqueous solutions (Kruil and Cole, 1996; Ramaswamy et al., 2005). Finally, we measured relative levels of both total apoE and apoE4 in e3/3 heterozygous cell lines and primary astrocytic cultures, as well as, in mouse plasma, CSF and brains, establishing a ratio of total apoE to apoE4 in each individual sample and thus providing a strong internal control. Together, our data show that the human e4 allele is preferentially degraded in astrocytes, leading to a reduced level of apoE protein and cholesterol secretion and low steady state brain and CSF levels of apoE in transgenic animals.

Although the mechanisms that lead to increases in CNS, apoE2 levels have been previously explored (Mann et al., 2004; Fryer et al., 2005) and appear to be related to the inability of apoE2 to bind to the LDL receptor (Fryer et al., 2005), the mechanisms that leads to low levels of CNS apoE4 are less clear. In vivo, the lower levels of apoE4 were not attributable to decreased mRNA levels, suggesting a post-translational mechanism. Indeed, we observed a consistent decrease in the level of apoE4 secreted into the media of primary astrocytes and human astrocytomas. This reduction in apoE4 levels was not apparently attributable to enhanced cellular retention of apoE4 but rather an accelerated intracellular degradation of newly synthesized apoE4. One potential mechanism to explain this observation is that the cellular quality control machinery recognizes apoE4 as a misfolded protein and targets it for degradation. Indeed, it is known from in vitro observations that apoE4 is the least stable of all the apoE isoforms: apoE4 denatures at lower concentrations of urea and at lower temperatures, whereas the denaturation pattern of apoE4 suggests that it can exist as a partially folded intermediate or a molten globule state (Morrow et al., 2002). Interestingly, the...
stabilization of a molten globule state caused by point mutations in the cystic fibrosis transmembrane conductance regulator gene is known to target this protein for enhanced degradation (San-tucci et al., 2008).

The main focus of research into the role of apoE4 in AD has concentrated on the ability of apoE to influence the metabolism of the peptidic neurotoxin, Aβ, that accumulates in the brain of AD patients. A large number of in vitro and in vivo studies have suggested that endogenous CNS-derived apoE-containing lipoprotein particles bind to Aβ and promote its clearance and degradation (DeMattos et al., 2004; Koistinaho et al., 2004; Manelli et al., 2004; Stratman et al., 2005; Trommer et al., 2005) and that apoE4 carriers have increased Aβ burden (Beffert et al., 1999; Corder et al., 2004; Fryer et al., 2005). Our data suggests that low brain levels of apoE4 relative to apoE3 may contribute to raised Aβ levels, because the low apoE levels in e4 carriers may reduce the clearance efficiency of the Aβ peptide and thus accelerate its subsequent deposition. Indeed, recent studies by our group and others have shown, that dosing of APP transgenic mice with LXR agonists, which increase murine CNS apoE levels, also decrease Aβ levels (Koldamova et al., 2005; Burns et al., 2006; Riddell et al., 2007). It will be interesting to determine whether the LXR compounds can reduce the Aβ burden in e4/4 mice crossed with the APP transgenic animals.

Although this apoE/Aβ interaction may account for the strong association of e4 with AD, it cannot fully explain the apparent association of apoE with a range of neurological disorders that do not exhibit an accumulation of the Aβ peptide. One alternative hypothesis recently proposed suggests that apoE4 is preferentially susceptible to proteolytic degradation into cytotoxic fragments (Huang et al., 2001; Brecht et al., 2004). Although degradation of apoE4 to cytotoxic N-terminal fragments is an attractive CNS-dependent mechanism to account both for the low levels of apoE detected in the brains of e4 mice and neurodegeneration, we could not detect the accumulation of any such fragments in our cultures or brain homogenates. Nevertheless, it should be noted that one cannot rule out the possibility that the apoE fragments may preferentially accumulate in the disease state and not in the healthy young animals examined in this study.

One additional important role that apoE may play in the CNS is in the delivery of cholesterol to neurons. The neuronal synapse is a cholesterol-rich organelle (Pfrieger, 2003) and astroglia-derived cholesterol has been shown to be essential in maintaining neuronal health by promoting synaptogenesis (Mauch et al., 2001) and neurite outgrowth (Handelmann et al., 1992; Nathan et al., 1994). Moreover, it has been hypothesized that because apoE is dramatically upregulated after neuronal injury, that the ability of apoE to deliver cholesterol to regenerating neurons may be an important factor in cognitive recovery from brain injury (Poirier, 1994). Multiple in vitro studies support this claim: for example, it has been demonstrated that apoE knock-out mice and e4/4 targeted replacement mice exhibit more severe neurological and cognitive deficits after closed head injury (Chen et al., 1997; Sabo et al., 2000), experimental stroke (Laskowitz et al., 1997; Sheng et al., 1998, 1999), entorhinal cortex lesions (Fagan et al., 1998; White et al., 2001) and experimental allergic encephalomyelitis (Karussis et al., 2003) than controls (wild-type and e3/3 mice, respectively). Moreover, it has also been noted that the presence of the e4 allele correlates with a reduced number of dendritic spines in transgenic mice and AD patients (Ji et al., 2003; Lai et al., 2006). These studies suggest that e4/4 mice display a similar phenotype to the apoE knock-out and that, at least with respect to plasticity, apoE4 has characteristics of a "loss of function" mutation. Our data, together with the work of Ramaswamy et al. (2005), suggests that the preferential degradation of apoE4 relative to apoE3 in astrocytes leads to a reduced capacity for the delivery of cholesterol the neurons for repair and that this phenotype may ultimately underlie this plasticity loss of function. Indeed, the diverse array of neurological conditions that exhibit very distinct neuropathology but implicate e4 as a risk factor, imply that the apoE4 protein may not harbor any direct pathological actions contributing to the underlying neurodegenerative processes but rather that apoE4 lacks the protective effects of apoE3. Although additional studies are required to identify the exact mechanism leading to the enhanced degradation of intracellular apoE4, these data suggest that pharmacological approaches targeted at stabilizing apoE4 or promoting apoE and/or cholesterol secretion from astrocytes may be potential disease modifying therapies for Alzheimer's disease.

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