

# The Lipoprotein Receptor LR11 Regulates Amyloid $\beta$ Production and Amyloid Precursor Protein Traffic in Endosomal Compartments

Katrin Offe,<sup>1,2</sup> Sara E. Dodson,<sup>1,2</sup> James T. Shoemaker,<sup>1,4</sup> Jason J. Fritz,<sup>1,4</sup> Marla Gearing,<sup>1,3</sup> Allan I. Levey,<sup>1,4</sup> and James J. Lah<sup>1,4</sup>

<sup>1</sup>Center for Neurodegenerative Disease, <sup>2</sup>Graduate Program in Neuroscience, and Departments of <sup>3</sup>Pathology and <sup>4</sup>Neurology, Emory University, Atlanta, Georgia 30322

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline and neuropathological changes, including the deposition of amyloid  $\beta$  ( $A\beta$ ) in senile plaques. The mechanisms causing the disease and  $A\beta$  accumulation are not well understood, but important genetic associations with apolipoprotein E genotype and involvement of lipoprotein receptors have become apparent. LR11 (also known as SorLA), a member of the low-density lipoprotein receptor family, has been identified previously as an altered transcript in microarray analyses of samples from human AD cases. Here, we show neuronal expression of the lipoprotein receptor LR11 in control brain in regions vulnerable to AD neuropathology and marked reduction of LR11 expression in these regions in AD brains before cell death. Overexpression of LR11 drastically reduces levels of extracellular  $A\beta$  and also lowers levels of total cellular amyloid precursor protein (APP). LR11 colocalizes with APP and regulates its trafficking in endocytic compartments, which are important intracellular sites for APP processing and  $A\beta$  generation. Endogenous LR11 localizes to neuronal multivesicular bodies in both rat and human brain. The robust correlation between reduced LR11 expression and AD neuropathology and its potent effects on extracellular  $A\beta$  levels suggest that this neuronal lipoprotein receptor could play an important role in AD pathogenesis.

**Key words:** LR11; sorLA; VPS10; lipoprotein receptors; amyloid  $\beta$ ; amyloid precursor protein; Alzheimer's disease; endocytic pathway

## Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia in adults. The mechanisms underlying the disease are still poorly understood, but deposition of senile plaques composed of fibrillar aggregates of amyloid  $\beta$  peptide ( $A\beta$ ) is a characteristic hallmark of the disease and believed to play a fundamental role in disease pathogenesis (Selkoe, 2000, 2001).

$A\beta$  is produced by two sequential proteolytic cleavages of the amyloid precursor protein (APP): cleavage by  $\beta$ -secretase releases a large N-terminal APP ectodomain fragment ( $APP_{\beta}$ ), and subsequent action of  $\gamma$ -secretase on the membrane-bound C-terminal portion of APP produces  $A\beta$ . Alternatively, APP can be processed by  $\alpha$ -secretase, which releases  $APP_{\alpha}$  and cleaves within the  $A\beta$  sequence, thus precluding  $A\beta$  formation. The processing of APP along these pathways is determined by its intracellular trafficking and exposure to secretase activities. Although  $APP_{\alpha}$  is mostly produced at or near the cell surface, both the

early secretory pathway and the endosomal system have been reported as sites of  $A\beta$  production (Sisodia, 1992; Koo and Squazzo, 1994; Hartmann et al., 1997; Xu et al., 1997).

Several molecules involved in lipid metabolism have been implicated in AD and, more specifically, in the regulation of  $A\beta$  levels. Apolipoprotein E (ApoE) is the main lipoprotein expressed in the brain, and the *APOE*  $\epsilon 4$  allele is a major genetic risk factor for AD (Corder et al., 1993; Strittmatter et al., 1993). Members of the low-density lipoprotein receptor (LDLR) family, which bind ApoE, have also been genetically associated with AD risk, and the LDLR-related protein (LRP) interacts with APP and ApoE (Okuizumi et al., 1995; Kang et al., 1997, 2000; Ulery et al., 2000; Pietrzik et al., 2002). LR11 is another member of the LDLR family with a unique multidomain structure including 11 LDLR class A ligand binding repeats (Yamazaki et al., 1996). Structural elements in LR11 also place it in the vacuolar protein sorting 10 protein (VPS10p) homology domain family of intracellular sorting receptors (Jacobsen et al., 1996), and LR11 appears to interact with GGA adaptor proteins, which are important in Golgi to endosome trafficking (Jacobsen et al., 2002). LR11 is expressed primarily in the brain (Taira et al., 2001), and we have previously shown LR11 transcript downregulation in lymphoblasts of AD patients using a cDNA microarray study (Scherzer et al., 2004).

Here, we show that LR11 protein in control brain concentrates in neurons in regions vulnerable to AD neuropathology and that neuronal LR11 expression in these regions is markedly

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Correspondence should be addressed to Dr. James J. Lah, Center for Neurodegenerative Disease, 615 Michael Street, Suite 505 South, Atlanta, GA 30322. E-mail: jlah@emory.edu.

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reduced in AD brains. Furthermore, manipulating LR11 levels results in dramatic changes in levels of extracellular A $\beta$ , which are accompanied by substantial effects of LR11 on APP distribution in the endosomal/lysosomal system. Additionally, ultrastructural studies localize endogenous LR11 to neuronal multivesicular bodies (MVBs). Its impact on APP and A $\beta$  identifies LR11 as a regulator of APP processing. Together with the observed changes in LR11 expression in vulnerable brain regions, these findings suggest that LR11 may play a major role in the pathogenesis of AD.

## Materials and Methods

**Antibodies.** The primary antibodies used were against LR11 [monoclonal mLR11 to VPS10 domain, kindly provided by Drs. H. Bujo (Department of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Chiba, Japan) and W. J. Schneider (Division of Molecular Genetics, Department of Medical Biochemistry, Medical University Vienna, Vienna, Austria) (Hirayama et al., 2000), and polyclonal 3850.7 to LR11 C terminus, kindly provided by Dr. C. Schaller (Zentrum für Molekulare Neurobiologie, Universität Hamburg, Hamburg, Germany) (Hampe et al., 2000)], C8 [APP C terminus, polyclonal; a gift from Dr. D. Selkoe (Center for Neurologic Diseases, Harvard University, Boston, MA) (Selkoe et al., 1988)], 22C11 (APP N terminus, monoclonal; Chemicon, Temecula, CA), EEA1 and GM130 (monoclonals; Transduction Laboratories, San Diego, CA), and EF-1 $\alpha$  (monoclonal; Upstate Biotechnology, Lake Placid, NY).

**Immunohistochemistry.** Blocks of the hippocampus, frontal cortex, basal ganglia, and cerebellum from 13 AD cases and eight controls were fixed for 24–48 h in 4% paraformaldehyde, embedded in paraffin or cryoprotected in 30% sucrose, and frozen. Paraffin-embedded blocks were cut into 8  $\mu$ m sections, deparaffinized, and pretreated with pepsin (Biomed, Foster City, CA). Frozen blocks were cut into 50  $\mu$ m sections. Sections were treated with hydrogen peroxide, washed in Tris buffer, blocked with normal serum, and incubated with anti-LR11 antibodies overnight at 4°C. On day 2, sections were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), followed by avidin–biotin–peroxidase complex (Elite ABC kit; Vector Laboratories). Color development was performed with 3,3'-diaminobenzidine. Paraffin-embedded sections were counterstained with hematoxylin. Control sections incubated without primary antibody showed negligible staining.

**Semiquantitative reverse transcription-PCR.** Human embryonic kidney 293 (HEK293) cells were transfected with an LR11 expression vector or empty vector (pcDNA3) as a control. Total RNA was isolated 72 h after transfection TRIzol (Sigma, St. Louis, MO) according to the manufacturer's recommended protocol. RNA was precipitated with isopropanol, resuspended in diethylpyrocarbonate-water, and treated with Turbo DNase Free (Ambion, Austin, TX) to remove any contaminating genomic DNA. Single-stranded cDNAs were generated with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) using oligo(dT). Subsequent PCR was performed using 2  $\mu$ l of the first-strand cDNA in a reaction mixture containing 50  $\mu$ M of sense and antisense primers, 200 mM dNTPs, and 1 U of *Taq* DNA polymerase (Promega, Madison, WI) in reaction buffer containing 2.5 mM MgCl<sub>2</sub>. Thermocycling was performed using the following protocol: (1) 95°C for 7 min; (2) designated cycles of 94°C for 1 min, 57°C for 2 min, and 72°C for 2 min; and (3) 72°C for 5 min. A suitable PCR cycle number for quantitative analysis of gene expression was determined by titrating from 18 to 40 cycles. For APP, 27 cycles of PCR were performed using 5'-AGGAGGGCATCCTGAGTATTGCCAAGAAG-3' (sense) and 5'-TGGCGACGGTGTGCCAGTGA-3' (antisense) as primers to yield a 267 bp product. For LR11, 28 cycles of PCR were performed using 5'-AAGCTCTTCAGGCTATCCATGGGGACG-3' (sense) and 5'-AACGCAGGCTGC CAGATCTACGGAT-3' (antisense) as primers to yield a 273 bp product. For  $\beta$ -actin, 21 cycles of PCR were performed using 5'-GGGGAGGTGATAGCATTGCTTTCGTG-3' (sense) and 5'-CCGAATGGACTGGTCTCAAGTCAGTGTA-3' (antisense) as primers to yield a 219 bp product. PCR products were separated on a 1%

agarose gel and stained with ethidium bromide. The bands were quantified using a Fluorochem 8800 gel documentation system densitometry software suite (Alpha Innotech Corporation, San Leandro, CA).

**Double-labeling immunofluorescence and colocalization analysis.** Control and LR11-transfected HEK293 cells were fixed in 2% paraformaldehyde. Rats were perfused with 4% paraformaldehyde, and frozen brains were cut into 50  $\mu$ m sections and treated with 3% hydrogen peroxide. Samples were rinsed, blocked, and incubated with both primary antibodies overnight. Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were either directly conjugated to fluorophores or biotinylated, followed by avidin–biotin–peroxidase complex and tyramide-conjugated fluorophores (PerkinElmer, Boston, MA). Control samples incubated with no or only one primary antibody demonstrated no significant cross-reactivity or background staining. Images were captured using a LSM510 confocal microscope (Zeiss, Thornwood, NY), and colocalization analysis was performed as described previously (Volpicelli et al., 2001).

**Immunogold electron microscopy.** After treatment with primary antibody (see above, Immunohistochemistry), sections were incubated with secondary antibody conjugated to ultra small gold particles (Aurion, Wageningen, The Netherlands). After rinsing, sections were fixed in glutaraldehyde for 1 h, enhanced with R-gent SE-EM (Aurion), and post-fixed in OsO<sub>4</sub>. Sections were dehydrated, embedded in Epon, and cut into ultra thin sections (60–70 nm). Thin sections were counterstained with uranyl acetate, followed by lead citrate, and examined using a Hitachi (Tokyo, Japan) H-7500 electron microscope.

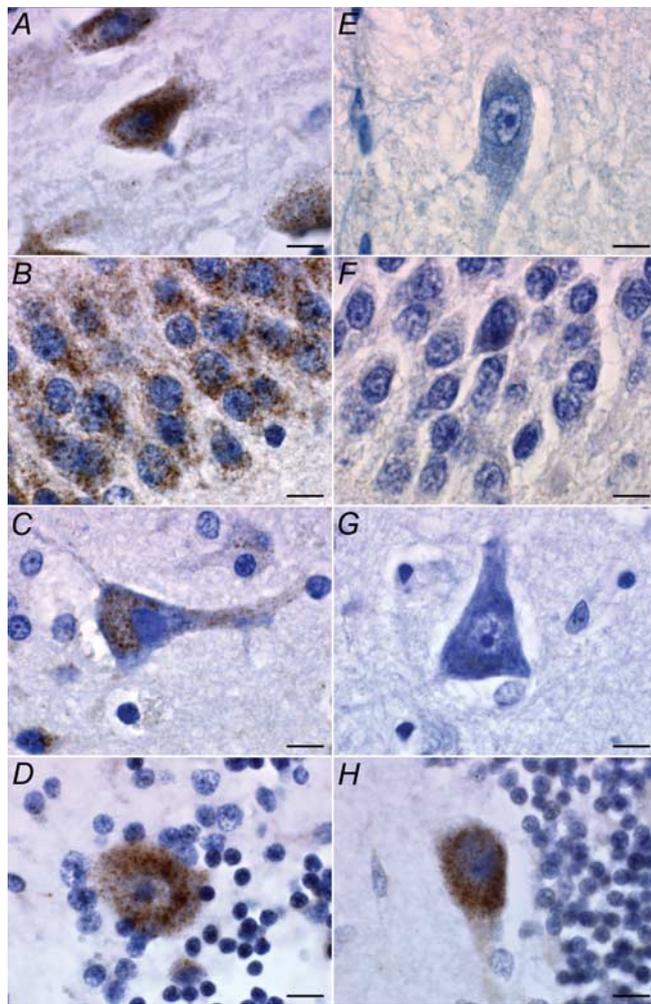
**Western blotting.** Cell extracts were separated by SDS-PAGE and transferred overnight to Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked at room temperature for 30 min, probed with primary antibodies overnight at 4°C, rinsed, and incubated with secondary antibodies (1:10,000) conjugated to fluorophores (Molecular Probes, Eugene, OR) for 1 h at room temperature. Images were captured, and band intensities were quantified using an Odyssey Image Station (LI-COR, Lincoln, NE). Band intensities were normalized to loading controls.

**A $\beta$  ELISA.** HEK293 cells were transfected with an LR11 expression vector or empty vector (pcDNA3) as a control. For endogenous A $\beta$  measurements, medium was conditioned for 48 h beginning 24 h after transfection. Seventy-two hours after transfection, conditioned medium was collected and cells were harvested. A $\beta$  levels were measured using the human  $\beta$  amyloid 1–40 ELISA kit (Biosource, Camarillo, CA) according to the manufacturer's instructions and normalized to total cellular protein. Measurement of A $\beta$ 1–42 was attempted, but the levels of endogenously produced A $\beta$ 1–42 were below reliable detection limits. Plates were read at 450 nm on a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA). Experiments were performed in triplicate. Cell extracts were blotted for LR11 and APP and conditioned media samples were blotted for APPs as described above (see Western blotting).

## Results

We have previously reported downregulation of LR11 expression in AD patients (Scherzer et al., 2004). To identify potentially important regional differences, we examined LR11 expression patterns in the brain with respect to regions vulnerable in AD. LR11 immunostaining was evaluated in two regions strongly affected by AD pathology, the hippocampal formation and frontal cortex, and two less affected regions, the basal ganglia and cerebellum. In control brain, strongly immunolabeled cytoplasmic puncta were observed in CA1–CA3 pyramidal neurons extending through the cell body and into basal dendrites (Fig. 1A). Similar staining was present throughout the cytoplasm of dentate granule cells (Fig. 1B). In the frontal cortex, intense punctate staining was found in cytoplasm and proximal dendrites of large pyramidal neurons (Fig. 1C). In the cerebellum, only Purkinje cells showed significant LR11 immunoreactivity (Fig. 1D), and little or no detectable staining was found in most basal ganglia neurons (data not shown).

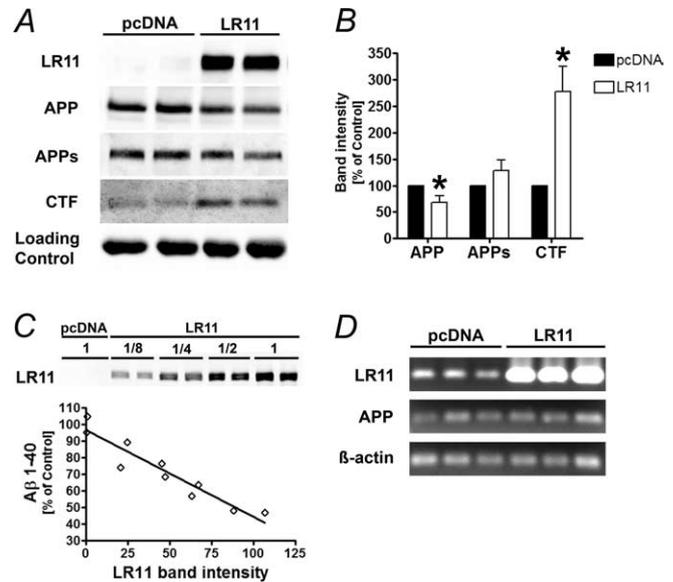
In striking contrast to control brain, CA1–CA3 pyramidal



**Figure 1.** LR11 is selectively lost in neurons and vulnerable brain regions in AD. **A–C**, In control brains, strong punctate immunolabeling was found in CA1–CA3 pyramidal neurons (**A**), dentate granule cells (**B**), and frontal cortex pyramidal neurons (**C**). **D**, LR11 immunoreactivity was also detected in Purkinje cells of the cerebellum. **E–G**, In AD brains, LR11 immunoreactivity is absent in CA1–CA3 pyramidal neurons (**E**), dentate granule cells (**F**), and frontal cortex pyramidal neurons (**G**). Hematoxylin counterstain shows otherwise healthy appearing neurons. **H**, LR11 is preserved in Purkinje cells of the cerebellum in AD patients. Scale bars, 10  $\mu$ m.

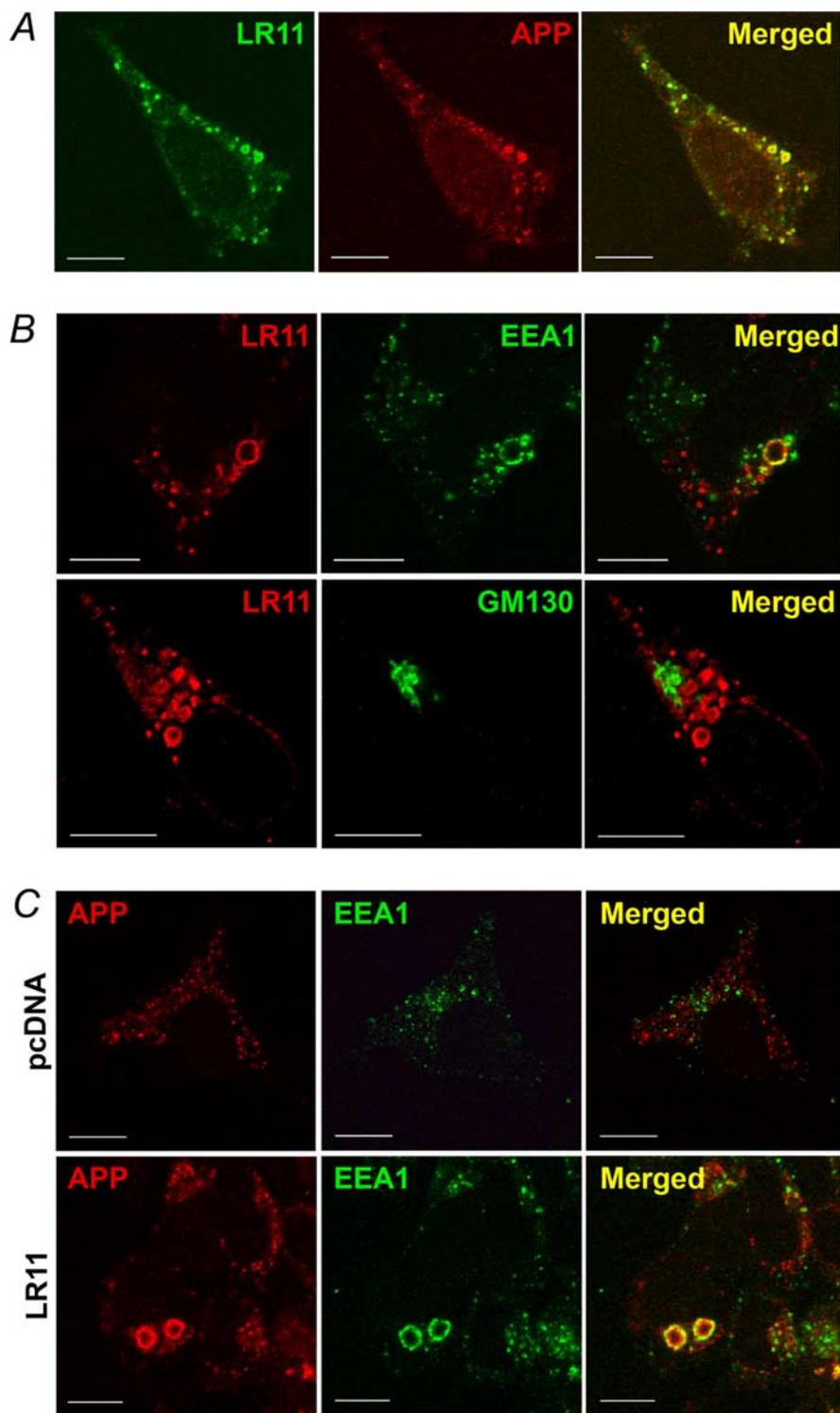
neurons in AD brain exhibited scarce LR11 immunoreactivity (Fig. 1*E*), and staining was nearly absent in dentate granule cells (Fig. 1*F*). In the frontal cortex, LR11 immunoreactivity was barely detectable in pyramidal neurons (Fig. 1*G*). This loss of immunoreactivity was not simply attributable to cell loss, because hematoxylin counterstaining showed many otherwise healthy appearing hippocampal and cortical neurons (Fig. 1*E–G*). As in control brains, AD brains showed good immunolabeling in cerebellar Purkinje cells (Fig. 1*H*) and little or no staining in basal ganglia neurons (data not shown). These findings were remarkably consistent across all AD and control brains and indicate selective loss of LR11 expression in AD brain in regions vulnerable to the disease, the hippocampal formation and frontal cortex, whereas staining in relatively unaffected regions, the cerebellum and basal ganglia, was unchanged.

To assess the functional importance of LR11, we studied the effect of LR11 overexpression on levels of full-length APP, C-terminal APP fragments (CTFs), APPs, and A $\beta$ . Although these experiments involved artificial overexpression of LR11 in cells that normally express very little endogenous receptor, the



**Figure 2.** LR11 reduces levels of cellular APP and extracellular A $\beta$ . **A**, Western blots of control and LR11-overexpressing cells show high LR11 expression in LR11-transfected cells and a decrease in total APP in cell extracts. Secreted APPs in conditioned media is not altered, whereas levels of cellular CTFs are increased. Loading control EF-1 $\alpha$  is unchanged. **B**, Quantification of bands shows a significant difference between control and LR11-transfected cells for APP (LR11 mean,  $68.5 \pm 11.7\%$  of control;  $p = 0.0432$ ) and CTFs (LR11 mean,  $278 \pm 47.39\%$  of control;  $p = 0.0228$ ) but not for APPs (LR11 mean,  $127.9 \pm 20.33\%$  of control;  $p = 0.2342$ ). **C**, Western blots show increasing levels of LR11 protein expression after transfection with pcDNA or increasing amounts of LR11 DNA. The numbers reflect the copy number of LR11 plasmid used relative to control plasmid. Levels of extracellular A $\beta$  1–40 significantly correlate with LR11 protein expression ( $R^2 = 0.8975$ ;  $p < 0.0001$ ). **D**, Semiquantitative RT-PCR shows that mRNA levels of LR11 are increased after LR11 transfection, but levels of APP and  $\beta$ -actin (control) mRNA remain unchanged. Data are shown as percentage of control. Error bars represent SD.

impact on APP and A $\beta$  were measured using endogenously expressed APP to avoid potential artifacts from overexpression of exogenous APP. HEK293 cells were transiently transfected with LR11 or control DNA, cell extracts were blotted for APP and total CTFs, and conditioned media were blotted for total APPs. Most of the APPs and CTFs produced by these cells result from  $\alpha$ -secretase cleavage (data not shown), and the relatively low endogenous levels of APP expression did not allow us to separately measure the  $\beta$ -cleaved forms. In this study, we therefore report total levels of APPs and CTFs. Cells were monitored for accumulation of endogenously produced A $\beta$  1–40 in the media using an ELISA. LR11 overexpression significantly changed levels of full-length APP, CTFs, and A $\beta$ . Levels of full-length APP in the cell extracts of LR11-transfected cells were reduced by 30% ( $p = 0.0432$ ), whereas levels of secreted APPs in the conditioned media were not significantly altered ( $p = 0.1407$ ) when normalized to cellular APP. Levels of CTFs in LR11-overexpressing cells were increased  $\sim 2.5$ -fold after normalization to full-length APP ( $p = 0.0228$ ) (Fig. 2*A, B*). A major change between LR11 and control conditions was observed in the levels of extracellular A $\beta$ . A $\beta$  1–40 levels were reduced by  $\sim 50\%$  in the media samples collected from LR11-transfected cells compared with controls (LR11 mean,  $57.3 \pm 3.5\%$  of control;  $p = 0.0023$ ). Transfection efficiency in these cells is  $\sim 60\%$ , suggesting that the effects on individual LR11-expressing cells is likely substantially greater than the overall effects measured in these experiments. To more firmly establish a relationship between LR11 expression and A $\beta$  levels, we transiently transfected HEK293 cells with control DNA or increasing amounts of LR11 DNA. Endogenous A $\beta$  levels in the



**Figure 3.** LR11 colocalizes with APP and alters APP localization to the endosomal system. *A*, LR11 staining in transfected HEK293 cells shows a punctate pattern (green), immunoreactivity for endogenous APP also shows intracellular punctate staining (red), and the merged image shows substantial colocalization (yellow) of LR11 and APP. *B*, LR11 (red) colocalizes with the endosomal marker EEA1 (green) as shown by yellow labeling in the merged image, but no colocalization of LR11 was found with the Golgi marker GM130. *C*, APP staining (red) shows little overlap with EEA1 (green) staining in pcDNA-transfected HEK293 cells. Colocalization (yellow) is increased significantly after LR11 transfection for both APP colocalization with EEA1 (pcDNA mean,  $3.6 \pm 0.8\%$ ; LR11 mean,  $9.5 \pm 6.0\%$ ;  $p = 0.0064$ ) and EEA1 colocalization with APP (pcDNA mean,  $4.5 \pm 1.1\%$ ; LR11 mean,  $16.8 \pm 8.8\%$ ;  $p = 0.0005$ ). Scale bars, 10  $\mu\text{m}$ .

media were measured by ELISA, and LR11 expression levels were determined by Western blot. LR11 expression increased with the plasmid copy number, whereas A $\beta$  levels decreased and negatively correlated with LR11 expression ( $R^2 = 0.8975$ ;  $p < 0.0001$ )

(Fig. 2C). To assess whether the difference in cellular APP was attributable to a down-regulation of APP gene expression, we compared levels of APP mRNA using semiquantitative reverse transcription-PCR (RT-PCR) (Fig. 2D). APP mRNA expression was unchanged between control and LR11-transfected cells, indicating that the observed reduction in APP levels occurs posttranscriptionally. These results show that LR11 expression regulates levels of both secreted A $\beta$  and cell-associated full-length APP and CTFs, suggesting an important dose-dependent effect of LR11 on APP processing.

To gain insight into the mechanism by which LR11 might regulate APP and A $\beta$ , we analyzed the subcellular localization of LR11 and the effects of LR11 expression on APP distribution. Both LR11 and APP localize to intracellular vesicular structures in LR11-transfected HEK293 cells (Fig. 3A). Quantitative analysis of confocal images revealed that 22.6% of LR11 staining overlapped with APP and, conversely, 32.4% of the APP staining in these cells colocalized with LR11. Analysis of its subcellular localization showed significant colocalization of LR11 with early endosomal autoantigen 1 (EEA1), a marker of early endosomes (Fig. 3B), and quantification of staining distribution revealed that 24.0% of LR11 was present in EEA1-positive compartments. In contrast to previous studies (Jacobsen et al., 1996, 2001; Andersen et al., 2005), we found poor colocalization of LR11 immunoreactivity with Golgi markers (Fig. 3B). Because APP processing depends on its exposure to the different secretases in various subcellular compartments, we tested the possibility that expression of LR11 may affect the trafficking of APP. In control transfected cells, 3.6% of endogenous APP colocalized with EEA1, but after LR11 transfection, colocalization of APP and EEA1 increased to 9.5% ( $p = 0.0064$ ;  $n = 12$ ). Similarly, 4.5% of EEA1 colocalized with APP in control cells compared with 16.8% in LR11-transfected cells ( $p = 0.0005$ ) (Fig. 3C). Although the total percentage of APP colocalization with EEA1 is low, LR11 overexpression resulted in a twofold to threefold increase in colocalization showing its ability to potentially alter APP trafficking within early endosomes.

Because the overlap with EEA1 accounts for a minority of LR11, we sought to identify additional compartments, in which LR11 might function to influence APP processing. In addition to altering APP distribution in the endosomal system, another frequently observed effect of LR11 over-

expression is the formation of large vesicular structures ( $\sim 1\text{--}5\ \mu\text{m}$  diameter) in transfected cells (Fig. 3*B*). Many of these show colocalization of immunoreactivity for LR11, APP, and EEA1 (data not shown). These structures were not seen in control transfected HEK cells or in human brain, where LR11 immunoreactivity consistently shows a staining pattern of small cytoplasmic puncta (Fig. 1). These exaggerated vacuoles are clearly linked to overexpression of LR11, similar to structures seen under other conditions in which normal trafficking is disrupted by overexpression of key endosomal trafficking proteins (Komada et al., 1997; Ceresa et al., 2001).

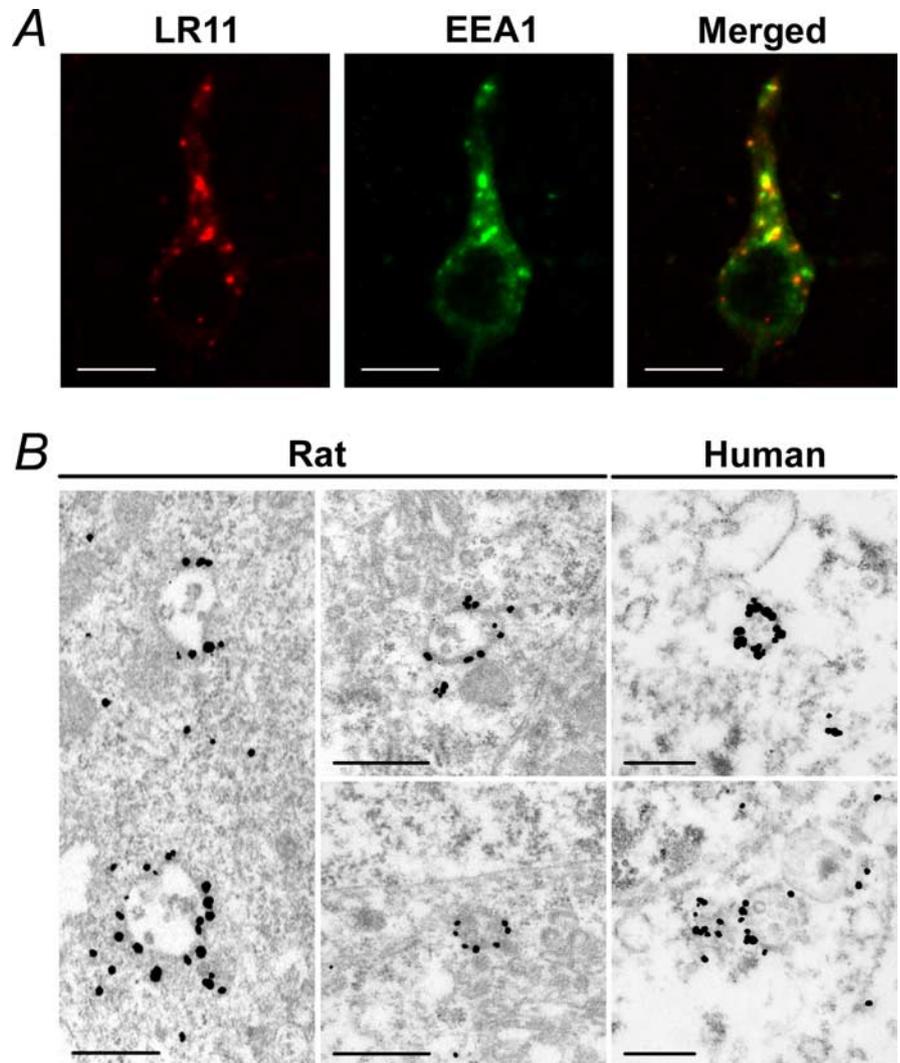
To more precisely define the normal subcellular localization of LR11, we first used double-labeling immunofluorescence on rat cortical sections. Using antibodies for LR11 and markers for subcellular compartments, we again found colocalization of LR11 with EEA1 (Fig. 4*A*). Next, we performed immunogold electron microscopy on rat and human brain sections. In both the rat and human cortex, immunogold particles revealed LR11 distribution within neuronal somata. Clusters of immunogold particles frequently concentrated around vesicular structures ( $\sim 300\text{--}500\ \text{nm}$  diameter) containing smaller intraluminal vesicles, identifying them as MVBs (Fig. 4*B*). LR11 staining using an antibody directed to the C terminal of the protein consistently labeled the cytoplasmic face of the limiting membranes of MVBs but not internal membranes, suggesting association with recycling membranes (Felder et al., 1990). These ultrastructural observations support the light microscopic findings showing LR11 distribution in endosomal compartments and suggest that a substantial portion of cellular LR11 is found in late endosomes. Our subcellular localization studies indicate a functionally important endosomal distribution of LR11, and the impact of LR11 on APP processing may be mediated through regulation of APP trafficking in endosomal compartments.

## Discussion

Our findings link the lipoprotein receptor LR11 to AD pathologically based on its highly consistent region-specific reduction in vulnerable regions of AD brains, and its potent effects on A $\beta$  production link it to a fundamental mechanism of AD pathogenesis. In agreement with previous studies (Motoi et al., 1999), we detected LR11 expression in neurons in the neocortex, limbic cortex, and cerebellum and less prominent expression in brain regions such as the thalamus and basal ganglia. In AD brains, LR11 immunoreactivity was selectively lost with remarkable consistency before cell death in neuronal populations of brain regions most severely affected in AD, such as the hippocampus, whereas it was preserved in unaffected brain regions such as the cerebellum. The consistency and magnitude of LR11 loss specifi-

cally in affected regions suggests that LR11 reduction may play an important role in AD pathogenesis.

The observed loss of LR11 in AD brains is particularly interesting in the context of its ability to reduce levels of extracellular A $\beta$  in experimental systems. The fact that LR11 overexpression also reduces levels of full-length APP and increases levels of CTFs, without affecting APP transcript levels, suggests that the reduction in A $\beta$  levels is attributable to effects on APP processing. Several attempts to identify altered clearance of extracellular A $\beta$  based on LR11 expression have yielded negative results (K. Offe, data not shown). The proteolytic processing of APP depends on its exposure to three secretase activities, each with its own complex subcellular distribution. ADAM10, a metalloprotease shown to exhibit  $\alpha$ -secretase activity is found primarily at the plasma membrane (Lammich et al., 1999) in accordance with previous reports of  $\alpha$ -secretase activity at this site (Sisodia, 1992). An aspartyl protease named BACE ( $\beta$ -site APP cleaving enzyme) has been identified as the primary  $\beta$ -secretase in the brain (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999), and it localizes mainly to the trans-Golgi network and endosomes (Vassar et al., 1999; Huse et al., 2000; Walter et al., 2001; Yan et al., 2001; Kinoshita et al.,



**Figure 4.** LR11 is localized to the endosomal system in the brain. *A*, Endogenous LR11 in a rat cortical neuron shows intracellular punctate staining (red) that colocalizes with EEA1 staining (green), as indicated by yellow staining in the merged image. *B*, immunogold electron microscopy of the rat and human cortex shows LR11 labeling on MVBs. Scale bars: *A*, 10  $\mu\text{m}$ ; *B*, 500 nm.

2003). The third secretase activity,  $\gamma$ -secretase, responsible for the final cleavage that releases A $\beta$ , is mediated by a large multiprotein complex, which includes presenilin-1 (De Strooper et al., 1998; Wolfe et al., 1999) (for review, see Iwatsubo, 2004). The intracellular localization, maturation, and trafficking of  $\gamma$ -secretase is particularly complex, but its functional distribution includes activity in endosomal compartments (Lah and Levey, 2000). Because of this complex distribution of the secretases in various subcellular compartments, the trafficking of APP to these compartments is of great importance in regulating the production of A $\beta$ . In addition, regulation of intracellular traffic in endosomal/lysosomal compartments also plays a crucial role in the degradation of APP and A $\beta$ .

The colocalization of LR11 with APP in early endosomes suggests that LR11 regulates APP processing through effects on its trafficking in endosomal/lysosomal compartments. Endosomes have been shown to be a major site of A $\beta$  generation, and A $\beta$  produced in endosomes can subsequently be recycled to the cell surface where it is secreted (Koo and Squazzo, 1994). Disruption of the endosomal system, specifically the enlargement of early endosomes, is one of the earliest neuronal changes seen in AD brains and occurs before A $\beta$  deposition (Cataldo et al., 1996, 1997, 2000; Troncoso et al., 1998). LR11 expression strongly alters APP trafficking within early endosomes, dramatically increasing colocalization of APP and EEA1 when LR11 is overexpressed (Fig. 3). However, although the effect is substantial, distribution in EEA1-positive early endosomes only accounts for a portion of the cellular pools of LR11 and APP, and additional details of the role of LR11 in regulating endosomal/lysosomal trafficking of APP remain to be defined.

Our immunogold electron microscopic data reveal a strong association of endogenous LR11 with MVBs in the rat and human cortex, an intermediate compartment of the endosomal/lysosomal system. Recent evidence identifies MVBs as a key intracellular compartment in A $\beta$  metabolism. Although  $\beta$ - and  $\gamma$ -secretases have not specifically been identified in these structures, immunoelectron microscopic studies have localized A $\beta$ 1–42 and oligomeric A $\beta$  to MVBs in the human brain and Tg2576 transgenic mice (Takahashi et al., 2002, 2004), and both A $\beta$  and APP CTFs were localized to MVBs in APP/PS1 double-transgenic mice (Langui et al., 2004). Aside from these links to A $\beta$  metabolism, MVBs play a key role in sorting internalized receptors, which proceed from MVBs to lysosomes for degradation (Futter et al., 1996).

Based on its localization to MVBs, its effects on endosomal APP trafficking, and its ability to regulate A $\beta$  levels, we propose that LR11 modulates APP processing and A $\beta$  production by enhancing the flow of APP and APP CTFs toward MVBs. Trafficking of APP and APP CTFs to MVBs rather than recycling endosomal compartments would alter their interactions with secretases and reduce A $\beta$  secretion into the extracellular space. This effect of LR11 is consistent with both the observed accumulation of CTFs in cell extracts and the reduction in A $\beta$  in the conditioned medium. Such a trafficking function for LR11 is supported by its unique multidomain structure, which suggests a potential role as an intracellular sorting receptor (Jacobsen et al., 1996). In addition to motifs common to LDLR family members, LR11 contains a Vps10p-homology domain and a GGA-binding domain, both of which are usually involved in trafficking from the Golgi to the endosomal/lysosomal system (Marcusson et al., 1994; Jacobsen et al., 1996, 2002; Yamazaki et al., 1996; Puertollano et al., 2001). Additional studies will need to address whether the interaction between

LR11 and APP occurs in the Golgi to endosome pathway or by altering the trafficking of APP after delivery and internalization from the cell surface.

Connections between sporadic AD and lipid metabolism have become increasingly apparent. These include the robust genetic susceptibility conferred by the *APOE*  $\epsilon$ 4 allele as well as epidemiological studies showing the potential effect of certain cholesterol-lowering drugs on disease risk (Wolozin et al., 2000). Our studies of the ApoE receptor LR11 have now established that (1) neuronal LR11 expression is diminished in patients with AD (Scherzer et al., 2004), (2) there is selective loss of LR11 in regions that are vulnerable to AD pathology, (3) endogenous brain LR11 concentrates in MVBs, (4) LR11 influences APP trafficking in endosomes, and (5) LR11 expression inversely correlates with A $\beta$  levels. These findings echo results from the study of LRP, which established strong associations between this ApoE receptor and AD (Kang et al., 1997, 2000; Ulery et al., 2000; Pietrzik et al., 2002). Although additional research is needed, the identification of two distinct ApoE receptors, which appear to regulate amyloidogenesis by influencing intracellular trafficking of APP suggests that this represents a generally important mechanism in the pathogenesis of common sporadic forms of AD.

Collectively, our findings have important implications for AD pathogenesis. The ability of LR11 to lower A $\beta$  levels along with the dramatic loss of LR11 in vulnerable brain regions in AD suggest that changes in LR11 promotes amyloid deposition and may play a central role in disease pathogenesis. Shortly after submission of this study, another report was published, in which interactions between LR11 (alternatively referred to as sorLA) and APP were examined (Andersen et al., 2005). This study corroborated our previous observation that LR11 protein is lost in the brains of AD patients. In addition, Anderson et al. (2005) offer nice data indicating interactions between LR11 and APP. The few relatively minor inconsistencies between our studies, including differences in subcellular localization of the LR11–APP interactions, may be attributable to the cell types used and effects introduced by overexpression of target proteins. Importantly, Anderson et al. (2005) reach the same fundamental conclusion that interactions with LR11 produce significant changes in APP processing and amyloid production. Together, the available data support an important role for LR11 in AD pathogenesis and identify this receptor as a potential novel therapeutic target for treatment of late-onset sporadic AD.

## References

- Andersen OM, Reiche J, Schmidt V, Gotthardt M, Spoelgen R, Behlke J, von Arnim CA, Breiderhoff T, Jansen P, Wu X, Bales KR, Cappai R, Masters CL, Gliemann J, Mufson EJ, Hyman BT, Paul SM, Nykjaer A, Willnow TE (2005) Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. *Proc Natl Acad Sci USA* 102:13461–13466.
- Cataldo AM, Hamilton DJ, Barnett JL, Paskevich PA, Nixon RA (1996) Properties of the endosomal–lysosomal system in the human central nervous system: disturbances mark most neurons in populations at risk to degenerate in Alzheimer's disease. *J Neurosci* 16:186–199.
- Cataldo AM, Barnett JL, Pieroni C, Nixon RA (1997) Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer's disease: neuropathologic evidence for a mechanism of increased  $\beta$ -amyloidogenesis. *J Neurosci* 17:6142–6151.
- Cataldo AM, Peterhoff CM, Troncoso JC, Gomez-Isla T, Hyman BT, Nixon RA (2000) Endocytic pathway abnormalities precede amyloid beta deposition in sporadic Alzheimer's disease and Down syndrome: differential effects of APOE genotype and presenilin mutations. *Am J Pathol* 157:277–286.
- Ceresa BP, Lotscher M, Schmid SL (2001) Receptor and membrane recycling

- cling can occur with unaltered efficiency despite dramatic Rab5(q79l)-induced changes in endosome geometry. *J Biol Chem* 276:9649–9654.
- Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261:921–923.
- De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 391:387–390.
- Felder K, Miller K, Moehren G, Ullrich A, Schlessinger J, Hopkins CR (1990) Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. *Cell* 61:623–634.
- Futter CE, Pearse A, Hewlett LJ, Hopkins CR (1996) Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *J Cell Biol* 132:1011–1023.
- Hampe W, Riedel IB, Lintzel J, Bader CO, Franke I, Schaller HC (2000) Ectodomain shedding, translocation and synthesis of SorLA are stimulated by its ligand head activator. *J Cell Sci* 113:4475–4485.
- Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, Roberts GW, Masters CL, Dotti CG, Unsicker K, Beyreuther K (1997) Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. *Nat Med* 3:1016–1020.
- Hirayama S, Bujo H, Yamazaki H, Kanaki T, Takahashi K, Kobayashi J, Schneider WJ, Saito Y (2000) Differential expression of LR11 during proliferation and differentiation of cultured neuroblastoma cells. *Biochem Biophys Res Commun* 275:365–373.
- Huse JT, Pijak DS, Leslie GJ, Lee VM, Doms RW (2000) Maturation and endosomal targeting of beta-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease beta-secretase. *J Biol Chem* 275:33729–33737.
- Iwatsubo T (2004) The gamma-secretase complex: machinery for intramembrane proteolysis. *Curr Opin Neurobiol* 14:379–383.
- Jacobsen L, Madsen P, Moestrup SK, Lund AH, Tommerup N, Nykjaer A, Sottrup-Jensen L, Gliemann J, Petersen CM (1996) Molecular characterization of a novel human hybrid-type receptor that binds the alpha2-macroglobulin receptor-associated protein. *J Biol Chem* 271:31379–31383.
- Jacobsen L, Madsen P, Jacobsen C, Nielsen MS, Gliemann J, Petersen CM (2001) Activation and functional characterization of the mosaic receptor SorLA/LR11. *J Biol Chem* 276:22788–22796.
- Jacobsen L, Madsen P, Nielsen MS, Geraerts WP, Gliemann J, Smit AB, Petersen CM (2002) The sorLA cytoplasmic domain interacts with GGA1 and -2 and defines minimum requirements for GGA binding. *FEBS Lett* 511:155–158.
- Kang DE, Saitoh T, Chen X, Xia Y, Maslah E, Hansen LA, Thomas RG, Thal LJ, Katzman R (1997) Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurology* 49:56–61.
- Kang DE, Pietrzik CU, Baum L, Chevallier N, Merriam DE, Kounnas MZ, Wagner SL, Troncoso JC, Kawas CH, Katzman R, Koo EH (2000) Modulation of amyloid beta-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. *J Clin Invest* 106:1159–1166.
- Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT (2003) Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *J Cell Sci* 116:3339–3346.
- Komada M, Masaki R, Yamamoto A, Kitamura N (1997) Hrs, a tyrosine kinase substrate with a conserved double zinc finger domain, is localized to the cytoplasmic surface of early endosomes. *J Biol Chem* 272:20538–20544.
- Koo EH, Squazzo SL (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J Biol Chem* 269:17386–17389.
- Lah JJ, Levey AI (2000) Endogenous presenilin-1 targets to endocytic rather than biosynthetic compartments. *Mol Cell Neurosci* 16:111–126.
- Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F (1999) Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc Natl Acad Sci USA* 96:3922–3927.
- Languid D, Girardot N, El Hachimi KH, Allinquant B, Blanchard V, Pradier L, Duyckaerts C (2004) Subcellular topography of neuronal Abeta peptide in APPxPS1 transgenic mice. *Am J Pathol* 165:1465–1477.
- Marcusson EG, Horadzovsky BF, Cereghino JL, Gharakhanian E, Emr SD (1994) The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the VPS10 gene. *Cell* 77:579–586.
- Motoi Y, Aizawa T, Haga S, Nakamura S, Namba Y, Ikeda K (1999) Neuronal localization of a novel mosaic apolipoprotein E receptor, LR11, in rat and human brain. *Brain Res* 833:209–215.
- Okuizumi K, Onodera O, Namba Y, Ikeda K, Yamamoto T, Seki K, Ueki A, Nanko S, Tanaka H, Takahashi H, Oyanagi K, Mizusawa H, Kanazawa I, Tsuji S (1995) Genetic association of the very low density lipoprotein (VLDL) receptor gene with sporadic Alzheimer's disease. *Nat Genet* 11:207–209.
- Pietrzik CU, Busse T, Merriam DE, Weggen S, Koo EH (2002) The cytoplasmic domain of the LDL receptor-related protein regulates multiple steps in APP processing. *EMBO J* 21:5691–5700.
- Puertollano R, Aguilar RC, Gorshkova I, Crouch RJ, Bonifacino JS (2001) Sorting of mannose 6-phosphate receptors mediated by the GGAs. *Science* 292:1712–1716.
- Scherzer CR, Offe K, Gearing M, Rees HD, Fang G, Heilman CJ, Schaller C, Bujo H, Levey AI, Lah JJ (2004) Loss of apolipoprotein E receptor LR11 in Alzheimer disease. *Arch Neurol* 61:1200–1205.
- Selkoe DJ (2000) Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. *Ann NY Acad Sci* 924:17–25.
- Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 81:741–766.
- Selkoe DJ, Podlisky MB, Joachim CL, Vickers EA, Lee G, Fritz LC, Oltersdorf T (1988) Beta-amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and non-neural tissues. *Proc Natl Acad Sci USA* 85:7341–7345.
- Sinha S, Anderson JP, Barbour R, Basl GS, Caccavello R, Davis, D, Doan M, Dovey HF, Frigon N, Hong J, Jacobson-Croak K, Jewett N, Keim P, Knops J, Lieberburg I, Power M, Tan H, Tatsuno G, Tung J, Schenk D, et al. (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature* 402:537–540.
- Sisodia SS (1992) Beta-amyloid precursor protein cleavage by a membrane-bound protease. *Proc Natl Acad Sci USA* 89:6075–6079.
- Strittmatter WJ, Weisgraber KH, Huang DY, Dong LM, Salvesen GS, Pericak-Vance M, Schmechel D, Saunders AM, Goldgaber D, Roses AD (1993) Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. *Proc Natl Acad Sci USA* 90:8098–8102.
- Taira K, Bujo H, Hirayama S, Yamazaki H, Kanaki T, Takahashi K, Ishii I, Miida T, Schneider WJ, Saito Y (2001) LR11, a mosaic LDL receptor family member, mediates the uptake of ApoE-rich lipoproteins in vitro. *Arterioscler Thromb Vasc Biol* 21:1501–1506.
- Takahashi RH, Milner TA, Li F, Nam EE, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK (2002) Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. *Am J Pathol* 161:1869–1879.
- Takahashi RH, Almeida CG, Kearney PF, Yu F, Lin MT, Milner TA, Gouras GK (2004) Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain. *J Neurosci* 24:3592–3599.
- Troncoso JC, Cataldo AM, Nixon RA, Barnett JL, Lee MK, Checler F, Fowler DR, Smialek JE, Crain B, Martin LJ, Kawas CH (1998) Neuropathology of preclinical and clinical late-onset Alzheimer's disease. *Ann Neurol* 43:673–676.
- Ulery PG, Beers J, Mikhailenko I, Tanzi RE, Rebeck GW, Hyman BT, Strickland DK (2000) Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer's disease. *J Biol Chem* 275:7410–7415.
- Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 286:735–741.
- Volpicelli LA, Lah JJ, Levey AI (2001) Rab5-dependent trafficking of the m4 muscarinic acetylcholine receptor to the plasma membrane, early endosomes, and multivesicular bodies. *J Biol Chem* 276:47590–47598.

- Walter J, Fluhrer R, Hartung B, Willem M, Kaether C, Capell A, Lammich S, Multhaup G, Haass C (2001) Phosphorylation regulates intracellular trafficking of beta-secretase. *J Biol Chem* 276:14634–14641.
- Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. *Nature* 398:513–517.
- Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G (2000) Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Arch Neurol* 57:1439–1443.
- Xu H, Sweeney D, Wang R, Thinakaran G, Lo AC, Sisodia SS, Greengard P, Gandy S (1997) Generation of Alzheimer beta-amyloid protein in the trans-Golgi network in the apparent absence of vesicle formation. *Proc Natl Acad Sci USA* 94:3748–3752.
- Yamazaki H, Bujo H, Kusunoki J, Seimiya K, Kanaki T, Morisaki N, Schneider WJ, Saito Y (1996) Elements of neural adhesion molecules and a yeast vacuolar protein sorting receptor are present in a novel mammalian low density lipoprotein receptor family member. *J Biol Chem* 271:24761–24768.
- Yan R, Bienkowski MJ, Shuck ME, Miao H, Tory MC, Pauley AM, Brashier JR, Stratman NC, Mathews WR, Buhl AE, Carter DB, Tomasselli AG, Parodi LA, Heinrichson RL, Gurney ME (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. *Nature* 402:533–537.
- Yan R, Han P, Miao H, Greengard P, Xu H (2001) The transmembrane domain of the Alzheimer's beta-secretase (BACE1) determines its late Golgi localization and access to beta-amyloid precursor protein (APP) substrate. *J Biol Chem* 276:36788–36796.