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DISSERTATION

Titel der Dissertation

Regulation and differential functions of ApoER2 and VLDL receptor in Reelin signaling

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Doktorin der Naturwissenschaften (Dr. rer. nat.)

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Abstract

The Reelin signaling cascade is an important pathway in the development of the brain of vertebrates, guaranteeing proper lamination of cerebellum, cerebral cortex, and hippocampus. The matrix protein Reelin controls positioning of migrating neuroblasts by binding to two receptors on their cell surface, ApoER2 and VLDL receptor. Both receptors transmit the signal into the cell which results in phosphorylation of the intracellular adapter protein Dab1. To a certain extent, both receptors can compensate for each other, and only the loss of both results in a phenotype similar to that of *reeler* mice which lack funtional Reelin. Nevertheless, both receptors also have specific distinct functions, as corroborated by analyses of the subtle phenotypes displayed in mice lacking either ApoER2 or VLDL receptor. This study focusses on those functional differences between the two Reelin receptors. ApoER2 and VLDLR were studied and compared in terms of sorting within the plasma membrane, mechanism of endocytosis, Reelin internalization rate, intracellular traffic, degradation, and proteolytic processing. Moreover, regulation of ApoER2 and VLDL receptor levels by a liver X receptor mediated degradation pathway recently described for related receptors was investigated. Using a panel of chimeric receptors, composed of extracellular, transmembrane and intracellular domains of ApoER2 and VLDL receptor in diverse combinations, it was furthermore analysed if differential behaviour is structurally determined or based on specific sorting of the receptors to lipid raft or non-raft domains of the plasma membrane. The different fates of ApoER2 and VLDL receptor after Reelin binding which are demonstrated here could affect further signaling and might therefore constitute a mechanism for finetuning of the Reelin pathway. VLDL receptor, residing in the non-raft domain, internalizes and destines Reelin for degradation via the clathrin-coated pit/clathrin-coated vesicle/endosome pathway without being degraded to a significant extent. Binding of Reelin to ApoER2, resident of rafts, leads to the production of particular receptor fragments with specific functions of their own and to degradation of ApoER2 via lysosomes. These features contribute to a novel model that emphasizes negative feedback loops specifically mediated by ApoER2 and VLDL receptor, respectively.

Zusammenfassung

Der Reelin Signalweg gewährleistet in der Hirnentwicklung von Vertebraten die korrekte Schichtung des Cerebellums, des cerebralen Cortex und des Hippocampus. Das Matrix-Protein Reelin steuert dabei wandernde Neuroblasten, indem es an zwei ihrer Membranrezeptoren, ApoER2 und VLDL Rezeptor, bindet. Beide Rezeptoren übermitteln dieses Signal in die Zelle, wo es zu Phosphorilierung des Adapterproteins Dab1 führt. Bis zu einem gewissen Grad können ApoER2 und VLDL Rezeptor den Verlust des jeweils anderen kompensieren. Daher führt nur der Verlust beider Rezeptoren zum *Reeler* Phänotyp, der auch bei Mäusen mit fehlerhaftem Reelin Protein auftritt. Dennoch haben beide Rezeptoren zusätzliche individuelle Funktionen, wie die weniger ausgeprägten, aber spezifischen Phänotypen von ApoER2- oder VLDL Rezeptor-defizienten Mäusen vermuten lassen. Diese Arbeit konzentriert sich auf ebendiese funktionellen Unterschiede der Reelin Rezeptoren. ApoER2 und VLDL Rezeptor wurden in Hinblick auf Lokalisierung in der Plasmamembran, Endozytosemechanismus, zelluläre Reelin-Aufnahme, intrazelluläre Sortierung, Prozessierung und Abbau verglichen. Weiters wurde die Regulation der Rezeptormenge durch einen erst kürzlich beschriebenen Liver-X-Rezeptor-abhängigen Abbauweg untersucht. Mit Hilfe von chimären Rezeptoren aus den Extrazellulär-, Transmembran- und Intrazellulärdomänen von ApoER2 und VLDL Rezeptor wurden des Weiteren strukturelle Eigenschaften sowie Membranlokalisation innerhalb oder ausserhalb von Lipid Rafts als mögliche Ursachen für die rezeptorspezifischen Besonderheiten gegenübergestellt. Die hier demonstrierten, unterschiedlichen Wege von ApoER2 und VLDL Rezeptor nach der Bindung von Reelin könnten eine Feinabstimmung des Reelin Signalwegs darstellen. VLDL Rezeptor, welcher ausserhalb der Lipid Raft-Fraktion der Plasmamembran zu finden ist, internalisiert Reelin über den Clathrin-coated pit/Clathrin-coated vesicle/Endosom-Weg und führt es so dem Abbau zu, ohne selbst abgebaut zu werden. Bindung von Reelin an den Lipid Raft-assoziierten ApoER2 führt zur Produktion von spezifischen Rezeptorfragmenten und zum lysosomalen Abbau des Rezeptors. Anhand dieser Ergebnisse wurde ein neues Modell des Reelin Signalwegs entwickelt, welches spezifische Rückkopplungs-Effekte durch ApoER2 und VLDL Rezeptor miteinbezieht.

Contents

1 Introduction

1.1 The LDL receptor family in endocytosis and signaling

The low density lipoprotein (LDL) receptor family comprises several closely related transmembrane proteins which are involved in both molecular transport and signal transduction in a wide range of physiological functions in different tissues (reviewed in *May et al.*, 2007; *Nykjaer and Willnow*, 2002). The major domain pattern common to all members of this protein family can be found in LDL receptor relatives throughout a variety of eukaryotic multicellular species from C.elegans (*Yochem and Greenwald*, 1993) to rat (*Saito et al.*, 1994) and human (*Herz et al.*, 1988), which highlights the evolutionary conservation and importance of this ancient gene family.

Originally, members of this gene family were solely known for their endocytic function. First and foremost, the eponymous family member, LDL receptor (LDLR), plays an important role in cholesterol homeostasis since it binds lipoprotein particles from the bloodstream and mediates their uptake into and lysosomal degradation within cells of the liver and peripheral organs. It was this receptor, study of which first led to the understanding of the mechanism of receptor-mediated endocytosis (*Anderson*, 1977; *Brown and Goldstein*, 1986; *Goldstein et al.*, 1985). In 1985, Michael S. Brown and Joseph L. Goldstein were awarded the Nobel prize in Medicine for revealing the implications of the LDL receptor pathway on the regulation of cholesterol metabolism. Meanwhile, however, the functions of the LDL receptor family were extended to participation in signaling cascades by transmitting extracellular signals to intracellular adapter proteins. They play important additional roles in health and disease and are for example involved in regulation of neuronal migration in embryonic and adult brain (*Andrade et al.*, 2007; *Blake et al.*, 2008; *Herz et al.*, 2000), synaptic plasticity and memory (*D'Arcangelo*, 2005; *Qiu et al.*, 2002), development of Alzheimer's disease (*Ulery et al.*, 2000) and atherosclerosis (*Boucher et al.*, 2003), tumor progression (*Liu et al.*, 2000), and digit differentiation (*Johnson et al.*, 2005).

1.1.1 Structural and functional features of LDL receptor relatives

To date, seven core members of the mammalian LDL receptor family have been reported. In addition to LDLR itself, these are apolipoprotein E receptor 2 (ApoER2, also referred to as LRP8), very low density lipoprotein receptor (VLDLR), the LDLR-related protein (LRP, also referred to as LRP1), LRP1b, MEGF7 (also referred to as LRP4) and Megalin (also referred to as gp330 or LRP2). More distantly related members of the family which do not completely fit into the tight structural definition of the family core, are LRP5, LRP6, and SorLA (also referred to as LR11). The core members of the receptor family share a common modular structure which is composed of five distinct domains: (i) an extracellular ligand binding domain, (ii) an epidermal growth factor (EGF) precursor homology domain, (iii) an O-linked sugar domain, (iv) a transmembrane domain, and (v) a cytoplasmic domain (reviewed in *Gent and Braakman*, 2004) (Fig. 1.1).

The extracellular ligand binding domain is composed of multiple LDL receptor type A (LA) repeats, each of which consists of approximately 40 amino acids including six cysteine residues forming disulfide bonds. In addition, each LA repeat comprises a conserved sequence rich in acidic amino acids which can bind a calcium ion (*Fass et al.*, 1997). This is essential for functionality of the extracellular domain since the incorporation of calcium is necessary for correct disulfide bond formation and maintenance of the structural integrity of the domain (*Atkins et al.*, 1998; *Blacklow and Kim*, 1996) and is a prerequisite for ligand binding (*Daniel et al.*, 1983; *Schneider et al.*, 1979). Residues involved in binding and coordination of the ion become surface-exposed, thereby creating a characteristic site of negative surface potential which can establish electrostatic interactions with complementary charged surfaces on the respective ligands (*Fisher et al.*, 2006).

Prevention of premature ligand association during receptor synthesis and trafficking to the plasma membrane is an essential task, since cells which pack and export lipoproteins, the major binding partners for the gene family, also express LDLR and its relatives (*Twisk et al.*, 2000). It was shown that the receptor-associated protein (RAP), an endoplasmic reticulum (ER) resident protein of 39 kDa, is at neutral pH able to bind the LA repeats of all core members of the LDLR family examined to date (*Battey et al.*, 1994; *Iadonato et al.*, 1993; *Kounnas et al.*, 1992; *Medh et al.*, 1995; *Stockinger et al.*, 1998). In the cell, RAP binding blocks any unspecific and undesirable interactions with other ligands and thereby facilitating receptor trafficking within the early secretory pathway and delivery to the cell

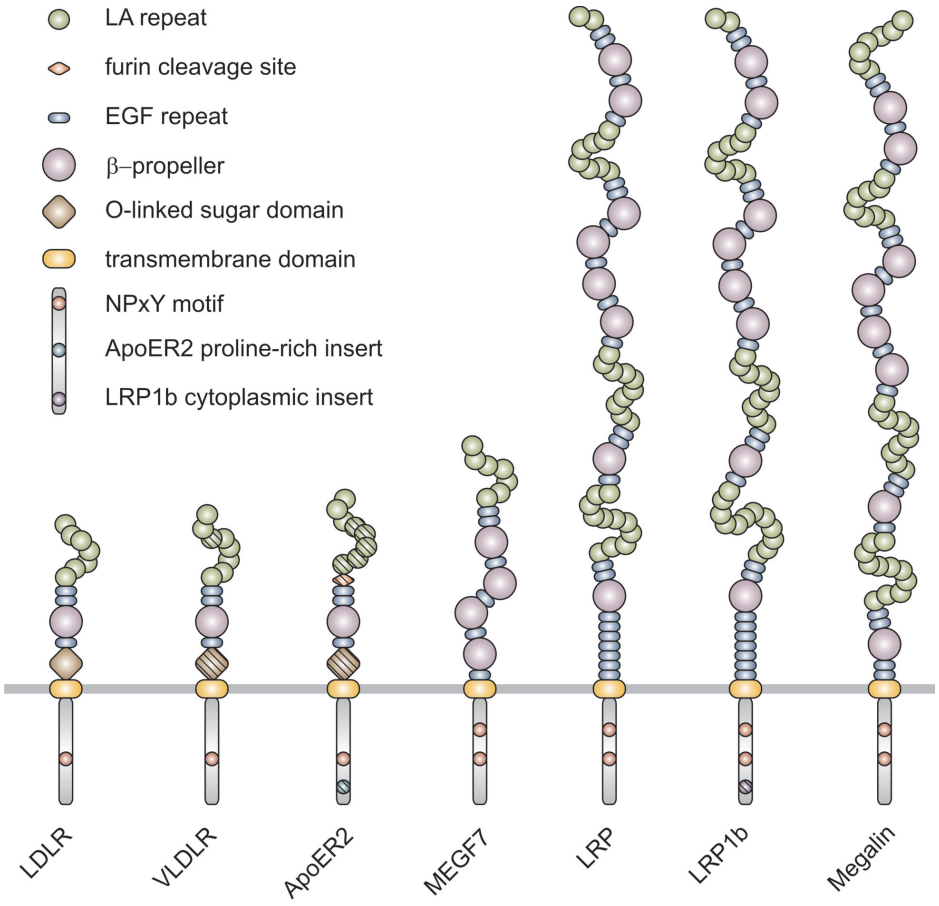

Figure 1.1 The modular structure of LDL receptor family members. These receptors contain five distinct modules: LA-repeats, EGF precursor homology domains, O-linked sugar domain, transmembrane domain, and a cytoplasmic tail harboring one or more NPxY motifs. Differentially spliced structures are depicted hatched.

surface (*Bu et al.*, 1995; *Willnow et al.*, 1996b). When the RAP-receptor complex reaches the Golgi apparatus, the low pH condition there induces dissociation of the binding partners (*Bu et al.*, 1995). In addition to its function as an escort protein, RAP may also assist in the folding of the receptors (*Bu and Rennke*, 1996)

In all LDLR family core members, the ligand binding domain is followed by one or more EGF precursor homology domains, also referred to as LDL receptor type B repeats. These modules include cysteine-rich growth factor repeats of about 40 amino acids (EGF-like repeats) which are flanking the major components of these domains, six YWTD motifs (containing the consensus tetrapeptide Tyr-Trp-Thr-Asp). These motifs enable the buildup of a six-bladed β-propeller structure (*Jeon et al.*, 2001; *Springer*, 1998) (Fig. 1.2) which is implicated in the

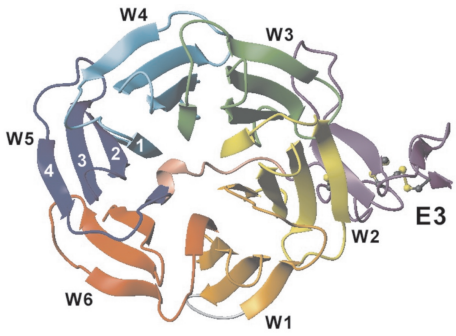

Figure 1.2 Ribbon representation of the six-bladed β-propeller structure and adjacent C-terminal EGF-like module (E3) of the LDL receptor.. View down the central axis of the propeller. Each blade of the propeller consists of four antiparallel β-strands. Each second innermost strand (labeled 2) contains a YWTD consensus sequence. (Taken from *Jeon et al.*, 2001).

pH-dependent dissociation of receptor and ligand in endosomes (*Davis et al.*, 1987; *Rudenko et al.*, 2002).

LDL binding to LDL receptor essentially occurs at ligand binding repeats 4 and 5. By structural analysis it was shown that at endosomal pH, these two repeats intramolecularly fold back toward the β-propeller (*Rudenko et al.*, 2002). This results in a closed conformation not able to sustain LDL binding (Fig. 1.3). Therefore, the current model implies that this competition for LA repeats is responsible for the release of bound ligands from LDL receptor family members at low pH in the endosomal compartment.

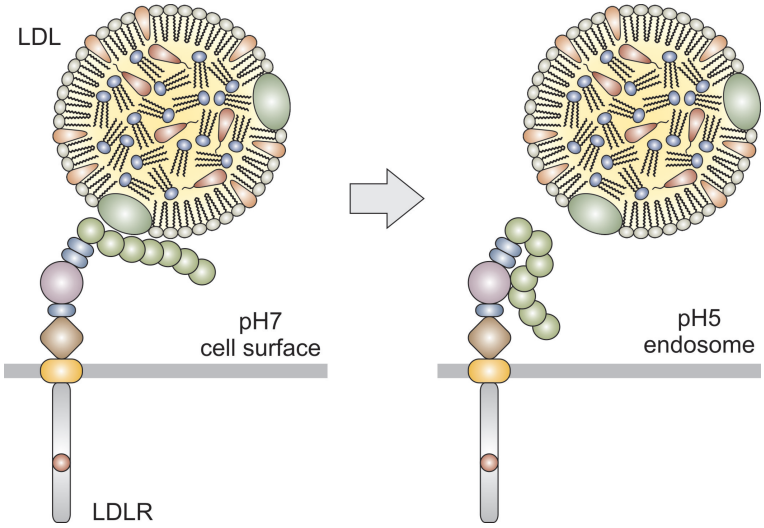

Figure 1.3 Model for the pH-dependent release of LDL from its receptor. Ligand binding modules 3-7 participate in binding of LDL at neutral pH, whereas modules 4 and 5 are engaged in intramolecular contacts with the YWTD β-propeller domain at endosomal pH, leading to release of the lipoprotein.

Adjacent to the EGF precursor homology domain, the so-called O-linked sugar domain (OLSD) can be found in some of the receptor family members, i.e. LDLR, and splice variants of VLDLR and ApoER2. This short region is rich in serine and threonine residues

which can undergo O-linked glycosylation. As it was shown, receptors glycosylated in this way are less prone to proteolytic processing (*Magrané et al.*, 1999; *May et al.*, 2003) which could imply differential biological functions of distinct receptor variants.

Following a single transmembrane domain of about 20 amino acids, all family members contain a short cytoplasmic domain harboring one or more NPXY motifs (Asp-Pro-Xxx-Tyr, where Xxx denotes any amino acid) (*Chen et al.*, 1990). As they constitute the binding sites for intracellular adapter proteins (*Chen et al.*, 1990), these motifs are not only essential for receptor-mediated signal transduction but also for internalization of ligands.

For clathrin-mediated endocytosis of membrane proteins, recruitment to clathrin-coated pits in the plasma membrane by binding of adapter proteins is essential. These specialized membrane regions are defined by the concentrated presence of the coat protein clathrin which occurs in the form of triskelia, three-legged complexes built up from heavy and light chains (*Brodsky et al.*, 2001; *Kirchhausen*, 2000). Together with Adapter protein complex 2 (AP2), these structures form polygonal cages at the plasma membrane (coated pits) which are able to invaginate and pinch off the membrane to form clathrin-coated vesicles (CCVs). This is facilitated by the GTPase dynamin which is believed to form a collar around the neck of the invaginated pit (Fig. 1.4). CCVs are released into the cytoplasm and deliver their cargo to the endosomal system after shedding the clathrin coat.

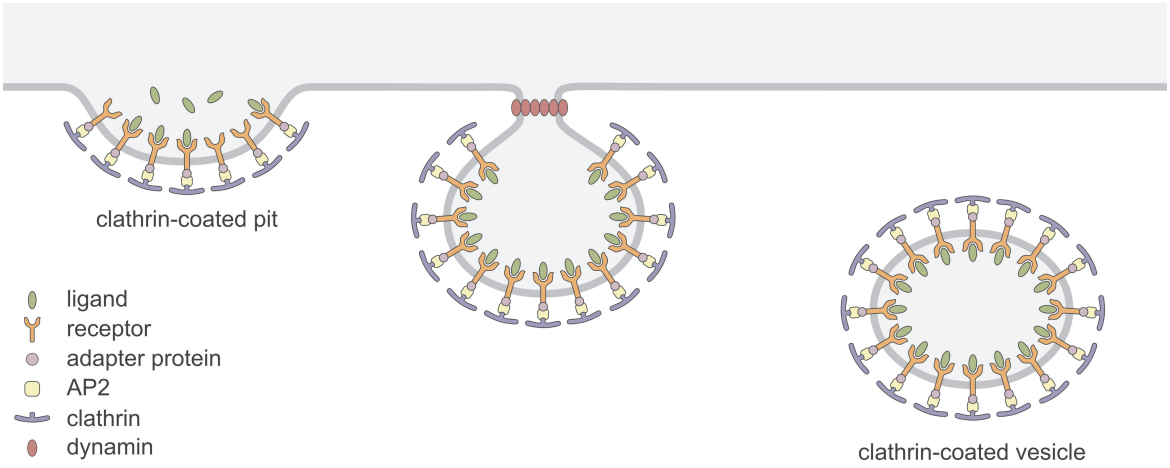

Figure 1.4 Clathrin-mediated endocytosis. Cargo-loaded receptors are recruited to coated pits where they interact with clathrin via binding of AP2 and adapter proteins, i.e. ARH in the case of LDLR. Clathrin triskelions crosslink to build a polygonal structure which facilitates invagination of the coated pit. At the neck of the invaginated pit, dynamin assembles to form a collar and drive membrane fission and release of the coated vesicle into the cytoplasm.

In the case of LDLR family members, adapter proteins enabling clathrin-mediated endocytosis are disabled 2 (Dab2) and autosomal recessive hypercholesterolemia (ARH), which in turn assemble with AP2 (*Mishra et al.*, 2002; *Morris and Cooper*, 2001) to recruit the receptors to coated pits. Additionally, the NPXY motif also binds adapter molecules responsible for relaying a signal into the cell, as it is the case for disabled 1 (Dab1) transmitting the Reelin signal (see section 1.2).

1.1.2 The LDL receptor in lipid metabolism

LDL receptor was the first member of the gene family to be discovered and is the best studied one, so far. This receptor is implicated in the maintainance of cholesterol homeostasis by mediating cellular uptake and catabolism of plasma cholesterol.

In order to enable transportation within the circulation, cholesterol and other lipids are packaged into lipoprotein particles assembled from lipids and proteins. These particles consist of a lipid core composed of esterified cholesterol and triglycerides, surrounded by a monolayer of phospholipids and unesterified cholesterol (Fig. 1.5). Depending on their triglyceride proportion and their buoyant density, lipoprotein particles are grouped into five subcategories: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL), in the order of increasing density and decreasing triglyceride portion. Associated with the surface of lipoprotein particles are apolipoproteins which are essential for recognition of lipoprotein particles by enzymes, receptors, and for interaction with one another.

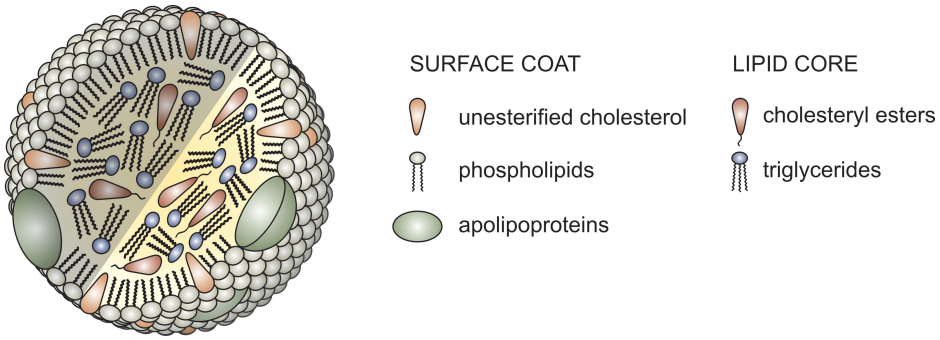

Figure 1.5 Structure of lipoproteins. Lipoproteinparticles are composed of a lipid core containing cholesteryl esters and triglycerides, and a surface coat of phospholipids, unesterified cholesterol and apolipoproteins.

Lipid metabolism in mammals employs two interacting pathways which control the fate of lipids delivered by diet (exogenous pathway) and newly synthesized lipids (endogenous pathway). In the exogenous pathway, dietary lipids are taken up by epithelial cells of the intestinal mucosa, packaged into chylomicrons and released into the circulation via the lymph. In the blood stream, part of the chylomicrons' triglycerides are extracted and hydrolyzed by lipoprotein lipase (LPL), thereby releasing glycerol and fatty acids which can be taken up by cells of peripheral tissues, especially muscle, for energy generation and stored by adipose tissue. The triglyceride-deprived particles, now called chylomicron remnants, bind to receptors (LDLR and LRP) on hepatocytes and are internalized via receptor-mediated endocytosis and degraded within lysosomes to provide the cells with glycerol, fatty acids, and cholesterol.

Endogenous lipids, i.e. triglycerids and cholesterol originating from the liver, are released to the circulation in the form of VLDL particles. In a manner similar to the fate of chylomicrons, VLDL donates triglycerides to peripheral tissues. The particle itself is thereby converted to VLDL remnants, called IDL, and LDL by further triglyceride extraction. LDL particles, whose lipid portion is mainly composed of cholesterol and phospholipids now, are absorbed by peripheral tissues or circulate back to the liver. In both cases, apoliporotein B-100 (apoB-100) on the surface of LDL particles binds to LDL receptor on target cells and the lipoprotein is internalized via receptor-mediated endocytosis. Excess cholesterol is collected from the tissues by HDL and delivered back to the liver.

Internalization of LDL particles after binding to LDLR occurs via clathrin-mediated endocytosis (originally referred to as receptor-mediated endocytosis, *Goldstein et al.*, 1985). After delivery of receptor and ligand to the endosomal compartment, the slightly acidic pH environment causes the receptor to change its conformation and to release its ligand (as described in section 12). The vesicles where this process takes place are therefore known as compartment of uncoupling of receptor and ligand (CURL). While LDLR recycles back to the plasma membrane, LDL is delivered to the lysosome for degradation. Released cholesterol is important for use in the buildup of cell membranes and synthesis of bile acids and steroid hormones. The LDL receptor pathway is summarized in Fig. 1.6.

As a response to the release of cholesterol from lipoprotein particles, the cell initiates several feedback pathways to prevent overacculmulation of cholesterol (*Brown and Goldstein*, 1986). To shut down further lipoprotein uptake, LDL receptor expression is downregulated. Furthermore, 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) synthase and HMG-CoA

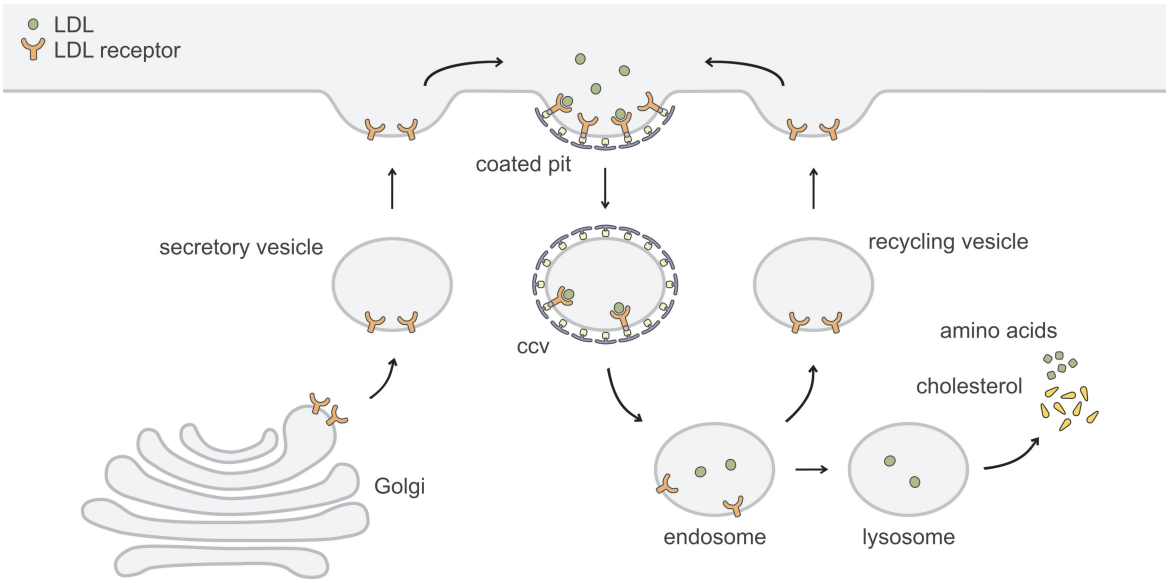

Figure 1.6 The LDL receptor pathway. Mature LDL receptor is released from the Golgi body and transported to the plasma membrane by the secretory pathway. Upon binding of its ligand LDL, the receptor is recruited to clathrin coated pits and internalized via clathrin mediated endocytosis. In the endosome, pH change leads to release of LDL which is subsequently degraded in the lysosome to release cholesterol and amino acids. LDL receptor is recycled back to the plasma membrane where it is available for internalization of more LDL from the circulation. Ccv, clathrin coated vesicle.

reductase, two important enzymes in the biosynthesis of cholesterol, are suppressed, and acyl-CoA-cholesterol:acyltransferase (ACAT) which facilitates the storage of excess cholesterol as cholesterol esters is activated.

Regulation of the genes for LDL receptor and enzymes neccessary for cholesterol synthesis is mediated by sterol response element binding proteins (SREBPs) which normally bind to sterol response elemens in the DNA to activate gene transcription. In the situation of high intracellular cholesterol levels, SREBPs cannot undergo the proteolytical cleavage which is neccessary for their activation, resulting in downregulation of gene expression and reduced cholesterol uptake and synthesis (reviewed in *Weber et al.*, 2004).

By delivery of cholesterol to the liver and peripheral tissues, LDL receptor not only supplies cells with cholesterol but also ensures efficient clearance of lipoproteins from the circulation. Disruption of this major task by genetically altered receptor molecules causes a major problem for systemic lipid metabolism. The resulting disorder is called familial hypercholesterolemia (FH) in humans and is characterized by accumulation of LDL in the circulation

due to defective uptake into tissues. Symptoms caused by elevated plasma LDL levels are atherosclerosis and heart attacks.

FH can be caused by mutations affecting the synthesis of LDL receptor in the endoplasmic reticulum (ER), transport to and modification in the Golgi body, binding of LDL, endocytosis of the receptor-ligand complex, and recycling of the receptor after ligand internalization. Also mutations in the genes for ARH or ApoB, or gain-of-function mutations of the proprotein convertase subtilisin/kexin type 9 (PCSK9) can cause FH (reviewed in *Soutar and Naoumova*, 2007). PCSK9 induces lysosomal degradation of LDL receptor by binding to LDLR's first EGF precursor homology domain which results in interference with its recycling by a yet unknown mechanism (*Zhang et al.*, 2007).

Recently, another regulatory mechanism for LDL receptor availability involving liver X receptors (LXRs) and Mylip/Idol has been reported (*Zelcer et al.*, 2009).

LXRs (LXRα and LXRβ) are nuclear receptors which act as transcription factors upon binding of an activating ligand. LXR forms a heterodimer with retinoid X receptor (RXR) (*Apfel et al.*, 1994). In a resting state, the receptor complex binds to LXR-responsive elements (LXREs) on the promoter of target genes (*Wiebel and Gustafsson*, 1997) and suppresses gene transcription by recruiting corepressors such as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (NCOR) to the DNA (*Chen and Evans*, 1995; *Hörlein et al.*, 1995). Upon binding of a ligand to either LXR or RXR (*Wiebel and Gustafsson*, 1997), conformational changes lead to release of these corepressors and interaction with specific coactivators, resulting in activation of gene expression (*Glass and Rosenfeld*, 2000).

LXRs are involved in the regulation of several physiological functions in lipid, bile acid and glucose metabolism, immune and inflammatory responses, and energy balance (reviewed in *Baranowski*, 2008; *Gabbi et al.*, 2009; *Zelcer and Tontonoz*, 2006).

One prominent role of LXRs is systemic cholesterol homeostasis. More specifically, this includes hepatic excretion of cholesterol into bile (*Peet et al.*, 1998; *Yu et al.*, 2003), intestinal cholesterol absorption (*Repa et al.*, 2002, 2000), de novo cholesterol synthesis (*Peet et al.*, 1998; *Schultz et al.*, 2000), reverse cholesterol transport from peripheral cells to the liver via HDL particles (*Lewis and Rader*, 2005; *Naik et al.*, 2006), and, as recently reported, LDLR degradation (*Zelcer et al.*, 2009).

In the regulation of cellular LDL receptor levels, LXR is activated by binding of metabolized cholesterol derivates (oxysterols) (*Lehmann et al.*, 1997) and induces expression of genes which prevent excess cholesterol accumulation, among them the inducible degrader of the LDLR (Idol) (*Zelcer et al.*, 2009).

Idol, also known as Mylip, is an E3 ubiquitin ligase which upon LXR-driven expression, induces ubiquitination of LDL receptor and its subsequent degradation (*Zelcer et al.*, 2009). Although generally, ubiquitination leads to proteasomal degradation, in this case LDL receptor is broken down in the lysosome in response to Idol action (*Zelcer et al.*, 2009).

Since excess cholesterol in the cell activates both PCSK9 and the LXR/Idol pathway, leading to the same result - lysosomal degradation of LDLR - the two mechanisms may cooperate. One possibility would be PCSK9-dependent ubiquitination of LDLR by Idol (*Sawamura*, 2009). This model however, is only hypothetical and to date, lacks any experimental evidence.

1.1.3 VLDL receptor

VLDL receptor is most closely related to LDLR. The two proteins share an almost identical domain structure except for one additional LA repeat in VLDLR. This difference however, determines slightly different binding characteristics of VLDLR. Whereas LDLR preferably interacts with LDL, VLDL receptor recognizes triglyceride-rich apoE-containing lipoproteins, i.e. VLDL, IDL, and chylomicrons but not LDL. Furthermore, VLDL receptor also binds ligands which are not involved in lipid metabolism which is currently ruled out in case of LDLR. These ligands include urokinase plasminogen activator (uPA)/plasminogen activator inhibitor-1 complex (*Battey*, 1995), lipoprotein lipase (*Battey*, 1995), tissue factor pathway inhibitor (TFPI) (*Hembrough et al.*, 2001), Reelin (*Hiesberger et al.*, 1999) and Thrombospondin (*Mikhailenko et al.*, 1997).

Mammalian VLDLR mRNA was found to be differentially spliced, generating receptor variants containing or lacking the O-linked sugar domain with the shorter isoforms seeming to be less stable at the cell surface (*Magrané et al.*, 1999). In addition, a brain-specific isoform with seven LA repeats was identified which lacks exon 4 (corresponding to LA repeat 3) (*Christie et al.*, 1996; *Takahashi et al.*, 2004). Only isoforms with eight LA repeats occur in chicken. Here, VLDLR containing the OLSD can be found in somatic cells, predominantly

heart and muscle, while receptors lacking the domain are expressed by oocytes (*Bujo et al.*, 1995a).

In mammals, VLDLR expression is highest in skeletal muscle, heart, and adipose tissue, less in brain, and very low in liver (*Wyne et al.*, 1996). Due to this expression pattern and the receptor's strong interaction with apoE, its physiological role was believed to be the delivery of triglyceride-rich particles such as VLDL to peripheral tissues (*Wyne et al.*, 1996) or the immobilization of these particles on the surface of endothelial cells in order to facilitate LPL action facilitating delivery of free fatty acids to the adjacent tissue. This was suggested by the findings of LPL-induced enhancement of VLDLR-VLDL-interaction (*Takahashi et al.*, 1995), enhancement of LPL action by VLDLR (*Goudriaan et al.*, 2004), and involvement of VLDLR in LPL transcytosis across endothelial cells (*Obunike et al.*, 2001). Surprisingly, it was shown that VLDLR deficiency did not result in altered lipoprotein profiles in mice, nor in significant changes in body weight or adipose tissue mass (*Frykman et al.*, 1995). Nevertheless, a role for VLDLR in fatty aicd uptake by adipose tissue could be concluded from the partial resitance of knockout mice to diet-induced obesity (*Goudriaan et al.*, 2001). A significant increase in serum triglyceride levels however, could only be detected in LDLR/VLDLR double knockout mice on a high-fat diet (*Tacken et al.*, 2000), pointing to a partial compensation by LDLR in VLDLR knockout mice.

As suggested by the wide variety of ligands binding to VLDLR, the receptor is also implicated in many different functions beyond lipoprotein metabolism, and was found to play important roles in signal transduction, angiogensis, and tumor growth. Together with Thrombospondins, for example, VLDLR mediates a nonapoptotic pathway to inhibit cell division in microvascular endothelial cells, by inhibiting thymidine uptake (*Oganesian et al.*, 2008). In mammalian brain development and in the postnatal brain, the receptor acts as a signal transducer in the Reelin pathway together with ApoER2 (see section 1.2). In men, VLDLR deficiency was recently linked to dysequilibrium syndrome with ataxia and mental retardation which results from profound cerebellar hypoplasia (*Boycott et al.*, 2009; *Moheb et al.*, 2008; *Ozcelik et al.*, 2008; *Türkmen et al.*, 2008).

In avian species, VLDL receptor is expressed in the same tissues as in mammals, but additionally plays an important role in oocyte development by acting as an endocytic receptor for yolk precursors.

When an oocyte is chosen for final maturation and ovulation in the chicken ovulatory cycle,

a phase of rapid growth, uptake of plasma components from the circulation, and their deposition as yolk are initiated (*Perry et al.*, 1978). One major part of the yolk are lipoproteins, mainly VLDL and vitellogenin (VTG) which are synthesized by the liver, secreted to the circulation, and taken up by growing oocytes via VLDL receptor-mediated endocytosis. Seven days after growth phase initiation, when the oocyte has reached its full size, it is released from the follicle, i.e. it ovulates, which finally causes egg production.

In the absence of VLDLR, the oocyte is not able to enter the rapid growth phase, and egg-laying is disturbed. Additionally, the lipoproteins not transported to the growing oocyte remain in the circulation of the hen which in turn shows signs of hyperlipidemia and atherosclerosis (*Bujo et al.*, 1995b).

Yolk precursors extracted from the circulation have to pass the theca cell layer (composed of theca externa and interna), the basement membrane, an epitheloid monolayer of granulosa cells, and the zona pellucida before reaching VLDLR which mediates their uptake into the growing oocyte in order to form yolk (Fig. 1.7).

Passing of the theca layers is possibly facilitated by perlecan, originally known as a basement membrane protein in other species which, in chicken, is found in the extracellular matrix (ECM) of theca externa cells (*Hummel et al.*, 2004). It is able to bind VLDL and is probably used by the follicle to import and store VLDL within the ECM for subsequent uptake into the oocyte (*Barber et al.*, 1991; *Perry and Gilbert*, 1979; *Shen et al.*, 1993) (reviewed in *Schneider*, 2009). For this purpose, VLDL might then be transported to and through the basement membrane via interaction with the protein ggBM1 (*Hummel et al.*, 2007). Passage through the monolayered granulosa cells is accomplished by diffusion through gaps between the cells.

1.1.4 ApoE receptor type 2

ApoER2 is almost exclusively expressed in brain in chicken, and brain, testis, and placenta in mammals (*Brandes et al.*, 1997). Homozygous knockout mice show no obvious severe phenotype but male animals are sterile, suggesting that ApoER2 might be involved in sperm production (*Trommsdorff et al.*, 1999). As the very low endocytosis rate of ApoER2 compared to other members of the LDLR family suggests, the primary function of this receptor seems to lie in signaling rather than receptor-mediated endocytosis (*Li et al.*, 2001). Indeed,

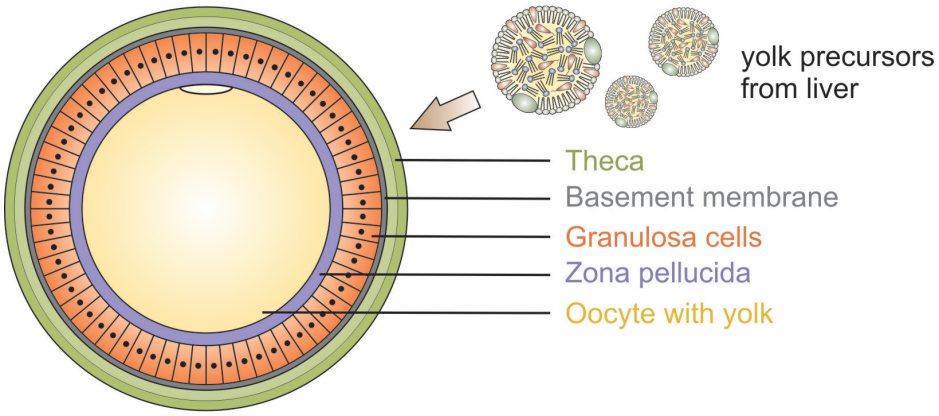

Figure 1.7 Uptake of yolk precursors to the chicken follicle in the pre-ovulatory growth phase. Yolk precursors, transported from the liver to the follicle via the circulation traverse theca interna and externa, basement membrane, granulosa cell layer, and zona pellucida before passing the oocyte membrane by receptor mediated endocytosis.

it is one of the two receptors mediating the Reelin signaling pathway in mammalian brain development (see section 1.2).

ApoER2 can be found in a multitude of splice variants, harbouring different numbers of LA repeats and containing or lacking the O-linked sugar domain, a furin cleavage site and a short proline-rich insert in the cytoplasmic tail.

Depending on the species and organ expressing it, ApoER2 can contain three to eight LA repeats. Murine ApoER2 has beed found in several isoforms, all lacking exon 5 which codes for LA-repeats 4-6 and is constitutively spliced in this species. Therefore the variant with the largest ligand binding domain contains the five remaining repeats 1-3, 7, and 8. In other ApoER2 molecules, LA repeat 8 is either missing or replaced by a 13 amino acid long furin consensus cleavage site (*Brandes et al.*, 2001). By cleavage by the serine endoprotease furin, a soluble receptor fragment containing the entire ligand binding domain consisting of four LA-repeats is produced (Fig. 1.8). The secreted fragment is able to block Reelin signaling in primary neurons (*Koch et al.*, 2002), which suggests a role for this splice variant in negative feedback-regulation of the signaling pathway *in vivo*. Also in human ApoER2, an alternatively spliced furin site was reported (*Brandes et al.*, 1997). Splicing of the ligand binding domain in men produces variants with LA repeats 1-3, 1-3 and 7, but also the whole seven repeats (*Kim et al.*, 1997). In chicken, isoforms with seven or eight LA repeats have

been found. Both do not contain a furin cleavage site (*Brandes et al.*, 1997).

Just like VLDL receptor, ApoER2 has been found to differentially express an O-linked sugar domain in mice which as expected is more susceptable to proteolytic processing, too (*May et al.*, 2003). In response to ligand binding, ApoER2 variants containing this domain can be cleaved by α-secretase at a site just outside the plasma membrane to generate a soluble extracellular fragment (N-terminal fragment, NTF) and a membrane-bound C-terminal fragment (CTF) which can be further processed by γ-secretase to release a soluble intracellular fragment (ICD) (*Hoe and Rebeck*, 2005; *May et al.*, 2003) (Fig. 1.8). Further trafficking or functions of NTF and ICD are not quite clear yet. However, the NTF might act as negative regulator for the Reelin pathway, as it was shown for the furin-generated fragment. The ICD might either be efficiently and rapidly degraded or shuttled to the nucleus by Dab1. Regulation of gene expression by proteolytic fragments has been shown in the case of notch (*Okochi et al.*, 2002), APP (*Gao and Pimplikar*, 2001), and others. Either way, the ICD seems to be removed from the cytoplasm very quickly, since it is constantly hard to detect there.

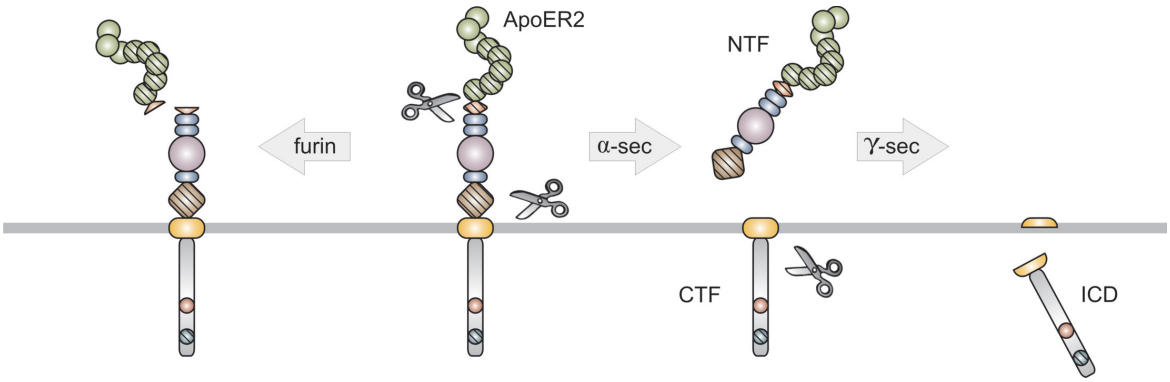

Figure 1.8 Secretase-mediated processing of ApoER2. ApoER2 variants containing the furin cleavage site can be processed to release the ligand binding domain to the extracellular space. Upon ligand binding, ApoER2 is subjected to two consecutive cleavage events. By α-secretase processing, a soluble N-terminal and membrane-bound C-terminal fragment (NTF and CTF, respectively) are produced. Further fragmentation by γ-secretase releases the soluble intracellular domain (ICD) from the membrane into the cytoplasm.

Another feature is exclusively known for ApoER2: a 59 amino acid long proline-rich insert in the receptor's cytoplasmic tail, encoded by a single exon, occurs in some isoforms of the murine and human (but no chicken) receptor (*Brandes et al.*, 1997). Binding of JIP-1 (JNK interaction protein 1) and JIP-2 has been shown to occur at this region (*Stockinger et al.*, 2000). JIP and c-jun N-terminal kinase (JNK) are major proteins of the MAP kinase

pathway (*Yasuda et al.*, 1999). Another adaptor protein binding to the proline-rich insert is the postsynaptic density protein PSD-95 which interacts with both ApoER2 and the N-methyl-D-aspartate (NMDA) type gutamate receptor simultaneously in the presence of Reelin. This interaction plays a role in synaptic plasticity and memory in the adult brain by regulating synaptic calcium influx via the NMDA receptor (*D'Arcangelo*, 2005).

As it was show recently, ApoER2 is mainly found in lipids rafts (*Mayer et al.*, 2006; *Riddell et al.*, 2001), small specialized cholesterol- and sphingolipid-rich microdomains of the plasma membrane (see Fig. 1.9), moving freely within the cell surface (*Brown and London*, 2000). This, is in contrast to LDLR and VLDLR which are strictly excluded from these membrane domains. A special type or subdomain of lipid rafts are caveolae (*Anderson*, 1998; *Kurzchalia and Parton*, 1999). The composition of caveolae is similar to that of lipid rafts, exept for the additional presence of caveolin, a structural protein which occurs in homo- and hetero-oligomers (*Sargiacomo et al.*, 1995; *Scherer et al.*, 1997) and is able to associate with cholesterol (*Murata et al.*, 1995) and glycophospholipids (*Fra et al.*, 1995). These interactions are believed to determine the observed flask-like ivaginated shape of caveolae (*Sargiacomo et al.*, 1995) (Fig. 1.9).

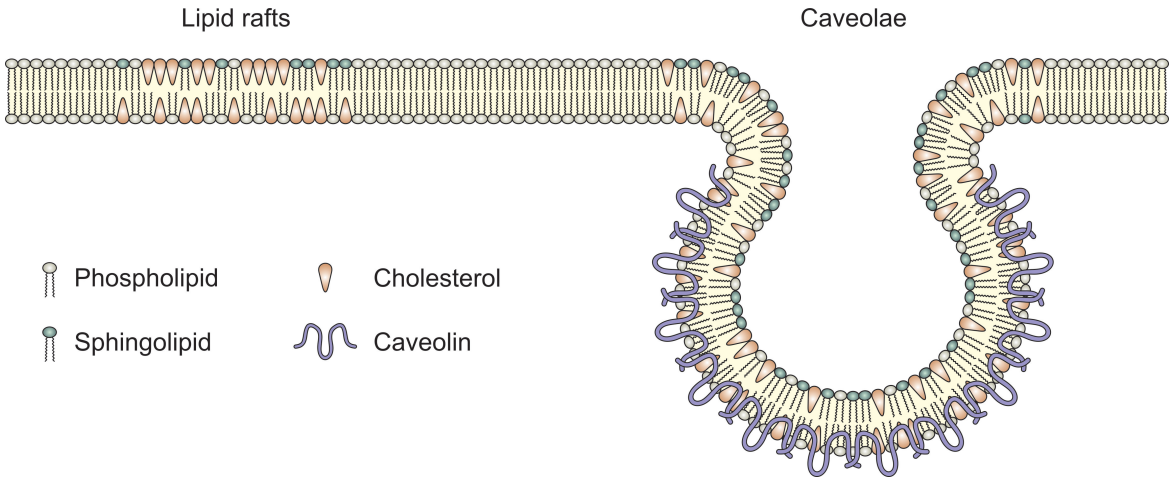

Figure 1.9 Schematic representation of lipid rafts and caveolae. Lipid rafts are freely moving domains of the plasma membrane. They show a high concentration of cholesterol and sphingolipids. Caveolae possess a similar lipid composition but are characterized by the additional presence of caveolin which associates with the membrane to facilitate invagination.

Lipid rafts and especially caveolae are supposed to be implicated in signal transduction rather than bulk endocytosis (*Anderson*, 1998; *Razani et al.*, 2002), since caveolae show a low internalization rate with a halftime of more than 20 minutes and carry little volume.

Furthermore, many signaling receptors and membrane transporters are clustered in these regions. Further evidence for a (negative) regulatory role of caveolin rather than importance for the uptake machinery was supplied by the finding that experimentally reduced caveolin levels tend to slightly accelerate raft-mediated uptake (*Nabi and Le*, 2003). The reason for this could be stabilization of rafts by caveolin, as suggested by the observation that caveolae are largely static.

However, endocytosis of proteins associated to lipid rafts, can occur by caveolae-dependent or -independent mechanisms (*Kirkham and Parton*, 2005). In the case of ApoER2, internalization occurs via clathrin-mediated endocytosis, arranged by binding of Dab2, despite the receptor's localization to rafts (*Cuitino et al.*, 2005). Nevertheless, ApoER2 endocytosis could still be regulated by the presence of caveolin.

1.1.5 LDLR-related protein

LRP is a ubiquitously expressed large glycoprotein of about 600 kDa. After synthesis, it is cleaved by furin in the trans-Golgi network which generates an extracellular and a transmembrane fragment remaining associated with one another (*Herz et al.*, 1990). Furthermore, LRP can be specifically processed like Notch and APP, generating a soluble extracellular and intracellular fragment, likely to be involved in signaling mechanisms (*May et al.*, 2002).

Homozygous LRP knockout mice die at early embryonic age which seemingly results from an essential role of LRP during implantation of the embryo, when it mediates uptake and degradation of urokinase-type plasminogen activator/plasminogen activator-inhibitor 1 complexes (*Herz*, 1992).

Since this prevented study of LRP deficiency effects *in vivo*, Herz and collegues came up with a mouse model permitting inducible, tissue specific disruption of the LRP gene using Cre/loxP recombination (*Rohlmann et al.*, 1998).

Using this model, it was shown that LRP mediates clearance of chylomicron remnants from the plasma (*Rohlmann et al.*, 1998) which is in accordance with the original identification of the receptor as an endocytic receptor for apoE-rich lipoproteins in the liver (*Beisiegel et al.*, 1989). In addition, LRP mediates uptake and intracellular degradation of several more ligands, i.e. activated α_2-macroglobulin (α_2-M*), factor VIII, and serpin-enzyme complexes to the liver and is of great importance for diverse physiological functions in a broad range

of tissues. Functions of LRP involve regulation of vascular permeability, regulation of cell migration, control of cellular entry of bacterial toxins and viruses, and modulation of the integrity of the blood-brain-barrier (reviewed in *Lillis et al.*, 2008).

LRP expressed in the central nervous system binds α_2-M*, which enables it to influence synaptic plasticity via PSD-95 mediated interaction with NMDA receptors (*Qiu et al.*, 2002). Another important role of LRP in the brain lies in keeping the balance between production and clearance of β-amyloid (Aβ), a proteolytic fragment of amyloid precursor protein (APP), which is implicated in the pathology of Alzheimer's disease in aggregates called amyloid plaques. Cells deficient in LRP show increased cell surface levels of APP and reduced Aβ amounts (*Pietrzik et al.*, 2002; *Ulery et al.*, 2000) which increases the risk of amyloid plaques.

Although it could be determined that binding of LRP to APP occurs via the cytoplasmic adapter protein Fe65 (*Pietrzik et al.*, 2004), the pathway leading from LRP-APP interaction to increased Aβ production is not entirely clear at present. According to the currently proposed mechanism, association of APP with LRP leads to increased trafficking of APP through the endosomal compartments where BACE and presenilin-1 (PS-1), the two cleaving enzymes necessary for Aβ production are known to reside. This would lead to enhanced proteolysis of APP (Fig. 1.10). As it was shown, in response to this, the intracellular domain of APP, released after generation of Aβ, is able to suppress LRP transcription in a complex with Fe65 and Tip60 (*Liu et al.*, 2007).

In 2003, another role of LRP was discoverd in the maintainance of blood-brain-barrier (BBB) function (*Yepes et al.*, 2003). Here, tissue-type plasminogen activator (tPA)-regulated BBB permeability seems to be regulated by LRP. This mechanism may also contribute to a role of LRP in Alzheimer's, since Aβ may be cleared from the brain through the BBB by LRP (*Shibata et al.*, 2000).

Moreover, LRP is essential for protection of the vasculature from atherosclerosis. Apart from its role for lipoprotein uptake to the liver, it counteracts the development of atherosclerosis also through other mechanisms triggered in vascular smooth muscle cells and macrophages. While it was shown that LRP suppresses platelet-derived growth factor (PDGF) signaling pathways in smooth muscle cells (*Boucher et al.*, 2003) for this purpose, the pathway by which LRP in macrophages alters the progression of atherosclerosis is not known.

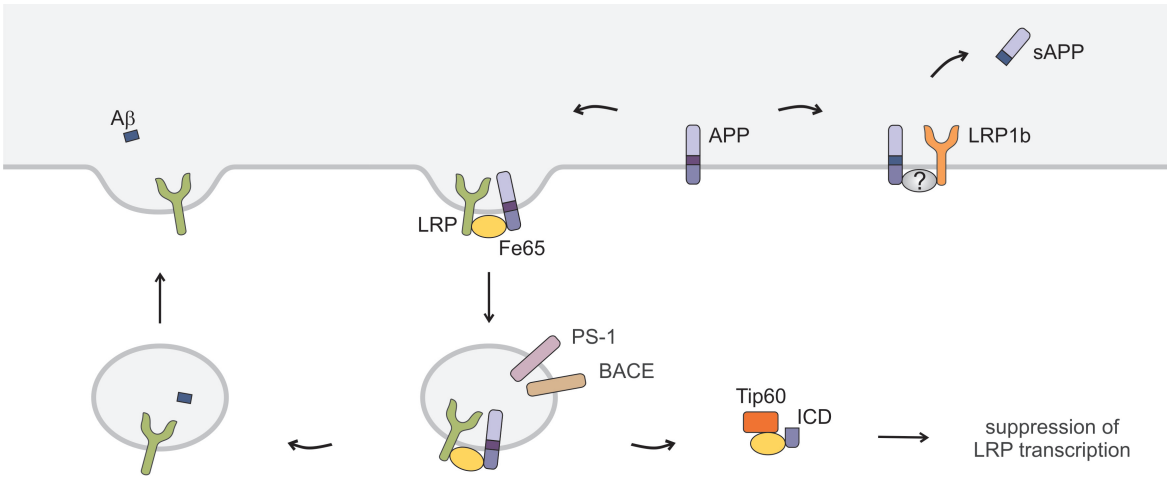

Figure 1.10 Proposed effects of LRP and LRP1b in APP trafficking and processing. LRP is able to form a complex with APP via Fe65 and enhance its internalization. In the endosomal compartment, APP is available for processing by BACE and PS-1 which generates both the Aβ peptide and releases the APP intracellular domain (ICD) into the cytoplasm. While LRP and Aβ are shuttled back to the cell surface, APP ICD stays associated with Fe65 and, together with Tip60, forms a complex able to suppress further transcription of LRP. APP binding to LRP1b (possibly by a yet unidentified adapter protein) is not preferentially endocytosed but stays at the plasma membrane. Here, it is subjected to different processing events resulting in the release of sAPP which does not contribute to progression of Alzheimer's.

1.1.6 LRP1b

LRP1b is highly homologous to LRP, except for an additional LA repeat in LRP1b. Additionally, a splice variant of LRP1b contains a unique exon coding for a 33 amino acid long insert in the receptor's cytoplasmic tail (*Liu et al.*, 2000). LRP1b was originally identified as a putative tumor suppressor which is inactivated in 40% of non-small lung cancer cell lines (*Liu et al.*, 2000). Later on, mutations in the LRP1b gene were also found to be linked with several other kinds of cancer (*Hirai et al.*, 2004; *Langbein et al.*, 2002; *Pineau et al.*, 2003; *Sonoda et al.*, 2004). The mechanisms by which LRP1b interferes with tumor progression might be based on its ability to impair urokinase receptor regeneration on the cell surface and inhibit cell migration (*Li et al.*, 2003).

Although human LRP1b expression seems to be widespread with highest levels in brain, adrenal gland, salivary gland, and testis, the almost exclusive expression site of LRP1b in mice is the central nervous sytem (*Li et al.*, 2005). In the brain, LRP1b is involved in development of Alzheimer's disease, too. An antagonistic effect of LRP1b for LRP-mediated

pathways was suggested, since LRP1b binds the same ligands as LRP but exhibits a much slower internalization rate (*Liu et al.*, 2001) (Fig. 1.10). Confirming this hypothesis, it was shown that the slow endocytosis rate of LRP1b leads to accumulation of APP on the cell surface together with a reduction of Aβ production (*Cam et al.*, 2004).

1.1.7 Megalin

Megalin is an endocytic receptor which interacts with several signaling pathways by regulation of the availability of the respective extracellular ligands, including BMP4, vitamin D, and sex hormones.

Expression levels of megalin in the adult organism are highest in the proximal renal tubule and intestinal epithelium (*Müller et al.*, 2003), more specifically at the apical surface of these epithelial borders (*Christensen et al.*, 1995). In the epithelium of the proximal renal tubule, megalin is responsible for reabsorption of serveral vitamins from the primary urine (*Farese and Herz*, 1998). This was first noticed when loss of megalin was shown to result in increased excretion of 25-hydroxy-vitamin D and vitamin D binding protein (*Nykjaer et al.*, 1999), leading to vitamin D deficiency and in consequence, bone mineralization defects. Similar to reuptake of vitamin D/vitamin D binding protein, also vitamin A and vitamin B12 systemic balance were found to be dependent on megalin function (*Christensen et al.*, 1999; *Moestrup et al.*, 1996).

Homozygous loss of megalin in the brain leads to impaired bone morphogenic protein 4 (BMP4) signaling, since megalin was shown to bind and deliver BMP4 to the lysosome for degradation. Therefore, in the absence of megalin, BMP4 accumulates which negatively regulates sonic hedgehog (Shh) signaling, resulting in holoprosencephaly, a malformation of the forebrain (*Farese and Herz*, 1998; *Willnow et al.*, 1996a).

Furthermore, defective sex steroid signaling resulting from megalin deficiency was identified as a source for malformations in male and female reproductive organs (*Hammes et al.*, 2005).

More recently, mutations in the megalin gene have been linked to Donnai-Barrow syndrome, a rare autosomal recessive disorder causing agenesis of the corpus callosum, diaphragmatic hernia, facial anomalities, ocular anomalies, sensorineural deafness, and cognitive impairment (*Kantarci et al.*, 2007).

In addition, megalin was also proposed to be involved more directly in signaling pathways in the proximal renal tubule, since like LRP, it is processed by a metalloproteinase to generate a soluble extracellular fragment and a membrane-bound C-terminal fragment which can most likely be further cleaved to release a soluble intracellular domain (*Zou et al.*, 2004). The potential role of the generated fragment in transcriptional regulation or signaling pathways is not yet elucidated, however.

1.1.8 MEGF7

Multiple EGF-like domain containing protein 7 (MEGF7) is the most recent member added to the LDLR family, being identified not until 1998 in a screen for large proteins containing EGF repeats in the mammalian brain (*Nakayama et al.*, 1998).

MEGF7 deficient mice show developmental defects including most prominently, growth retardation and polysyndactyly, combined with mild craniofacial and tooth abnormalities (*Johnson et al.*, 2005). Polysyndactyly, the fusion and duplication of digits at fore and hind limbs, is the result of inappropriate digit formation due to disruption of a network of essential signaling pathways including fibroblast growth factors (FGF), BMPs, Wnts, and Shh. Indeed, MEGF7 was found to modulate these pathways by regulating expression of the respective signaling proteins (FGF8, BMP2, BMP4, Wnt7a, Shh). This leads to abnormal growth of the early embryonic limb bud, the major site of MEGF7 expression in the embryo (*Johnson et al.*, 2005). The essential role of MEGF7 in digit differentiation was confirmed more recently when two new mutations of the gene were found to result in the same phenotype of polysyndactyly as described for homozygous knockout mice (*Simon-Chazottes et al.*, 2006).

In the nervous system, MEGF7 deficiency manifests in a block of the early development of neuromuscular junctions (NMJ) in the postsynaptic endplate region of muscles (*Weatherbee et al.*, 2006). Furthermore, MEGF7 was shown to interact with PSD-95 and influence its regulation by calmodulin-dependent protein kinase II (CAMKII) (*Tian et al.*, 2006).

1.2 The Reelin signaling pathway

1.2.1 Physiological significance of the Reelin signal

Reelin is a large glycoprotein of the extracellular matrix (ECM) which is mainly found in the central nervous system (CNS). Via binding to transmembrane receptors and subsequent phsophorylation of intracellular proteins, Reelin transmits a signal into neuroblasts of the developing cerebellum, cerebral cortex, and hippocampus, guiding their migration to their proper destinations. There, lack of Reelin results in disturbed neuronal layer formation and general developmental deficits (Fig. 1.11). Its direction-giving role persists to the postnatal brain, when the Reelin cascade controls migration of neuronal precursors from the subventricular zone through the rostral migratory stream for the continous supply of the olfactory bulb (OB) (*Hack et al.*, 2002) and from the subgranular zone to the granule cell layer in the dentate gyrus (*Frotscher et al.*, 2003). Furthermore, it is involved in synaptic plasticity (*D'Arcangelo*, 2005; *Weeber et al.*, 2002) and development of dendrites (*Niu et al.*, 2004) and dendritic spines (*Niu et al.*, 2008).

Reelin is not restricted to the brain but it can also be detected in the blood stream, liver, and adrenal gland (*Smalheiser et al.*, 2000), spinal cord (*Yip et al.*, 2004), eyes (*Pulido et al.*, 2007), lymphatic capillaries (*Samama and Boehm*, 2005), and in the dental pulp (*Buchaille et al.*, 2000). Knowledge of the physiological functions of Reelin in these tissues however, is still scarce. Suggestions include roles for Reelin in nociceptive processing (*Akopians et al.*, 2008; *Villeda et al.*, 2006), response to injury of the eye and liver (*Kobold et al.*, 2002; *Pulido et al.*, 2007), peripheral nerve regeneration (*Lorenzetto et al.*, 2008), and lymphangiogenesis (*Samama and Boehm*, 2005).

Reelin deficiency in mice leads to motoric defects, most likely arising from problems in cerebellar development. The main characteristics of this phenotype are ataxia, tremors, imbalance, and a reeling gait. These deficits were first observed by Douglas Scott Falconer in 1951 in mice he descriptively named *reeler* due to their behaviour (*Falconer*, 1951). The gene responsible for the *reeler* phenotype was cloned and identified more than forty years later (*D'Arcangelo et al.*, 1995).

In men, complete disruption of the Reelin signaling pathway is very rarely observed. Still, two human disorders have been shown to arise from malfunctioning of this signaling cascade. Norman-Roberts syndrome, a form of lissencephaly, was first described in 1976 (*Norman*

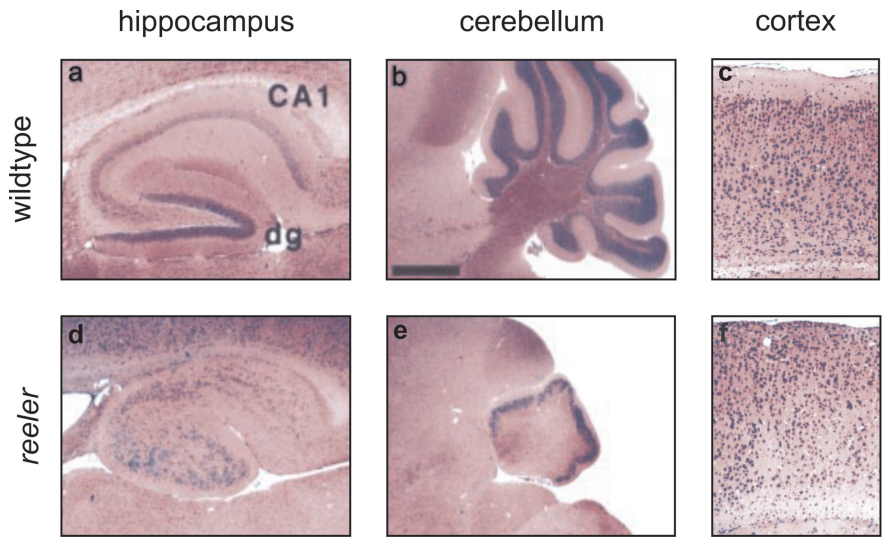

Figure 1.11 Laminar defects in *reeler* mice. In the *reeler* brain, layered structures are disorganized and degenerated. Reelin deficiency is most obvious in the hippocampus (d), cerebellum (e), and cerebral cortex (f) when compared to the corresponding structures of the wildtype brain (a-c). (Adapted from *Trommsdorff et al.*, 1999).

et al., 1976) and is characterized by severe cerebellar hypoplasia, cognitive delay, craniofacial features, and seizures. It is attributed to a mutation in the Reelin gene (*Hong et al.*, 2000) and is inherited as an autosomal recessive trait.

The second Reelin signaling associated disorder, a subgroup of dysequilibrium syndrome, arises from disruption of the VLDLR gene (*Boycott et al.*, 2005; *Moheb et al.*, 2008), coding for one of the Reelin receptors, and is also known as VLDLR-associated cerebellar hypoplasia (VLDLR-CH). Although dysequilibrium syndrome was first described in 1973 (*Sanner*, 1973), the connection between this condition and VLDL receptor was first identified in 2005 in several individuals of the Hutterite population in the USA and Canada (*Boycott et al.*, 2005; *Glass et al.*, 2005; *Schurig et al.*, 1981), and a number of Iranian (*Moheb et al.*, 2008) and Turkish (*Ozcelik et al.*, 2008; *Türkmen et al.*, 2008) families. Patients present with ataxia, mental retardation, and occasional seizures. In addition, quadrupedal locomotion was observed in affected families from Turkey, which however, seems to be a behavioural adaptation (*Türkmen et al.*, 2008). Like Norman-Roberts Syndrome, also VLDLR-CH is inherited in an autsomal recessive manner.

Epigenetic regulation and polymorphisms of the Reelin gene were also implicated in several psychological diseases, including autism (reviewed in *Fatemi*, 2005; *Kelemenova and*

Ostatnikova, 2009), bipolar disorder (*Guidotti et al.*, 2000), and schoziphrenia (reviewed in *Fatemi*, 2005; *Grayson et al.*, 2006). Furthermore, a strong link between the Reelin pathway and Alzheimer's disease was suggested, since Reelin expression and glycosylation is altered in patients (*Botella-López et al.*, 2006) and gene variants correlate with pathogenesis of AD (*Seripa et al.*, 2008). Furthermore, Reelin was shown to influence tau phosphorylation (*Hiesberger et al.*, 1999; *Ohkubo et al.*, 2003), affect APP trafficking and processing (*Hoe et al.*, 2006), and antagonize Aβ effects at the synapse (*Durakoglugil et al.*, 2009).

1.2.2 Components and mechanism of Reelin signaling

Reelin is a large glycoprotein of about 450 kDa which is secreted by specialized cells into the extracellular matrix. It can act both as a serine protease (*Quattrocchi et al.*, 2002) and as a signaling molecule in the Reelin pathway.

The structure of the Reelin protein is composed of several modules (see Fig. 1.12). At the N-terminus, Reelin contains a cleavable signaling peptide (S), followed by a region showing similarity to F-Spondin (SP), and a unique region only found in Reelin to date (H). This N-terminal part of Reelin has been shown to be essential for Reelin dimerization or multimerization (*Kubo et al.*, 2002).

The major part of Reelin is composed of 8 consecutive repeats each of which contains two subdomains (A and B), separated by an EGF-like motif (*D'Arcangelo et al.*, 1995).

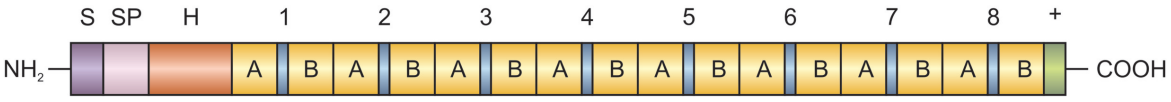

Figure 1.12 Schematic representation of the Reelin protein. Reelin comprises a short signal peptide (S), an F-Spondin homology domain (SP), a unique region (H), eight consecutive Reelin repeats (1-8), and C-terminal strongly basic region (+). Each Reelin repeat is composed of two subdomains (A and B), separated by an EGF motif (depicted in blue). (Adapted from *Tissir and Goffinet*, 2003).

The highly conserved C-terminal region of Reelin (+) contains basic residues and was suggested to be essential for secretion of the protein, since mice with mutant Reelin lacking this domain (*reeler* Orleans) accumulate Reelin in the cytoplasm (*de Bergeyck et al.*, 1997). More recently however, this view was refuted when it was proposed that instead, the C-terminal region is necessary for efficient downstream signaling by binding tightly to Reelin repeat 8,

thereby altering its structure and augmenting the interaction with Reelin receptors on the cell surface (*Kohno et al.*, 2009b; *Nakano et al.*, 2007).

In vivo, Reelin is processed by yet unknown metalloproteinases at two sites between repeats 2 and 3, and 6 and 7 (*Lambert De Rouvroit et al.*, 1999), thereby generating three fragments (N-R2, R3-6, and R7-8) and two intermediates (N-R6 and R3-8) (Fig. 1.13). The function of the individual fragments is not entirely clear yet although the central fragment of Reelin (R3-6) was proposed to be necessary and sufficient for binding to its receptors and stimulation of the Reelin pathway (*Jossin et al.*, 2004; *Yasui et al.*, 2007). The N-terminal fragment N-R2 was recently proposed to be generated within target cells which have internalized Reelin and to be subsequently resecreted into the extracellular space. The amount of secreted N-R2 fragment however, does not seem to have any influence on the signaling activity of Reelin (*Hibi and Hattori*, 2009).

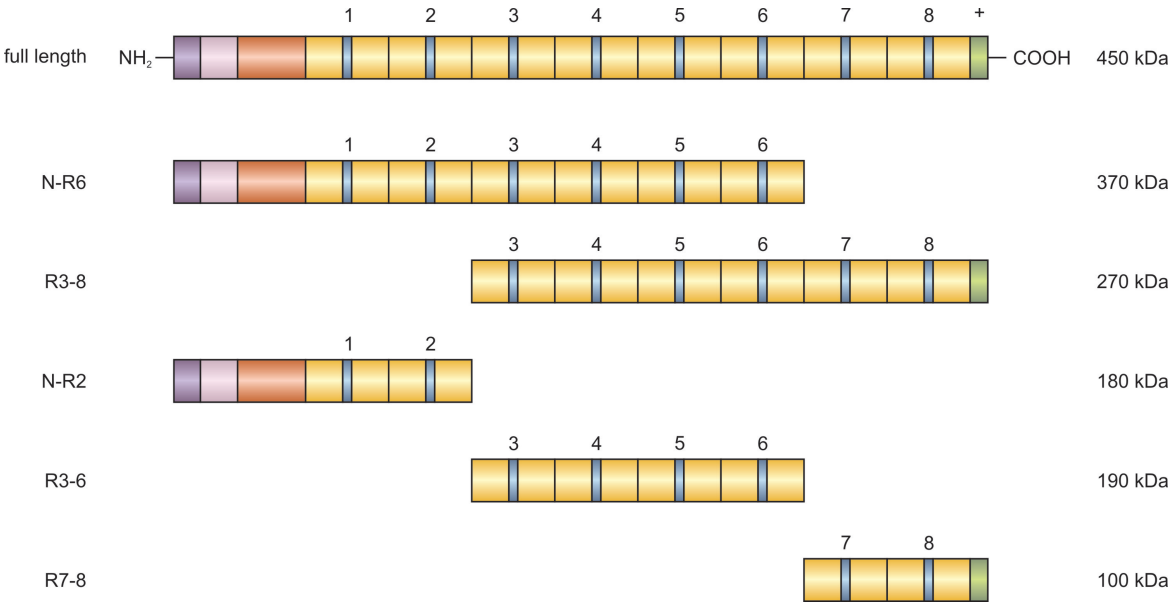

Figure 1.13 Proteolytic fragments of Reelin. Reelin is processed at two sites at the borders between repeats 2/3 and 6/7. Single cleavages produce two intermediate fragments, N-R6 and R3-8, while complete processing generates three final fragments, N-R2, R3-6, and R7-8.

While *in vitro*, processing of Reelin does not have any effect on or even reduces its signaling activity (*Jossin et al.*, 2004; *Kohno et al.*, 2009a), it seems to be a crucial step for functional signaling in cultured embryonic brain slices (*Jossin et al.*, 2007). This contradiction could be explained by a requirement of Reelin, being anchored to the ECM, to be released by proteolytic cleavage in order to reach and stimulate target cells. Release would not be

necessary for neuronal cultures *in vitro* where all cells are exposed to soluble Reelin and its fragments (*Jossin et al.*, 2007).

To stimulate the signaling cascade (Fig. 1.14), Reelin has to be available as disulfide-linked homodimers which are formed by interaction of the N-terminal domains of the proteins (*Kubo et al.*, 2002). Reelin dimers interact with the extracellular ligand binding domains of two or more ApoER2 and/or VLDLR molecules (*D'Arcangelo et al.*, 1999). The receptors are thereby clustered which allows them to induce phosphorylation of the intracellular adapter protein Dab1 (*Strasser et al.*, 2004) being bound to the NPXY motifs in the receptors' cytoplasmic tails via its phosphotyrosine binding (PTB) domain (*Howell et al.*, 1999).

Dab1 phosphorylation can occur on five tyrosine residues (Y185, Y198, Y200, Y220, and Y232) with Y198 and Y220 seeming to be the major phosphorylation sites responding to Reelin stimulation (*Keshvara et al.*, 2001). Involvement of Y185 and Y232 remains controversial (*Feng and Cooper*, 2009; *Keshvara et al.*, 2001; *Morimura and Ogawa*, 2009). Recently, it was disparately proposed that distinct phosphorylated tyrosine residues might either be important for different downstream signaling events mediated by Dab1 (*Morimura and Ogawa*, 2009) or might be able to compensate for each other (*Feng and Cooper*, 2009).

As a prerequisite for tyrosine phosphorylation however, Dab1 has to be serine/threonine phosphorylated by cyclin-dependent kinase 5 (Cdk5) which acts independently of Reelin signaling (*Keshvara et al.*, 2002). Only in this state, Dab1 is a substrate for tyrosine phosphorylation by members of the Src family of kinases (SFK). The main kinase for Dab1 was shown to be Fyn although also Src and, to a lesser extent, Yes act on Dab1 in response to Reelin (*Arnaud et al.*, 2003b; *Kuo et al.*, 2005).

The respective kinase might be recruited to the signaling complex by interaction of Reelin with a co-receptor. Members of the integrin family of adhesion molecules mediating interactions between neurons and glia cells (*Anton et al.*, 1999), would be possible candidates for this. $\alpha 3\beta 1$-integrin is able to associate with SFKs at its intracellular tail (*Schwartz*, 2001) and Reelin at the extracellular domain (*Dulabon et al.*, 2000). It was therefore proposed to be recruited to ApoER2/VLDLR-bound Dab1 upon Reelin stimulation, thereby contributing to Dab1 phosphorylation (*Gupta et al.*, 2002; *Schmid et al.*, 2005). As it was shown in our lab however, clustering of ApoER2 or VLDLR by antibodies against the respective ligand binding domains is sufficient for Dab1 phosphorylation (*Strasser et al.*, 2004). So, at least the primary Reelin signaling event is independent of a potential co-receptor.

The initial components of the pathway seem to constitute a linear signal transmission, since mice lacking either Reelin, ApoER2 and VLDLR, or Dab1 show a similar phenotype. This was observed in naturally occuring mutants, including *reeler*, and *scrambler* and *yotari* mice, both lacking functional Dab1 (*Sheldon et al.*, 1997), in knockout mice for ApoER2 and VLDLR (*Trommsdorff et al.*, 1999), or Dab1 (*Howell et al.*, 1997), and in mice expressing a mutated phosphorylation-incompetent Dab1 protein (*Howell et al.*, 2000).

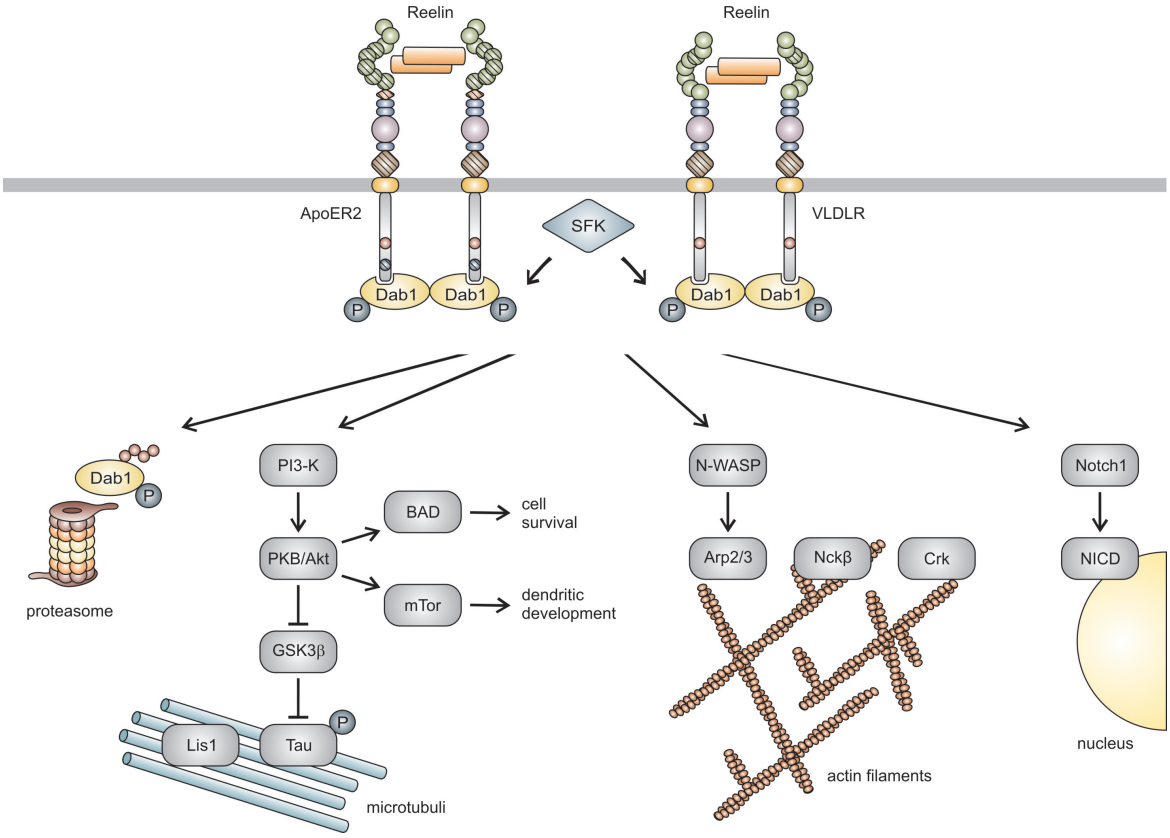

Figure 1.14 Transmission of the Reelin signal. Reelin bind to its receptors ApoER2 and VLDLR which causes tyrosine phosphorylation of Dab1 by Src family kinases (SFK). Phosphorylated Dab1 has several downstream targets involved in microtubule rearrangement (PI3-K pathway, Lis1), regulation of actin polymerization (N-WASP, Nckβ, Crk), cell survival (BAD), dendritic development (mTor), and gene expression (Notch1). Dab1 itself is ubiquitinated and degraded by the proteasome upon activation of the signaling pathway.

Signaling downstream of Dab1 is more diverse and less well characterized than the initial steps of the cascade. One pathway is based on interaction of phosphorylated Dab1 with phosphatidylinositol-3-kinase (PI3-K), leading to activation of protein kinase B (PKB)/Akt, thereby blocking action of glycogen synthase kinase 3β(GSK3β) (*Beffert et al.*, 2002; *Bock*

et al., 2003). This results in reduced phosphorylation of the microtubule-associated protein tau (*Beffert et al.*, 2002; *Ohkubo et al.*, 2003). Another downstream target of PKB/Akt is mammalian target of rapamycin (mTor) which was also reported to be regulated in response to Reelin signaling. This branch of the signaling cascade seems to be of special importance for the role of Reelin in dendritic development (*Jossin and Goffinet*, 2007). Furthermore, Reelin-induced PI3-K/Akt signaling might participate in cell survival via phosphorylation of Bcl-2/Bcl-XL associated death promoter (BAD) (*Ohkubo et al.*, 2007). Obviously, Reelin stimulation might as well influence other targets of the PI3-K pathway, although these effects are not clarified yet.

Dab1 was also shown to regulate neuronal Wiscott-Aldrich Syndrome protein (N-WASP), leading to activation of actin-related protein 2/3 (Arp2/3) complex and actin polymerization at the leading edge of migrating neuroblasts (*Winder*, 2004). This enables the formation of filopodia and cellular motility.

Moreover, as it was recently shown, the Reelin pathway seems to employ Notch signaling for proper neuronal migration in the developing cortex (*Hashimoto-Torii et al.*, 2008). Dab1 which co-immunoprecipitates with Notch-1 and Notch intracellular domain (NICD, the activated form of the receptor), likely is the signal mediator here, too (*Keilani and Sugaya*, 2008).

Other interaction partners of tyrosine-phosphorylated Dab1 are lissencephaly 1 (Lis1) (*Assadi et al.*, 2003), Nck (noncatalytic region of tyrosine kinase) adapter protein (Nckβ) (*Pramatarova et al.*, 2003), and Crk family members (*Ballif et al.*, 2004; *Chen et al.*, 2004; *Huang et al.*, 2004). The common characteristic of these signaling targets is their involvment in cytoskeletal rearrangements of both microtubuli and actin filaments, and cell migration.

Dab1 itself is subjected to ubiquitination and subsequent proteasomal degradation upon Reelin-induced phosphorylation as a feedback mechanism (*Arnaud et al.*, 2003a). Ubiquitination is carried out by an E3 ubiquitin ligase. Selection of phosphorylated Dab1 relies on the ubiquitinase's component Cullin 5 (Cul5) which only binds to Dab1 if it is phosphorylated on Y198 (*Feng et al.*, 2007). In the absence of Cul5, Dab1 accumulates, resulting in excess migration of neuroblasts in the cortical plate (*Feng et al.*, 2007).

1.2.3 Reelin signaling in neocortical development

The mammalian neocortex is the tissue best studied in terms of Reelin signaling. The neocortex is the evolutionary youngest part of the brain, constituting the outermost layer of the cerebral cortex.

Horizontally, it is organized in several distinct regions being specialized in complex processes like memory, learning, and sensory functions. The vertical structure reveals six cellular layers, composed of pyramidal neurons, with different morphologies and functions. These neuronal layers are built by sequential radial migration of neuroblasts from the germinal center, the ventricular zone (VZ) along glial fibers to the pial surface of the brain. This happens in an "inside-out-manner", meaning that earliest born neurons populate the innermost layers whereas later born cells settle in more superficial layers.

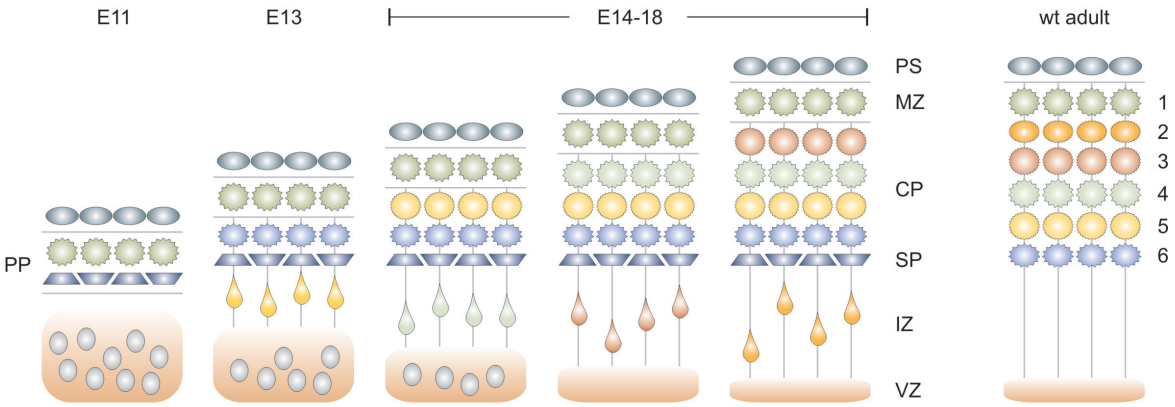

Figure 1.15 Development of the six-layered structure of the neocortex. Cortical postmitotic neurons start migration from the ventricular zone (VZ) to the pial surface (PS) of the cortex at E11, establishing the preplate (PP). At E13, this structure is split by the second wave of neurons into the subplate (SP) and marginal zone (MZ). Further neuronal waves pass the intermediate zone (IZ) by glia-guided migration and populate the cortical plate (CP) in an inside-out pattern, meaning that each batch of neurons bypasses all earlier layers to settle above them, just underneath the MZ. At E18, when the cortical plate is established, the subplate degenerates, leaving behind the final six-layered structure of the neocortex. (Adapted from *Andrade et al.*, 2007).

In the mouse, this process is initiated at embryonic day 11 (E11) when the first batch of neuroblasts starts migrating and form the so-called preplate (PP), a temporary layer of the developing cortex containing Cajal-Retzius cells which have been shown to be the Reelin

secreting cells in the mammalian cortex (*D'Arcangelo et al.*, 1997). The second wave of neuoblasts starts migration at E13 and splits the preplate into the marginal zone (MZ) with Cajal-Retzius neurons and the subplate (SP). These cells constitute the first layer of the new cortical plate. The following neuronal waves have to pass not only the subplate but also the earlier neuronal layers in order to reach their proper positions just beneath the marginal zone. Once all six layers have been established around E18, the subplate degenerates (*Gupta et al.*, 2002) (Fig. 1.15) and the remaining structure persists into adulthood.

In the Reelin deficient brain, migrating neuroblasts are unable to traverse the existing layers of older neurons. In particular, even splitting of the preplate does not occur, leaving a structure called the superplate (SPP). Later on, this defect results in an inverted "outside-in" and disorganized layering of the cortical plate (*Gupta et al.*, 2002) (Fig. 1.16).

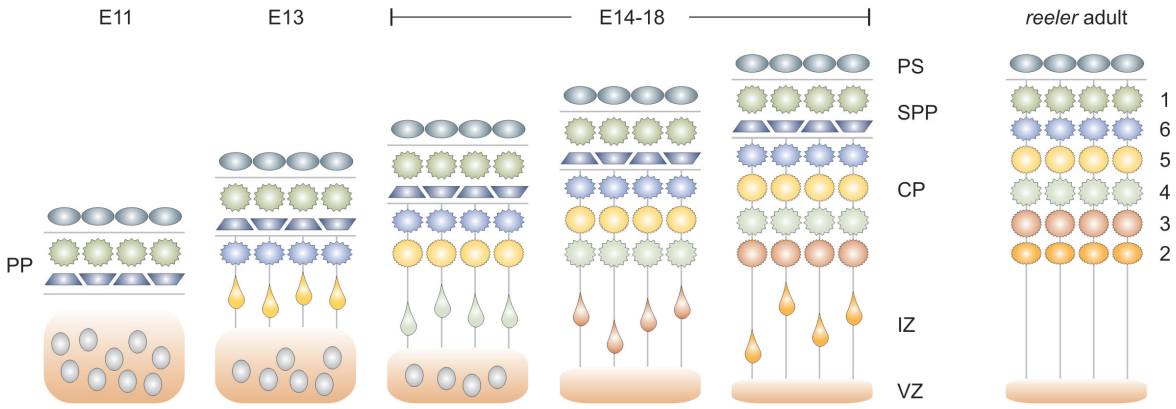

Figure 1.16 Developmental defect in the *reeler* neocortex. In *reeler* mice, migrating neuroblasts are unable to pass existing neuronal layers. This results in defective preplate splitting which leaves the double-layered so-called superplate (SPP) to reside beneath the MZ. Consequently, later neuronal waves are equally unable to traverse their predecessors, leading to an inverted cortical layering since later neurons settle beneath earlier ones. (Adapted from *Andrade et al.*, 2007).

Several hypotheses concerning the actual role of Reelin in this layering mechanism have been discussed. Reelin was proposed to act as a chemokine, to constitute a repellant cue for migrating neuroblasts (*Schiffmann et al.*, 1997), to transmit a stop signal to cells which have reached their final destination (*Frotscher*, 1997), or to facilitate dissociation of neuroblasts from radial glia (*Dulabon et al.*, 2000).

The most conclusive integrative model for Reelin action was presented recently (*Cooper*,

2008). This "detach and go" model is based on the occurance of two distinct modes of migration occuring during formation of the cortical plate (*Nadarajah et al.*, 2001).

Glia-giuded locomotion describes the movement of the entire neuronal cell along radial glial fibers which constitute a scaffold for the migrating neuroblast. Somal translocation is a glia-independent mechanism. Here, the migrating cell first extends its leading edge towards the marginal zone and, after encountering Reelin, shortens its process which is anchored to the MZ, in order to pull the cell soma through the preexisting neuronal layers (Fig. 1.17).

During corticogenesis, somal translocation seems to be preferentially used by neuroblasts of early waves, whereas the majority of later neurons who have to bypass greater distances and a growing number of neuronal layers, employs locomotion (*Nadarajah et al.*, 2003).

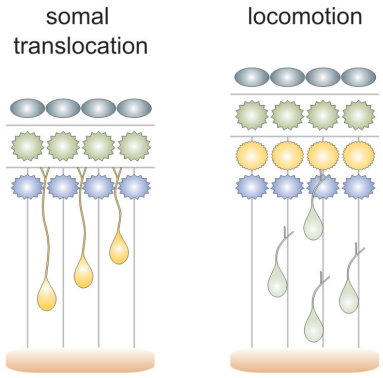

Figure 1.17 Two modes of neuronal migration. Radial migration of neurons occurs by two different mechanisms. During somal translocation, the cell pulls its soma along its previously extended leading edge which is attached to the MZ. Cells migrating by locomotion use radial glial fibers as a scaffold and move the entire cell at once. (Adapted from *Andrade et al.*, 2007).

The "detach and go" model states that when the extended leading processes of the first waves of migrating neurons reach the MZ, Reelin initiates somal translocation, either for splitting the preplate or for transition through the subplate. Later neurons use locomotion along glia fibers to reach the MZ and encounter Reelin. There, Reelin induces detachment from radial glia and again somal translocation to enable these neurons to bypass preexisting layers. In the absence of Reelin, the failure of this mechanism would therefore lead to an unsplit preplate. Later neuronal waves would still be able to migrate along radial glial fibres but would get stuck beneath the first neuronal layer, unable to initiate somal translocation (*Cooper*, 2008) (Fig. 1.18). These defects are in accordance with the observed *reeler* phenotype which strongly supports this model for Reelin action.

The action of Reelin might be extended by additional aspects facilitating migration of neuroblasts. First, Reelin might use its serine protease activity (*Quattrocchi et al.*, 2002) to affect cellular adhesion and thereby assist in the migration process.

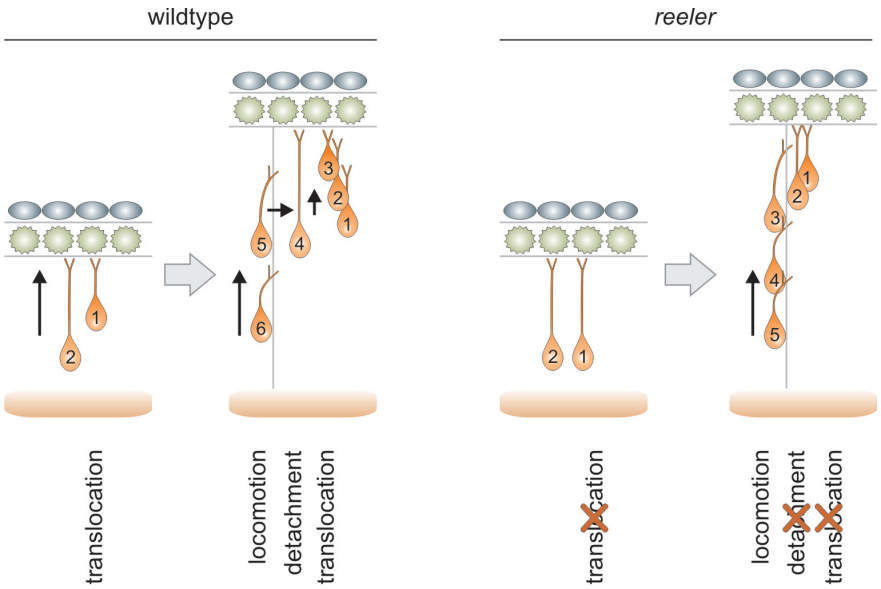

Figure 1.18 The detach and go model. According to this model, early neurons (1, 2) use somal translocation to reach their destined layer when they encounter Reelin in the marginal zone. Later neurons (3-6) overcome the distance to the MZ by glia-guided locomotion. Upon Reelin binding, they detach from the glial fibers and bypass the earlier layers by somal translocation. In Reelin deficient brains, early neurons are not able to translocate while later neurons still travel along the glial fibers but fail to detach and pass the pre-existing layers. This leads to an inverted and disorganized laminar structure. (Modified from *Cooper*, 2008).

Second, since it was shown to interact with α3β1-integrin, Reelin could further enhance dissociation of neurons from radial glia by removal of α3β1-integrin from the cell surface via endocytosis (*Dulabon et al.*, 2000).

Third, Reelin was reported to induce phosphorylation of n-cofilin, a protein involved in actin disassembly, which leads to stabilzation of the cytoskeleton. This might constitute a mechanism for anchoring the leading processes of migrating neuroblasts to the MZ when they encounter Reelin (*Chai et al.*, 2009).

Interestingly, n-cofilin phosphorylation requires Reelin, ApoER2, Dab1, PI3K, and SFKs but not VLDLR (*Chai et al.*, 2009). Previously, it was reported that late migrating neuroblasts in the neocortex depend only on ApoER2 but not VLDLR (*Hack et al.*, 2007), leading to the assumption that these neurons of the outer cortical layers may especially need n-cofilin-induced stabilization of their leading edge, possibly for giving the orientation needed for somal translocation (*Chai et al.*, 2009).

2 Aims of this study

The Reelin signal can be transmitted into target cells by both ApoER2 and VLDLR. Both receptors are able to phosphorylate the primary Reelin target, Dab1, with equal efficiencies. This explains why these receptors can, to a large extent, compensate for each other as corroborated by analysis of single receptor knockout mice. Only loss of both ApoER2 and VLDLR results in a phenotype which is also observed in *reeler* mice, lacking functional Reelin. Nevertheless, loss of either one of the Reelin receptors leads to distinct phenotypes in mice. Both single receptor knockouts show disorganized cortical layers. However, while the *reeler*-typical inversion of cortical lamination can be found in ApoER2 but not VLDLR knockout mice, loss of VLDLR leads to overmigration of cortical neuroblasts (*Hack et al.*, 2007). Moreover, VLDLR seems to be essential for the structural development of the cerebellum, whereas the primary role of ApoER2 lies in cortical lamination (*Trommsdorff et al.*, 1999).

Observation of these differences led to the question which structural and functional features of ApoER2 and VLDLR discriminate them and specify their distinct function(s). Since initiation of the signaling cascade does not seem to be influenced by the type of receptor mediating it, this study aimed at the detailed analysis of the fate of ApoER2 and VLDLR after binding of Reelin and Dab1 phosphorylation. As it was previously shown, ApoER2 and VLDLR reside in different microdomains of the plasma membrane. Based on this finding, endocytotic behaviour, intracellular trafficking, degradation, and processing of both repetors should be closely investigated and compared here. In addition, chimeric receptors built of ApoER2 and VLDLR domains and fractionation of plasma membrane microdomains should be used to link differences between the receptors to structural features or differential sorting on the cell surface.

3 Results and Discussion

3.1 Differential functions of ApoER2 and VLDL receptor in Reelin signaling

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Differential Functions of ApoER2 and Very Low Density Lipoprotein Receptor in Reelin Signaling Depend on Differential Sorting of the Receptors*[S]

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ApoER2 and very low density lipoprotein (VLDL) receptor transmit the Reelin signal into target cells of the central nervous system. To a certain extent, both receptors can compensate for each other, and only the loss of both receptors results in the *reeler* phenotype, which is characterized by a gross defect in the architecture of laminated brain structures. Nevertheless, both receptors also have specific distinct functions, as corroborated by analyses of the subtle phenotypes displayed in mice lacking either ApoER2 or VLDL receptor. The differences in their function(s), however, have not been defined at the cellular level. Here, using a panel of chimeric receptors, we demonstrate that endocytosis of Reelin and the fate of the individual receptors upon stimulation are linked to their specific sorting to raft *versus* non-raft domains of the plasma membrane. VLDL receptor residing in the non-raft domain endocytoses and destines Reelin for degradation via the clathrin-coated pit/clathrin-coated vesicle/endosome pathway without being degraded to a significant extent. Binding of Reelin to ApoER2, a resident of rafts, leads to the production of specific receptor fragments with specific functions of their own and to degradation of ApoER2 via lysosomes. These features contribute to a receptor-specific fine tuning of the Reelin signal, leading to a novel model that emphasizes negative feedback loops specifically mediated by ApoER2 and VLDL receptor, respectively.

Defective Reelin signaling causes lamination defects in many areas of the cerebral cortex, hippocampus, and cerebellum (1, 2). The major abnormality in the cortex arises during embryogenesis from the inability of radially migrating neurons to invade and split the preplate. These neurons settle beneath the preplate, which is shifted toward the pial surface, where it forms a "superplate" in affected animals. Consequently, later born neurons cannot bypass earlier neurons that have settled beneath the superplate so that consecutive waves of neurons generated in the subventricular zone and migrating outwards establish a pattern of inverted neuronal layers. The role of Reelin in the correct lamination of certain brain structures was recently compiled in the "detach and go" model (3), where Reelin was proposed to promote detachment of migrating neurons from glial fibers and their translocation to the outermost area of the developing cortical plate. Despite recent progress in understanding molecular events in the Reelin signaling pathway (reviewed in Refs. 4 and 5), our knowledge about the modulation of the initial signal and downstream events guiding migration and positioning of neurons or modulating other processes like dendrite development is still scarce (6, 7). Proposed mechanisms involve Lis1 (8), Nckβ (9), and differential modulation of phosphatidylinositol 3-kinase-downstream pathways employing mTOR or GSK3β (10). The key events indispensable for triggering these pathways are binding of Reelin to ApoER2 and VLDL[2] receptor (VLDLR) and subsequent phosphorylation of Dab1. Obviously, Reelin-mediated receptor clustering triggers tyrosine phosphorylation of Dab1 (11). This event, however, does not seem to be sufficient to evoke the full Reelin signal, because anti-receptor antibodies that trigger Dab1 phosphorylation by receptor clustering do not rescue the *reeler* phenotype in brain slice cultures (12). In addition, thrombospondin 1, which is another functional ligand for ApoER2 and VLDLR in the brain, promotes Dab1 phosphorylation without eliciting the canonical Reelin signaling pathway (13).

Dab1 phosphorylation is mediated by members of the Src family of kinases (14–16). Mice lacking both Fyn and Src develop a phenotype similar to that of the Dab1-deficient scrambler mice (17). Dab1 binds to the NFDNP*X*Y sequence motif present in the cytosolic domains of ApoER2 and VLDLR (18). This domain is indispensable for Reelin signaling, since mice lacking VLDLR and carrying mutant alleles for ApoER2 coding for an altered NFDNPVY motif that does not bind Dab1 develop a *reeler* phenotype (19). This domain is also present in other members of the LDL receptor gene family, such as LDL receptor, LRP1, and LRP2 (20), and plays a key role in clathrin-mediated endocytosis of these receptors. In a Dab1-overexpressing cell model system, Dab1 decreases the endocytosis rate of the LDL receptor by interfering with the formation of the endo-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4.

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[2] The abbreviations used are: VLDL, very low density lipoprotein; VLDLR, VLDL receptor; MES, 4-morpholineethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; RCM, Reelin conditioned medium; MCM, mock conditioned medium; Ab, antibody; HRP, horseradish peroxidase; IOD, integrated optical density; CLM, caveolin-rich light membrane(s); PBS, phosphate-buffered saline; CCV, clathrin-coated vesicle; NCM, non-caveolae membrane(s); CDX, methyl-β-cyclodextrin; CTF, C-terminal fragment; EEA1, early endosomal antigen 1.

cytosis complex (21). However, because Dab1 is brain-specific, the interaction with LDL receptor, LRP1, and LRP2 and the interference with endocytosis of these receptors most likely are not of physiological relevance. Our own studies and others have demonstrated that ApoER2 associates with lipid rafts, whereas VLDLR is strictly excluded from these microdomains (22–24). Despite its raft association, ApoER2 is endocytosed via the clathrin-mediated process, apparently through its association with Dab2 (22). It is still unclear, however, how important endocytosis of ApoER2 and VLDLR is in Reelin signaling.

With the identification of ApoER2 and VLDLR as functional Reelin receptors, it became evident that both receptors can compensate for each other to a certain extent, since only the lack of both receptors causes the *reeler* phenotype (25). Loss of ApoER2 or VLDLR alone causes subtle but distinguishable phenotypes, pointing to VLDLR being more important for the development of the cerebellum and ApoER2 for lamination of the cortex. This fact was recently corroborated by detailed studies on the divergent roles of ApoER2 and VLDLR in the migration of cortical neurons (26), which demonstrated that ApoER2 is indispensable for the correct migration of late generated neurons, whereas the VLDLR-mediated Reelin signal prevents neurons from entering the marginal zone.

Because both receptors mediate Dab1 phosphorylation, the question of the molecular substrate for their individual functions arises. Using chimeric receptor constructs and a fibroblast model system, we now demonstrate that endocytosis and cellular trafficking differ between ApoER2 and VLDLR and relate to raft *versus* non-raft localization of these receptors.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Conditioned Media—NIH 3T3 and 293 HEK cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), and penicillin/streptomycin (Invitrogen) at 37 °C and 7.5% CO_2. Stable NIH 3T3-based cell lines expressing murine ApoER2 harboring LA repeats 1–3, 7, and 8 and containing the proline-rich cytoplasmic insert (3T3 A), murine VLDLR lacking the O-linked sugar domain (3T3 V), or either receptor and murine Dab1 (3T3 A/D and 3T3 V/D) (23) were kept under puromycin selection (0.75 μg/ml). Stable cell lines expressing chimeric receptors were generated as described for 3T3 A and 3T3 V (23) and were grown under the same conditions. 24 h before the experiment, puromycin-resistant cells were switched to growth medium lacking puromycin. Reelin-expressing 293 HEK cells were cultivated and used for production of Reelin conditioned medium (RCM) as described before (27). Mock conditioned medium (MCM) was prepared from untransfected 293 HEK cells using the same procedure. Primary rat neuronal cultures were obtained from embryonic day 16.5 rat embryonic brains and were kept in DMEM/F-12 (Invitrogen) containing B27 supplement (Invitrogen) and penicillin/streptomycin at 37 °C and 5% CO_2 for 72 h before use as described (11). Transient transfection of 3T3 was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cloning of Chimeric Receptors—The following primers were used for generation of chimeric receptor constructs. VLDLR and ApoER2 primer pairs, spanning the respective full-length murine cDNAs, each containing an EcoRI restriction site (underlined), were as follows: VLDLR sense primer (primer 1) (5′-CGG AAT TCA TGG GCA CGT CCG CGC GC-3′), VLDLR antisense primer (primer 2) (5′-ATG AAT TCA AGC CAG ATC ATC ATC TGT GCT TAC-3′), ApoER2 sense primer (primer 3) (5′-ATG AAT TCA TGG GCC GCC CAG AAC TGG-3′), and ApoER2 antisense primer (primer 4) (5′-ATG AAT TCT CAG GGC AGT CCA TCA TCT TC-3′). ApoER2-VLDLR chimeric primers, annealing to ApoER2 at their 5′ region (shown in italic type) and to VLDLR at their 3′ region (underlined) were as follows: sense primer (primer 5) (5′-*GGT ACC TCA TCT GGA GGA A*TT GGC AAC-3′) and antisense primer (primer 6) (5′-*CGC TTC CAG TTC CTC CAC* ATC AAG TAG CC-3′), recognizing the junction of transmembrane and intracellular domain, and sense primer (primer 9) (5′-*CAA CAG TCA CCG CTG CTG* CTG CCT GGG-3′) and antisense primer (primer 10) (5′-*CCC AAT GAC* TGA AGT CCC TTT TGG GGG AAC-3′), recognizing the junction of extracellular and transmembrane domain. VLDLR-ApoER2 chimeric primers, annealing to VLDLR at their 5′ region (underlined) and to ApoER2 at their 3′ region (italic type) were as follows: sense primer (primer 7) (5′-GGC TAC TTG ATG *TGG AGG AAC TGG AAG CG*-3′) and antisense primer (primer 8) (5′-GTT GCC AAT *TCC TCC AGA TGA GGT AAC CAC*-3′), recognizing the junction of transmembrane and intracellular domain, and sense primer (primer 11) (5′-CCA AAA GGG ACT TCA *GTC ATT GGG GTC ATC GTG C*-3′) and antisense primer (primer 12) (5′-GAT GGC CCA GGC AG*C AGC AGC GGT GAC TGT TGA GC*-3′), recognizing the junction of extracellular and transmembrane domain.

For the first round of PCR amplification, fragments of ApoER2 and VLDLR were amplified from pMSCVpuro-ApoER2 and pMSCVpuro-VLDLR (23), using the following primers: primers 1 and 10 for VLDLR extracellular domain, primers 3 and 12 for ApoER2 extracellular domain, primers 1 and 6 for VLDLR extracellular and transmembrane domain, primers 3 and 8 for ApoER2 extracellular and transmembrane domain, primers 9 and 2 for VLDLR transmembrane and intracellular domain, primers 11 and 4 for ApoER2 transmembrane and intracellular domain, primers 5 and 2 for VLDLR intracellular domain, and primers 7 and 4 for ApoER2 intracellular domain. The obtained fragments were purified and used as templates for another round of PCR amplification. Fragments harboring the VLDLR extracellular and transmembrane domain were mixed with fragments harboring the ApoER2 intracellular domain and *vice versa*, and fragments harboring the VLDLR extracellular domain were mixed with fragments harboring the ApoER2 transmembrane and intracellular domain and *vice versa*. Primers used for amplification were primers 1 and 4 for the VVA and VAA constructs and primers 3 and 2 for the AAV and AVV constructs. The PCR products obtained in this second PCR amplification step were cloned into the pMSCVpuro backbone using the EcoRI restriction site.

Antibodies—Antibodies against ApoER2 were Ab 186, raised against the entire ligand-binding domain (11); Ab 220, raised against the first ligand-binding repeat (28); and Ab 20, raised against the

cytoplasmic tail (29). The ligand-binding domain of VLDLR was detected using Ab 74, which was raised in rabbits using a maltose-binding protein fusion protein containing the first ligand binding repeat of VLDLR. Ab 187 (11) was used for detection of VLDLR in immunofluorescence assays. For immunoprecipitation of Dab1, Ab 48 and Ab 54 (11) against the short splice variant of murine Dab1 were used. Mouse anti-Dab1 (D4) and mouse anti-Reelin (G10) antibodies were kind gifts of Andre Goffinet (University of Louvain, Belgium). Mouse anti-Lis1 was obtained from Orly Reiner (Weizmann Institute of Science, Rehovot, Israel). The following antibodies were purchased from the indicated sources: mouse anti-VLDLR (6A6) and mouse anti-phosphotyrosine (PY99), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse anti-HA (HA.11), Covance; mouse anti-clathrin heavy chain and rabbit anti-Caveolin, BD Transduction Laboratories; rabbit anti-early endosomal antigen 1, Affinity BioReagents; secondary HRP-conjugated anti-mouse and anti-rabbit antibodies, Jackson ImmunoResearch; Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit, Molecular Probes.

Preparation of Cell Extracts, SDS-PAGE, and Western Blotting—Cells were washed twice with ice-cold PBS and lysed in Hunt buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) supplemented with proteinase inhibitor mix (Complete™, Roche Applied Science). Cell debris was removed by centrifugation for 5 min at 20,000 × *g*. Proteins were separated by reducing SDS-PAGE and transferred onto nitrocellulose membranes by semidry blotting. Membranes were blocked in PBS containing 0.1% Tween 20 and either 5% bovine serum albumin or 5% nonfat dry milk and incubated with primary and HRP-conjugated secondary antibodies. For detection, enhanced chemiluminescence solution (Pierce) was used. For quantification of Western blot results, integrated optical density (IOD) values of the bands were calculated using Gel-Pro analyzer software (Media Cybernetics) and normalized to IOD of the loading control. For ligand blots, cell extracts were separated by non-reducing SDS-PAGE and Western blotting, and membranes were blocked in TBS containing 5% bovine serum albumin and 2 mM $CaCl_2$ and incubated with RCM diluted with Opti-MEM (1:2) containing 2 mM $CaCl_2$ for 2 h before incubation with primary (G10) and secondary antibodies.

Reelin Uptake and Degradation Assay—NIH 3T3 cells expressing ApoER2 or VLDLR were precooled at 4 °C for 30 min and incubated with RCM at 4 °C for 1 h to allow for Reelin binding to the cells. After extensive washing with TBS, cells were covered with Opti-MEM (Invitrogen) and shifted to a 37 °C water bath. After the indicated periods, cell extracts were prepared using Hunt buffer and analyzed by Western blotting. Relative Reelin amounts were calculated from IOD values.

Dab1 Phosphorylation Assay—Cells expressing Dab1 and one of the receptors were starved for 1 h in plain DMEM and incubated for 1 h with RCM or MCM. Cell extracts were prepared in Hunt buffer containing protease inhibitor mix and phosphatase inhibitors (50 mM NaF and 2 mM Na_3VO_4) and used for immunoprecipitation. Extracts were incubated with anti-Dab1 antiserum overnight at 4 °C, 40 μl of a protein A-Sepharose bead slurry (Zymed Laboratories Inc.) were added, and samples were incubated for 1 h at 4 °C again. Beads were collected by centrifugation at 500 × *g* for 1 min and washed three times using Hunt buffer. Samples were analyzed by Western blotting.

Receptor Degradation and Fragmentation Assays—To analyze degradation and secretase-mediated fragmentation of ApoER2, receptor-expressing cells were starved for 1 h in plain DMEM or DMEM containing 20 μg/ml cycloheximide and incubated for 4 or 6 h with medium containing cycloheximide and the indicated ligands and supplements. Cell extracts were prepared in Hunt buffer and analyzed by Western blotting.

Isolation of Caveolin-rich Light Membranes (CLM)—CLM were prepared from stable NIH 3T3 fibroblasts grown to confluence in 15-cm dishes or from WT mouse embryonic brains isolated at embryonic day 15. All procedures were carried out at 4 °C. Briefly, cells were washed with TBS and pelleted by centrifugation (5 min, 1400 × *g*). The supernatant was removed, and cells were solubilized in TBS containing 2% Brij 78P (Fluka) and Complete™ protease inhibitors (Roche Applied Science) by passaging the cells 10 times through a 23-gauge needle. Cell debris was removed by centrifugation (10 min, 21,000 × *g*) and the lysate (0.6 ml) was mixed with 0.6 ml of 90% (w/v) sucrose in MBS (MES-buffered saline; 25 mM MES, pH 6.5, 150 mM NaCl) and transferred to an ultracentrifuge tube. A discontinuous sucrose gradient was formed above the homogenate by adding 2.5 ml of 35% (w/v) sucrose in MBS, followed by 0.6 ml of 5% (w/v) sucrose in MBS. After centrifugation at 160,000 × *g* for 20 h in a Beckman SW60Ti rotor at 4 °C, 0.44-ml fractions were collected from the top of the tube. Fraction 2 at the interface between the 5 and 35% sucrose boundaries was designated the CLM fraction.

Preparation of Clathrin-coated Vesicles—Coated vesicles were prepared from NIH 3T3 cells expressing either receptor grown to near confluence or primary rat neurons cultured for 72 h using a ^2H_2O, 8% sucrose gradient (30). Cells were washed twice with PBS and once with MES buffer (100 mM MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 3 mM NaN_3, Complete™ proteinase inhibitor mixture). All steps were carried out at 4 °C. Cells were scraped in MES buffer and homogenized using a Potter tissue grinder. The homogenate was centrifuged at 5000 × *g* for 5 min. The pellet was resuspended in MES buffer and centrifuged at 5000 × *g* for 5 min. The supernatants from the two centrifugations were combined and centrifuged at 100,000 × *g* for 60 min. The resulting pellet was resuspended in MES buffer and centrifuged at 10,000 × *g* for 10 min. The resulting pellet was resuspended in MES buffer again, and the centrifugation was repeated. The supernatants from the two centrifugation steps were combined and centrifuged at 100,000 × *g* for 60 min. The pellet was resuspended in 1 ml of MES buffer and centrifuged at 10,000 × *g* for 10 min; the resulting pellet was again resuspended using 1 ml of MES buffer and centrifuged at 10,000 × *g* for 10 min. The combined supernatants were loaded on the top of 2 ml of 8% sucrose in ^2H_2O and centrifuged at 80,000 × *g* for 2 h. The pellet, resuspended in MES buffer, was centrifuged at 20,000 × *g* for 10 min. The supernatant was recovered and designated as clathrin-coated vesicle (CCV) fraction. Protein concentration was determined using the Coomassie Plus protein assay reagent (Pierce) accord-

ing to the manufacturer's protocol, and equal protein amounts of cell lysates and CCV were subjected to SDS-PAGE and Western blotting.

Immunofluorescence Assays and Microscopy—Sterile glass coverslips were coated with 40 μg/ml poly-L-lysine in PBS for 1 h at room temperature. NIH 3T3 cells expressing ApoER2 or VLDLR were grown on the coverslips for 24 h using standard fibroblast growth medium. Cells were cooled to 4 °C, washed with ice-cold PBS, and overlaid with RCM. After 1 h of incubation, RCM was removed, and cells were washed and incubated with Opti-MEM at either 4 or 37 °C for 10 min. Subsequently, cells were washed and fixed with 4% paraformaldehyde fixative. Fixed cells were washed with PBS containing 100 mM glycine and incubated with 0.1% Triton X-100 in PBS for 2 min to permeabilize the plasma membrane. Cells were washed again with PBS, blocked for 30 min with blocking solution (1% bovine serum albumin in phosphate-buffered saline) at room temperature, and incubated with primary and secondary antibodies diluted in blocking solution for 1 h each. Coverslips were washed again and mounted on glass slides using DAKO fluorescent mounting medium (Dako Corp.). Slides were analyzed using a confocal fluorescence microscope (laser-scanning microscope 510, Zeiss) and the corresponding software (Zeiss LSM Image Browser). Antibodies used for detection were Ab 186 for ApoER2, Ab 187 for VLDLR, G10 for Reelin, anti-early endosomal antigen 1, and secondary Alexa Fluor-coupled anti-mouse and anti-rabbit antibodies.

RESULTS

Expression and Subcellular Sorting of Chimeric Receptors—We have recently demonstrated that (i) ApoER2 and VLDLR reside in distinct subdomains of the plasma membrane and (ii) independently of this localization, both receptors mediate Reelin-induced Dab1 phosphorylation (23). Thus, we reasoned that recently unraveled functional differences of ApoER2 and VLDLR in migration of cortical neurons (26) could be due to differences in ligand endocytosis and intracellular trafficking of the receptors. Such differences might be caused either by differential sorting of the receptors to raft *versus* non-raft domains or by intrinsic properties of the receptor molecules independent of their sorting. To distinguish between these possibilities, we constructed a panel of chimeric receptors by swapping the respective intracellular, transmembrane (*TM*), and extracellular domains as detailed in Fig. 1*A*. The resulting chimeric receptors termed VVA, AAV, VAA, and AVV (V, derived from VLDLR; A, derived from ApoER2; in the order from left to right: extracellular domain, transmembrane, cytoplasmic domain), were expressed in 3T3 mouse fibroblasts and tested for functionality. As demonstrated in supplemental Fig. 1*A*, all constructs were expressed at comparable levels and were recognized by the appropriate antibodies (corresponding epitopes for the antibodies used are indicated in Fig. 1*A*). The faint double band produced by Ab 74 in cells expressing constructs not containing the extracellular domain of VLDLR must be due to cross-reactivity with an unrelated protein because it is also present in mock-transfected 3T3 cells. Furthermore, all constructs containing the extracellular domain derived from ApoER2 produce a double band, which is characteristic for ApoER2 and represents the precursor and the mature form of the receptor (23). All chimeric receptors bind Reelin, as tested by ligand blotting (supplemental Fig. 1*B*), and 3T3 fibroblasts expressing Dab1 (23), and any one of the chimeric receptors respond to Reelin with robust Dab1 phosphorylation as the WT receptors do (supplemental Fig. 1*C*). Next, we compared the subcellular localization of the chimeras with those of the WT receptors by separating the cell membranes into CLM and heavy membrane fractions containing ER membranes and non-raft fractions of the plasma membrane (non-caveolae membranes (NCM)) as described (23). As demonstrated previously in fibroblasts (23), mature ApoER2 is predominantly present in the CLM fraction, characterized by the presence of caveolin, whereas the immature form of ApoER2 (present in the ER) and VLDLR are exclusively found in the heavy non-raft membrane fraction (Fig. 1*B*). This particular subcellular distribution is not a specific effect seen in fibroblasts but is also evident *in vivo* because the receptors follow the same distribution in membranes prepared from embryonic mouse brain (Fig. 1*C*). As demonstrated in Fig. 1*B*, chimeras expressed in fibroblasts and comprising the extracellular domain of VLDLR (VVA, VAA) are found in the NCM. In contrast, chimeras containing the extracellular domain of ApoER2 (AAV, AVV) follow the distribution of WT ApoER2 independently of the composition of the remaining parts of the receptors. Thus, the extracellular domain of ApoER2 determines its sorting to raft domains of the cell membrane.

This model system provides us with the opportunity to test whether Reelin endocytosis, receptor degradation, or specific receptor cleavage are determined by (i) specific molecular features of the respective intracellular domains of the receptors or (ii) their localization to raft or non-raft domains of the cell membrane.

Endocytosis—To evaluate the efficiency of ApoER2 and VLDLR to endocytose and degrade Reelin, we used fibroblast cell lines engineered to express selected components of the Reelin signaling pathway (23). The cells (3T3 V; 3T3 A) were incubated with Reelin at 4 °C to allow binding of the ligand. After washing the cells, Reelin-free medium was added, the cells were shifted to 37 °C, and at the indicated time points, cell-associated Reelin was measured by Western blotting using antibody G10, which interacts with full-length Reelin and the proteolytic fragments NR6 and NR2 (see supplemental Fig. 2). Because endocytosis and degradation of full-length Reelin and both fragments follow a similar kinetic (supplemental Fig. 2), only full-length Reelin is shown in the following figures. As demonstrated in Fig. 2, cells expressing VLDLR (3T3 V) degrade associated Reelin extremely quickly. After 12 min, more than 60%, and after 24 min, all of the detectable cell-associated Reelin was lost. In cells expressing ApoER2 (3T3 A), however, bound Reelin remained stably associated with the cells, slowly dropping to 75% of the starting level after 24 min of incubation at 37 °C (Fig. 2*B*). As control, we used the parental 3T3 cells not expressing any of the two receptors. These cells do not interact with Reelin, demonstrating that binding and subsequent loss of Reelin is dependent on the presence of the receptors. To test whether the effect is cell-specific, we transiently transfected HeLa cells with ApoER2 and VLDLR and

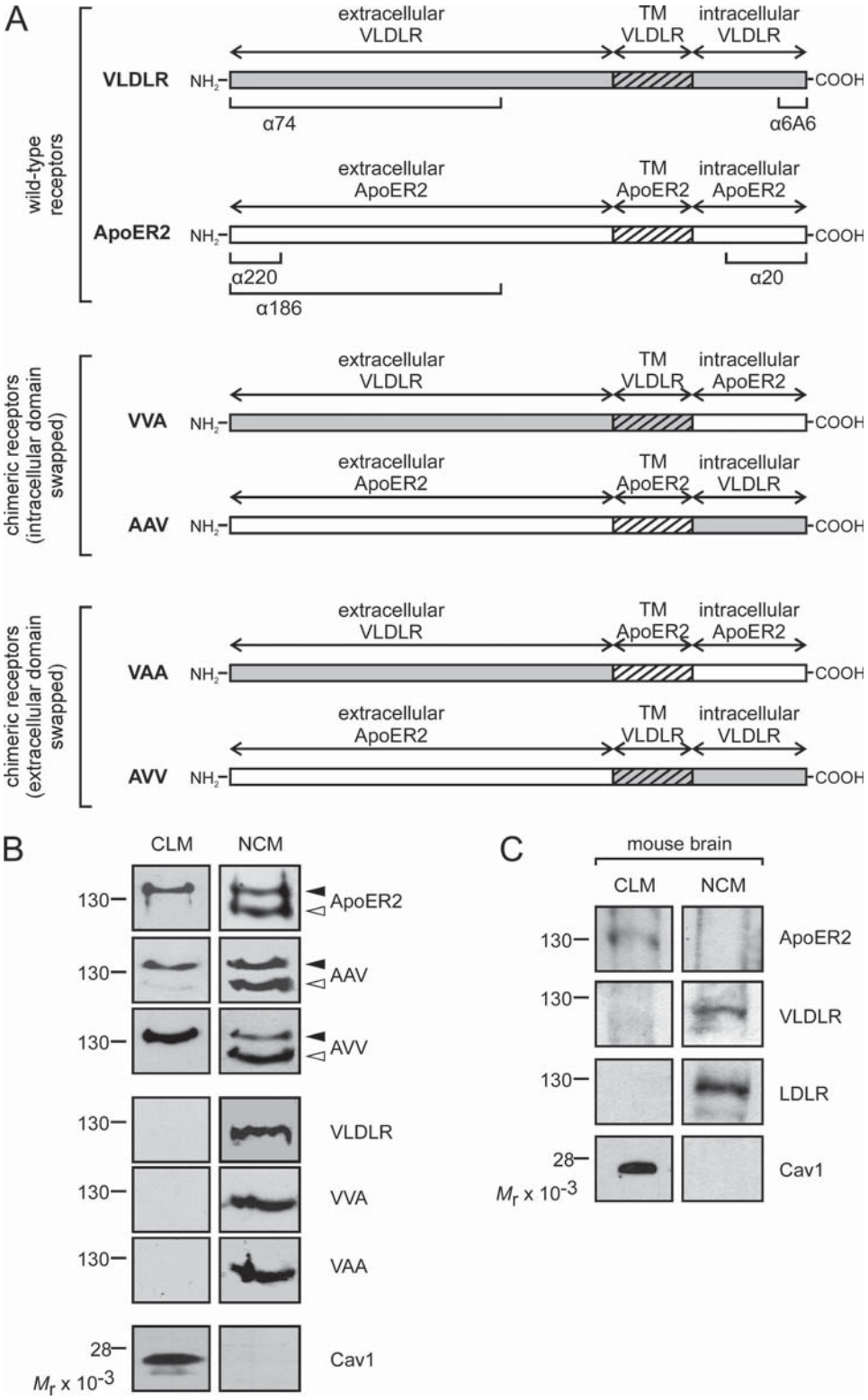


FIGURE 1. **Localization of ApoER2, VLDLR, and chimeric receptors within the plasma membrane.** *A*, schematic presentation of chimeric receptors consisting of intracellular, transmembrane (*TM*), and extracellular domains of ApoER2 (*A*) and VLDLR (*V*), respectively. Epitopes of the antibodies used are marked in the schematic diagram of the WT receptors. *B*, 3T3 cells expressing either of the receptors were fractionated by density centrifugation to prepare CLM and NCM fractions as described under "Experimental Procedures." The presence of the respective receptor was analyzed by Western blotting using Ab 20 for detection of ApoER2, VVA, and VAA and Ab 6A6 for detection of VLDLR, AAV, and AVV. *Filled* and *open arrows* indicate mature ApoER2, AAV, and AVV receptors and their unglycosylated precursors, respectively. The quality of the fractionation procedure was controlled by analyzing the presence of caveolin 1 by Western blotting using an anti-caveolin antibody. *C*, total brain extracts of embryonic WT mice (embryonic day 15; *E15*) were treated and analyzed as described above to detect the indicated proteins in CLM and NCM.

performed the same set of experiments. ApoER2- and VLDLR-mediated Reelin degradation followed the same kinetic as demonstrated for fibroblasts (data not shown). Thus, independent of cell type, VLDLR exhibits a high internalization rate leading to a fast removal of the ligand from the cell surface. In contrast, ApoER2, which resides in rafts, mediates very little Reelin degradation within the time frame relevant to Reelin signaling (20 min).

To evaluate whether Reelin is endocytosed by VLDLR and ApoER2 via the same intracellular pathway, we prepared CCVs from cell lysates and analyzed the content of this preparation by Western blotting. 3T3 cells expressing either ApoER2 or VLDLR and cultured primary neurons from WT rats were incubated in the presence of Reelin for 60 min and washed, and the CCVs were prepared and tested for the presence of the respective receptor and Reelin (Fig. 3). In 3T3 cells, Reelin and the corresponding receptor were always present in the CCV-enriched fraction, independent of the receptor expressed (Fig. 3*A*). Note that in CCV, only the mature form of ApoER2 is present, as expected. In primary neurons derived from WT rats that express VLDLR and ApoER2, Reelin and both receptors were present in the CCV preparation. In an alternative approach, we studied this process by immunofluorescence microscopy (Fig. 4). 3T3A and 3T3V cells were incubated at 4 °C with Reelin to allow receptor binding of the ligand in the absence of membrane-dependent endocytosis. Then the cells were incubated in Reelin-free medium for 10 min at 4 °C or 37 °C and fixed and processed for immunostaining with antibodies against Reelin, the respective receptor, and EEA1, respectively. As demonstrated in Fig. 4*A*, cells expressing ApoER2 or VLDLR incubated with Reelin at 4 °C exhibit prominent staining for Reelin outlining the cell surface. This staining is not continuous but shows a punctuate pattern that co-localizes with that obtained

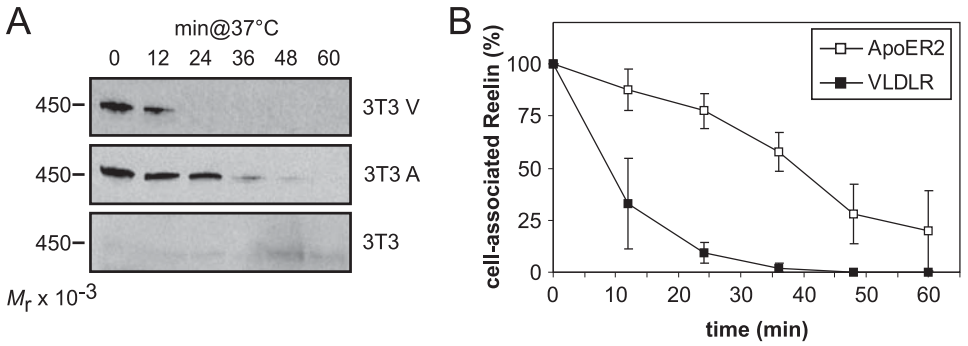


FIGURE 2. **VLDLR mediates Reelin endocytosis much more efficiently than ApoER2.** *A*, 3T3 cells expressing ApoER2 or VLDLR or mock-transfected 3T3 cells were incubated with RCM at 4 °C to allow binding of Reelin to the respective receptors. Cells were then shifted to 37 °C for the indicated time periods to allow internalization and degradation of the ligand. Extracts were prepared and analyzed for cell-associated Reelin by Western blotting using Ab G10 in combination with an HRP-coupled goat anti-mouse antibody. *B*, Western blots of *A* and two identical independent experiments were quantified by densitometry, and IOD values of the bands were normalized to the density of the band corresponding to the first time point. *Error bars*, S.E. ($n = 3$).

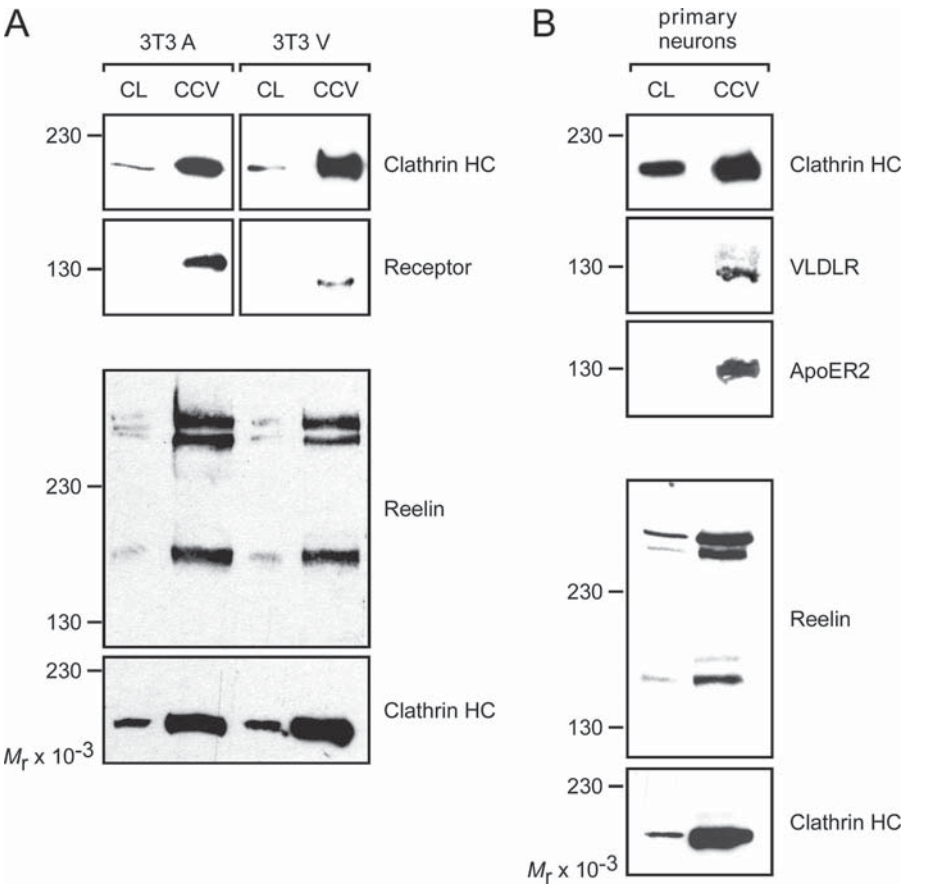


FIGURE 3. **ApoER2 and VLDLR internalize Reelin via clathrin-mediated endocytosis.** *A*, 3T3 cells expressing either ApoER2 or VLDLR were incubated with RCM for 1 h, and CCVs were prepared from total cell lysates (*CL*) as described under "Experimental Procedures." The presence of Reelin and the respective receptors was analyzed by Western blotting using the appropriate antibodies and the corresponding HRP-coupled secondary antibodies. Clathrin heavy chain was detected as control for enrichment of CCVs. *B*, CCVs were prepared from primary rat neurons and analyzed as described above.

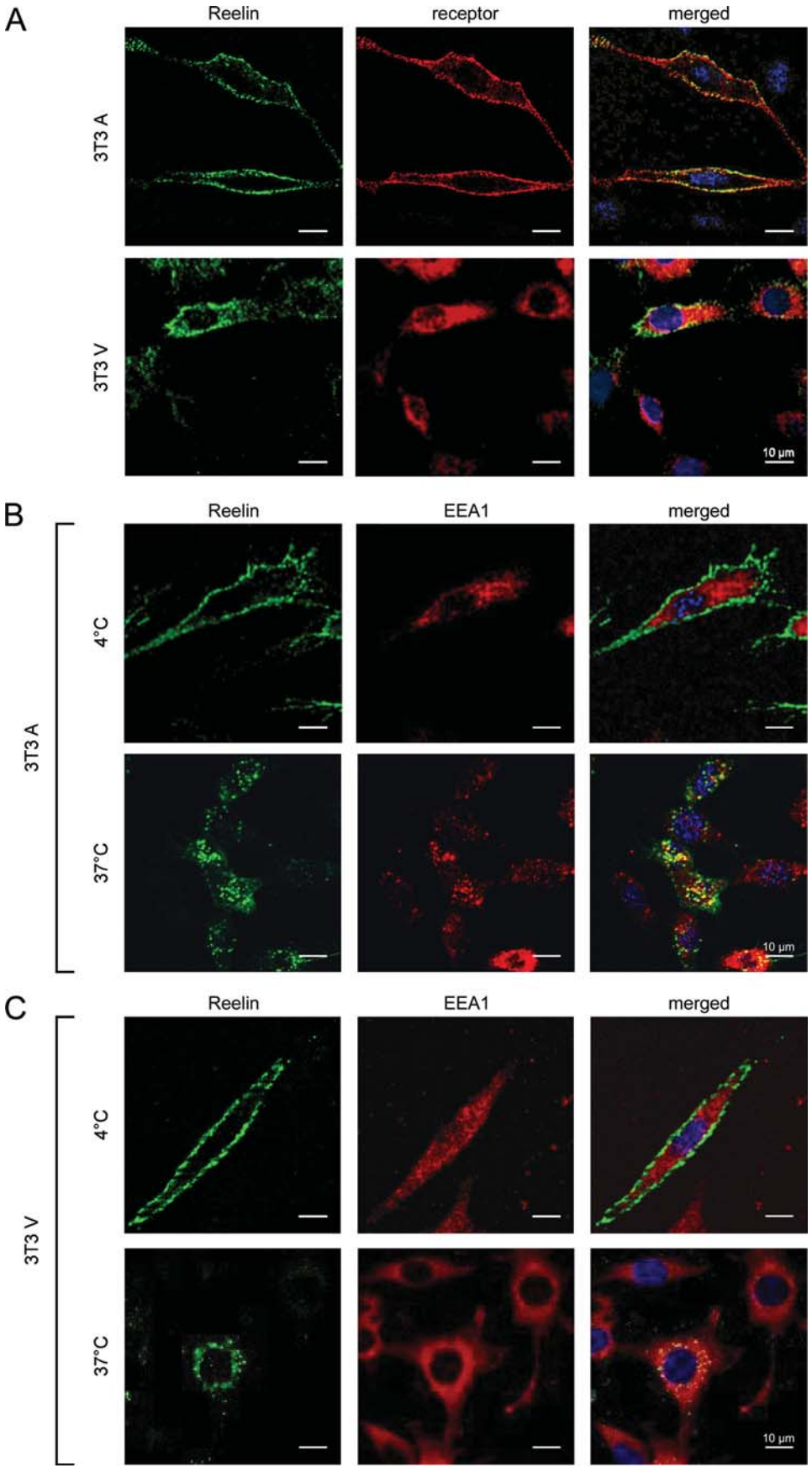
with antibodies against the respective receptors. Under these conditions (endocytosis is blocked at 4 °C), Reelin does not co-localize with intracellular EEA1 (Fig. 4, *B* and *C*, 4 °C). After 10 min at 37 °C, Reelin appears in vesicular structures co-localizing with EEA1, independently of the receptor expressed. Together with the results obtained from the analysis of coated vesicles (Fig. 3), these data suggested that Reelin endocytosed via ApoER2 follows the same route as Reelin taken up by VLDLR (*i.e.* via clathrin-coated vesicles).

Having established that internalized ApoER2 and VLDLR follow the same pathway, we set out to investigate whether the different endocytosis rates for ApoER2 and VLDLR are due to their distinct membrane localization. Thus, internalization rates of Reelin were determined as performed for the WT receptors (Fig. 2) using cells expressing the different chimeric receptor constructs described in the legend to Fig. 1. As demonstrated in Fig. 5*A*, chimeric receptors containing the extracellular domain derived from ApoER2 exhibited the same endocytosis kinetics as WT ApoER2. The chimeras containing the extracellular domain of VLDLR, however, removed Reelin from the cell surface with the same rate as WT VLDLR (Fig. 5*B*). Thus, the receptor domains responsible for specific membrane localization of the receptors also determine their endocytosis rates. To test this notion further, cells expressing ApoER2 were treated with methyl-β-cyclodextrin (CDX), which removes cholesterol from the cell surface, thereby dissolving the raft structures. Because depletion of cholesterol also interferes with clathrin-mediated endocytosis (31), we determined the concentration of CDX (5 mM) that did not affect VLDLR-mediated Reelin endocytosis (data not shown). As previously reported (23), this treatment completely shifts ApoER2 into the non-raft fraction, and as demonstrated here (Fig. 5*C*), it increased the ApoER2-mediated Reelin endocytosis rate to that observed with VLDLR. These results demonstrate that the localization to rafts, rather than an intrinsic feature of the receptor, determines the slower endocytosis rate of ApoER2 in comparison with VLDLR.

Reelin-mediated Dab1 phosphorylation is necessary (32) but not sufficient to trigger the Reelin response in neurons (12). To test whether Reelin endocytosis is linked to or necessary for Dab1 phosphorylation, 3T3 cells expressing Dab1 and either ApoER2 or VLDLR (23) were exposed to Reelin at

37 or 4 °C. ApoER2 and VLDLR were both able to mediate Dab1 phosphorylation not only at 37 °C but importantly also at 4 °C (supplemental Fig. 3), demonstrating that Reelin endocytosis is not necessary for the primary signaling event.

Receptor Degradation and Processing—We have previously observed that in 3T3 cells expressing ApoER2, the receptor becomes dramatically down-regulated/degraded upon Reelin stimulation (23). To test whether the loss of ApoER2 is a specific feature of the 3T3 cell system and is mediated by the Reelin signaling cascade, we studied this effect in more detail. Primary rat neurons were stimulated for 5 h with Reelin, and the levels of ApoER2 and VLDLR were subsequently assessed by Western blotting (Fig. 6*A*, *lanes 1* and *4*). This treatment resulted in a significant loss of ApoER2, whereas VLDLR levels remained unchanged when compared with treatment with mock medium. Next, neurons and 3T3A cells were treated with Ab 186, which targets the extracellular domain of ApoER2 (see Fig. 1*A*) and induces Dab1 phosphorylation via receptor-clustering (23). As demonstrated in Fig. 6, *A* (*lane 3*; neurons) and *B* (*lane 2*; 3T3), treatment with Ab 186 also led to a dramatic loss of ApoER2. The effect of this antibody is specific because it could be blocked by the addition of soluble receptor fragment (Fig. 6*B*, *lane 3*, *MBP-ApoER2*), and an antibody against the intracellular domain of ApoER2 (Ab 20) had no effect (Fig. 6*B*, *lane 1*). The addition of receptor-associated protein, which binds to the receptors without inducing clustering and Dab1 phosphorylation had no effect (Fig. 6*B*, *lane 4*). These findings indicate that the loss of ApoER2 is mediated by receptor clustering and not merely by ligand binding and is a specific feature of ApoER2 but not of VLDLR. To test whether Reelin-mediated degradation of ApoER2 occurs via the lysosomal or the proteasomal pathway, we used specific inhibitors. The



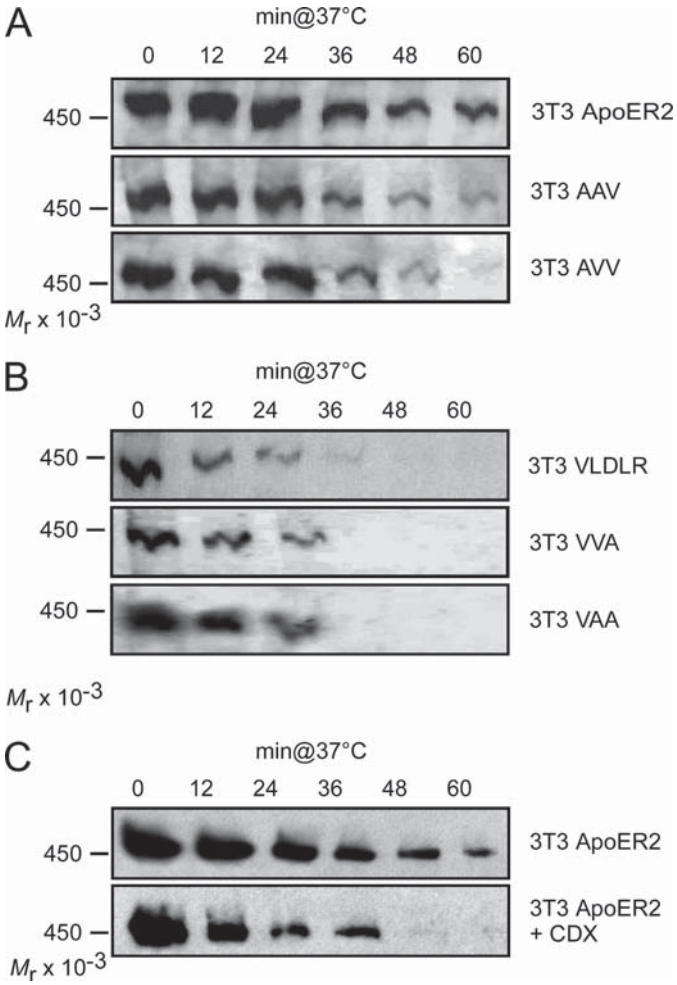


FIGURE 5. **Reelin internalization and degradation rates of ApoER2 and VLDLR depend on their sorting within the plasma membrane.** 3T3 cells expressing ApoER2 or one of the chimeric receptors containing the extracellular domain of ApoER2 (*A*) or VLDLR or one of the chimeric receptors containing the extracellular domain of VLDLR (*B*) were incubated with RCM at 4 °C to allow binding of Reelin to the respective receptors. Cells were then shifted to 37 °C for the indicated time periods to allow internalization and degradation of the ligand. After washing the cells, extracts were prepared and analyzed for cell-associated Reelin by Western blotting using Ab G10 in combination with an HRP-coupled goat anti-mouse antibody. *C*, 3T3 cells expressing ApoER2 were treated as described for *A* in the presence (*lower panel*) or absence (*upper panel*) of the raft-disrupting agent CDX (5 mM). Cell extracts were analyzed as described for *A*.

addition of the lysosomal blockers NH_4Cl and chloroquine reduced the degradation of ApoER2 (Fig. 6*C*), whereas MG132, an inhibitor of proteasomes, did not. From the results presented in Fig. 6 and those showing that ApoER2 is endocytosed via coated vesicles and early endosomes (Figs. 3 and 4), we conclude that ApoER2 is endocytosed via the coated pit/coated vesicle/endosome pathway and becomes degraded in the lysosome despite its localization in raft domains. Next, we evaluated the stability of the chimeric receptors. 3T3 cells expressing the individual chimeric receptors were treated with Reelin, and the fate of the receptors was evaluated by Western blotting. As demonstrated in Fig. 7*A*, all receptor variants containing the extracellular domain of ApoER2 became degraded upon Reelin stimulation, whereas variants containing the corresponding domain of VLDLR did not change their abundance. Reelin-induced degradation is not specific for raft-associated ApoER2, however, because raft-dissolving agents like CDX (Fig. 7*B*) or nystatin (Fig. 7*C*) did not inhibit this process.

Another aspect of the Reelin signaling cascade is the Reelin-induced cleavage of ApoER2, which produces a soluble extracellular (*NTF*) and a soluble intracellular fragment (intracellular domain; *ICD*) by the sequential action of α- and γ-secretase, respectively (33, 34) (Fig. 8*A*). Because the production of the intracellular domain cannot be monitored directly, most likely due to its inherent instability (34), we followed the production of the membrane-bound precursor CTF by Western blotting. Incubation of 3T3 cells expressing WT ApoER2 with Reelin resulted in a significant increase of the 25-kDa CTF (supplemental Fig. 4, *lane 1*), which was not observed upon the addition of receptor-associated protein (*lane 3*). Again, CTF production could be triggered by the bivalent agent Ab 186 (*lane 5*), and this was blocked by the addition of soluble receptor fragment (*lane 6*). For controls, the cells were incubated with mock-conditioned medium (*lane 2*) and Ab 20 (directed against the intracellular domain; *lane 4*). Under these conditions, small amounts of CTF were detected, apparently produced even when the cells are not stimulated. The properties of the chimeric receptors in regard to the specific fragmentation process (Fig. 8*B*) were more complex than those described above for receptor degradation and endocytosis. Although VLDLR does not undergo fragmentation at all (Fig. 8*C*, *lane 1*), chimeras containing the extracellular domain of ApoER2 produced significant amounts of CTF upon Reelin treatment (Fig. 8*C*, *lanes 3* and *5*). It should be noted that the antibody used (6A6) to detect the CTF containing the respective domains of VLDLR consistently gives weaker signals than Ab 20 directed against the intracellular domain of ApoER2. Chimeras containing the transmembrane plus intracellular domains (VAA) or only the intracellular domain of ApoER2 (VVA) are processed to a small extent, but this was not enhanced by Reelin (Fig. 8*B*, *lanes 3–6*). Disruption of rafts using CDX significantly blocked the Reelin-induced production of CTF from WT ApoER2 (Fig. 8*D*).

DISCUSSION

Inactivation of the genes for both Reelin receptors, ApoER2 and VLDLR, leads to a *reeler* phenotype in mice (25). Single knock-out mice lacking the gene either for ApoER2 or for VLDLR display only subtle abnormalities in their brain architectures. This suggests that ApoER2 and VLDLR can at least partially compensate for each other, at least in respect to their function in establishing laminated brain structures. This was confirmed in primary neurons and in a fibroblast cell model by

FIGURE 4. **ApoER2 and VLDLR internalize Reelin via the early endosomal compartment.** *A*, Reelin co-localizes with ApoER2 and VLDLR at 4 °C. 3T3 cells expressing ApoER2 (*upper panel*) or VLDLR (*lower panel*) were incubated with RCM at 4 °C to allow binding of Reelin to the receptors. Cells were fixed, permeabilized, and stained using Ab 186 for detection of ApoER2, Ab 187 for VLDLR, and Ab G10 for Reelin. *B* and *C*, internalized Reelin co-localizes with EEA1. 3T3 cells expressing ApoER2 (*B*) or VLDLR (*C*) were incubated with RCM at 4 °C for 1 h, washed, and incubated with Opti-MEM for 10 min at 4 °C (*upper panels*) or 37 °C (*lower panels*), respectively. Cells were fixed, permeabilized, and stained using Ab G10 for detection of Reelin and an antibody against EEA1 for staining of the early endosomal compartment. *Scale bars*, 10 μm.

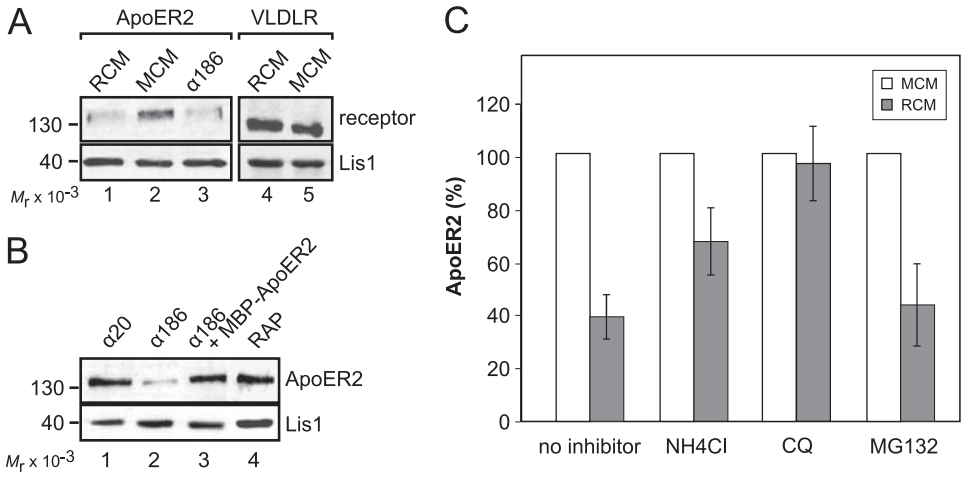


FIGURE 6. **ApoER2 degradation is induced by multivalent ligands and is mediated by the lysosomal pathway.** Primary rat neurons (*A*) and 3T3 cells expressing ApoER2 (*B*) were incubated for 5 h with RCM, MCM, Ab 186 (1:100; targets ApoER2 ligand binding domain), Ab 20 (1:100; targets ApoER2 intracellular domain), Ab 186 and recombinant ApoER2 N-terminal fragment *(MBP-ApoER2)*, or 20 μg/ml recombinant receptor-associated protein (*RAP*). Total cell extracts were analyzed by Western blotting using Ab 20 (ApoER2) and Ab 6A6 (VLDLR) and an antibody against Lis1 as a loading control in combination with the corresponding HRP-coupled secondary antibodies. *C*, ApoER2-expressing 3T3 cells were incubated with RCM or MCM. Lysosomal degradation was blocked by the addition of 10 mM NH4Cl or 25 μM chloroquine; proteasomal degradation was blocked using 25 μM MG132. Cell extracts were analyzed as described for *A* and *B*, and results were quantified by densitometry. IOD values of ApoER2 bands were normalized to the density of Lis1 bands. *Error bars*, S.E. ($n = 3$).

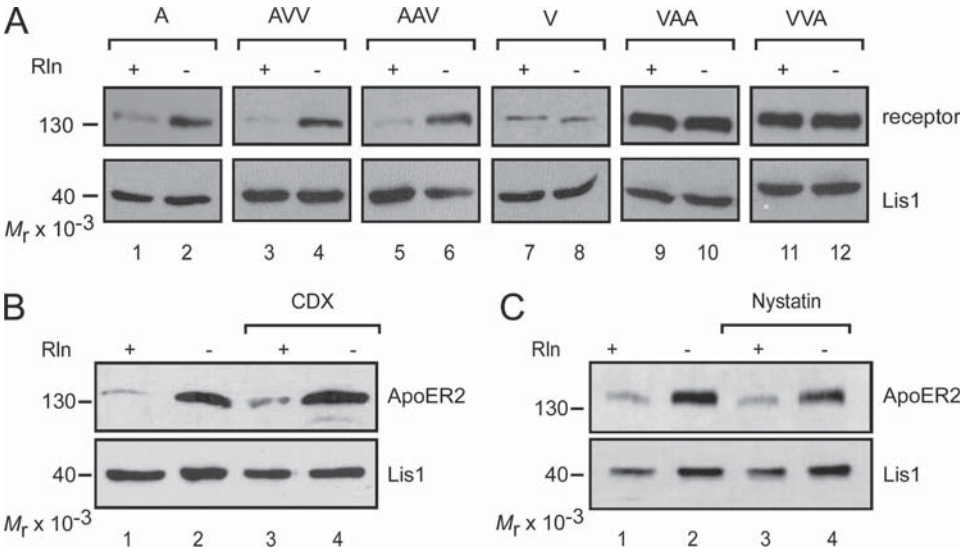


FIGURE 7. **The extracellular domain of ApoER2 but not its sorting to lipid rafts is required for Reelin-induced lysosomal receptor degradation.** *A*, 3T3 cells expressing one of the wild type (*A* and *V*) or chimeric receptors (*AVV*, *AAV*, *VAA*, and *VVA*) were stimulated with RCM (*lanes 1*, *3*, *5*, *7*, *9*, and *11*) or MCM (*lanes 2*, *4*, *6*, *8*, *10*, and *12*) for 5 h and analyzed for receptor degradation by Western blotting using Ab 20 for ApoER2, VAA, and VVA; Ab 220 for AAV and AVV; and Ab 74 for VLDLR. Lis1 was highlighted using an anti-Lis1 antibody and used as a loading control. *B*, ApoER2-expressing 3T3 fibroblasts were incubated with RCM (*lanes 1* and *3*) or MCM (*lanes 2* and *4*) and the raft-disrupting agent CDX (5 mM; *lanes 3* and *4*) for 5 h. Cell extracts were analyzed for ApoER2 degradation by Western blotting using Ab 20 in combination with an HRP-coupled goat-anti-rabbit antibody. Lis1 was highlighted using an anti-Lis1 antibody and used as a loading control. *C*, cells were treated and analyzed as described for *B*, except that 15 μg/ml nystatin was used for disruption of rafts.

demonstrating that the initial event of the Reelin signaling pathway (*i.e.* phosphorylation of Dab1) is equally well supported by ApoER2 and VLDLR (11, 23). Divergent roles for ApoER2 and VLDLR have been corroborated recently. In neuronal migration, VLDLR mediates a stop signal for migrating neurons, whereas ApoER2 plays a distinct role in the migration of neocortical neurons generated late (26). The specific phenotypes of the single knock-out animals and the observed distinct roles for both receptors might be in part caused by selective expression of the receptors in distinct areas of the brain (35). On the other hand, ApoER2 and VLDLR may have distinct functions in the Reelin pathway, apart from the primary phosphorylation event. Other processes in the central nervous system seem to depend on specific variants of ApoER2 only and involve the modulation of synaptic plasticity and memory (36), the control of neuronal survival (37), and selenium uptake (38, 39). One reason for differential functions of these receptors is structural differences in the respective intracellular domains (40) linking ApoER2 to adapters that do not interact with VLDLR (41). Other features could be selective expression in distinct subdomains of the cell membrane and/or their divergent endocytic competence. As shown in our laboratory, ApoER2 is prevalently present in raft domains, whereas VLDLR is not (23). The avian ortholog of VLDLR is expressed on growing oocytes where it efficiently mediates receptor-mediated endocytosis of yolk precursors, thus playing a pivotal role in follicle development (42), whereas ApoER2 was demonstrated to exert an extremely slow endocytosis rate in comparison with other members of the LDL receptor family (43). As recently postulated, VLDLR and/or ApoER2-mediated endocytosis of full-length Reelin or its central fragment produced by metalloproteinases might play an important role in the Reelin signaling pathway by controlling the strength of the signal via modulating the availability of the ligand (44). Thus, we set out to study structural features of the receptors that might be responsible for different fates and/or functions of the receptors in terms of cellular sorting, endocytosis, and receptor trafficking.

For most of the present studies, we used a recently established fibroblast-based cell system in which the Reelin signaling pathway has been partially reconstituted by expressing Dab1 and either ApoER2 or VLDLR (23). These cells respond to Reelin stimulation with Dab1 phosphorylation and phosphatidylinositol 3-kinase activation, leading to protein kinase B/Akt phosphorylation undistinguishable from primary neurons. In

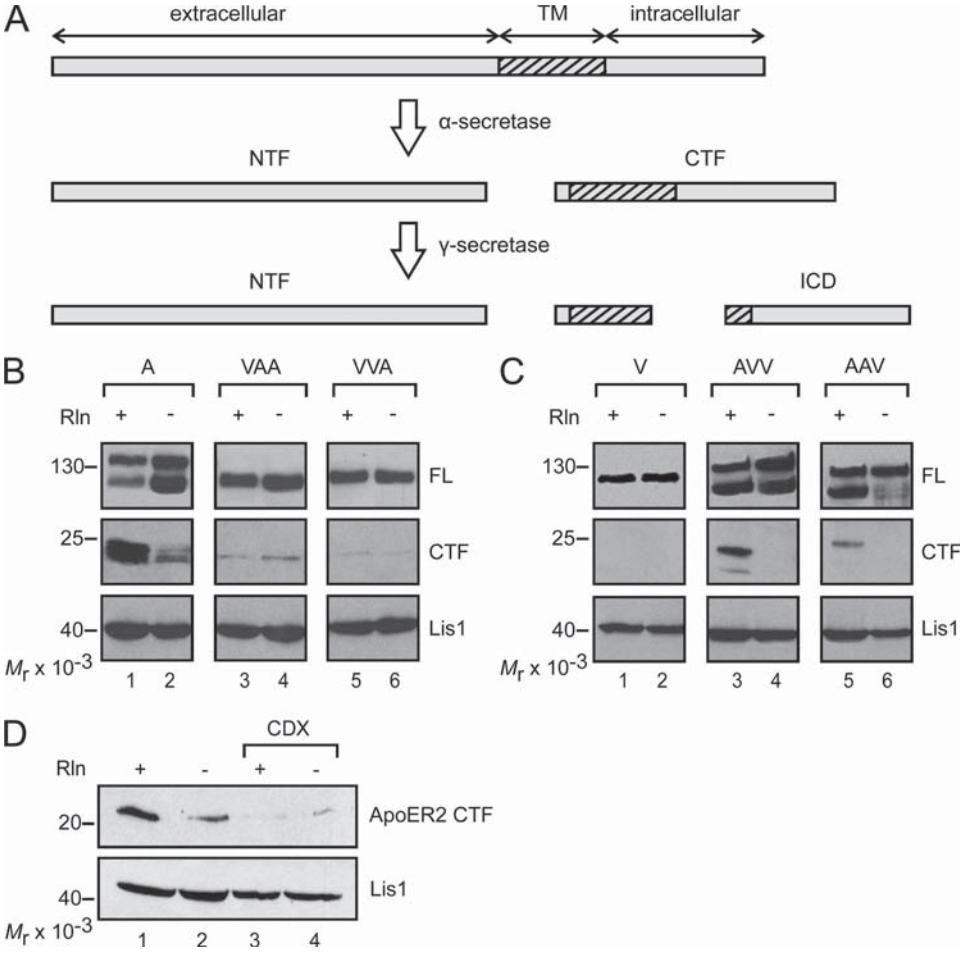


FIGURE 8. **Secretase-mediated cleavage of ApoER2 depends on the sorting of the receptor to lipid rafts.** *A*, ApoER2 is subjected to cleavage by α-secretase upon Reelin stimulation, thereby producing a soluble extracellular *N*-terminal fragment (*NTF*) and a membrane-bound C-terminal fragment (*CTF*). The latter is further processed by α-secretase to release a soluble intracellular domain (*ICD*). *B* and *C*, 3T3 fibroblasts expressing one of the WT (*A* and *V*) or chimeric receptors (*VAA*, *VVA*, *AVV*, and *AAV*) were stimulated with RCM or MCM. Cell extracts were analyzed by Western blotting using Ab 20 for detection of the CTF derived from A, VAA, and VVA (*B*) and Ab 6A6 for the CTF derived from V, AVV, and AAV (*C*). *D*, 3T3 cells expressing ApoER2 were treated as described for *B* and *C* in the presence (*lanes 3* and *4*) or absence (*lanes 1* and *2*) of CDX. Production of the CTF was analyzed as described for *B*. Lis1 was highlighted using an anti-Lis1 antibody and used as a loading control. *FL*, full-length.

addition, we have now used a panel of genetically engineered receptor chimeras between ApoER2 and VLDLR (Fig. 1*A*) to define the structural elements in the receptors responsible for differential sorting of the receptors. These chimeric receptors were also used to determine whether functional differences are caused by selective expression in raft *versus* non-raft domains or by intrinsic structural differences of the respective receptors.

As previously demonstrated by analysis of whole cell extracts (23), ApoER2 migrates as a distinct double band representing the precursor (lower band) and the fully glycosylated mature form (upper band), which is present in the raft domains of the plasma membrane. Expression of the chimeric receptors corroborates these findings, as the extracellular part of ApoER2 containing the *O*-linked sugar domain (45) directs the receptors to the raft fraction, whereas all constructs containing the extracellular domain of VLDLR did not show this behavior (Fig. 1*B*). Ligand blotting and evaluation of Reelin-induced and receptor-mediated Dab1 phosphorylation demonstrated that all chimeric receptor constructs are Reelin binding-competent (supplemental Fig. 1*B*) and transmit the signal into the cells equally well (supplemental Fig. 1*C*). Membrane fractionation studies allowed us to identify the extracellular domain of ApoER2 as the one responsible for directing the receptor to caveolae/rafts. This is reminiscent of epidermal growth factor receptor, which is also sorted to rafts; there, the responsible region was identified to be within 60 amino acids of the extracellular domain juxtaposed to the cell membrane representing the *O*-linked sugar domain (46). As reported in the same paper, glycosylation of this domain is indeed the key to such sorting, since when glycosylation is blocked, epidermal growth factor receptor is targeted to non-raft membrane domains. This is an interesting observation, because the predominant variant of VLDLR expressed in the brain lacks exactly this domain and is absent from the raft fraction (23). The fact that the sorting signal derives from the extracellular domain of the receptors allowed us to dissect whether specific functions of the receptors are due to their differential sorting or to structural differences in their respective intracellular domains.

The major finding of these studies is that VLDLR indeed internalizes Reelin very efficiently, resulting in a local depletion of Reelin. ApoER2, in contrast, endocytoses Reelin with very low efficiency, and cells expressing ApoER2 do not significantly decrease the concentration of Reelin in the medium. Although localized in the raft fraction of the plasma membrane, slow endocytosis of ApoER2 takes place via the classic endocytosis pathway involving coated pits/coated vesicles/early endosomes (Figs. 3 and 4). This is in agreement with previous findings demonstrating that ApoER2 is endocytosed independent of its raft association by a clathrin-mediated process involving the adapter Dab2 (22). It was demonstrated that the central fragment (R3-6) is critical to exert the signaling function of Reelin, suggesting that this domain of Reelin interacts with ApoER2 and VLDLR (12). The presence of NR2, which lacks the central part of Reelin (R3-6), in coated vesicles suggests that this fragment either stays associated with full-length Reelin via the oligomerization domain present in the N-terminal region (47) or that NR2 is produced after endocytosis. Using the panel of chimeric receptors and raft-dissolving agents like CDX, we demonstrate that it is the raft association of ApoER2 that causes its slow endocytosis rate. As soon as

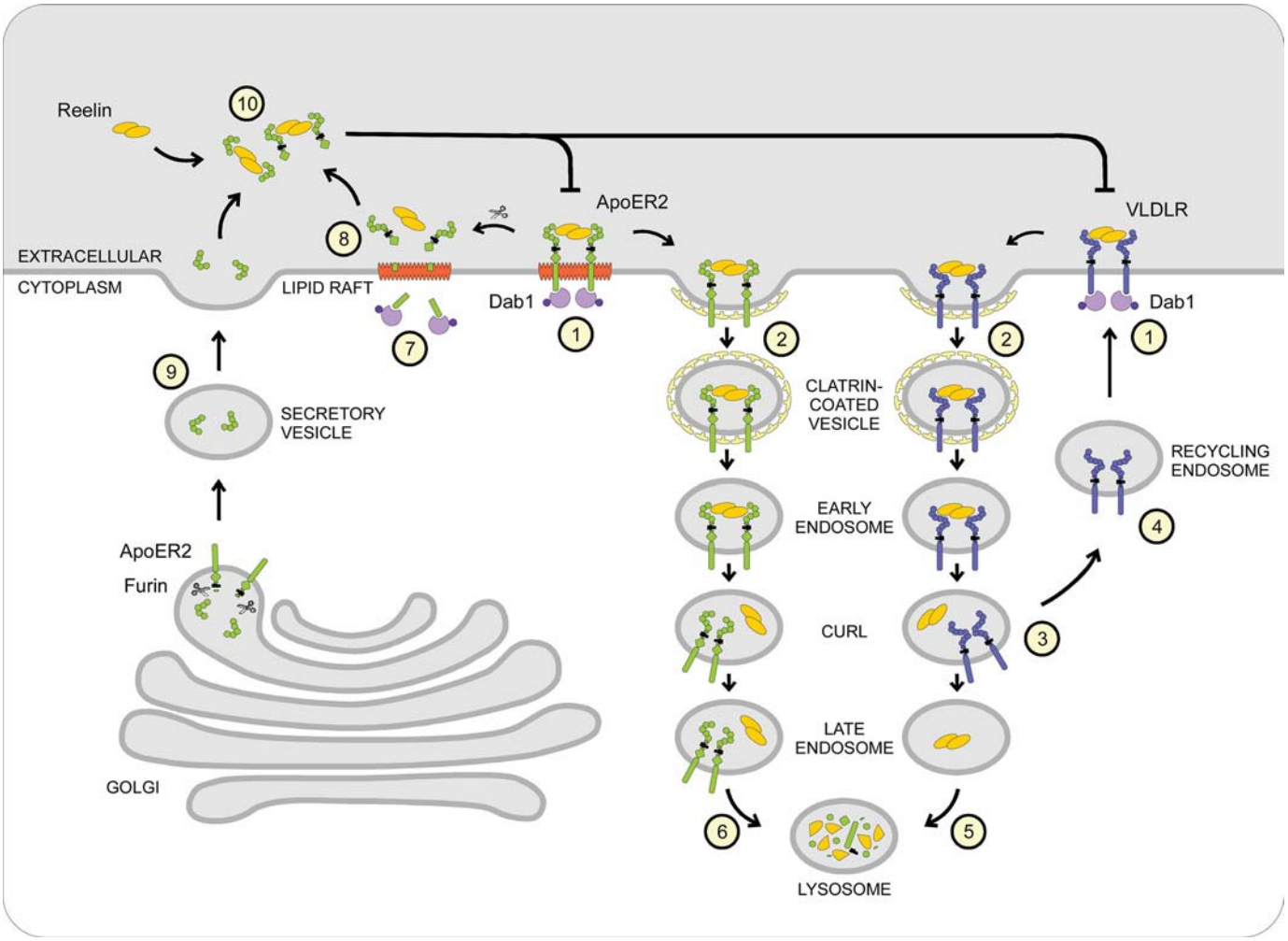


FIGURE 9. **Model of the intracellular fates of ApoER2 and VLDLR upon Reelin stimulation.** Upon binding of Reelin, both ApoER2 and VLDLR mediate phosphorylation of Dab1 (*step 1*). VLDLR internalizes Reelin rapidly via clathrin-mediated endocytosis (*step 2*) and is separated from Reelin in the compartment of uncoupling of receptor and ligand (*step 3*). VLDLR then recycles back to the plasma membrane (*step 4*), whereas Reelin is delivered to the lysosome for degradation (*step 5*). ApoER2 internalizes Reelin via the same pathway (*step 2*), although the receptor originally resides in lipid rafts and endocytoses its ligand at a much slower rate. In contrast to VLDLR, ApoER2 is not recycled but ends up in the lysosome together with Reelin (*step 6*). As an additional feedback mechanism, Reelin stimulation induces secretase-mediated cleavage of ApoER2, thereby generating a soluble intracellular fragment (ICD) (*step 7*), the function of which is not defined yet, and a soluble extracellular fragment containing the ligand binding domain (*step 8*). This fragment can, together with another N-terminal fragment produced from an ApoER2 isoform by furin cleavage (*step 9*), inhibit the Reelin signal by sequestering free Reelin in the surroundings of the cell (*step 10*).

ApoER2 loses its association with rafts, the endocytosis rate of the receptor increases significantly. This effect might even be underestimated by the experiment using CDX to dissolve the rafts because this agent also blocks clathrin-dependent endocytosis by generally depleting the membrane of cholesterol (31). Thus, slow translocation from rafts to non-raft domains of the membrane might be the rate-limiting step of ApoER2-mediated endocytosis.

The second significant difference between ApoER2 and VLDLR is the fact that the levels of ApoER2, but not of VLDLR, significantly drop in the presence of Reelin (23). As demonstrated here, Reelin-induced degradation of ApoER2 occurs via the lysosomal pathway and depends on the presence of receptor-clustering ligands. Again, the extracellular domain of ApoER2 determines the fate of the receptor upon binding of Reelin. Disruption of rafts by CDX or nystatin had no influence on receptor degradation, demonstrating that neither the caveolin-mediated pathway originating from raft structures nor the targeting of raft-associated receptors to endosomes (48) is responsible for receptor degradation. Thus, ApoER2 degradation most likely occurs by clathrin-mediated endocytosis and subsequent sorting to lysosomes. Structural differences in the extracellular domains of ApoER2 and VLDLR must be responsible for directing ApoER2 predominantly to lysosomes and VLDLR into the recycling pathway back to the plasma membrane. For the LDL receptor, it was shown that a distinct region of the extracellular domain (*i.e.* the cysteine-rich growth factor repeats (epidermal growth factor repeats)) is responsible for uncoupling of receptor and ligand within the endocytic pathway (49). When this domain is deleted, the receptor no longer releases its ligand and becomes degraded in the lysosome. Thus, we speculate that differences in the epidermal growth factor repeats between ApoER2 and VLDLR are responsible for the distinct behavior of the receptors.

Parallel to lysosomal degradation of ApoER2, specific breakdown products of this receptor are produced upon Reelin stim-

ulation (33, 34). Here we demonstrate that this effect (i) is not simply induced by binding of a ligand but requires the presence of a clustering ligand, such as Reelin, or antibodies against the extracellular domain, which also induce Dab1 phosphorylation; (ii) is dependent on the presence of the extracellular domain of ApoER2; and (iii) is dependent on the presence of rafts. VLDLR variants carrying the extracellular domain of ApoER2 are cleaved like ApoER2 itself, whereas intact VLDLR completely escapes this process. This is in agreement with previous findings that the presence of the *O*-linked sugar domain promotes γ-secretase-mediated cleavage of the receptor (34) and that the γ-secretase complex is associated with rafts (50, 51). Thus, most likely, the entire processing (α- and γ-secretase-mediated) takes place within the raft domain of the plasma membrane. Whether clustering of the receptors or Dab1 phosphorylation or both actually triggers the induction of the cleavage is still an open question.

These results allow us to propose a model for describing both the interrelated and independent functions of ApoER2 and VLDLR in Reelin signaling (Fig. 9). VLDLR present in non-raft domains of the plasma membrane binds Reelin, which results in Dab1 phosphorylation (Fig. 9, *step 1*). The Reelin-receptor complex is rapidly internalized by clathrin-mediated endocytosis (Fig. 9, *step 2*), and Reelin is uncoupled from the receptor (Fig. 9, *step 3*) and sorted to lysosomes (Fig. 9, *step 5*), whereas VLDLR recycles back to the cell membrane (Fig. 9, *step 4*). This reduces the amount of extracellular Reelin significantly, thus shutting off the persistence of the signal without rendering the cell refractory to VLDLR-mediated actions. Binding of Reelin to ApoER2 present in rafts also leads to immediate phosphorylation of Dab1 (Fig. 9, *step 1*) but not to a significant reduction of extracellular Reelin, because ApoER2-mediated endocytosis is slow. The Reelin signal itself can be turned off via degradation of phosphorylated Dab1, which occurs independently of the signaling receptor (52). Selective Reelin-mediated loss of ApoER2 depends on clathrin-mediated endocytosis and lysosomal degradation of the receptor (Fig. 9, *step 6*). As shown here, this process is much slower than for VLDLR but in the long run renders the target cell refractory to further Reelin stimulation until new receptor is synthesized. In parallel, specific fragmentation by α- and γ-secretases leads to the production of soluble intracellular receptor fragment (Fig. 9, *step 7*), which might exert its action within the nucleus (34), and a soluble extracellular fragment (Fig. 9, *step 8*). The extracellular fragment containing the ligand binding domain, together with secreted soluble ApoER2 fragments (Fig. 9, *step 9*) produced by the action of furin from certain splice variants of the receptor (28), bind Reelin and thereby attenuate the entire pathway (Fig. 9, *step 10*). Thus, in regions of the brain where mostly ApoER2 is expressed, the Reelin-induced activation of target cells is not accompanied by a reduction of Reelin. Thus, Reelin might keep exerting other functions without further inducing the canonical Reelin signal, which is efficiently turned off by Dab1 degradation and production of dominant negative receptor fragments. Whether the production of soluble intracellular fragments derived from ApoER2 by γ-secretase cleavage represents an independent signal within the cell as proposed (34) awaits clarification.

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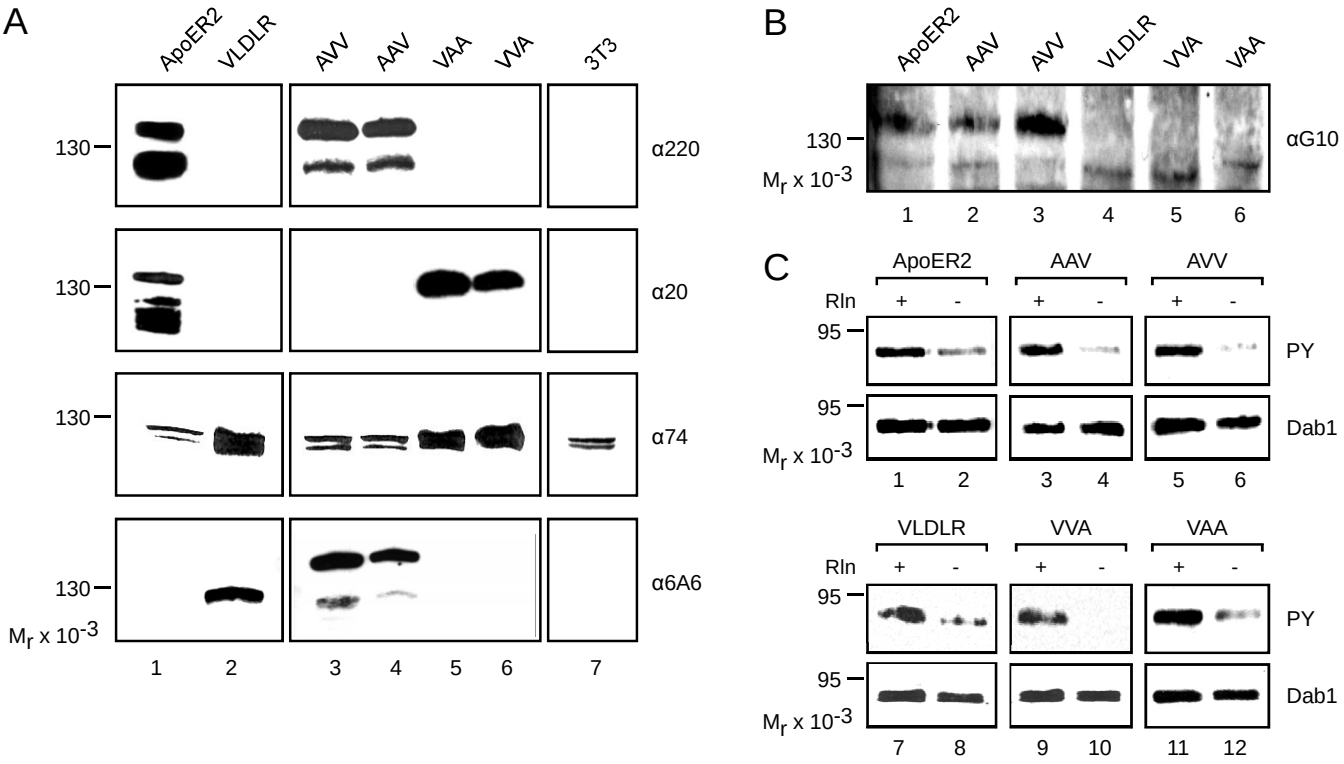
Supplemental figure 1



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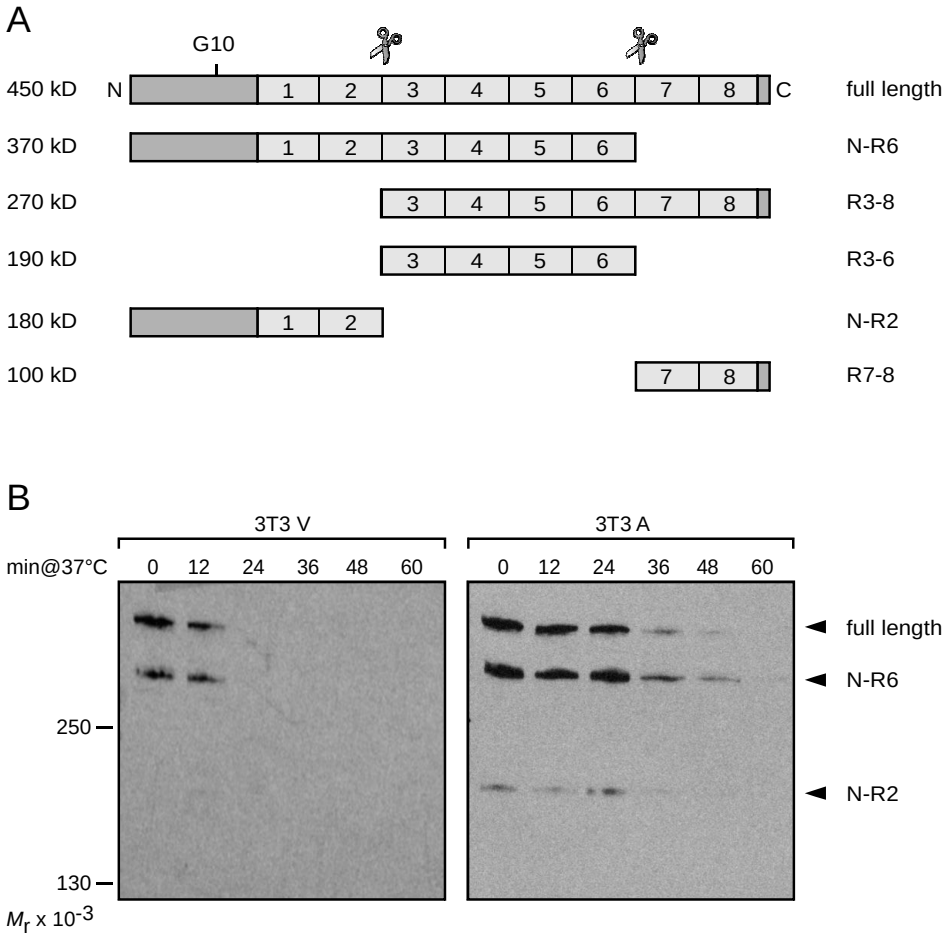

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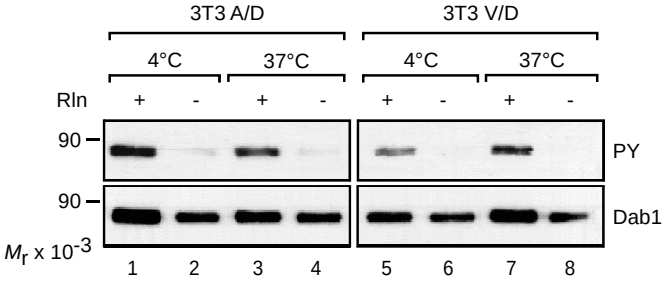

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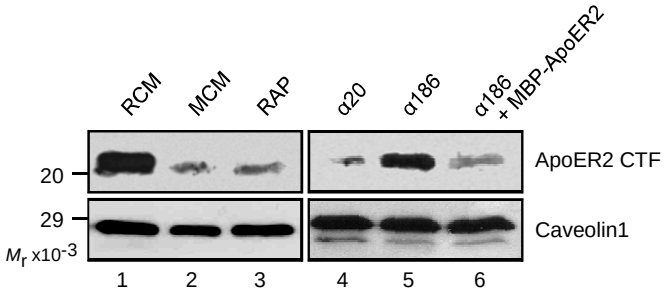

Supplemental Figure 1: Expression and functional characterization of chimeric receptors. **(A)** *wt* receptors (lanes 1 and 2) and chimeric receptors (lanes 3-6) were expressed in 3T3 fibroblasts, total cell extracts were separated by SDS-PAGE and the receptors analyzed by Western blotting using the indicated antibodies in combination with the appropriate HRP-coupled secondary antibodies. Mock transfected 3T3 fibroblasts (lane 7) were used as control. **(B)** Total cell extracts derived from cells expressing ApoER2 (lane 1), VLDLR (lane 4) or the respective chimeric receptors (lanes 2, 3, 5, 6) were separated by SDS-PAGE and the receptors analyzed by ligand blotting using Reelin as ligand. Bound Reelin was detected by incubating the blots with an antibody against Reelin (G10) in combination with an HRP-coupled goat-anti-mouse antibody. **(C)** Dab1 phosphorylation mediated by wt (lane 1, 2, 7, and 8) and chimeric receptors (lanes 3-6 and 9-12). 3T3 cells expressing one of the receptors and Dab1 were incubated with RCM or MCM for 30 minutes and Dab1 was immunoprecipitated from cell extracts and analyzed by Western blotting using antibodies against Dab1 and phosphorylated tyrosine in combination with an HRP-coupled goat-anti-mouse antibody.

Supplemental Figure 2: Full-length Reelin and Reelin fragments show similar endocytosis and degradation kinetics. **(A)** Reelin is cleaved at two sites, generating three fragments and two intermediates. Reelin antibody G10 recognizes N-R6 and N-R2 in addition to full length Reelin. **(B)** Western blot partially presented in Fig. 3A. 3T3 cells expressing ApoER2 or VLDLR were incubated with RCM at 4°C to allow binding of Reelin to the respective receptors. Cells were then shifted to 37°C for the indicated time periods to allow internalization and degradation of the ligand. Cell extracts were prepared and analyzed for cell-associated Reelin by Western blotting using Ab G10 in combination with an HRP-coupled goat-anti-mouse antibody. Black arrows indicate the respective Reelin fragments.

Suppemental Figure 3: Dab1 phosphorylation is independent of Reelin internalization. 3T3 cells expressing Dab1 and either ApoER2 (lanes 1-4) or VLDLR (lanes 5-8) were incubated with RCM or MCM for 30 minutes at 4°C or 37°C to inhibit or allow endocytosis, respectively. Dab1-phosphorylation was measured by immuno-precipitating Dab1 from total cell extracts and analyzing the precipitate by Western blotting using antibodies against Dab1 and phosphorylated tyrosine in combination with an HRP-coupled goat-anti-mouse antibody.

Supplemental Figure 4: Secretase-mediated cleavage of ApoER2 is induced by multivalent ligands. ApoER2-expresssing 3T3 fibroblasts were incubated for 5 h with RCM (lane 1), MCM (lane 2), 20 µg/ml recombinant RAP (lane 3), Ab 20 (lane 4, 1:100, targets ApoER2 intracellular domain), Ab 186 (lane 5, 1:100, targets ApoER2 ligand binding domain), or Ab 186 and recombinant ApoER2 N-terminal fragment (lane 6, MBP-ApoER2) and total cell extracts were analyzed by Western blotting for ApoER2-CTF (Ab 20 in combination with an HRP-coupled goat-anti-rabbit antibody). Caveolin1 was highlighted using a caveolin1 antibody and used as a loading control.

3.2 Regulation of Reelin receptors by Idol

3.2.1 Materials and Methods

Cell lines, conditioned media, and antibodies were described in section 3.1. Cell culture, cell extract preparation, SDS-PAGE, Western blotting were done as described ibidem.

GW3965 was purchased from Sigma and dissolved to a stock concentration of 1 mM in DMSO.

RNA was prepared from cultured cells using TRI reagent (Molecular Research Center, Inc.) and reverse transcribed to cDNA using the DyNAmo cDNA synthesis kit (Finnzymes) according to the manufacturers' protocols. cDNA was used for PCR with GoTaq Green polymerase Master Mix (Promega). Primers for detection of Abca1 and Idol of mouse and rat origin were designed using primerblast (NCBI) and purchased from VBC Biotech. The primers were Abca1-S: 5'-CAGCAGCCTGTGGGGGCATC-3', Abca1-AS: 5'-TGGCTGGAACCAG GGTGGCT-3', Idol-S: 5'-TGGCAGGCCACCTCCAGTGT-3', and Idol-AS: 5'-GGGTGGC CATCTGCACCACA-3'.

3.2.2 Results

It was recently shown that activation of liver X receptors leads to enhanced expression of the ubiquitin ligase Idol and subsequent ubiquitination and degradation of LDL receptor (*Zelcer et al.*, 2009). Other members of the LDL receptor genefamily, namely LRP, MEGF7 and SorLa were not affected by Idol expression, ApoER2 only marginally.

To investigate a possible LXR-dependent regulation of the two Reelin receptors ApoER2 and VLDLR, the fibroblast model system previously established in our lab was employed. 3T3 cells expressing ApoER2 were incubated with or without the synthetic LXR ligand GW3965 for 6 h or 24 h, respectively and analysed for ApoER2 levels by Western blotting. As it is shown in figure 3.1(A), LXR activation by GW3965 indeed led to a significant reduction of receptor levels after 24 h of incubation (lane 3), compared to the negative control (DMSO, lane 4). 6 h after addition of GW3965, only a slight reduction in ApoER2 levels could be observed (lanes 1 and 2).

As expected, GW3965-induced degradation of ApoER2 was found to only decrease the mature, fully glycosylated form of the receptor (upper bands) but not its ER-resident precursor (lower bands). This confirms a role of the LXR-mediated pathway in posttranslational receptor degradation rather than targeting ApoER2 expression.

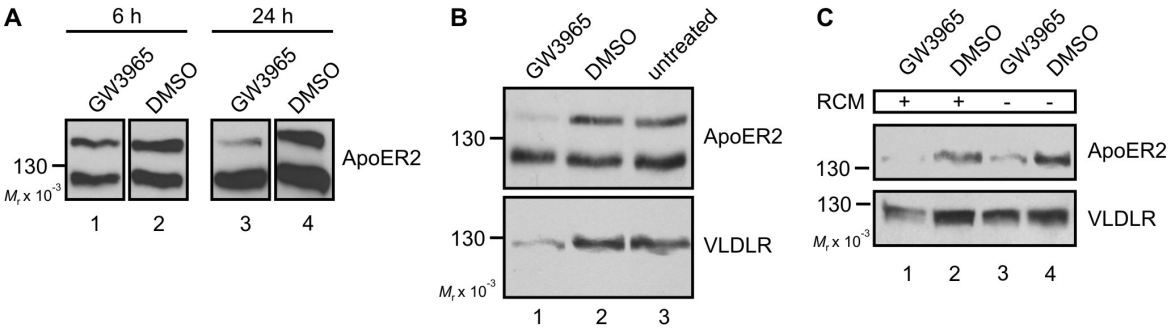

Figure 3.1 Influence of GW3965 on receptor degradation in fibroblasts. (A) 24 h incubation is sufficient for GW3965-induced degradation of ApoER2. 3T3 fibroblasts stably expressing murine ApoER2 were incubated with 1 µM GW3965 in DMSO (lanes 1, 3) or DMSO (lanes 2, 4) for 6 h or 24 h. Cell extracts were analysed by SDS-PAGE and Western blotting using AB 20. The two bands of ApoER2 represent mature receptor (upper band) and its unglycosylated precursor (lower band). **(B)** GW3965 induces degradation of both ApoER2 and VLDLR. 3T3 fibroblasts stably expressing murine ApoER2 (upper panel) or VLDLR (lower panel) respectively, were incubated for 24 h with 1 µM GW3965 in DMSO (lane 1), DMSO (lane 2) or were left untreated (lane 3). Cell extracts were analysed as described above using AB 20 for detection of ApoER2 and Ab74 for detection of VLDLR. **(C)** Reelin-driven receptor degradation adds to GW3965-induced degradation. 3T3 cells expressing one of the receptors were incubated for 24 h with GW3965 or DMSO and subsequently stimulated with Reelin- or mock-conditioned medium containing either GW3965 or DMSO, respectively for another 5 h. Cell extracts were analysed as described above.

Since 24 h of incubation with GW3965 were shown to be sufficient for induction of substensial ApoER2 degradation, a possible effect of LXR activation on VLDLR was analysed using the same time frame. As shown in figure 3.1(B), this treatment led to robust decrease of VLDLR levels, similar to ApoER2 (compare lanes 1 and 2). To exclude any unspecific effect of DMSO, receptor levels of cells incubated with or without DMSO were compared. Clearly, amounts of ApoER2 and VLDLR could be shown to be unaffected by DMSO (compare lanes 2 and 3).

Previous results showed that upon endocytosis of Reelin, ApoER2 is delivered to the lysosome for degradation while VLDLR is recycled to the plasma membrane (section 3.1). The mechanism for targeting ApoER2 to the lysosomal compartment is not yet clarified. However, since Idol-mediated degradation of LDLR has been shown to employ the lysosomal

pathway, too (*Zelcer et al.*, 2009), LXR/Idol-dependent ubiquitination is a candidate mechanism for Reelin-induced ApoER2 degradation. Reelin and LXR signaling might either be required to cooperate to enable this regulative mechanism or Idol action might be a straight downstream effect of Dab1 phosphorylation. The latter possibility is improbable however, since cell lines used to demonstrate LXR-dependent degradation of ApoER2 and VLDLR (figure 3.1(A and B)) do not express Dab1.

To investigate a possible link of LXR-mediated decrease of ApoER2 and VLDLR to Reelin-induced degradation, receptor-expressing 3T3 cells were again exposed to 24 h treatment with GW3965 or DMSO, followed by stimulation with Reelin- or mock-conditioned medium (RCM/MCM, respectively) under continued supplementation with GW3965 or DMSO for five more hours. For ApoER2 as well as VLDLR, GW3956 induced receptor degradation both under Reelin stimulation and mock treatment (figure 3.1(C), compare lanes 1 and 2 for RCM, and 3 and 4 for MCM). VLDLR levels were unaffected by Reelin stimulation which is in accordance with previous results. In the case of ApoER2 (upper panel), Reelin induced degradation in DMSO-treated samples (compare lanes 2 and 4) as expected. Supplementation with GW3965 however, led to further reduction of ApoER2 levels (lanes 2 and 4). This additive effect of LXR- and Reelin-mediated receptor degradation suggests a Reelin-independent role of LXRs in the regulation of ApoER2 and VLDLR.

To study the effects of active LXR signaling on the Reelin pathway, the primary signaling event, phosphorylation of Dab1 was examined. 3T3 cells expressing Dab1 and one of the Reelin receptors were treated with GW3965/DMSO and RCM/MCM as described in figure 3.1(C). Dab1 was immunoprecipitated from protein extracts and precipitates were analysed for phosphorylated Dab1 and total Dab1. As shown in figure 3.2, total Dab1 levels were constant throughout preparations for cells expressing ApoER2 and Dab1 (A) and or VLDLR and Dab1 (B). In both cases, Dab1 phosphorylation was induced by Reelin stimulation in the control samples (DMSO) but was abolished in the presence of GW3965. This can be explained by the dramatic LXR-induced decrease of available receptor able to relay the Reelin signal to Dab1.

To verify LXR-driven degradation of ApoER2 and VLDLR in their native cell system, analysis was repeated using primary neurons prepared from E16.5 rat embryonic brains. Surprisingly, no receptor loss could be observed in neuronal cultures treated with GW3965 (figure 3.3(A)).

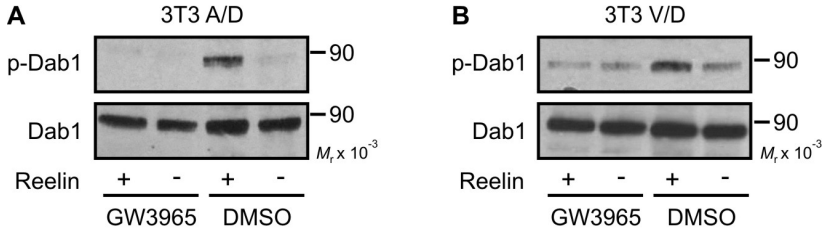

Figure 3.2 Influence of GW3965 on Dab1 phosphorylation in fibroblasts. 3T3 cells expressing either **(A)** ApoER2 or **(B)** VLDLR and Dab1 were incubated with 1 µM GW3965 in DMSO or DMSO alone for 24 h, starved in OptiMEM containing GW3965 or DMSO for 1 h, and stimulated with Reelin- or mock-conditioned medium containing GW3965 in DMSO or DMSO for 40 min. Protein extracts were prepared and Dab1 was immunoprecipitated. Phosphorylated Dab1 and total Dab1 were detected by Western blotting using antibodies D4 for Dab1 and PY99 for phosphorylated tyrosine.

The unability of GW3965 to induce ApoER2 and VLDLR degradation in neuronal cells could arise from disfunction of several steps in the mechanism. GW3965 might not be able to activate LXRs in neurons, LXRs or Idol might not be expressed in these cells, or Idol-mediated ubiquitination might be impaired.

To verify Idol expression in neuronal cultures, cDNA from neurons and 3T3 cells was subjected to PCR using Idol-specific primers designed to target mRNA of both mouse and rat origin, since neuronal cultures were prepared from rat embryos and 3T3 cells originate from mouse. As shown in figure 3.3(B), Idol transcripts can be detected in both cell types.

To analyse the differences between 3T3 cells and neurons in terms of LXR activation, PCR analysis using primers specific for rat and mouse Abca1, a classical target of LXRs, was performed using cDNA from both cell types after incubation with or without GW3965.

As shown in figure 3.3(C), the Abca1 PCR product could be detected at the expected size in all 3T3 and neuron samples. GW3965 supplementation however was only able to increase Abca1 expression in 3T3 cells but not in samples from cultured neurons. This led to the conclusion that activation of LXRs by GW3965 in neuronal cells is disturbed. PCR analysis using primers specific for Idol was also done but has not yet been conclusive.

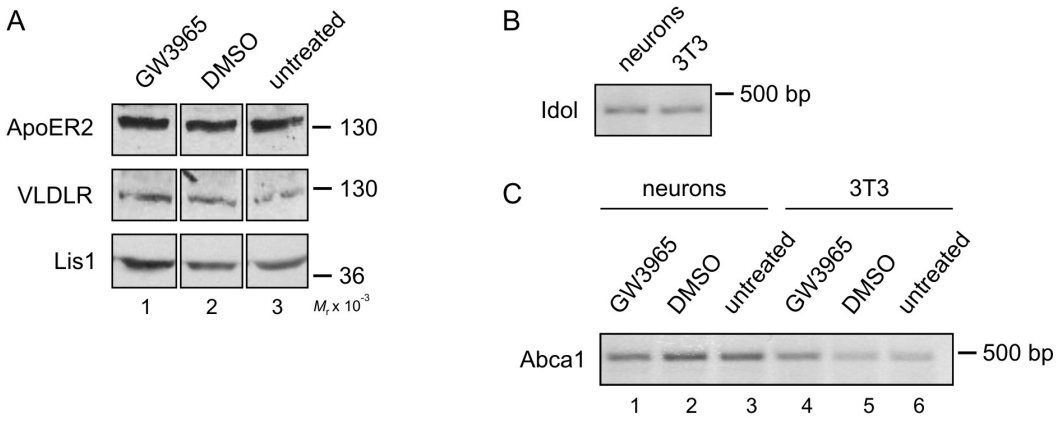

Figure 3.3 No influence of GW3965 on primary neuronal cultures. (A) GW3965 does not induce degradation of ApoER2 or VLDLR. Primary neuronal cultures were incubated for 24 h with 1 µM GW3965 in DMSO (lane 1), DMSO (lane 2) or were left untreated (lane 3). Cell extracts were analysed by SDS-PAGE and Western blotting using AB 20 for detection of ApoER2 and Ab6A6 for detection of VLDLR. **(B)** Neuronal cultures express Idol. cDNA from cultured neurons (lane 1) and 3T3 cells (lane 2) was used for PCR analysis with primers Idol-S/Idol-AS (see section 3.2.1). **(C)** Abca1 expression is not induced by GW3965 in neurons. cDNA from cultured neurons (lanes 1-3) and 3T3 cells (lanes 4-6) incubated with GW3965 (lanes 1 and 4) or DMSO (lanes 2 and 5) or without supplement (lanes 3 and 6) was used for PCR analysis with primers Abca1-S/Abca1-AS (see section 3.2.1).

3.2.3 Discussion

Summarising the results generated with our fibroblast model system, it was shown that stimulation of LXRs leads to a significant decrease of both ApoER2 and VLDLR levels, most likely mediated by Idol-dependent ubiquitination and lysosomal degradation as proposed for LDLR since this regulation does not target receptor expression but happens posttranslationally.

Furthermore, the decrease of ApoER2 and VLDLR blocks the transmission of the Reelin signal into the cell. Therefore, the LXR/Idol pathway could well constitute an additional mechanism regulating the Reelin signaling cascade.

The original factor inducing this putative regulation cascade however, is not yet clear. Nevertheless, a role of the LXR pathway as a feedback mechanism initiated by the canonical Reelin pathway can be ruled out, since it can as well be induced in the absence of the primary signaling target of Reelin, Dab1. This leaves two possibilities for an involvement in

the regulation of the Reelin pathway. First, LXR could be a downstream target of a parallel Dab1-independent, not yet defined route of Reelin signaling, and second, LXR might be activated independently of Reelin in a pathway blocking the Reelin signaling cascade by receptor degradation.

According to the data presented here, it seems likely that initiation of LXR-dependent degradation of ApoER2 and VLDLR occurs Reelin-independently since it is cumulative to receptor degradation caused by Reelin. Nevertheless, the latter could also be explained by inefficient stimulation of LXRs present in the cell using the given GW3965 concentration, so that Reelin signaling would be able to activate the remaining LXRs by a Dab1-independent mechanism and thereby further enhance Idol expression. To analyse this possibility, Reelin-induced receptor degradation would have to be examined in a situation where LXR or Idol activity is blocked. This could be achieved by addition of a specific blocking reagent or by downregulation of LXR expression using RNAi. If Reelin-mediated receptor loss was not changed under these conditions, it would obviously not employ the LXR/Idol mechanism.

Although these results strongly support a regulative effect of LXR activation on the Reelin signaling pathway, it is as well possible that *in vivo*, LXR and Idol do not play any role in the regulation of the Reelin receptors. At least in primary neuronal cultures, no effect of GW3965 supplementation on the receptor levels could be observed, although Idol mRNA is present. Presumably, this might be due to the inability of GW3965 to activate LXRs in neuronal cultures, since levels of Abca1, a classical LXR target were not affected either by treatment with GW3956. This result has to be verified however, using a quantitative method like realtime PCR. Furthermore, quantitative analysis using primers for Idol would be important, since this is the relevant LXR target for the problem at hand. Under the assumption that GW3965 is indeed unable to activate LXRs here, this could be explained by a putative co-inducer present in 3T3 cells but not in neurons which is needed for LXR activation. This hypothesis however remains to be investigated.

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5 List of Figures

6 Curriculum vitae

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Publications

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Participation in scientific meetings

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