

LIPOPROTEIN RECEPTOR-RELATED PROTEIN IN BRAIN AND IN CULTURED NEURONS OF MICE DEFICIENT IN RECEPTOR-ASSOCIATED PROTEIN AND TRANSGENIC FOR APOLIPOPROTEIN E4 OR AMYLOID PRECURSOR PROTEIN

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Abstract—The role of the receptor-associated protein in controlling the expression of the low-density lipoprotein receptor-related protein was analysed in brain and in cultured neurons of receptor-associated protein $-/-$ mice. In addition, the effect of two important ligands of lipoprotein receptor-related protein in brain, i.e. apolipoprotein E and amyloid precursor protein, was examined by crossing the receptor-associated protein $-/-$ mice with transgenic mice overexpressing these proteins specifically in neurons. The immunohistochemical localization of lipoprotein receptor-related protein and receptor-associated protein in wild-type mouse brain was demonstrated to be congruent over all structures, including the cortex and hippocampus. In primary hippocampal neurons, lipoprotein receptor-related protein was distributed somatodendritically and receptor-associated protein was concentrated perinuclearly. In hippocampal neurons from receptor-associated protein $-/-$ mice, lipoprotein receptor-related protein was redistributed over the cell body at the expense of the dendrites. In the absence of receptor-associated protein, maturation of lipoprotein receptor-related protein is slow, resulting in accumulation of the uncleaved 600,000 mol. wt precursor. Neither the added expression of apolipoprotein E4 nor that of amyloid precursor protein in cultured neurons influenced the maturation of lipoprotein receptor-related protein, in either the presence or absence of receptor-associated protein. This result shows that receptor-associated protein is not needed to allow co-expression of lipoprotein receptor-related protein with these ligands in neurons. Furthermore, the typical ramified neuronal morphology of cultured primary neurons and the histology and architecture of the brain were normal in receptor-associated protein $-/-$ mice and in all of the double transgenic mice. Finally, we demonstrated that the survival of receptor-associated protein $-/-$ hippocampal neurons was normal and unaffected by the genotype of the glial feeder cells, whether they were derived from wild-type mice or from mice deficient in receptor-associated protein or apolipoprotein E.

These results show that, despite the dramatic effect on maturation and cellular localization of lipoprotein receptor-related protein, the absence of receptor-associated protein did not result in any notable physiological, functional or morphological effects. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: transgenic mice, brain, hippocampal neurons, receptor-associated protein, lipoprotein receptor-related protein, apolipoprotein E.

The low-density lipoprotein receptor-related protein (LRP) is a typical member of the low-density lipoprotein receptor gene family, i.e. containing a single membrane-spanning receptor and composed of a large number of only a few structurally different elements or domains. LRP is the most multifunctional receptor known because it binds a curious diversity of ligands, including proteinases, proteinase inhibitors and their complexes, apolipoprotein E (ApoE)-enriched lipoproteins, lipoprotein lipase and many others.^{15,21,26} In contrast to other members of the family, LRP is essential for mouse embryogenesis, since targeted inactivation of the LRP gene is lethal in the second week of pregnancy.¹⁴

Co-expressed with these receptors is a 40,000 mol. wt protein named receptor-associated protein (RAP). Functionally, RAP is defined as either an endogenous ligand or a chaperone that binds with high affinity to multiple sites on LRP^{13,48} and megalin.^{19,32,49} RAP also binds tightly to the very-low-density lipoprotein receptor,² while interaction with the classical low-density lipoprotein receptor is weak.²⁷ RAP is retained in and retrieved to the endoplasmic

reticulum (ER) and probably functions by transient interaction with the receptors, proposed to confer or maintain inactive or protected ligand-binding sites.^{7,8,50,51} Mice deficient in RAP were phenotypically normal, while levels of LRP were reduced *in vivo*, but not in cultured embryonic fibroblasts derived from RAP $-/-$ mice, which was interpreted as evidence for tissue- and cell-specific requirements for RAP in receptor biosynthesis and transport.⁵¹ Only massive overexpression of ApoE in the fibroblast cultures reduced their LRP levels, corroborating the hypothesis that ApoE effectively competes for RAP binding sites on LRP.⁵¹

LRP and RAP mRNAs were ubiquitously but not identically expressed in mouse organs, including the central and peripheral nervous systems.^{6,22,23} The precise function of LRP in the brain is not clear as a major neuronal receptor for ApoE-containing lipoproteins, an aspect that has gained momentum since the identification of the ApoE4 allele as the major genetic risk factor in Alzheimer's disease (AD).^{9,36} Many studies have attempted to isolate a specific "ApoE4 effect" in either receptor binding or interactions with cells, i.e. neurite extension and outgrowth.^{4,11,31,33} Inhibition by external RAP and by antibodies to LRP indicated a possible involvement of LRP.^{16,29} Immunoreactive LRP, and many of its ligands, such as ApoE, α_2 -macroglobulin (A2M) and amyloid precursor protein (APP), are found in the diagnostic amyloid or senile plaques in AD brain.^{34,38,41} In addition to ApoE lipoproteins, LRP mediates endocytosis of specified secreted isoforms of APP²⁰ and of A2M.⁴² Since the amyloid

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Abbreviations: AD, Alzheimer's disease; A2M, α_2 -macroglobulin; ApoE, apolipoprotein E; APP, amyloid precursor protein; ER, endoplasmic reticulum; LRP, lipoprotein receptor-related protein; MAP2, microtubule-associated protein-2; RAP, receptor-associated protein.

peptides bind to A2M, its endocytosis by LRP provides a clearing pathway for the amyloid peptides from extracellular space.³⁰ In addition to the functional connection, the encoding genes are genetically implicated in the pathogenesis of AD, as it is well known that mutations in the APP gene cause early-onset AD and that the ApoE4 allele is the most common genetic risk factor for AD. The recently reported association of the LRP gene on chromosome 12q12 to AD is yet to be functionally clarified,^{46,47} while the most recent addition, i.e. the A2M gene,⁵ remains to be conferred. Undoubtedly, this functional cluster of genes constitutes an important area of future research in normal development of the CNS and in pathological conditions such as AD.

The cloning of the human genes coding for LRP and RAP^{44,45} has led to further genetic analysis in humans by detecting mutations.^{46,47} Experimentally, we approached the problem in mice by inactivation of the RAP gene, which confirmed⁵⁰ that the absence of RAP did not cause major phenotypic problems. The only observation, as yet unreported, was a problem with breeding which became evident in our RAP $-/-$ breeding stock. Nevertheless, the normal development of RAP $-/-$ mice allowed us to study the regulation of expression of LRP in adult brain and in cultured hippocampal neurons by immunohistochemistry and immunofluorescence, which is described here. Primary hippocampal neurons survived and differentiated normally without endogenous expression of RAP, despite an important reduction in the levels of mature LRP. Transgenic mice that overexpress specifically in neurons, either human ApoE4 or human APP with a clinical AD mutation, displayed normal LRP expression, distribution and maturation. In addition, the neuronal expression of ApoE4 and APP did not aggravate the effect of RAP deficiency on LRP maturation. Survival of primary cultured neurons was unaffected by their genotype or by that of the glial feeder layer used, unlike reported results.²⁹

EXPERIMENTAL PROCEDURES

Antibodies

Affinity-purified goat antiserum (R777) specific for the 515,000 mol. wt subunit of LRP was generously provided by D. Strickland (American Red Cross, Rockville, MD, U.S.A.). A polyclonal antiserum against the 85,000 mol. wt subunit of LRP and the 600,000 mol. wt precursor form was prepared by immunizing rabbits with a synthetic peptide corresponding to the carboxyl terminus of the cytoplasmic domain (residues 4513–4525) conjugated to KLH. Rabbit antiserum against mouse RAP was kindly provided by Masayuki Ozawa (Kagoshima, Japan). Antibodies against ApoE and synaptophysin were purchased from Dako (Glostrup, Denmark) and monoclonal antibody against microtubule-associated protein-2 (MAP2) from Sigma (St Louis, MO, U.S.A.).

Animals

Mice deficient in RAP were generated with a construct based on the mouse RAP gene⁴⁵ by homologous recombination in ES cells according to standard procedures,⁴⁰ and their characterization will be described elsewhere. Offspring were crossed into the C57Bl/6 background and the mice used in the current experiments were of generations 4 and 5. The APP transgenic mice were generated with a modified mouse thy.1 gene construct²⁴ containing the human APP695 cDNA carrying the London mutation (V717I).²⁵ The transgenic ApoE4 mice were generated using the same modified mouse thy.1 gene promoter with the human ApoE4 gene embedded (unpublished results). All mice were housed in the same room in the animal house with free access to food and water, with a light cycle from 07.00 to 20.00, at 21°C and 60% relative humidity. The mice were regularly

monitored for health and behavior, and all experimental procedures were supervised by a veterinary doctor and performed according to the Flemish, Belgian, European and international standards and regulations.

Analysis of lipoprotein receptor-related protein and receptor-associated protein

For western blotting, frozen brain tissue was mechanically homogenized in 3 ml buffer containing 250 mM sucrose, 5 mM Tris, 1 mM EGTA and a mixture of proteinase inhibitors.²⁴ After clearing (2500 × g, 10 min), the supernatant was centrifuged (100,000 × g, 3 h) and pellets were resuspended in phosphate-buffered saline containing proteinase inhibitors. Samples were denatured by the addition of sodium dodecyl sulfate (2.3% final concentration), boiled for 10 min and reduced with 2-mercaptoethanol (1% final concentration). After separation by homogeneous 4% or 4–20% gradient Tris–glycine polyacrylamide gel electrophoresis, proteins were transferred electrophoretically (Hybond-C membranes, Amersham). Inactivation with fat-free milk was followed by sequential incubation with the specified primary antiserum and the specified secondary antiserum, and immune complexes were visualized (ECL, Amersham).

Immunocytochemical analysis of LRP and RAP was performed on primary hippocampal neurons cultured for 18–21 days. Cells were fixed in methanol for 10 min and incubated in blocking buffer, i.e. 2% fetal calf serum, 2% bovine serum albumin and 0.2% fish-skin gelatin, for 10 min. After incubation with specified primary antibodies, cells were washed and treated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G or rhodamine isothiocyanate-coupled goat anti-rabbit immunoglobulin G for 1 h at room temperature, rinsed and mounted.

Specimens were viewed through a Nikon Diaphot 300 connected to the MRC1024 confocal microscope (BioRad, U.K.). Images were captured by LaserSharp (version 2.0) on a Compaq Prosignia 300 workstation and finally processed using Adobe Photoshop 4.0 (Adobe Systems, CA, U.S.A.).

Immunohistochemical analysis of LRP and RAP in the brain was performed after mice were perfused with saline and 1% paraformaldehyde in 0.07 M sodium phosphate buffer (pH 7). Brain was removed and stored in cold phosphate-buffered saline containing 20% sucrose. After embedding, sections (10 μm) were cut and stained with specified antibodies. Endogenous peroxidase activity was inactivated with 0.6% H₂O₂ in methanol/Tris-buffered saline, before sections were incubated with streptavidin–biotin complex and immune complexes were visualized with diaminobenzidine and H₂O₂.²³

Primary cultures of hippocampal neurons

Embryos at day 18 from wild-type, RAP $-/-$ and ApoE $-/-$ mice were isolated, and primary cultures prepared¹ from hippocampi dissected and dissociated with trypsin (15 min, 37°C). Cells were plated on to poly-lysine-coated dishes and coverslips at a density of 10⁶ per dish (35 mm). When cells were attached, the coverslips were grown in the presence of a confluent monolayer of cortical glia cells, isolated from the same embryos. The culture medium was a commercial neurobasal medium supplemented with B27 and 0.5 mM glutamine (Life Technologies S.A., Merelbeke, Belgium), while glial proliferation was inhibited by adding cytosine arabinoside (5 μM) to the medium after one day in culture.

RESULTS

Lipoprotein receptor-related protein and receptor-associated protein in primary neurons and brain of wild-type and receptor-associated protein $-/-$ mice

The localization of LRP and RAP in neurons of wild-type mice is totally different. LRP was located very similarly to MAP2, with fluorescence in and over the cell body and the dendrites, and no staining of the axon (Fig. 1A, B). Signals for RAP were weaker and present mainly within the perinuclear region (Fig. 1D). In neurons from RAP $-/-$ mice, this signal is absent, while LRP was redistributed to the cell body with almost negligible

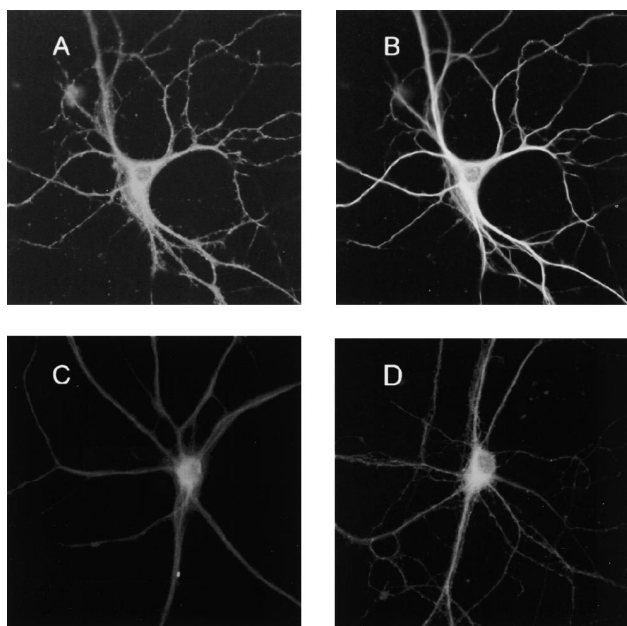


Fig. 1. Immunofluorescent localization of LRP and RAP protein in embryonic hippocampal neurons. Cultured hippocampal neurons from wild-type (A, B, D) and *RAP*^{-/-} (C) mice were immunolabeled after 18 days in culture, with antiserum R777 for LRP (A, C), with a monoclonal antibody for MAP2 (B) and for RAP (D). Magnification: $\times 200$.

staining along the dendrites (Fig. 1C; compare with Fig. 1A). The decrease in the total amount of the 600,000 mol. wt precursor and the 515,000 mol. wt subunit, recognized by the R777 antibody, in *RAP*^{-/-} hippocampal neurons compared with wild-type neurons, was measured by western blotting of total hippocampi (see below). The fluorescent signal obtained for LRP was granular or vesicular in aspect, as opposed to the smooth signal also obtained for MAP2 in *RAP*^{-/-} neurons, indicating that LRP remained contained in small vesicles, even when maturation and proteolytic processing were heavily compromised (see below).

Immunohistochemically, expression of LRP and RAP in brains of wild-type mice was evident in all regions, including the cortex, hippocampus, thalamus and hypothalamus. Reaction was most intense in neurons of the CA region (Fig. 2A) and in the cortex (Fig. 2C), with a granular staining pattern over the cell bodies and along the dendrites (Fig. 2C). Immune reaction was less intense in the dentate gyrus (Fig. 2B) and in the striatum, while even less was detectable over glial cells of the white matter and in astrocytes. The immunolocalization of RAP was essentially similar to that of LRP, demonstrating that RAP and LRP are co-expressed in the same cells in the brain (Fig. 2D–F), unlike in other tissues.^{22,23} This might simply reflect the fact that neurons are the primary cell type in the brain that express members of this receptor family. However, if the expression pattern of RAP is interpreted to reflect the combined expression of all receptors to which it can bind, then the results point to a direct functional relation of RAP to LRP in the brain, which is not evident in other organs, as discussed before.²³ The absence of RAP in the brain of *RAP*^{-/-} mice weakened the LRP signal considerably, but the tissue distribution remained similar to that in wild-type mice (Fig. 2G–I).

Lipoprotein receptor-related protein in brains of receptor-associated protein -/- and of apolipoprotein E4 and amyloid precursor protein transgenic mice

The levels of LRP mRNA were very similar, as measured by northern blotting, in the cerebrum and cerebellum of wild-type and *RAP*^{-/-} mice (results not shown), demonstrating the effect on LRP to be a post-translational event. The level of the 515,000 mol. wt subunit was considerably decreased in *RAP*^{-/-} mice compared with wild-type mice (Fig. 3A). Western blotting was used to quantify the 600,000 mol. wt LRP precursor and the 85,000 mol. wt transmembrane subunit resulting from cleavage by furin, in extracts of the cerebrum, cerebellum and hippocampus of wild-type and *RAP*^{-/-} mice. This revealed that, in the absence of RAP, the level of the 85,000 mol. wt LRP subunit was reduced to about 30% in *RAP*^{-/-} mice, while the level of the 600,000 mol. wt LRP precursor was increased about 2.5-fold (Fig. 3B, C). Proteolytic processing and maturation of LRP is thus confirmed to be markedly slowed down in the absence of RAP.^{50,51}

The hypothesis that co-expression of LRP with a ligand, in this case ApoE, would reduce its expression has been tested in cultured fibroblasts.⁵¹ Since the requirement for RAP in functional receptor expression appears to be cell or tissue specific, we have analysed overexpression in neurons as an important paradigm. The test included human ApoE4, as an important ligand of LRP in the brain (see Introduction) and, in addition, in the context of and in relation to the pathology in AD, we tested the potential of co-expressed APP on the maturation of LRP. This strategy was employed because, whereas APP is normally synthesized and secreted by neurons, ApoE is not, and both interact with LRP.^{3,20} Transgenic mice overexpressing the London mutant APP isoform were used, since the APP protein was expressed at high levels in this transgenic line.²⁵

The experiment was conducted by crossing *RAP*^{-/-} mice independently with two transgenic mouse strains, one expressing the human ApoE4 allele and the other human APP mutated at codon 717, i.e. valine to isoleucine, to reflect the London mutation.¹² In both transgenic mouse strains, expression of the transgene is controlled by using a construct based on the mouse thy-1 gene adapted to result in neuron-specific expression.²⁴ The phenotypic analysis of these transgenic mice is discussed elsewhere.²⁵ The dissected hippocampus and primary cultures of hippocampal neurons ApoE4 and APP/London transgenic mice were analysed by immunoblotting, as above. Neither the 600,000 mol. wt LRP precursor nor the 85,000 mol. wt processed LRP subunit was changed in concentration in extracts from brain or in primary neurons in either of these transgenic mice (Fig. 3B, C). F2 offspring of the double transgenic mice were genotyped and selected to be homozygously deficient in RAP and to be heterozygously transgenic for either ApoE4 or APP/London. Analysis of LRP in the brain did not reveal an additional aggravation of the situation relative to *RAP*^{-/-} mice with regard to the levels of the 600,000 mol. wt LRP precursor and its 85,000 mol. wt furin-derived product (Fig. 3B). Thus, co-expression of ApoE or APP did not appreciably influence LRP biosynthesis or maturation in either genotype, i.e. with or without RAP. The tissue-specific and ligand-dependent requirements for RAP in LRP biosynthesis and transport thereby appeared much more subtle than anticipated.

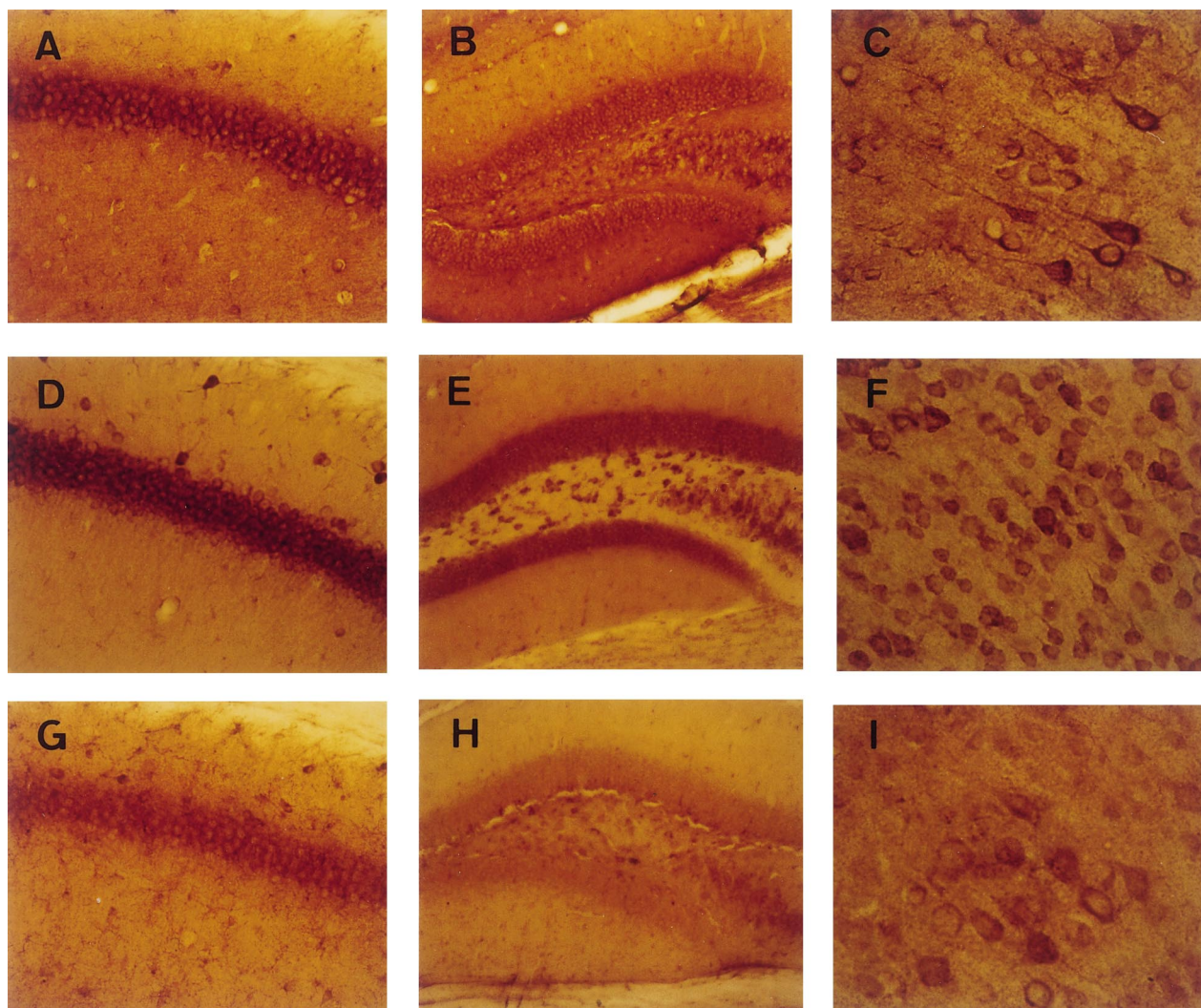


Fig. 2. Immunohistochemical localization of LRP and RAP protein in brains of wild-type and RAP^{-/-} mice. (A–C) Immunostaining for LRP with antiserum R777 in wild-type mice in the CA2 region (A; $\times 20$), dentate gyrus (B; $\times 10$) and cortex (C; $\times 40$). (D–F) Immunostaining for RAP in wild-type mice in the CA2 region (D; $\times 20$), dentate gyrus (E; $\times 10$) and cortex (F; $\times 40$). (G–I) Immunostaining for LRP with antiserum R777 in RAP^{-/-} mice in the CA2 region (G; $\times 20$), dentate gyrus (H; $\times 10$) and cortex (I; $\times 40$).

Survival of neurons is not affected by their genotype or by the genotype of co-cultured glial cells

Primary cultures of murine hippocampal neurons, plated in dishes without glial cells, normally survive for 10–14 days (Fig. 4). In co-cultures with glial cells, plated on to different glass coverslips, the neuron layers can survive for about one month (Fig. 4). To test whether either intracellular RAP or secreted ApoE lipoproteins, either directly or indirectly, affected neuronal survival and neurite outgrowth, neurons derived from wild-type or RAP^{-/-} mice were co-cultured with glial cells derived from mice that were deficient in either RAP or ApoE.

Survival of hippocampal neurons was largely unaffected by the absence of RAP (Fig. 4). Neurons died extensively from day 24 onwards and never survived longer than 33 days (Fig. 4). This was observed with both wild-type and RAP^{-/-} derived neurons, while it also proved to be independent of whether the neurons were co-cultured with glial cells derived from wild-type, RAP^{-/-} or ApoE^{-/-} mice (Fig. 4). The gross and fine morphology of all primary neurons in culture was very similar overall, and dendritic ramification was

independent of their genotype and also unaffected by the genotype of the co-cultured glial cells (Fig. 1; results not shown).

DISCUSSION

Mice were generated that lack a functional RAP gene to gain insight into the physiological role of this chaperone *in vivo*, specifically with regard to neuronal functioning and the brain. The RAP-deficient mice were phenotypically normal and fertile, confirming independent studies that focussed on liver and peripheral cholesterol metabolism.^{50,51} Here, we present results obtained in RAP^{-/-} mice, and additionally crossed with ApoE and with APP transgenic mice, and we concentrated on the brain and analysed primary cultures of hippocampal neurons derived from single and double transgenic mice.

Previous results from *in situ* hybridizations^{22,23} are now confirmed immunohistochemically, demonstrating that LRP and RAP mRNA and protein coincided in localization in murine brain, embryonic or adult. The wide and abundant expression of LRP in the brain and in peripheral neurons

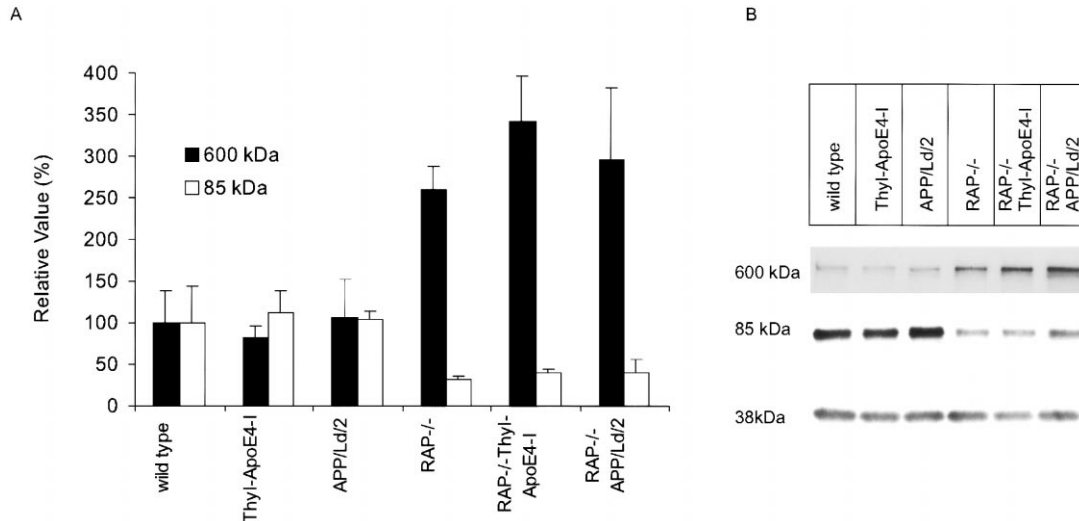


Fig. 3. Analysis of the 600,000 mol. wt precursor and the 515,000 and 85,000 mol. wt subunits of LRP in the brain of specified deficient and transgenic mice. (A) Western blotting with the R777 antibody, recognizing the 600,000 and 515,000 mol. wt forms. Analysis of the brain of wild-type mice (lane 1) and RAP^{-/-} mice (lane 2). (B) Western blotting with the C-terminal antibody (LRP-F36), recognizing the 600,000 mol. wt precursor and the 85,000 mol. wt subunit. Synaptophysin was used as internal marker for quantitation and normalization after scanning and densitometric analysis. Analyses of the brain of wild-type (lane 1), ApoE4 (lane 2), APP/London (lane 3), RAP^{-/-} (lane 4), RAP^{-/-} ApoE4 (lane 5) and RAP^{-/-} APP/London (lane 6) mice are shown. (C) Histograms representing quantitation by densitometric scanning after western blotting normalized to the synaptophysin signal in each lane on the same blot. Three mice of each of the different genotypes indicated were analysed and the mean value (with S.E.M.) is shown relative to the content of the 85,000 mol. wt (black lanes) and 600,000 mol. wt (white lanes) LRP subunits in the brain of wild-type mice.

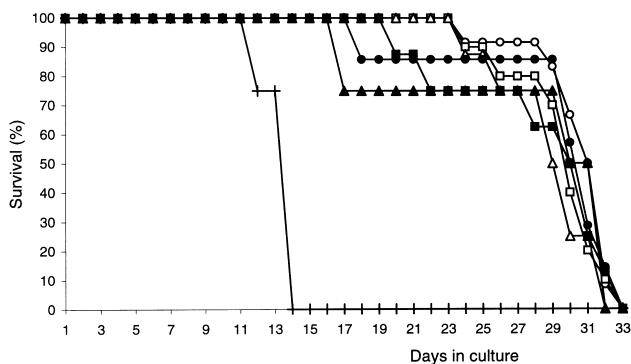


Fig. 4. Survival of primary cultures of hippocampal neurons derived from wild-type and RAP^{-/-} mice in co-culture with specified glial cells derived from wild-type, RAP^{-/-} or ApoE^{-/-} mice. (+) Wild-type neuron, no glia; (○) wild-type neuron, wild-type glia; (□) wild-type neuron, RAP^{-/-} glia; (△) wild-type neuron, ApoE glia; (●) RAP^{-/-} neuron, wild-type glia; (■) RAP^{-/-} neuron, RAP^{-/-} glia; (▲) RAP^{-/-} neuron, ApoE glia.

suggests an important function within the adult CNS,⁶ but the embryonal lethality following targeted inactivation of LRP¹⁴ prevented analysis of the brain in adult and ageing LRP^{-/-} mice. RAP^{-/-} mice were thought to offer an indirect route to this problem, but the present results demonstrated unequivocally that the functioning and regulation in neurons and in the brain *in vivo* are both more complex and more simple than was predicted from cellular studies. An additional burden of this system was introduced by crossing RAP^{-/-} mice with transgenic mice that we engineered to overexpress human ApoE4 or human APP specifically in neurons. Overexpression of these proteins in RAP^{-/-} neurons allowed analysis, both in the brain and in primary neuronal cultures, of the cellular expression, localization and maturation of LRP.

The most important outcome of these experiments was that, despite the dramatic effect on maturation and cellular localization of LRP, the absence of RAP did not result in any notable

physiological or functional effect, either morphological or functional. The overall expression pattern of LRP in the brain was normal, while RAP^{-/-} mice were functionally normal. In contrast to wild-type neurons, where LRP is located in and over the cell body and the dendrites, in RAP^{-/-} neurons, LRP is localized mainly within the perinuclear region, with almost negligible staining along the dendrites. This staining pattern and the results obtained by western blotting demonstrate that, in RAP^{-/-} neurons, the levels of the mature 85,000 and 515,000 mol. wt subunits are reduced, resulting in only a weak staining for LRP in the dendrites. LRP staining in the RAP^{-/-} neurons resembles RAP staining in wild-type neurons, suggesting that unprocessed LRP is retained in the ER instead of being transported to the cell surface. This is consistent with the data of Ko *et al.*,¹⁸ who demonstrated that a mutation in the furin cleavage site of LRP abolishes furin-mediated post-translational endoproteolysis of LRP, resulting in a retarded exit of LRP from the ER.

Additional overexpression in neurons of human ApoE4 and human APP did not affect LRP biosynthesis or maturation, either in the presence or in the absence of RAP. This convincingly demonstrated that the function of RAP as a chaperone during the biosynthesis of LRP did not include "permission" of co-expression of ligands such as ApoE and APP. This result further strengthens the notion that simple extrapolation from *in vitro* analysis of any cell type⁵¹ to neurons should be carefully examined and experimentally verified.

It was demonstrated that LRP binds only to the KPI-containing form of APP and not to the 695 isoform.²⁰ This suggests that, in neurons, only a very low amount of APP interacts with LRP, since the KPI-containing APP isoforms are expressed predominantly in astroglial cells, while the expression of the APP695 isoform predominates in neurons.¹⁰ Very recently, however, it was demonstrated that FE65, a cytosolic adaptor protein, contains two distinct protein interaction domains that interact with LRP and APP,³⁹ and both APP and FE65 co-localize in the ER/Golgi and possibly in

endosomes.³⁵ The binding of FE65 to the cytoplasmic tails of LRP and APP suggests that the London mutant APP isoform interacts with LRP in the same way as wild-type APP. These data indicate a potential general biochemical mechanism by which ligands for LRP could modulate the cellular processes leading to neurodegeneration. The fact that RAP is not needed for co-expression of LRP with ApoE or APP in neurons is therefore an important issue for any further study that approaches the involvement of LRP in AD.

The reported effects of exogenous and heterologous lipoproteins and particles on neurons and neurite outgrowth^{16,29,31,37} may involve LRP, as indicated by its inhibition by RAP and antibodies to LRP.²⁹ The present results demonstrated that neurons displaying less than one-third of the normal amount of mature LRP survived, differentiated and branched normally. Additional tests with co-cultures of glial cells with a different genotype, i.e. deficient in RAP or in ApoE, did not affect either wild-type or RAP $-/-$ neurons. The vital importance of glial cells for long-term survival of these neurons can therefore not be ascribed to secreted ApoE lipoprotein particles. Whether other LRP ligands and other receptors are (in)dispensable in this situation is being investigated. A potential candidate promoting neurite outgrowth is the wide-spectrum proteinase, cytokine and growth factor scavenger, A2M.^{17,28,43} Although many growth factors important in the brain bind to A2M, the question remains whether uptake by LRP is required, while production of A2M has not yet been demonstrated in mouse brain, in any of its constituent cells or in any condition at any age, a situation different from human brain and which is also under investigation.

One physiological problem observed with RAP $-/-$ mice, not yet reported, concerned their breeding performance, which was below expectation relative to and based on nearly a decade of experience with different knockout mouse strains, generated from the same ES cell line by the same technology and maintained in the same genetic backgrounds, i.e. 129 and

C57Bl, housed and fed identically.⁴⁰ The cause of this lower than standard reproduction of the RAP $-/-$ mice is currently being examined.

CONCLUSIONS

Our results underline the importance of RAP as a chaperone in the maturation process of LRP and, in turn, in determining its cellular localization in cultured neurons. However, at the same time, we demonstrate that this system is well buffered and displays a broad reserve in capacity of mature LRP, since the complement of only one-third of normal mature LRP results in normal survival and branching of hippocampal neurons. In addition, we demonstrate that the absence of RAP or ApoE in the glial support or feeder cells is of no importance for the same neuronal parameters in culture, corroborating the normal brain architecture and functional integrity of the brains of RAP $-/-$ and of ApoE $-/-$ mice. Additional overexpression in neurons of two important LRP ligands, i.e. human ApoE4 and human APP, did not affect the maturation of LRP, either in the presence or in the absence of RAP. These important conclusions and data demonstrate that neurons have specific rules for intracellular protein sorting and processing, and should be treated and studied directly, not by extrapolation.

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