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Modulation of amyloid β -protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway

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Susceptibility to Alzheimer's disease (AD) is governed by multiple genetic factors. Remarkably, the LDL receptor-related protein (LRP) and its ligands, apoE and α 2M, are all genetically associated with AD. In this study, we provide evidence for the involvement of the LRP pathway in amyloid deposition through sequestration and removal of soluble amyloid β -protein (A β). We demonstrate in vitro that LRP mediates the clearance of both A β 40 and A β 42 through a bona fide receptor-mediated uptake mechanism. In vivo, reduced LRP expression is associated with LRP genotypes and is correlated with enhanced soluble A β levels and amyloid deposition. Although LRP has been proposed to be a clearance pathway for A β , this work provides the first in vivo evidence that the LRP pathway may modulate A β deposition and AD susceptibility by regulating the removal of soluble A β .

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Introduction

The LDL receptor-related protein (LRP) is a multifunctional receptor that mediates the internalization and degradation of ligands involved in metabolic pathways of lipoproteins and protease/protease-inhibitor complexes (1), including α 2-macroglobulin (α 2M) (2), apoE (3), and Kunitz protease inhibitor (KPI) containing forms of amyloid precursor protein (APP) (4). Remarkably, the aforementioned ligands are all genetically associated with Alzheimer's disease (AD) (5, 6) and are found in senile plaques in brains of AD patients (7). To date, the strongest evidence directly implicating a role of LRP in AD is from genetic studies first reported by us (8) and subsequently confirmed in four independent case-control cohorts (9–12). In addition, another genetic polymorphism in LRP was found to be associated with AD (13), further evidence corroborating the LRP gene as an important AD-susceptibility locus. Our study reported a genetic polymorphism (C766T) in exon 3 of LRP that is under-represented in AD and associated with later age of disease onset. However, the underlying biologic relevance of the silent LRP C766T polymorphism is unclear. Moreover, the precise mechanisms by which LRP and its ligands may contribute to AD pathogenesis are unknown.

The generation of amyloid β -protein (A β) from APP and its subsequent deposition in the brain are believed to be key events in the pathogenesis of AD (14). Not surprisingly, the sequestration and clearance of A β recently has been hypothesized to be another potential key regulatory step in amyloid deposition (7, 8, 15). It has been shown that α 2M can complex with A β and subsequently be degraded through the LRP-mediated pathway in cultured cells (16, 17). In addition, because LRP is highly expressed in the central nervous system (CNS) (18), internalization of apoE-enriched lipoprotein particles by way of LRP may impact neuronal membrane remodeling (19). Thus, alterations in brain LRP expression or activity may impact both neuronal homeostasis and amyloid deposition and thereby modify the pathogenesis of AD. In this study, we demonstrate that the LRP pathway is critical for clearance of soluble A β in vitro and provide genetic and biochemical in vivo evidence that LRP modulates soluble A β levels, amyloid burden, and susceptibility to AD.

Methods

Cell lines and cDNA constructs. Control mouse fibroblasts (+/+; MEF-1) and fibroblasts heterozygous (+/-; PEA 10) or homozygous (-/-; PEA 13) for LRP deficiency were obtained from American Type Culture Collection

(Rockville, Maryland, USA) and cultured as described previously (20). Human APP695 was inserted into the pBabepuro retroviral expression vector (21) and transfected into GP+E86 packaging cell line. Stable transformants were selected with puromycin (5 µg/ml). After infection with recombinant viruses, mouse fibroblasts (*LRP*^{+/+}, *LRP*^{+/-}, and *LRP*^{-/-}) were selected with puromycin (2.5 µg/ml) and analyzed without clonal selection. Chinese hamster ovary (CHO) cells overexpressing APP751 with V717F FAD mutation were generated as described previously (22). A full-length RAP cDNA (GenBank accession no. M63959) was amplified by PCR from a human liver cDNA library (CLONTECH Laboratories, Palo Alto, California, USA) and subcloned into pGEX-4T (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). GST-RAP fusion protein was expressed in *Escherichia coli* and purified using a glutathione column, according to manufacturer's directions.

In vitro Aβ clearance assays. Purified α2M was obtained from Athens Research Laboratory (Athens, Georgia, USA) and activated by methylamine as described (4). Conditioned medium from CHO cells (IS-CHO; Irvine Scientific, Santa Ana, California, USA) overexpressing APP751 with V717F FAD mutation was added to confluent nontransfected *LRP*^{+/-} and *LRP*^{-/-} fibroblasts for 48 hours in the presence of 20 nM α2M, and Aβ40 and Aβ42 levels were measured by sandwich ELISA. For clearance of iodinated synthetic Aβ, ¹²⁵I-Aβ40 (Amersham Pharmacia Biotech) and α2M were incubated overnight at 37°C, and the incubation mix was added to confluent cultured cells for 24 hours. The medium was then collected and subjected to scintillation counting for γ radiation.

Human subjects and neuropathological evaluation. All subjects were unrelated white Americans of European descent. All pathology-confirmed AD subjects (National Institute of Neurological Disorders and Stroke [NINDS]/Alzheimer's Disease and Related Disorders Association [ADRD] criteria) were obtained from the Alzheimer's Disease Research Center (ADRC) at the University of California, San Diego (UCSD). Senile plaques were identified in thioflavin S-stained sections of the midfrontal cortex and counted under a ×10 objective and a ×10 ocular lens (field size, 1.6 mm²), as described previously (23). For quantitation of LRP levels, all *LRP* T allele-positive AD cases (*n* = 17) with pathological data and available frozen tissue from the appropriate brain region were entered into the study. Age-matched AD cases with C/C genotype were randomly selected for measurement of LRP levels (*n* = 20). For assessing amyloid burden, all available pathology-confirmed cases with known *LRP* genotypes from UCSD-ADRC were included (*n* = 103). All AD subjects were late-onset AD (≥60 years at onset of disease). All autopsied control subjects were obtained from the Johns Hopkins University ADRC and the Baltimore Longitudinal Study of Aging (24). *APOE* and *LRP* genotyping were performed as described previously (8).

Quantitation of LRP levels. Frozen brain tissues derived from the midfrontal cortex of pathologically confirmed control and AD subjects were homogenized in 1% NP40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP40, 100 µg/ml AEBSF, and 10 µg/ml leupeptin). For quantitation of LRP in brain, 50 µg of detergent-soluble protein was separated by SDS-PAGE and transferred onto nitrocellulose membranes. From the same blots, LRP was detected by a polyclonal Ab against the 85-kDa light chain of LRP (25), while actin (AC-40; Sigma Chemical Co., St. Louis, Missouri, USA) and synaptophysin (SY38; Roche, Indianapolis, Indiana, USA) were detected by specific mAb's. The primary Ab's were detected by incubation with biotinylated secondary Ab's, followed by ¹²⁵I-streptavidin. The signals were quantitated by phosphorimaging (Bio-Rad, Hercules, California, USA). Signals from quantitations were in a linear range, as determined from standards included with each experiment. The LRP, actin, and synaptophysin signal on each blot was first standardized to the internal control. Then the LRP signal was normalized to actin or synaptophysin.

Aβ measurements. Human plasma samples were collected from living subjects clinically diagnosed as probable AD at the UCSD-ADRC using NINDS/ADRD criteria. Plasma samples were collected in EDTA tubes and kept at 4°C until centrifugation. Aβ quantitation was performed using a standard sandwich ELISA. Briefly, microtiter wells were coated with a monospecific Ab that selectively recognizes the carboxy terminal of Aβ1-40 or Aβ1-42; the wells were then blocked with 1% BSA/PBS. Human plasma samples were diluted 1:1 in 0.1% CHAPS/PBS and then captured in Ab-coated wells for 18 hours at 4°C. After binding, wells were washed with PBS, and Aβ was detected with an anti-human Aβ1-12 mAb (26D6) conjugated to horseradish peroxidase (HRP). Each sample was assayed in triplicate and quantitated to a standard Aβ curve within the linear range. For immunoprecipitation of Aβ from medium of cultured cells, a polyclonal Ab to Aβ (3134) was used as described (26).

Statistics. For two-group comparisons between genotypes or disease status, a two-sided *t* test was used. Where SD was significantly different between groups, Welch's correction was applied to two-sided *t* tests. ANOVA, coupled with Tukey post hoc test, was used to assess the combinatorial effects of *LRP* and *APOE* genotypes on plasma Aβ levels. A χ^2 test for linear trend was used to assess *LRP* genotype distributions across ordered plaque-number categories among AD subjects. Linear regression analysis was used to assess the magnitude and significance of the correlation between LRP levels and age of subjects.

Results

LRP mediates the clearance of secreted Aβ without altering Aβ production from APP695. It has been demonstrated previously that LRP can serve as a cell surface-internalization

receptor for the KPI containing isoforms of secreted APP, including APP751 and APP770 (4). Moreover, LRP can associate with cellular APP751 at the cell surface and mediate its internalization (27). Recently, it was shown by Ulery and colleagues that LRP alters A β production from the KPI containing APP751, presumably through the KPI-LRP interaction (28). Thus, it is important to examine the possibility that LRP might be involved in the formation of A β from APP695, the major neuronal isoform lacking the KPI domain. To study the effects of LRP expression on A β production and clearance, we first established an in vitro cell-culture model using mouse fibroblasts genetically deficient in LRP (*LRP*^{-/-}) and corresponding LRP-expressing control cells (*LRP*^{+/+} and *LRP*^{+/-}). In these fibroblasts transfected with equivalent levels of human APP695, the levels of A β secreted into the medium in the absence of α 2M within 24 hours was equivalent between *LRP*^{+/-} and *LRP*^{-/-} cells as measured by immunoblotting and sandwich ELISA (Figure 1, a and b). Addition of RAP, a competitive antagonist of all ligands binding to LRP, had no effect on levels of secreted A β during this time period (Figure 1b). Thus, this indicated that different levels of LRP did not alter the secretion of A β from APP695, although the possible role of LRP in other aspects of APP processing cannot be excluded. Having established that LRP does not alter A β production within a 24-hour time period, we examined the clearance of endogenously secreted A β through the α 2M-LRP pathway. Consistent with recent observations in primary neurons (29), addition of activated α 2M directly to serum-free medium of *LRP*^{+/+} fibroblasts reduced A β levels by approximately 60% (Figure 1c) within 48 hours. In contrast, α 2M had no effect on A β levels in *LRP*^{-/-} cells, indicating that LRP is required for α 2M-mediated reduction in A β . Coincubation of α 2M with RAP completely blocked α 2M-mediated reduction of A β in LRP-expressing cells, confirming the specificity of the LRP pathway in removal of A β complexes (Figure 1c). Interestingly, RAP treatment resulted in higher A β levels compared with untreated controls in LRP-expressing cells, suggesting a basal level of RAP-sensitive A β clearance activity in untreated controls. In LRP-deficient cells, RAP did not affect the level of A β in the presence or absence of α 2M (Figure 1c), indicating that the effects of RAP are specific for LRP and not directly on secretion or removal of A β . These data clearly demonstrate that secreted A β is removed through the α 2M-LRP pathway and that LRP is absolutely required for α 2M-mediated uptake of A β . Moreover, A β production from APP695 is not affected by LRP.

LRP mediates the clearance of secreted A β 40 and A β 42 through a bona fide receptor-mediated mechanism. It has been suggested previously that LRP can mediate the internalization and degradation of A β complexes in vitro (16). However, it has not been directly demonstrated that uptake of A β complexes by cultured cells is through a bona fide receptor-mediated mechanism. This issue is highlighted by previous observations that A β can be internalized by fluid-phase pinocytosis and

by the scavenger-receptor pathway in macrophages (30, 31). Moreover, it is not known whether the LRP pathway is capable of mediating the clearance of A β 42, a minor but putatively pathogenic species of A β that are initially deposited in senile plaques (32). To test whether A β 42 is removed through the LRP pathway, conditioned medium from CHO cells overexpressing V717F FAD mutant APP was added to native (i.e., untransfected) *LRP*^{+/-} and *LRP*^{-/-} cells in the presence of α 2M. As shown in Figure 2a, α 2M significantly reduced the levels of soluble A β 40 and A β 42 in the medium of *LRP*^{+/-} cells to similar degrees. As expected, neither A β 40 nor A β 42 levels were affected by α 2M in *LRP*^{-/-} cells, indicating the requirement of LRP in α 2M-mediated clearance of A β . Approximately 65% of A β 40 and 60% of A β 42 was cleared from the medium within

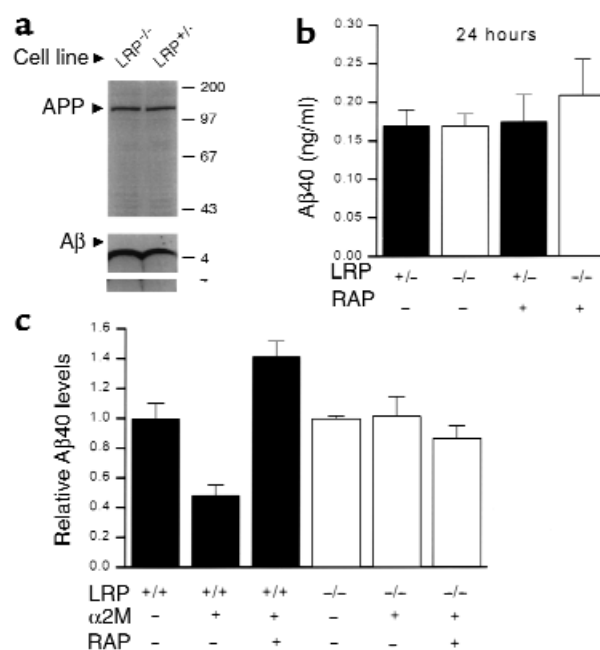


Figure 1

LRP does not alter the secretion of A β in cultured cells. *LRP*^{+/-} and *LRP*^{-/-} fibroblasts transfected with human APP695 were generated as described. (a) To determine whether LRP levels contribute to changes in production and secretion of A β , APP-transfected *LRP*^{+/-} and *LRP*^{-/-} cells were metabolically labeled with ³⁵S-methionine for 20 minutes and immunoprecipitated with an Ab specific for APP (upper panel). In parallel experiments, medium was conditioned for 24 hours, and the amount of secreted A β was analyzed by immunoprecipitation (3134), followed by Western blotting (26D6) (lower panel). (b) To distinguish between A β secretion and LRP-dependent A β clearance within a 24-hour period, APP overexpressing *LRP*^{+/-} and *LRP*^{-/-} cells were treated with or without RAP (1 μ M), and the medium was quantitated for the amount of A β by ELISA. Graph shows a representative experiment ($n = 3$) with means and SEM. (c) Confluent cultures of APP overexpressing *LRP*^{+/+} and *LRP*^{-/-} fibroblasts were incubated with or without activated α 2M (100 nM) in serum-free medium to induce uptake of A β through LRP. RAP (1 μ M), an antagonist for all known LRP ligands, was added to block α 2M-LRP-mediated effects. After 48 hours, medium was collected and analyzed for levels of A β by sandwich ELISA assay. A β levels are normalized to the no-treatment group of each cell line. Experiments were performed three times in triplicate. Error bars represent SEM.

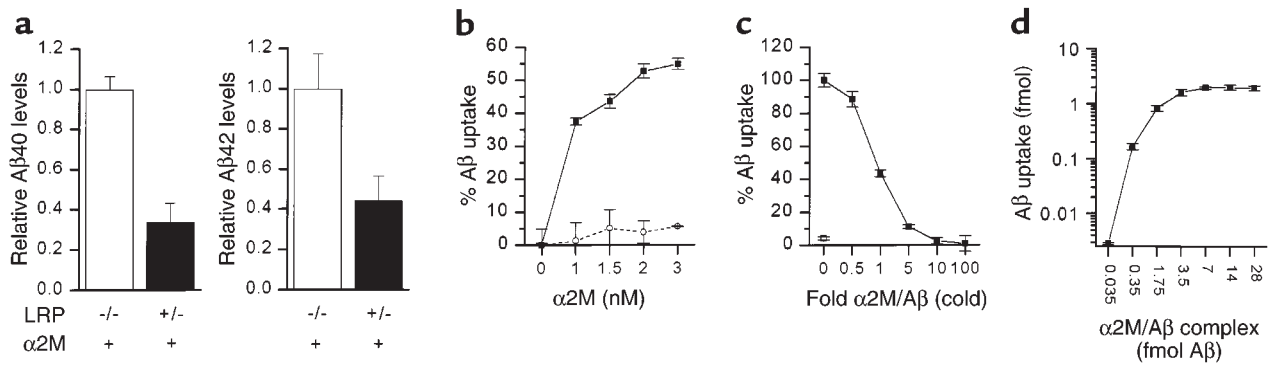


Figure 2

LRP mediates the clearance of both Aβ40 and Aβ42 through a receptor-mediated uptake mechanism. (a) CHO cells overexpressing APP751 with V717F FAD mutation were grown to confluency, and IS-CHO was collected for 48 hours. Conditioned medium was then added to confluent non-transfected *LRP^{-/-}* and *LRP^{+/-}* fibroblasts for 48 hours in the presence of 20 nM α2M, and Aβ40 and Aβ42 levels were measured by sandwich ELISA. Experiments were performed three times in triplicate. Error bars represent SEM. (b) To determine optimal Aβ uptake, mixtures of 0.1 nM ¹²⁵I-Aβ with 0, 1, 2, and 3 nM α2M were incubated overnight at 37°C, and the incubation mix was added to confluent *LRP^{-/-}* (filled squares) and *LRP^{+/-}* (open circles) fibroblasts for 24 hours. The medium was then collected and subjected to scintillation counting for γ radiation. Percentage of Aβ uptake reflects the proportion of counts lost from the input amount. (c) To determine whether Aβ uptake is subject to self-competition, a mixture of 0.1 nM ¹²⁵I-Aβ and 2 nM α2M was incubated overnight at 37°C, and the incubation mix was added to confluent *LRP^{-/-}* fibroblasts in the presence of increasing amounts of excess unlabeled α2M/Aβ complex for 24 hours (filled squares). In parallel experiments, 1 μM RAP was coinubated with the ¹²⁵I-Aβ/α2M mix (open squares). The medium was then collected and subjected to scintillation counting for γ radiation. Percentage of Aβ uptake is normalized to maximal Aβ uptake in the absence of unlabeled Aβ/α2M complex. (d) To determine whether Aβ uptake is subject to saturation, increasing amounts of ¹²⁵I-Aβ/α2M complex (mixed as before) were added to confluent *LRP^{-/-}* cells for 24 hours, and the medium was collected and subjected to scintillation counting for γ-radiation. Aβ uptake is calculated in femtomoles and represented in a log scale. All experiments were performed three times in triplicate, and a representative experiment is shown. Error bars represent SEM.

48 hours in *LRP^{+/-}* cells under conditions where cell-free degradation of Aβ was undetectable (Figure 2a). These data show that the LRP pathway efficiently mediates the uptake and degradation of the highly pathogenic Aβ42 species as well as Aβ40.

To demonstrate that Aβ uptake by the α2M-LRP pathway is through a bona fide receptor-dependent mechanism, we next assessed the uptake of synthetic ¹²⁵I-labeled Aβ in cultured fibroblasts. We first determined the optimal ratio of ¹²⁵I-α2M/Aβ for in vitro uptake of Aβ through LRP using a fixed physiological concentration of 0.1 nM Aβ. As shown in Figure 2b, an increasing amount of α2M facilitated the uptake of ¹²⁵I-Aβ in a concentration-dependent manner in *LRP^{+/-}* cells. Maximal uptake of Aβ was approximately 60% (Figure 2b), consistent with soluble Aβ produced from cultured cells. As anticipated, there was little to no uptake of ¹²⁵I-Aβ in *LRP^{-/-}* cells at any tested concentration of α2M (Figure 2b). Increasing amounts of unlabeled Aβ/α2M complex effectively blocked the removal of ¹²⁵I-Aβ in a concentration-dependent fashion, such that excess cold Aβ/α2M inhibited ¹²⁵I-Aβ uptake as effectively as the addition of RAP (Figure 2c). Finally, uptake of ¹²⁵I-Aβ/α2M complex was completely saturable with half-maximal uptake

at 50 pM ¹²⁵I-Aβ/1 nM α2M complex, an approximate K_d range (0.2–10 nM) that has been reported for binding of α2M to a variety of cell types (33) (Figure 2d).

Reduced LRP levels correlate with increased AD susceptibility. Having established that the level of LRP expression is critical for Aβ clearance in vitro, we next assessed whether LRP levels in the human brain might be altered during normal aging or disease. Measurement of LRP levels in the brain were performed by quantitative immunoblotting of the LRP 85-kDa light chain from the midfrontal cortex of AD and normal controls (NC). From pathology-confirmed AD and control age-matched subjects, LRP levels relative to actin were approximately twofold lower in AD brains compared with that of controls (Figure 3a: $t = 4.884$, $df = 76$, $P < 0.0001$; Table 1). Surprisingly, among control subjects there was a strong inverse correlation between age and LRP levels ($n = 39$, $r = -0.4905$, $P = 0.0015$), indicating that LRP expression normally declines with age. As shown in Table

Table 1

Relative LRP expression in AD and controls across age categories

	Total	LRP expression ± SD				
		≥65 years	40–64 years	65–74 years	75–84 years	≥85 years
Normal	0.57 ± 0.29 (n = 39)	0.50 ± 0.23 (n = 28)	0.76 ± 0.36 (n = 11)	0.54 ± 0.27 (n = 11)	0.53 ± 0.22 (n = 11)	0.37 ± 0.12 (n = 6)
AD	0.29 ± 0.21 (n = 39)	0.29 ± 0.21 (n = 39)	-	0.20 ± 0.18 (n = 9)	0.28 ± 0.19 (n = 22)	0.43 ± 0.23 (n = 8)
P value	<0.0001 ^A	0.0002 ^B				

LRP expression is normalized to actin. Ages are at death of subjects. Values represent means ± SD. ^ALRP level is significantly different between AD and controls as determined by two-tailed *t* test with Welch's correction by total comparison. ^BLRP level is significantly different between AD and controls when matched for age.

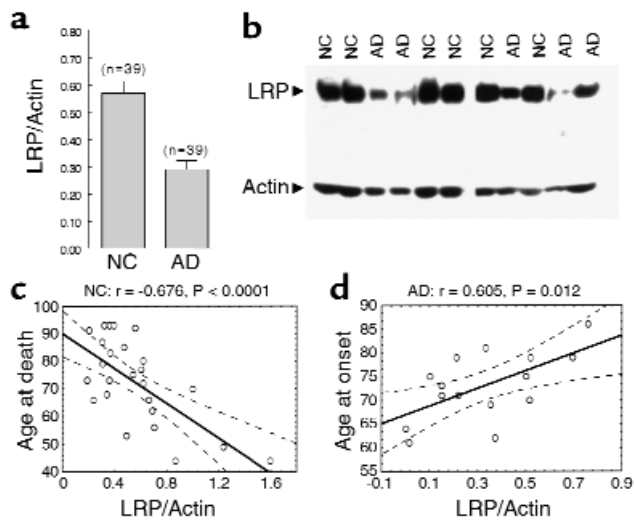


Figure 3

Association of brain LRP levels with AD susceptibility. LRP levels were quantitated by immunoblotting for the 85-kDa light chain of LRP and normalized to actin. (a) Comparison of AD and age-matched normal controls (NC) showed a significant difference in LRP levels ($t = 4.884$, $df = 76$, $P < 0.0001$). Error bars represent SEM. (b) Representative immunoblots containing LRP and actin signals from AD and NC samples are shown. (c) Levels of LRP in the brain are inversely correlated with age of control subjects (control subjects lacking *APOE* $\epsilon 4$ allele shown: $r = -0.6758$, $P < 0.0001$; all control subjects: $r = 0.4905$, $P = 0.0015$). (d) AD patients show a positive correlation between LRP levels and ages at onset of disease (AD subjects lacking *APOE* $\epsilon 4$ allele shown: $r = 0.6048$, $P = 0.0116$; all AD subjects: $r = 0.33465$, $P = 0.0429$). The regression slope (center line) and 95% confidence interval (two curved lines) are shown. The correlation coefficient (r) and P values are shown above the graph.

1, the average reduction in brain LRP level was approximately twofold between an age group of 40–64 (0.76 ± 0.11 SEM, $n = 11$) and those equal to or greater than 85 (0.37 ± 0.05 SEM, $n = 6$), with intermediate LRP expression among the age group of 65–84 (0.53 ± 0.06 SEM, $n = 22$). Interestingly, this inverse correlation was markedly stronger among noncarriers of the *APOE* $\epsilon 4$ allele (Figure 3c: $n = 28$, $r = -0.6758$, $P = 0.00008$). When LRP levels were normalized to synaptophysin instead of actin, a similar inverse correlation with age and LRP expression was observed among all control subjects ($n = 39$, $r = -0.3948$, $P = 0.0128$), indicating that the observed effect is not attributable to neuronal/synaptic loss.

In contrast to that observed in control subjects, higher LRP levels significantly correlated with later ages at onset of AD ($n = 37$, $r = 0.33465$, $P = 0.0429$) and death ($n = 37$, $r = 0.41032$, $P = 0.0169$). Importantly, LRP levels were still lower in AD patients after the age of 80 (0.34 ± 0.05 SEM, $n = 17$) compared with age-matched control subjects (0.48 ± 0.05 SEM, $n = 13$), although the largest differences were observed under the age of 85 (Table 1). Interestingly, the positive correlation between LRP levels and age of onset (Figure 3d: $n = 15$, $r = 0.60483$, $P = 0.0116$) and death ($n = 15$, $r = 0.63599$, $P = 0.0108$) resulted primarily from individuals lacking the *APOE* $\epsilon 4$ allele. Thus, the LRP effect is predominant among approximately 50% of AD

cases where currently no genetic susceptibility is attributed because pathogenic effects associated with *APOE* $\epsilon 4$ may be sufficient by itself to confer AD risk. Duration of disease from onset to death had no effect on LRP levels (graph not shown: $n = 37$, $r = 0.081$, $P = 0.6357$), indicating that the decline in LRP expression is unrelated to AD-related brain aberrations.

Genetic association of LRP with LRP levels, amyloid burden, and plasma A β . In view of the preceding observations, we next asked whether the correlation between LRP levels and AD susceptibility can be confirmed by a different analysis. The recently reported under-representation of the LRP exon 3 (C766T) polymorphism in late-onset AD has been demonstrated in five different populations (8–12), although the putative causative mutation/polymorphism in linkage disequilibrium with the C766T polymorphism remains to be identified. Because our current results demonstrated that the level of LRP in the brain is correlated with disease onset, we examined whether a manifestation of the C766T polymorphism is also reflected in LRP expression. Analysis of LRP geno-

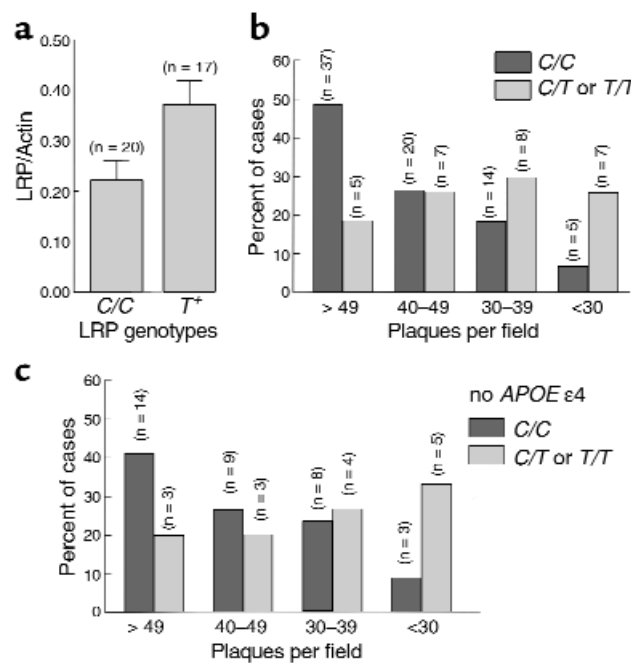


Figure 4

Association of LRP genotypes with LRP levels and amyloid burden in the AD brain. LRP levels were quantitated by immunoblotting for the 85-kDa light chain of LRP and normalized to actin. (a) AD patients harboring LRP *T* allele showed significantly higher levels of LRP in the brain ($t = 2.335$, $df = 35$, $P = 0.0254$). (b) AD brains were segregated into ordered categories of increasing amyloid burden, ranging from less than 30, 30–39, 40–49, and greater than 49 plaques per field and examined for association with LRP genotypes. The percentage and number of individuals within each plaque-per-field category are shown as a function of LRP genotypes. Statistical analysis shows an excessive overrepresentation of C/C genotypes across increasing plaques-per-field categories compared with T-positive genotypes (χ^2 for linear trend = 11.762, $df = 1$, $P = 0.0006$). (c) The LRP effect on amyloid burden is still observed among subjects that do not carry the *APOE* $\epsilon 4$ allele (χ^2 for linear trend = 6.135, $df = 1$, $P = 0.0133$).

Table 2

Association of *LRP* and *APOE* genotypes with plasma A β among clinically diagnosed AD patients

	Plasma A β (ng/ml) \pm SD (<i>n</i>)	<i>P</i> value
<i>LRP</i> genotypes		
C/C	0.49 \pm 0.37 (<i>n</i> = 43)	0.0109
C/T or T/T	0.31 \pm 0.15 (<i>n</i> = 23)	
<i>APOE</i> genotypes		
ϵ 4 ⁺	0.51 \pm 0.38 (<i>n</i> = 36)	0.0163
ϵ 4 ⁻	0.33 \pm 0.21 (<i>n</i> = 30)	
<i>LRP</i> and <i>APOE</i> genotypes		
C/C and ϵ 4 ⁺	0.56 \pm 0.42 (<i>n</i> = 26) ^A	<0.05 ^A
C/C and ϵ 4 ⁻	0.37 \pm 0.25 (<i>n</i> = 17)	
T ⁺ and ϵ 4 ⁺	0.37 \pm 0.20 (<i>n</i> = 10)	
T ⁺ and ϵ 4 ⁻	0.27 \pm 0.10 (<i>n</i> = 13) ^A	

Plasma A β measurements are performed by sandwich ELISA. Values represent means \pm SD with number of samples (*n*) in parenthesis. *P* values are derived from two-tailed *t* tests with Welch's correction. ^AFor combinations of *LRP* and *APOE* genotypes, ANOVA ($F = 3.0504$, $P = 0.03506$) is carried out with the post hoc Tukey test ($P < 0.05$).

type status revealed significantly higher *LRP* levels among AD cases harboring the C/T or T/T genotypes compared with carriers of the C/C genotype (Figure 4a: $t = 2.335$, $df = 35$, $P = 0.0254$), although still lower than that of control subjects. Because *LRP* T allele carriers have higher age at onset of AD than *LRP* C/C carriers as reported previously (8), we confined the analysis to comparable ages. In an age group (70–82 years) where there was no difference in ages at death between C/C ($n = 16$, 76.4 ± 0.18 SD) and T-positive genotypes ($n = 11$, 77.3 ± 0.15 SD), the same significant difference was observed ($t = 2.424$, $df = 25$, $P = 0.0229$). Higher *LRP* levels in AD are therefore associated with the protective effects of the T allele, consistent with under-representation of the T allele in AD (8). Different *APOE* genotypes, however, did not alter the level of *LRP* in AD brains (data not shown).

Next, we assessed whether the *LRP* T-allele associates with total amyloid burden in AD. Extending our previous analysis of neuritic plaques in brains of AD individuals (8), we assessed the total amount of thioflavin S-positive plaques, regardless of type, as an indicator of amyloid burden. *LRP* T allele carriers showed significantly fewer numbers of total senile plaques compared with C/C carriers, using *t*-test analysis ($t = 2.860$, $df = 101$, $P = 0.0051$). To examine the plaque-density profile of *LRP* T-positive versus C/C carriers, AD cases were segregated into four increasing levels of plaque-density categories. Statistical analysis revealed that the *LRP* T allele was excessively over-represented in the lower plaque-density categories as compared with the C/C genotype (Figure 4b: χ^2 for linear trend = 11.762, $df = 1$, $P = 0.0006$). The *LRP*-associated effects on amyloid burden were not dependent on the underlying *APOE* genetic status, because the same trend was observed among subjects that did not carry the *APOE* ϵ 4 allele (Figure 4c: χ^2 for linear trend = 6.135, $df = 1$, $P = 0.0133$).

Amyloid burden in postmortem AD brain tissues represents a terminal state that may not accurately reflect

the dynamic in vivo relationship between *LRP* expression and A β levels. Moreover, the measurements of A β in the brain is confounded by the presence of multiple pools with heterogeneous solubilities. Thus, we analyzed A β levels in human plasma to determine whether *LRP* genotypes associate with levels of soluble A β in vivo. Consistent with higher *LRP* levels among *LRP* T-allele carriers, A β in plasma was significantly decreased by 58% in carriers of the *LRP* T allele compared with carriers of the C/C genotype (Table 2: Welch's $t = 2.627$, $df = 60$, $P = 0.0109$). Surprisingly, plasma A β level was also significantly lower (by 54%) in non-*APOE* ϵ 4 carriers compared with carriers of the *APOE* ϵ 4 allele (Table 2: Welch's $t = 2.478$, $df = 55$, $P = 0.0163$), indicating that apoE4 not only increases amyloid burden in the brain (34), but also the amount of soluble A β systemically. This finding led us to ask whether there are any additive effects between *LRP* and *APOE* genotypes. The highest A β levels were found among carriers of both *LRP* C/C genotype and *APOE* ϵ 4 allele, which was greater than twofold more than carriers of both *LRP* T-allele and non-*APOE* ϵ 4 genotypes (Table 2: ANOVA, $F = 3.0504$, $P = 0.03506$; post hoc Tukey, $P < 0.05$).

Discussion

The genetic associations of *APOE*, α 2M, and *LRP* to late-onset AD are particularly intriguing in light of the fact that both apoE and α 2M are two key ligands for *LRP* (5, 6, 8). Both apoE and α 2M avidly bind A β in vitro and in vivo (5, 35). These observations, together with the finding that *LRP* and all of its ligands are present in senile plaques (7), strongly implicate the pathogenic importance of the *LRP* pathway in AD. We found that *LRP* levels are significantly reduced in AD, compared with healthy controls. Linear-regression analysis revealed that *LRP* levels progressively decline with the increasing age of control subjects (an inverse correlation) and are further reduced in AD subjects. Among AD patients, however, increased *LRP* levels were correlated with later age of disease onset, indicating that higher *LRP* levels might be protective against AD. This apparent protective effect was accentuated among noncarriers of the *APOE* ϵ 4 allele. As increasing age is the primary risk factor for AD, these data indicate that reduced *LRP* expression may be one factor contributing to AD susceptibility. This notion is highly consistent with the negative association of the *LRP* T allele to AD (8, 9, 11, 12) and increased *LRP* levels among T-allele carriers demonstrated in this study. Although the biological mode of the *LRP* T allele requires further characterization, we hypothesize that the C766T polymorphism is in linkage disequilibrium with a causative mutation or polymorphism that regulates *LRP* expression (for example, promoter/enhancer) in the aging brain.

In the current study, we provide compelling evidence that A β uptake via the α 2M-*LRP* pathway is through a bona fide receptor-mediated mechanism and not through nonspecific A β degradation or fluid-phase pinocytosis. This was shown by the competition of ¹²⁵I-

A β uptake with excess unlabeled A β complexes and the complete saturation of ¹²⁵I-A β uptake at physiological concentrations. So far, no other A β uptake pathway meets the criteria for a bona fide receptor-mediated mechanism. Although the scavenger receptor has been postulated to mediate the uptake of amyloid fibrils, such process is not subject to competition and saturation of the receptor (30, 31). At another level of A β catabolism, recent observations have indicated that neutral endopeptidase and insulin-degrading enzyme are both capable of degrading extracellular A β in a cell-autonomous manner (36, 37). Thus, it is likely that there are multiple ways of mediating A β degradation in vivo. Our study demonstrated that LRP does not alter the secretion of A β from APP695-expressing cells but is required for α 2M-mediated clearance of soluble A β . Because both LRP and APP695 are predominantly expressed in neurons, reduced LRP levels in the AD brain is predicted to negatively impact the clearance of soluble A β but not its production in neurons. However, it is important to note that LRP overexpression in LRP-deficient CHO cells results in altered trafficking of KPI containing APP751 (28), possibly through the APP-LRP physical interaction (4). Thus, it is possible that LRP also alters APP trafficking (i.e., internalization/recycling) and A β generation through other mechanisms. As APP isoforms are differentially expressed in neurons and glia, LRP-clearance activity versus altered APP trafficking might be differentially modulated across cell types.

The genetic association of *LRP T* polymorphism with both increased LRP expression and reduced amyloid deposition is intriguing in light of the in vitro evidence of A β clearance through the LRP pathway (16, 17). These observations are now further strengthened by genetic association of *LRP* with soluble A β levels in plasma. It is noteworthy that the pathogenic A β 42 species is as effectively cleared through the LRP pathway as A β 40 (Figure 1a), an activity that may dramatically impact amyloid deposition in vivo. Accordingly, we interpret these data to indicate that reduced LRP expression, at least in part, contributes to increased A β levels and amyloid deposition by negatively impacting A β clearance. This interpretation is consistent with our observation that reduced LRP expression is also correlated with increased AD susceptibility and earlier age of disease onset. In our cell-culture system, we demonstrated the requirement of LRP in the α 2M-mediated clearance of A β . However, it has been reported that apoE and lactoferrin, two other LRP ligands, also sequester A β and mediate its clearance (16, 38). Thus, reduced LRP levels may impede the clearance of various A β complexes. Interestingly, many LRP ligands, including apoE, α 2M, and lactoferrin, are produced from astrocytes, whereas LRP is largely expressed in neurons. Thus, it is likely that receptor-mediated uptake and clearance of soluble A β complexes occur in neurons, whereas uptake of fibrillar amyloid is mediated by microglia (30, 31). In this regard, downregulation of LRP expression has been linked to proinflammatory stimuli such as LPS and IFN- γ in cultured cells (39, 40).

We speculate that proinflammatory processes present in the AD brain may induce downregulation of LRP expression, further reducing A β clearance and enhancing amyloid deposition. Since LRP mediates the normal function of neuronal remodeling through internalization of apoE (19), reduced LRP expression in aging and disease may also compromise neuronal viability independent of the effects on A β clearance.

It has been demonstrated previously that the *APOE* ϵ 4 allele promotes amyloid deposition (34). Our unexpected finding that the *APOE* ϵ 4 allele is also associated with higher A β levels in plasma of AD subjects raises the possibility that different isoforms of apoE may also impact the removal of soluble A β . This is in agreement with the binding affinity of native apoE isoforms for A β (apoE2 > apoE3 > apoE4) (15, 41) and reduced LRP-dependent uptake of apoE4/A β complexes in CHO cells (38). However, the possibility that apoE4 interferes with A β clearance by accelerating A β aggregation cannot be excluded, consistent with the delayed amyloid deposition in apoE-deficient mice (42). Two recent studies surprisingly have shown that human apoE actually delays A β deposition in apoE-null mice, with apoE3 being more effective than apoE4. On the other hand, apoE is required for fibrillar A β deposits, and apoE4 converts A β to fibrillar deposits faster than apoE3 in a mouse model of human apoE expression (43, 44). These observations are consistent with the notion that apoE4 may both impede LRP-mediated A β clearance and promote A β fibrillogenesis.

The results of the current study provided the first in vivo evidence of the LRP-clearance pathway in AD pathogenesis. Our observations lead us to postulate that reduced LRP expression is a contributing risk factor for AD, possibly by impeding clearance of soluble A β complexes. Functional characterization of α 2M polymorphisms associated with AD (6) and future transgenic animal models of LRP and/or α 2M expression should further elucidate the mechanism of A β clearance and AD pathogenesis. The observation that AD risk or protection associated with LRP levels is strongest among noncarriers of the *APOE* ϵ 4 allele is particularly interesting in light of the ligand/receptor relationship between apoE and LRP. Because the receptor function of LRP obviously depends on intact activity of its ligands, we hypothesize that high levels of LRP cannot effectively rescue the pathogenic effects of apoE4, the latter operating at a step that negates the clearance mechanism. However, in the presence of apoE2 or apoE3, where the ligand complexes are not perturbed, alterations in LRP level and, in turn, clearance activity become highly consequential for AD pathogenesis. Since increased LRP expression may promote both neuronal survival mediated by apoE2 and apoE3 isoforms and also enhance the clearance of soluble A β complexes, the current data provide an alternative direction for AD therapeutic intervention by targeting the A β /LRP clearance pathway in non-*APOE* ϵ 4 carriers.

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