Role of SORLA in the brain and its relevance for Alzheimer disease

Dissertation zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie

der Freien Universität Berlin

vorgelegt von

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August, 2008

Die Doktorarbeit wurde am Max-Delbrück-Centrum für molekulare Medizin Berlin-Buch unter der Leitung von Prof. Dr. Thomas Willnow im Zeitraum seit dem 01.04.2005 angefertigt.

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Disputation am 03.12.2008

Introduction

1.1 Alzheimer disease

1.1.1 History and pathogenesis

Dementia illnesses are the most common diseases in industrialized countries. It is estimated that approximately one million people are affected by these illnesses in Germany alone. The neurodegenerative diseases among these are classified by the presence of neuronal loss in addition to mental dysfunctions. Within the neurodegenerative diseases Alzheimer disease (AD) is the most common disease entity with approximately 15 million people affected worldwide.

AD was described first by Alois Alzheimer in 1906. He correlated the mental dysfunctions of his patient Auguste Deter with the neuropathological finding of particular protein aggregates, the amyloid plaques and neurofibrillary tangles (NFT) in brain autopsy material (Goedert and Spillantini 2006). After the initial description by Alzheimer, it took 77 years until the main component of the amyloid plaques, the amyloid-β (Aβ), was identified (Allsop, Landon et al. 1983). Shortly thereafter it became apparent that Aβ originates from a longer precursor, the amyloid precursor protein (APP) (Kang, Lemaire et al. 1987). In parallel, the main component of the neurofibrillary tangles was identified as the hyperphosphorylated form of the so called tau protein (Grundke-Iqbal, Iqbal et al. 1986).

Amyloid plaques and neurofibrillary tangles are the pathological hallmarks of AD. All diseases marked by the presence of amyloid plaques and tau tangles are grouped into secondary tauopathies.

Both APP and tau have been under extensive investigation since their identification. Tau is one of the microtubule-associated proteins (MAPs) that stabilize neuronal microtubules for their function in the development of cell processes, establishment of cell polarity and intracellular transport (Mandelkow, Schweers et al. 1996). Tau is mainly expressed in axons. Its synthesis is unusual since tau mRNA is transported to the proximal axon where translation takes place (Litman, Barg et al. 1994). Tau can be phosphorylated at multiple sites, which regulate its microtubule-binding

properties. In AD patients, tau is found in its hyperphosphorylated form. Hyperphosphorylated tau shows a loss of microtubule binding as a consequence of the hyperphosphorylation (e.g. at site S262) (Biernat, Gustke et al. 1993). Hyperphosphorylated tau detaches from microtubules, which probably accounts for the disappearance of those in AD and results in the breakdown of intracellular traffic (Mandelkow and Mandelkow 1998).

Not all brain areas are affected in a similar way by protein aggregates (Arendt 2003). NFT's are mainly found in layer II of the entorhinal cortex, the subiculum and the CA1 region in the hippocampus. Neighbouring brain areas like the pre- and parasubiculum or the dentate gyrus in the hippocampus hardly show any NFT's. In later stages of disease, additional areas of the limbic system such as the nucleus olfactorius anterior, the amygdala and the nucleus basalis suffer from NFT's. Ultimately, NFT's affect the isocortex, appearing first in associative cortex followed by sensory- and motor cortex. The stepwise spreading of NFT's correlates with the severeness of the dementia and follows the route of cortical information integration in backward direction. There also exists a hierarchy of vulnerability within the cortex. In particular, the pyramidal cells of layer II and V in neocortex appear to be affected by NFT's (Arendt 2003).

In contrast to the NFT's, amyloid plaques are found more equally distributed throughout the brain, with most pronounced patterns in cortex and hippocampus (Arendt 2003). Neuronal cell loss in AD follows the same pattern as the spread of NFT's. It is first seen in the hippocampus and associative cortex (Arendt 2003).

Taken together, the brain areas mostly and firstly affected in AD are the phylogenetically young tissues, which are also of high synaptic plasticity (Arendt 2003). Synaptic loss correlates well with cognitive dysfunctions (Terry, Masliah et al. 1991; Selkoe 2002).

The most accepted theory for development of AD today is the so called amyloid hypothesis. According to this hypothesis, accumulation of Aβ in the brain is the primary insult driving AD pathogenesis, while other disease-related processes, including formation of NFT's, are a secondary result of an imbalance between Aβ production and Aβ clearance (Hardy and Allsop 1991; Hardy and Selkoe 2002). This model is supported by the fact that in familial AD (FAD), mutations leading to

increased Aβ production are the fundamental cause of early onset of the disease. So far more than 20 mutations in *App* and more than 160 mutations in enzymes processing APP (presenilin-1, -2) have been identified (also see www.alzforum.org).

1.1.2 The amyloid precursor protein

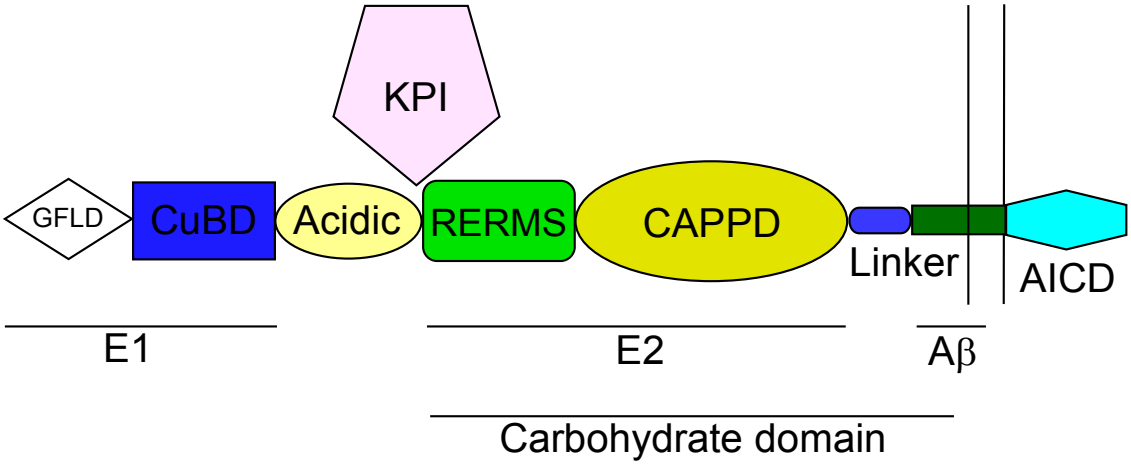


Figure 1: The structure of APP is composed of nine different protein domains.
At the amino-terminus the growth factor-like domain (GFLD) is located, followed by the copper binding domain (CuBD) - together also known as E1-region. Afterwards an acidic domain follows. In the case of APP_{751} and APP_{770} at this position a kunitz-type protease inhibitor domain (KPI) is found. Next a specific amino acid sequence is located (Arginin, Glutamin, Arginin, Methionin, Serin - RERMS-sequence), followed by the central APP domain (CAPPD), together also named E2-region. After this there is the linker domain that is together with the E2-region called carbohydrate domain. The single transmembrane domain of APP follows. Finally there is the APP intracellular domain (AICD) (Reinhard, Hebert et al. 2005).

APP is a member of an evolutionary conserved group of type-I transmembrane receptors (Figure 1). In total there are three members in this gene family: *App*, *Amyloid precursor protein-like protein 1* (*Aplp1*) and *Amyloid precursor protein-like protein 2* (*Aplp2*). Whereas vertebrates express all three receptors, invertebrates carry only one gene related to the gene family. For instance, *Caenorhabditis elegans* carries the *amyloid precursor-like 1 protein* (*apl-1*) (Walsh, Minogue et al. 2007).

1.1.3 The physiological function of APP

Through alternative splicing of pre-mRNA, three isoforms of APP are generated. APP_{770}, APP_{751} and APP_{695} vary in their relative amount in a cell type specific manner. In brain, APP_{770}, APP_{751} and APP_{695} occur in a ratio of 1:10:20 (Tanaka, Shiojiri et al. 1989). In neurons, APP was shown to traffick along the axon to the presynapse (Koo, Sisodia et al. 1990; Kins, Lauther et al. 2006). APP may serve as an adapter between the motor protein kinesin and transport vesicles (Kamal, Stokin et al. 2000; Kamal, Almenar-Queralt et al. 2001). The binding of APP to kinesin may be direct or via adater proteins such as the Jun n-terminal kinase (JNK) interacting protein (JIP-1) (Matsuda, Yasukawa et al. 2001; Scheinfeld, Roncarati et al. 2002; Inomata, Nakamura et al. 2003; Matsuda, Matsuda et al. 2003). *In vivo,* the axonal transport of APP to the presynapse was demonstrated in the rat optical nerve (Morin, Abraham et al. 1993).

As well as in the plasma membrane, APP is also found in the mitochondrial membrane. Elevated levels of APP interfere with energy metabolism and generate oxidative stress (Anandatheerthavarada and Devi 2007). Chronic *App* overexpression in the APP23 transgenic mouse model of AD was shown to reduce superoxide dismutase 1 (SOD-1) activity in brain, which could be compensated by a copper-enriched diet for the mice (Bayer, Schafer et al. 2003). SOD-1 facilitates the dismutation of oxygen radicals to hydrogen peroxide and requires binding of copper and zinc to potentiate its enzymatic activity. Copper levels in brain of AD patients were shown to be altered and APP was correlated to copper homeostasis (Multhaup 1997; Multhaup, Ruppert et al. 1997; Multhaup, Scheuermann et al. 2002; Bayer and Multhaup 2005; Kessler, Pajonk et al. 2005).

Furthermore, APP plays an important role in neuritogenesis as well as in neuronal regeneration after traumatic brain injury (Ikonomovic, Uryu et al. 2004; Jellinger 2004). Overexpression of human neuronal APP_{695} increases neuronal axogenesis in a dosage dependent manner. In *Drosophila*, injury of the brain results in dramatic upregulation of expression of the homolog APPL in affected brain areas (Chen and Tang 2006); Leyssen et al., 2005).

Finally, APP is involved in the development of the nervous system as deduced from studies on mouse models with simultanious deficiencies in *App*, *Aplp1* and *Aplp2*. By generating mice genetically deficient for *App, Aplp1* and *Aplp2* it was shown that

animals lacking either one of the three genes are viable and fertile. Animals lacking *Aplp2* and *App* and those lacking *Aplp2* and *Aplp1* each show a lethal phenotype (postnatal day 1), whereas mice deficient for *Aplp1* and *App* are apparently normal (Heber, Herms et al. 2000). The reason for the rather mild phenotypes of mice deficient for only one of the three may result from functional redundancy between *App*, *Aplp1* and *Aplp2* (Walsh, Minogue et al. 2007). In respect to their physiological function, *in vivo* and *in vitro* studies suggest that APP, APLP1 and APLP2 function to promote neurite outgrowth, neural cell migration and copper homoeostasis (Muller, Cristina et al. 1994; Zheng, Jiang et al. 1995; White, Reyes et al. 1999; Heber, Herms et al. 2000; Herms, Anliker et al. 2004). Mice deficient for *App*, *Aplp1* and *Aplp2* were shown to die *in utero* (Herms, Anliker et al. 2004). In 81% of the triple-deficient animals cranial defects were apparent, 68% of the animals showed lissencephaly, a cephalic disorder characterized by the loss of normal convolutions in the brain due to impaired neuronal migration, and a partial loss of Cajal Retzius cells was evident at embryonic day 18.5 (Herms, Anliker et al. 2004). In post-mitotic cells of the cortical ventricular zone reduction of APP expression using small-hairpin RNA applied *in utero* by electroporation, hindered the cells to migrate into the cortical plate (Young-Pearse, Bai et al. 2007). This defect was rescued when APP, APLP1 or APLP2 were overexpressed, demonstrating that all three proteins affect neuronal migration during forebrain development.

APP can be processed by several secretases producing a variety of processing products (Figure 2) with various functions. The two major processing pathways of APP are the amyloidogenic and the non-amyloidogenic processing pathway (Figure 2).

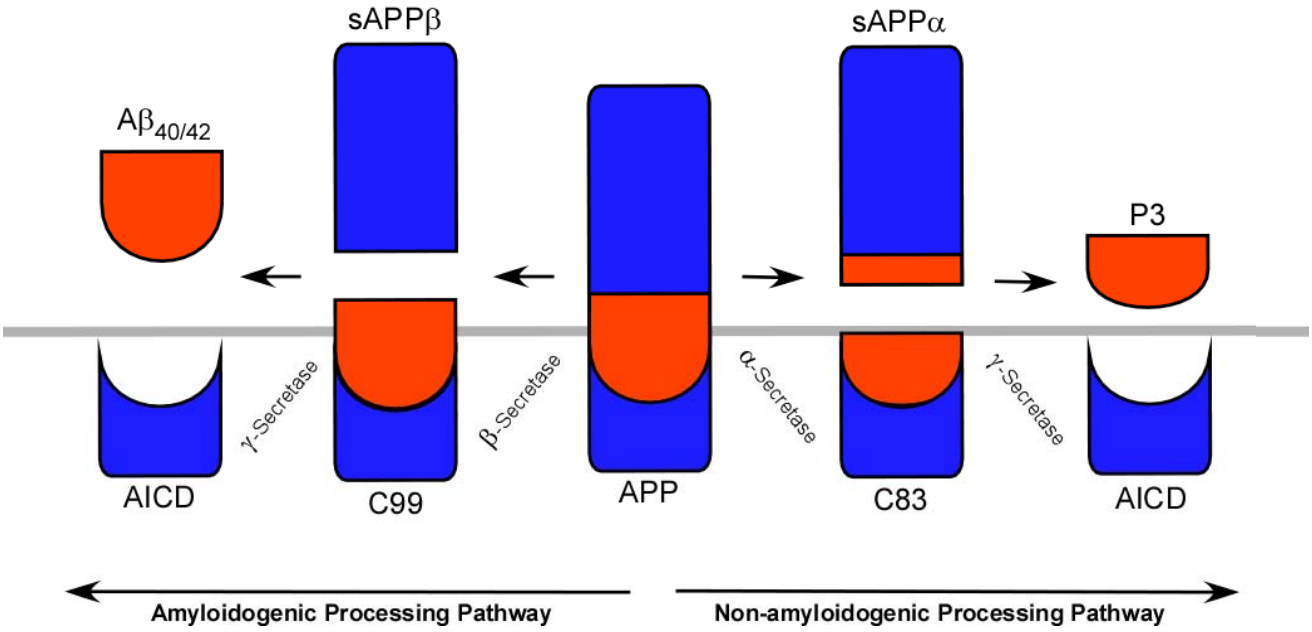


Figure 2: APP can be processed by several secretases.

The two major processing pathways for APP are the amyloidogenic and the non-amyloidogenic processing pathway. α-secretase cleavage generates soluble APPα (sAPPα) as well as the carboxy-terminal fragment C83. Subsequent cleavage of C83 by γ-secretase results in generation of P3 and APP intracellular domain (AICD). Alternatively, APP can first be cleaved by β-secretase leading to production of sAPPβ and C99. C99 can then be processed by γ-secretase producing AICD and Aβ.

The non-amyloidogenic processing pathway of APP

In the non-amyloidogenic processing pathway APP is cleaved consecutively by α- and γ-secretases. Through α-secretase cleavage soluble APPα (sAPPα) as well as the carboxy-terminal fragment (CTF) C83 are generated. Subsequent cleavage of C83 by γ-secretase results in generation of P3 and APP intracellular domain (AICD) (Seubert, Vigo-Pelfrey et al. 1992; Shoji, Golde et al. 1992; Haass, Hung et al. 1993).

The amyloidogenic processing pathway of APP

In the amyloidogenic processing pathway APP is first cleaved by β-secretase leading to the production of soluble APPβ (sAPPβ) and C99. C99 can then be processed by γ-secretase producing AICD and Aβ.

The localisation of APP within the cell

The processing fate of APP is defined by its subcellular localisation (Figure 3). Coming from the endoplasmic reticulum (ER), APP enters the Golgi apparatus, matures on its way through the Golgi and gets transported to the cell surface. En route to the cell surface, it is mainly processed by the non-amyloidogenic pathway starting with the cleavage by a disintegrin and metalloprotease (ADAM) or α-secretase. This cleavage mainly takes place in a post Golgi compartment or at the cell surface within the Aβ domain of APP disrupting the Aβ peptide sequence (Esch, Keim et al. 1990; Wang, Meschia et al. 1991).

Alternatively, APP may be endocytosed from the cell surface via clathrin-coated pits and mainly processed by the amyloidogenic pathway producing Aβ. This processing pathway starts mainly in acidic compartments such as endosomes or the trans Golgi network (TGN) with cleavage by β-site APP cleaving enzyme (BACE) producing sAPPβ and C99 (Yan, Han et al. 2001). BACE was shown to function as a homodimer (Schmechel, Strauss et al. 2004; Westmeyer, Willem et al. 2004; Multhaup 2006).

All CTF's (C99, C83) are processed by γ-secretase. The active γ-secretase complex consists of the four subunits, presenilin-1 (PS-1), anterior pharynx-defective 1 (APH-1), presenilin enhancer 2 (PEN-2) and nicastrin (NCSTN). PS-1 is endoproteolytically cleaved (Thinakaran, Borchelt et al. 1996) and the N- and C-terminal fragments were shown to exist as dimers in the catalytic core of γ-secretase (Cervantes, Gonzalez-Duarte et al. 2001; Schroeter, Ilagan et al. 2003; Cervantes, Saura et al. 2004). The amino termini of the CTF stubs are recognized by NCSTN, which functions as a γ-secretase substrate receptor (Edbauer, Winkler et al. 2003). The γ-secretase has been reported to cleave at variable sites, thus generating Aβ peptides of varying lengths (Qi-Takahara, Morishima-Kawashima et al. 2005; Zhao, Cui et al. 2005). This processing is mainly taking place in late endosomes, TGN and partly in ER (De Strooper 2003).

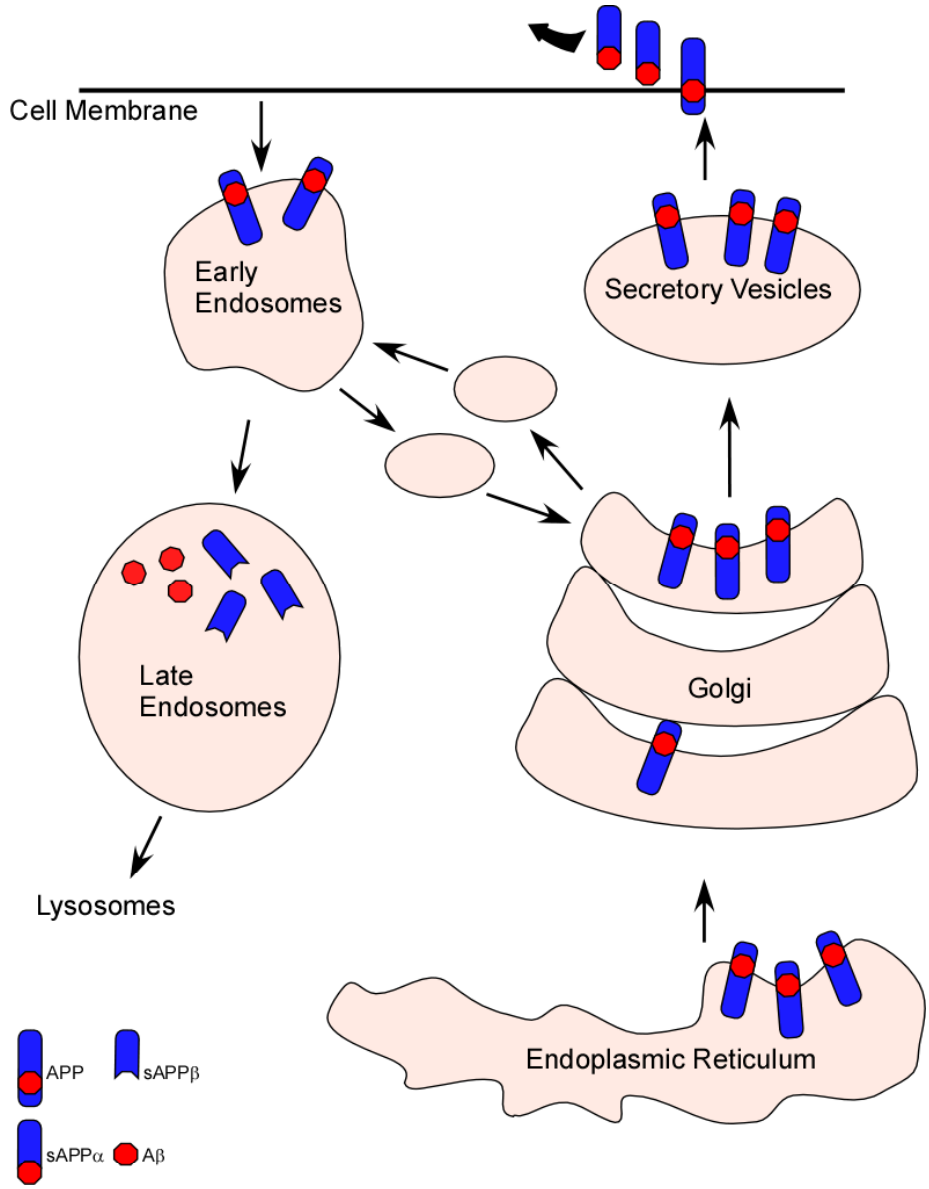


Figure 3: Localisation of APP within the cell determines its processing fate.

Newly synthesized APP molecules traverse the Golgi to the plasma membrane. Here some are cleaved to sAPPα. Non-processed precursors endocytose from the cell surface and move to late endosomal compartments for processing into sAPPβ and Aβ.

The Aβ peptide

Aβ has a length of 38 to 43 amino acids, whereby $A\beta_{40}$ is the most common to be produced. $A\beta_{42}$ peptides on the other hand are the pathologically most relevant form, as they form the core of amyloid plaques in the brains of AD patients (Iwatsubo, Odaka et al. 1994). Which species of Aβ is generated is regulated by an amino-acid motif GxxxG within the transmembrane sequence (TMS) of APP. These gly-

cine residues G29 and G33 are part of a dimerization site within the APP transmembrane sequence. Destabilization of the dimerization strength at this site was thereby found to determine the γ-secretase cleaving sites (Munter, Voigt et al. 2007).

The extracellular accumulation of Aβ is a first step in the amyloid cascade. Amyloid plaques have neurotoxic properties, leading to apoptosis of surrounding neurons. Neuronal apoptosis causes synaptic failure of affected brain areas (Yankner, Dawes et al. 1989; Takadera, Sakura et al. 1993; Behl, Davis et al. 1994). Most likely, it is the soluble oligomeric intermediate of Aβ that has the neurotoxic effects (Hardy and Selkoe 2002; Kawasumi, Hashimoto et al. 2002; Walsh, Klyubin et al. 2002). These oligomeric species correlate better with the degree of dementia in AD patients than the amyloid plaques. Oligomeric Aβ is known to bind to dendritic spines (a spine represents a synapse) and to change the morphology leading to destruction of the spine (Gong, Chang et al. 2003; Lacor, Buniel et al. 2004; Lacor, Buniel et al. 2007). Thus, oligomeric Aβ causes synaptic dysfunction that ultimately leads to dementia (Gralle and Ferreira 2007). However, it was also shown that soluble Aβ can have a trophic function in undifferentiated neurons and might even protect against neuronal hyperactivity (Yankner, Duffy et al. 1990; Kamenetz, Tomita et al. 2003).

It is interesting that APLP-1 and -2 do not carry the Aβ domain, so obviously it is not essential for the physiological main functions of the protein family. This between the species highly conserved domain more likely serves as an evolutionary advantage (Walsh, Minogue et al. 2007).

The sAPPα

The protective function of the non-amyloidogenic pathway is due to the neurotrophic effect of sAPPα. This action protects the neuron from dying and improves cognitive functions (Roch, Masliah et al. 1994; Furukawa, Sopher et al. 1996; Meziane, Dodart et al. 1998; Mattson, Guo et al. 1999). In addition, it was shown to regulate the morphology of neurons. Application of sAPPα to primary neurons resulted in neurite lengthening, this effect was dependent on the presence of cellular APP expression (Young-Pearse, Chen et al. 2008).

The AICD seems to be a nuclear transcription factor. It was shown to enter the nucleus and to trigger transcription (Kimberly, Zheng et al. 2001; Cao and Sudhof 2004; Chang, Kim et al. 2006). The physiological functions of sAPPβ and P3 remained elusive so far.

1.1.4 Interaction partners of APP

Sabo et al. (Sabo, Ikin et al. 2001) proved that overexpression of APP and the adapter protein FE65 led to an increase in cell motility in a cell culture system. It is believed that APP and FE65 modulate the actin cytoskeleton via interaction with the protein Mena.

The interaction of FE65 or its homologs FE65-like1 and FE65-like2 with APP increases the translocation of APP to cell surface, enhances γ-secretase-activity and elevates Aβ secretion (Sabo, Lanier et al. 1999; Chang, Tesco et al. 2003).

APP also interacts with other adhesion proteins like integrins on the surface of axons or with matrix molecules such as laminin (Storey, Beyreuther et al. 1996; Fossgreen, Bruckner et al. 1998).

Furthermore, APP interacts with several proteins implicated in signal transduction like Disabled-1 (Dab1) and X11/Mint-1 (M. McLoughlin and C.J. Miller 1996; Homayouni, Rice et al. 1999).

Various adapter proteins like the FE65 proteins, JIP-1b and Dab1 carry a phosphotyrosine-interacting domain (PID) which allows binding to the YENPTY amino acid sequence at the carboxy terminus of APP. This way, the translocation and processing of APP is modulated.

If JIP-1b binds to APP less sAPPβ and Aβ is secreted (Taru, Kirino et al. 2002). If mammalian Dab-1 (mDab-1) binds to APP, the intracellular levels of immature APP, of APP at the cell surface and of secretase activity are elevated (Trommsdorff, Borg et al. 1998; Howell, Herrick et al. 1999).

Interaction of the extracellular protein fibulin-1 and heparin sulfate proteoglycans (HSPG) with the E1 region of APP stimulates outgrowth of neurons (Small, Clarris et al. 1999) and inhibits the proliferative function of APP (Ohsawa, Takamura et al. 2001). Ho et al. (Ho and Sudhof 2004) have shown that the signaling molecule F-spondin binds to APP. Binding inhibits processing of APP by BACE. F-spondin is involved in differentiation of dorsal root ganglion cells (DRG) (Klar, Baldassare et al.

1992) and neurogenesis (Feinstein, Borrell et al. 1999).

Taken together APP and its processing products are involved in a multitude of processes such as cell migration and adhesion, development of the nervous system and signal transduction. Obviously, the impact of APP on these processes is determined by the balance of APP processing. Since the secretases processing APP are differentially located within the cell, the subcellular localisation of APP influences its processing fates in a major way.

1.1.5 Risk factors for AD

In FAD, mutations leading to increased Aβ production are the fundamental cause of early onset of AD. Today, more than 20 mutations in *App* and more than 160 mutations in enzymes processing APP have been identified. Duplication of the *App* locus in trisomy 21 and mutations at the β-cleavage site of *App* result in overall elevated Aβ levels and consequently in AD (Irie, Murakami et al. 2005; Rovelet-Lecrux, Hannequin et al. 2006). Mutations in *App* near the γ-cleavage site increase the relative production of $A\beta_{42}$ (Irie, Murakami et al. 2005).

Most mutations in presenilin-1, -2 also change the balance from $A\beta_{40}$ to $A\beta_{42}$ production, which is important as $A\beta_{42}$ tends to form amyloid plaques more easily (Ancolio, Dumanchin et al. 1999; Makarova, Williams et al. 2004). While mutations found in FAD were imformative for our understanding of the genes causative of AD processes, familial forms of the disease are rare. The majority of AD patients, in contrast, suffer from the sporadic form of AD (Weyerer 2005), which manifests itself later in life with a typical age of onset after 65 years (Blennow, de Leon et al. 2006). The environmental and genetic factors that cause sporadic AD remained elusive so far, but more than 30 gene loci have been proposed to raise the risk for late-onset AD (LOAD). The best characterized genetic predisposition is the polymorphism for APOE. Three isoforms exist in the human genome: E2, E3 and E4, which are encoded by different alleles ε2, ε3 and ε4. Most people carry isoform ε3. The rare form of ε4 is strongly correlated with an elevated risk for AD (Strittmatter, Saunders et al. 1993). This *Apoe* effect is gene dosage dependent. Homozygosity for *Apoe ε4* causes the highest risk to suffer from AD (Castano, Prelli et al. 1995; Hyman, Gomez-Isla et al. 1996; Holtzman, Bales et al. 2000). Coon et al. reported that

people with two *Apoe ε4* copies have a 25-fold increased risk for developing the disease compared to carriers homozygous for *Apoe ε3* (Coon, Myers et al. 2007). Other studies revealed an about 2.5-fold increased risk (Bertram, McQueen et al. 2007). In contrast *Apoe ε2* has a protective function (Corder, Saunders et al. 1993; Corder, Saunders et al. 1994).

Since the finding that the *Apoe* gene locus represents the most important risk factor for sporadic AD, the question how allelic variations of the gene may affect neurodegeneration has been in focus of research. Among other hypotheses, distinct effects of *Apoe* isoforms on APP processing (Irizarry, Deng et al. 2004; Hoe, Harris et al. 2005; Hoe, Pocivavsek et al. 2006), Aβ metabolism and aggregation (Bales, Dodart et al. 2002), or neuronal survival have been advanced (Poirier 2000; Martins, Hone et al. 2006). Loss of the low-density lipoprotein receptor (LDLR) function was shown to aggravate amyloid deposition and learning deficits in the Tg2576 mouse model of AD (Cao, Fukuchi et al. 2006). In contrast, breeding of *Ldlr*-deficient mice with the PDAPP transgenic line was reported to not significantly increase amyloid deposition (Fryer, Demattos et al. 2005). Thus, it is still unclear whether the LDLR itself plays a causal role in AD pathology. Given its function in directing uptake of cholesterol-rich lipoproteins into neurons, a role of APOE isoforms in cholesterol homeostasis in AD has been suggested. Functional interaction of APOE with its receptors is important for the control of cellular cholesterol homeostasis, as APOE receptors are crucial components of the cellular machinery that supplies cholesterol to neurons. Some studies provide evidence that cholesterol levels directly affect Aβ production rates (Simons, Keller et al. 1998; Abad-Rodriguez, Ledesma et al. 2004). Both, stimulatory and inhibitory effects of high cholesterol concentrations on amyloidogenesis have been reported, challenging the idea of cholesterol lowering therapies as beneficial treatment in AD (Abad-Rodriguez, Ledesma et al. 2004).

In addition, the formation of a stable complex of Aβ with APOE has been reported, suggesting a pathway for catabolism of the peptide via APOE receptors (Strittmatter, Saunders et al. 1993; Strittmatter, Weisgraber et al. 1993). Aβ internalized in complex with APOE could be subject to lysosomal degradation, thereby reducing the amount of molecules that form senile plaques. Interestingly, Aβ clearance rates have been reported to vary with *Apoe* species, being higher with ε2 and ε3, than ε4. These kinetics correlate well with the distinct binding affinities of the various

APOE/Aβ complexes to the low-density lipoprotein receptor-related protein 1 (LRP1), implicating this receptor in Aβ turnover (Jordan, Galindo et al. 1998; Yang, Small et al. 1999).

APOE has also been suggested to alter conformation of Aβ upon binding, acting as a chaperone that promotes amyloid plaque deposition. Such a model could also explain the isoform specific effects of APOE on AD with E4 promoting Aβ aggregation to a greater extent than E2 and E3 (Holtzman, Bales et al. 2000). In support of this concept, *Apoe*-deficient mice show a reduced plaque load when crossed with mouse models of AD (Bales, Verina et al. 1997; Bales, Verina et al. 1999). A similar role for apolipoprotein J (a ligand of LRP2 and APOER2) as facilitator of Aβ aggregation has been documented both alone (DeMattos, O'Dell M et al. 2002) and in combination with APOE (DeMattos, Cirrito et al. 2004).

In addition, APOE was reported to promote recruitment of astrocytes to regions in the brain with amyloid plaques inducing degradation of Aβ deposits (Koistinaho, Lin et al. 2004).

Besides APOE itself, several APOE receptors, belonging to the low-density lipoprotein receptor (*Ldlr*) gene family, have been associated with AD so far.

From genetic studies it is known that the very low-density lipoprotein receptor (*Vldlr)* correlates with AD (Yamanaka, Kamimura et al. 1998; Nakamura, Yamamoto et al. 2001; Carter 2007; Llorca, Rodriguez-Rodriguez et al. 2008), but a molecular mechanism explaining this correlation remains an open issue.

More is known about the implication of LRP1 in AD. LRP1 serves as receptor for APP (Kounnas, Moir et al. 1995; Knauer, Orlando et al. 1996) and is implicated in regulation of cellular transport of APP (reviewed in (Zerbinatti and Bu 2005; Bu, Cam et al. 2006; Waldron, Jaeger et al. 2006)). Because the first interaction between APP and LRP1, that has been characterized, involved the KPI domain of APP, which is not present in the neuronal variant of APP, the relevance of this association for AD-related processes has been questioned. Further investigations uncovered a second binding motif between LRP1 and APP in their cytoplasmic domains, present in all isoforms including APP_{695}. This association of APP and LRP1 is no direct protein-protein interaction, but requires linkage of the two proteins via the cy-

toplasmic adaptor protein FE65 (Trommsdorff, Borg et al. 1998; Gotthardt, Trommsdorff et al. 2000; Kinoshita, Whelan et al. 2001; Pietrzik, Yoon et al. 2004).

Most likely, LRP1 regulates the retention time of APP on the cell surface, with accelerated internalization and amyloidogenic processing in endocytic pathways as consequence (Ulery, Beers et al. 2000; Pietrzik, Busse et al. 2002; Cam, Zerbinatti et al. 2005; Yoon, Pietrzik et al. 2005). In a transgenic mouse model overexpression of LRP1 minireceptors in neurons resulted in increased Aβ levels in the brain, and in deficits in spatial learning and memory (Zerbinatti, Wozniak et al. 2004). Intriguingly, in *Lrp1*-transgenic mice, a distinct fraction of Aβ is associated with the plasma membrane, indicating that LRP1 may also increase intraneuronal amyloid accumulation (Zerbinatti, Wahrle et al. 2006). Taken together, changes in LRP1 expression levels may affect AD progression both by altering trafficking of APP and by clearance of Aβ.

The low-density lipoprotein receptor-related protein 1B (LRP1B) is a lipoprotein receptor closely related to LRP1 that also forms immunoprecipitable complexes with APP. In contrast to LRP1, its influence on APP processing is anti-amyloidogenic inasmuch as LRP1B retains APP molecules at the cell surface, impairing Aβ production (Cam, Zerbinatti et al. 2004). This effect could be explained by the much slower rates of endocytosis for LRP1B ($t_{½}$ > 10min) as compared to LRP1 ($t_{½}$ < 0.5min) (Li, Lu et al. 2001).

The APOE receptor-2 (APOER2) associates with APP both through extracellular and intracellular interactions. The interaction of APOER2 with the extracellular domain of APP is modulated by F-spondin, a secreted protein that functions in axonal path finding, cell-cell interactions, and neural regeneration (Hoe, Wessner et al. 2005). F-spondin has been implicated in AD previously because it affects amyloidogenic processing by decreasing β-secretase activity (Ho and Sudhof 2004). Recent findings indicate that F-spondin leads to retention of APP at the cell surface in association with APOER2, comparable to the situation seen for LRP1B (Hoe, Wessner et al. 2005). At the cytoplasmic site of the membrane, adaptors Dab1 and FE65 have been reported to physically link APP and APOER2 leading to a decrease in Aβ production (Hoe, Magill et al. 2006; Hoe, Pocivavsek et al. 2006). Interestingly, the adaptor X11 α/β was recently reported to work pro-endocytic and increase amyloid

production by accelerating APOER2-dependent APP internalization in the presence of APOE (He, Cooley et al. 2007).

Finally, the sorting protein related receptor containing A type repeats (SORLA) was identified as a major risk factor for AD (see below).

1.2 SORLA

SORLA was identified independently by biochemical purification of receptor-associated protein (RAP) binding proteins (Jacobsen, Madsen et al. 1996) and by a genetic screen for novel LDL receptor family members (Yamazaki, Bujo et al. 1996; Morwald, Yamazaki et al. 1997). So far it was identified in rabbit (Yamazaki, Bujo et al. 1996), chicken (Morwald, Yamazaki et al. 1997) and in human (Jacobsen, Madsen et al. 1996). The human gene encoding SORLA (*SORL1*) is located on chromosome 11q23/24. It encodes a protein with a molecular weight of 250 kDa (Jacobsen, Madsen et al. 1996). SORLA is expressed in several tissues but shows highest expression in cortex and hippocampus of the brain (Hermans-Borgmeyer, Hampe et al. 1998; Kanaki, Bujo et al. 1998). To a lower extent SORLA is also found in the spin, testes, lung, uterus, thyroid, lymph nodes and the kidney (Jacobsen, Madsen et al. 1996; Yamazaki, Bujo et al. 1996; Morwald, Yamazaki et al. 1997; Kanaki, Bujo et al. 1998).

Based on its structure, SORLA may be grouped into the *Ldlr* gene family as well as the family of vacuolar protein sorting 10 protein (VPS10P)-receptors (Figure 4).

Its extracellular domain consists of motifs that are shared with other members of the *Ldlr* gene family, e.g. complement-type repeat (CR)-domains and a ß-propeller (Jeon, Meng et al. 2001), whereas the VPS10P domain is only present in members of the *Vps10* gene family. This unique motif with homology to proteins involved in vacuolar protein sorting is present at the amino-terminus of the receptor suggesting a role for SORLA in intracellular sorting mechanisms. Indeed, the receptor has been shown to interact with the amyloid precursor protein (APP) and to regulate its intracellular trafficking (Andersen, Schmidt et al. 2006; Offe, Dodson et al. 2006).

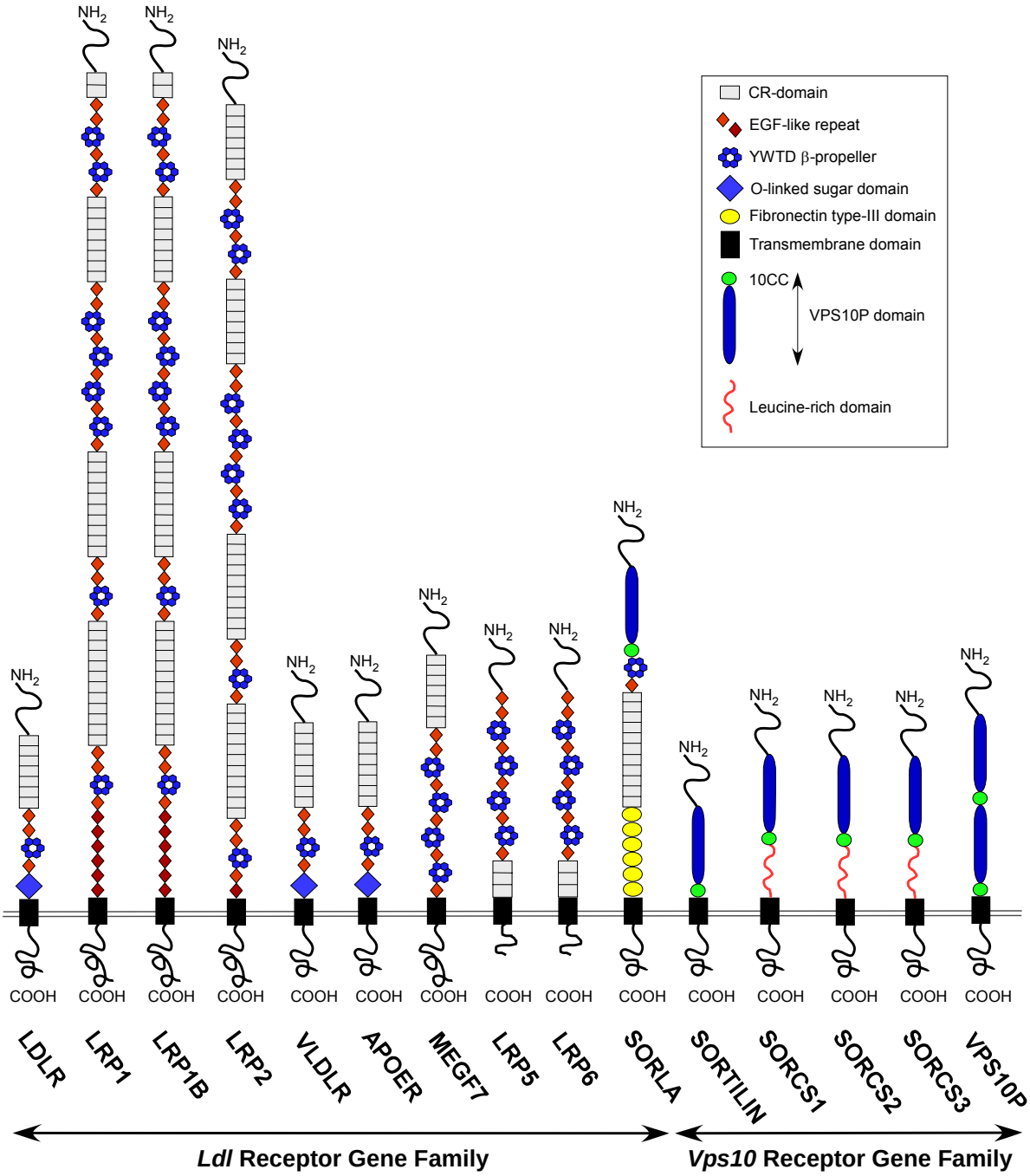


Figure 4: Shown are family members of the *Ldl* receptor gene family and the *Vps10* receptor gene family.
SORLA shares common motifs of both families, as seen. Abbreviations: LDLR, low-density lipoprotein receptor; LRP1, 1B, 2, low-density lipoprotein receptor related protein 1, 1B, 2; VLDLR, very low-density lipoprotein receptor; APOER2, apolipoprotein E receptor 2; MEGF7, multiple EGF-repeat-containing protein 7; LRP5, 6, low-density lipoprotein receptor related protein 5, 6; SORLA, sorting protein related receptor containing LDLR class A repeats; SORCS1-3, sortilin-related VPS10 domain containing receptor 1-3; VPS10P, vacuolar-protein-sorting 10 protein.

Structure of SORLA

SORLA is a type-I transmembrane receptor with a large extracellular aminoterminal part, one transmembrane domain and an intracellular carboxyterminal tail. SORLA consists of in total eight domains (Figure 4). Starting from the aminoterminus it includes the propeptide, the VPS10P domain, the beta-propeller, the epidermale growth factor precursor (EGF precursor) domain, 11 complement-type repeats / class-A repeats (CR-cluster), followed by six fibronectin type-III domains and the transmembrane anchor. Finally, the receptor harbors an intracellular tail consisting of 54 amino acids. Within this region there is a special amino acid sequence (phenylalanine–alanine–asparagine–serine–histidine–tyrosine, FANSHY), which is similar to FDNPxY or NPxY motifs found in many endocytic receptors. This motif is essential for clathrin-mediated endocytosis via interaction with the adaptor complex AP-2 (Chen, Goldstein et al. 1990).

SORLA has structural homology to the head activator binding protein (HAB) of *Chlorohydra viridissima* (Jacobsen, Madsen et al. 1996; Yamazaki, Bujo et al. 1996). HAB is the receptor for head activator protein (HA) (Hampe, Frank et al. 1996), which controls differentiation of the head and brain structures (Hampe, Urny et al. 1999). In comparison to HAB, SORLA has an identical combination of domains (Jacobsen, Madsen et al. 1996; Yamazaki, Bujo et al. 1996). Similiar to HAB, SORLA is synthesized as preproprotein (Jacobsen, Madsen et al. 1996; Hampe, Urny et al. 1999). After cleavage of the signaling peptide in endoplasmic reticulum, the propeptide gets processed in Golgi by furin (Hampe, Riedel et al. 2000; Jacobsen, Madsen et al. 2001).

APOE, lipoproteinlipase (LPL) and RAP are binding to the CR cluster of SORLA. The propeptide, HA and neurotensin bind to the VPS10P domain. Binding of the propeptide or RAP to SORLA inhibits binding of the other known ligands to the very same site (Jacobsen, Madsen et al. 2001).

Function of SORLA

Previosuly, the physiological function of SORLA was unclear. Limited insight into its possible function comes from *in vitro* studies. It was shown that only 10% of the receptor molecules are located at the cell surface, but 90% are present within the cell, mainly in trans-Golgi network (TGN) and endosomes (Jacobsen, Madsen et al.

2001). Hirayama et al. (Hirayama, Bujo et al. 2000) suggested involvement of SORLA in proliferation and differentiation of neuroblastoma cells, inducing these processes led to induction of SORLA on protein and mRNA levels. Particularly, the identification of a regulatory element by Hirayama et al. in the SORLA promoter region that binds CCAAT (cytidine-cytidine-adenosine-adenosine-thymidine)-enhancer-binding proteins (C/EBP), a family of transcription factors, raises interesting implications for receptor function, because elevation of C/EBP expression is associated with terminal differentiation in a number of cell types (Hirayama, Bujo et al. 2000). In addition, it was speculated that some cytokines might stimulate the induction of SORLA expression through the interaction of corresponding transcription factors and this sequence. Deleting the corresponding sequence of *Sorl1* revealed that the relative promoter activities are drastically reduced, suggesting that this region contains the key elements responsible for regulation.

In kidney, SORLA is expressed in the thick ascending limb of Henle's loop, distal convoluted tubules, and the collecting duct. In the latter cell type, the receptor locates to aquaporine positive vesicles (Riedel, Hermans-Borgmeyer et al. 2002). Aquaporines are water channels that, once activated, make the collecting duct water permeable to concentrate the primary urine.

First hints about a possible involvement of SORLA in AD came from a gene expression analysis of lymphoblasts from AD patients (Scherzer, Offe et al. 2004). In the control group, SORLA was found to be twice as highly expressed as in lymphoblasts from AD patients. Whether SORLA expression is downregulated as a consequence of neurodegenerative processes or whether loss of SORLA activity is a primary event in onset and progression of AD remained unclear.

A working model for the role of SORLA in AD came from a study by Andersen et al. (Andersen, Schmidt et al. 2006), which reported a direct interaction between SORLA and APP. Co-expression of APP with the receptor resulted in altered subcellular localisation of APP and in its trapping in Golgi/endosomal compartments (Figure 5). Altered localisation reduced overall processing rates of APP in cell culture. In contrast, in *Sorl1*-deficient mice APP processing was increased.

In addition, a reduction of protein levels of SORLA in cortex tissue from AD patients even further supported involvement of SORLA in AD.

Finally, the role of SORLA as Golgi retention factor for APP was further substantiated by Schmidt et al. (Schmidt, Sporbert et al. 2007). In cell culture experiments, variants of SORLA, lacking functional interaction sites with Golgi-localized, γ-adaptin ear homology domain, ADP-ribosylation factor-binding protein (GGA) and phosphofurin acidic cluster sorting protein 1 (PACS-1), adaptor proteins involved in protein transport to and from the TGN, revealed aberrant targeting of SORLA to the recycling compartment or the plasma membrane. This also caused faulty APP trafficking and imbalance in non-amyloidogenic and amyloidogenic processing fates.

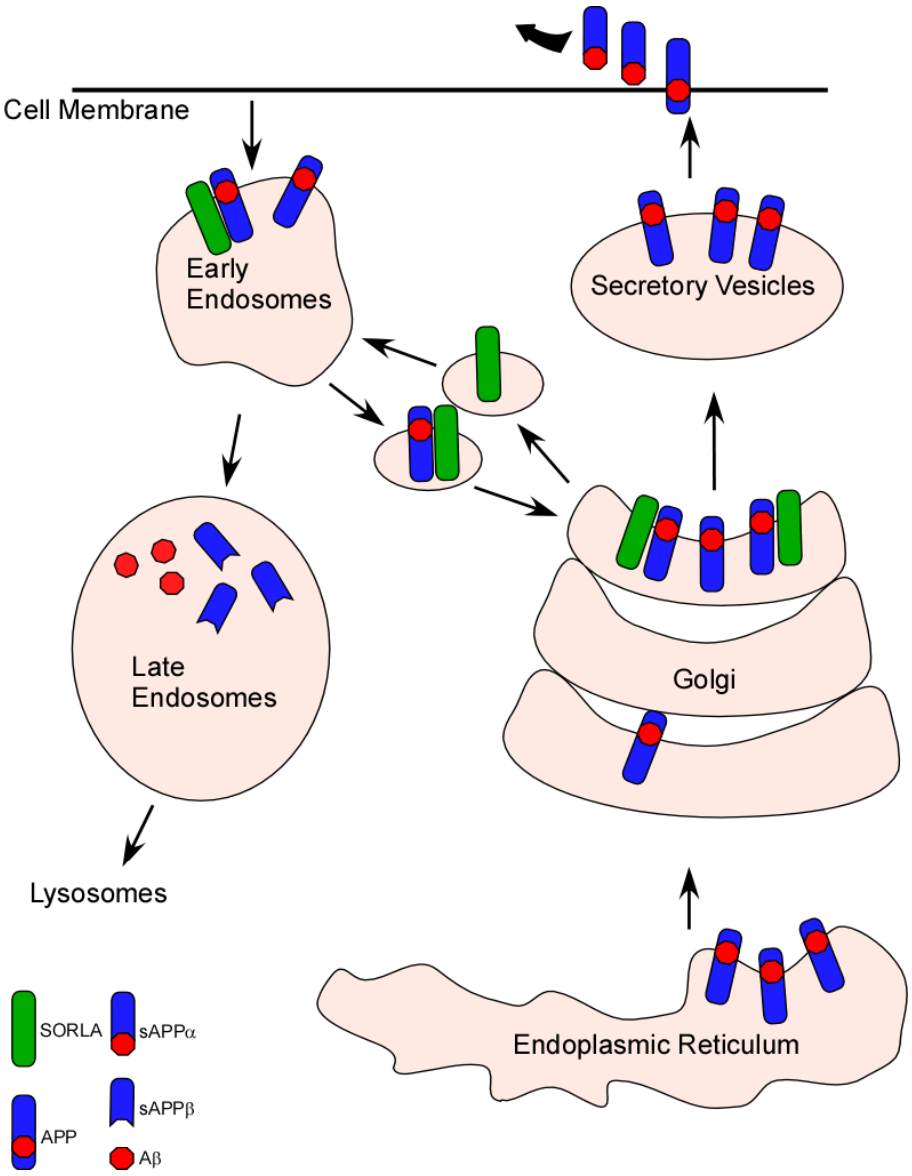


Figure 5: Proposed role for SORLA in APP processing
Newly synthesized APP molecules traverse the Golgi to the plasma membrane,

where some are cleaved to sAPPα. Non-processed precursors endocytose from the cell surface into late endosomal compartments for processing into sAPPβ and Aβ. SORLA acts as a sorting receptor that keeps APP in the Golgi/endosomal compartments, reducing the amount of APP that reaches the cell surface for processing.

Important *in vivo* prove of relevance for AD came from a study by Rogaeva et al. (Rogaeva, Meng et al. 2007) showing genetic association of SORLA and AD. Dodson et al. (Dodson, Gearing et al. 2006) in addition showed that SORLA expression is only affected in sporadic but not in familial AD.
Taken together, all experimental data from genetic studies in patients to cell culture models to *Sorl1*-deficient mouse models were consistent with an important role for *Sorl1* in sporadic Alzheimer disease by modulating the balance of APP processing, whether by changing its subcellular localisation or yet unknown additional processes.

1.3 Aim of this study

The aim of this thesis project was to shed more light on SORLA's function in brain *in vivo* in order to understand its role in normal brain physiology and in AD. This aim should be achieved by detailed analysis of the phenotype of *Sorl1*-deficient mice per se and by analysis of consequences of *Sorl1* deficiency in a mouse model of AD. In addition, the thesis was concerned with the identification of compounds that increase SORLA expression in neurons. Goal of these studies was the proof of concept that raising SORLA levels in the brain may represent a novel therapeutic concept to treat patients with AD.

2 Material and Methods

2.1 Animal experimentation

Mice were kept at standard conditions according to ethic committee regulations. All studies involving animals were performed in accordance with institutional guidelines. Wild type mice were of mixed genetic background (129SvEmcTer x C57BL/6N and 129SvEmcTer x Balb/c) and bred in house. *Sorl1*-deficient mouse lines SORLA18 (129SvEmcTer x Balb/c), SORLA27 (129SvEmcTer x C57BL/6N) and SORLA39 (129SvEmcTer x Balb/c) were generated by targeted gene disruption as described before (Andersen et al., 2005). A second receptor null line was generated independently through insertional mutagenesis of the murine *Sorl1* gene locus by a *lacZ* reporter gene by William C. Skarnes (Welcome Trust Sanger Center, UK). All lines gave identical results.

2.2 Immunohistology

Brains were fixed in situ in 4% formalin by transcardiac perfusion, post-fixed at 4 °C for 24 h and stored in 30% sucrose until further processing. For each animal, one hemibrain was cut into 40 µm thick free-floating sagittal sections, while the second hemibrain was embedded in paraffin and cut into 5 µm sections. Alternatively the entire brain was cut into 40 µm thick free-floating sections. Staining of paraffin sections with thionine (Nissl staining) or immunohistological detection of acetylcholine transferase (1:200; Chemicon, Hofheim, Germany), macrophage/microglial marker F4/80 (1:100; Serotec, Düsseldorf, Germany), astrocytic marker GFAP (glial fibrillary acidic protein) (1:1000 Advanced Immunochemical Inc., Long Beach, USA) on free-floating sections was performed according to standard protocols using biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, USA) and the ABC Elite kit (Vector Laboratories, Burlingame, USA) for visualization. For fluorescence microscopy (GFAP), sections were incubated with a secondary antibody conjugated with Alexa 488 (1:250; Molecular Probes, Leipzig, Germany) for 4 h, mounted with Vectashield (Vector Laboratories, Burlingame, USA) and viewed with

a laser scanning confocal microscope. DNA fragmentation in apoptotic nuclei was detected by labeling with the *in situ* cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's recommendations. For immunoelectron-microscopical detection of APP, dissected hippocampi of one wild type and one knockout mouse were fixed in 4% formaldehyde/ 0.5% glutaraldehyde in 0.1 M phosphate buffer, embedded in 10% gelatine and infiltrated with 2.3 M sucrose. Frozen samples were trimmed to the region of the neuronal cell layers and ultrathin cryosections were obtained according to Tokuyasu (Tokuyasu 1973). APP was detected by polyclonal anti-APP antibody 1227 (diluted 1:250). For signal detection, 12 nm colloidal gold-AffiniPure goat anti-rabbit IgG, EM grade (Jackson Immuno Research Lab., Inc., West Grove, USA) was used. Labeled Golgi fields were examined on an Zeiss 910 electron microscope equipped with an 1k x 1k CCD camera (Proscan, Lagerlechfeld, Germany) and analysed with the analySIS 3.2 software (Soft Imaging System, Münster, Germany) for counting grains of gold and Golgi area determination.

2.3 Quantification of neurogenesis

Proliferation and survival of newborn neurons were determined by 5-bromo-2-desoxy-uridine (BrdU) incorporation analysis following a 24 h or 4 weeks chase period, respectively. Animals received a single (proliferation) or a three days repeated (survival) intraperitoneal dose of 50 µg BrdU (Sigma-Aldrich, Seelze, Germany)/ g body weight at 10 mg/ml in 0.9% NaCl. Brains were fixed in 4% PFA, infiltrated in 30% sucrose, and sectioned at 40 µm. The number of BrdU-labeled cells was determined by immunohistology using rat anti-BrdU (1:500; Harlan Sera-Lab, Blackthorn, England), followed by biotinylated donkey anti-rat (1:500; Dianova, Hamburg, Germany) and the peroxidase detection system (ABC Elite kit; Vector Laboratories, Burlingame, USA) with nickel-intensified diaminobenzidine as chromogen. The sections were counted in a one-in-six series through a 40x objective, and multiplied by 6 to give an estimate for the total number of BrdU-positive cells per entire dentate gyrus.

2.4 Immunohistology of primary neurons

Hippocampal and cortical primary neurons were plated on coverslips coated with a mixture of poly-D-lysine (0.5 mg/ml, Sigma-Aldrich, Seelze, Germany) and collagen (4 mg/ml, BD, Heidelberg, Germany) solubilized in 17 mM acetic acid (ratio 1:1:3). Cells were fixed with 4% PFA for 8 min at room temperature and afterwards stored in PBS at 4 °C. For fluorescence microscopy, cells were stained with anti-SORLA and anti-APP (1227) antibodies (made in house) and incubated with secondary antibodies conjugated with Alexa 488, Alexa 555 or Alexa 647 (1:250; Molecular Probes, Leipzig, Germany) for 4 h. Thereafter, sections were mounted with Vectashield (Vector Laboratories, Burlingame, USA) and viewed with a laser scanning confocal microscope (Leica SP-5; Leica, Wetzlar, Germany).

2.5 Preparation of primary neurons

Solutions

Enzyme solution: 2 mg cysteine (Merck, Darmstadt, Germany), 1 mM $CaCl_2$, 0.5 mM EDTA in 10 ml DMEM (GIBCO, Karlsruhe, Germany) with 25 units/ml papain (Worthington, New Jersey, USA)

Dissociation solution: DMEM + 5 % FCS (Seromed, Vienna, Austria) + 100 U penicillin/ml + 0.1 mg streptomycin/ml (Boehringer/Roche, Mannheim, Germany)

Stop solution: 25 mg albumin (Sigma-Aldrich, Seelze, Germany) + 25 mg trypsin-inhibitor (Sigma-Aldrich, Seelze, Germany) in 10 ml dissociation solution

Neuronal medium: 100 ml NeurobasalA (GIBCO, Karlsruhe, Germany) + 2 ml B27 (GIBCO, Karlsruhe, Germany) + 1 ml Glutamax 100x (GIBCO, Karlsruhe, Germany) + 100 U penicillin/ml + 0.1 mg streptomycin/ml (Boehringer/Roche, Mannheim, Germany)

Preparation medium: HBSS (Hanks Balanced Salt Solution; GIBCO, Karlsruhe, Germany)

Procedure

Primary hippocampal and cortical neurons were prepared from newborn mice and rat (day 1-2 postnatal). Animals were sacrified by decapitation. Cortex or hippocampus were dissected in HBSS (4 °C), incubated in 1 ml enzyme solution (carbonized before for 15 min and preincubated for 1 h after adding papain) for 1 h at 37 °C shaking at 900 rpm. Afterwards the enzymatic reaction was stopped by incubating the tissue for 5 min at 37 °C (shaking with 900 rpm) in 1 ml stop solution. Then, cells were dissociated in 250 µl dissociation medium by pipetting the solution up and down with a 200 µl pipette several times avoiding air bubbles. The cells were collected by centrifugation for 10 min at 80 xg. The pellet was resuspended in neuronal medium and plated on poly-D-lysine/collagen coated preincubated plates (TPP, Trasadingen, Switzerland). The cells were kept at 37 °C with 5% CO_2 in atmosphere. If not stated differently, the neurons were kept for seven days for differentiation before starting the experiments. The medium was changed every 3-4 days by replacing half of the old medium. Neurons were not kept longer than 2 weeks in culture.

Harvest of cells

Cells were put on ice; the medium was replaced by PBS including protease inhibitors (Complete Protease Inhibitor Cocktail; Roche, Mannheim, Germnay) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail; Pierce/Perbio Science, Bonn, Germany). Cells were scraped off and collected by centrifugation at 2700 xg for 10 min at 4 °C. The cell pellet was resuspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% deoxycholate, 1% NP40, 0.1% SDS, including Complete protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail) and incubated on ice for 1 h for complete lysis. The protein content was measured by Bradford (Bradford 1976).

2.6 Protein biochemistry

2.6.1 Membrane extraction

To enrich membrane-associated proteins, the sample tissue was homogenized us-

ing an Ultra Turrax (IKA, St. Augustin, Germany) in a minimal volume of solution A (20 mM Tris-HCl, 2 mM $MgCl_2$, 0.25 mM Sucrose, pH 7.5 including Complete Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail). The cellular debris was removed by centrifugation at a low g-force (2300 xg, 10 min, 4 °C). Nuclei were removed by an additional centrifugation step at 13000 xg (8 min, 4 °C). Finally, the supernatant was ultracentrifuged at a high g-force (100000 xg, 45 min, 4 °C) to pellet the membrane fraction. The pellet was resuspended in RIPA buffer containing protease and phosphatase inhibitors (Complete Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail) and stored at -80°C. For western blotting 50 µg protein were mixed with Laemmli buffer containing 5% mercaptoethanol and heated to 94 °C for 3 min. Samples were stored at 4 °C for up to 1 week.

2.6.2 Full protein lysate

In order to generate full protein lysates, tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitors using an Ultra Turrax, incubated on ice for 1 h and cleared by centrifugation 15000 xg for 10 min at 4 °C. The supernatant was stored at -80°C or, preferentially, directly mixed with Laemmli buffer containing 5% mercaptoethanol and heated to 94 °C for 3 min. These samples were stored at 4 °C for up to one week.

2.6.3 Protein concentration determination

Protein concentration was determined spectrophotometrically at a wavelength of 595 nm (OD_{595}) as described by Bradford (Bradford 1976). Samples containing known concentrations of bovine serum albumin (BSA) were used to generate a standard curve. Protein Assay Dye Concentrate (Bio-Rad, München, Germany) was used for determination.

2.6.4 SDS PAGE (polyacrylamide gel electrophoresis)

Proteins were separated depending on their molecular weight on continuous or gradient gels containing polyacrylamide (PA). If not stated otherwise, 50 µg of protein were mixed with sample buffer and incubated at 94 °C for 3 min. Gel run was carried out at 45-95 V in SDS-PAGE running buffer (196 mM glycine, 0.1% SDS, 50 mM Tris-HCl, pH 8.4). After electrophoresis the gels were subjected to western

blotting.

2.6.5 Western blotting

Standard western blotting procedure was used for immunodetection of proteins separated by SDS-PAGE. Transfer was carried out in standard buffer (25 mM Tris-HCl, 192 mM Glycine; pH 8.4) using a commercial transfer chamber (Bio-Rad, München, Germany). The transfer was carried out at 95 V for 2 h or at 20 V overnight. The efficiency of transfer was checked by staining with Ponceau S (Sigma-Aldrich, Seelze, Germany) staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) to visualize the protein bands.

The membrane was then incubated with blocking solution (133 mM NaCl, 1.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.08% Tween 20, 5% fetal calf serum, 5% dry milk powder; pH 7.4) for 1 h at RT. The primary antibody was applied in binding buffer (133 mM NaCl, 1.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.08% Tween 20, 5% dry milk powder; pH 7.4) at a dilution of 1:500-1:1000. Incubation with the primary antibody was carried out at 4 °C overnight on a rocking platform. Unspecifically bound antibodies were removed the next day by washing the membrane once 15 min in washing buffer I (133 mM NaCl, 1.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.08% Tween 20, 0.1%SDS, 1% NP-40; pH 7.4) and twice 15 min in washingbuffer II (133 mM NaCl, 1.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.08% Tween 20; pH 7.4). The membrane was then incubated with peroxidase-conjugated secondary antibody at a dilution of 1:1500 in binding buffer at room temperature for 1 h. After washing once for 15 min with washing buffer I and twice 15 min with washing buffer II, the membrane was incubated with detection solution (Super Signal West PicoStable Peroxide/Luminol Enhancer solution or Super Signal West FemtoStable Peroxide/Luminol Enhancer solution, Pierce/Perbio Science, Bonn, Germany). Bands were detected using a CCD-camera (Fujifilm LAS-1000/ Intelligent Dark Box, Fujifilm, Düsseldorf, Germany).

2.6.6 ELISA - Enzyme-linked immunosorbent assay

For detection of $A\beta_{40}$ and $A\beta_{42}$ ELISA was applied. For detection of $A\beta_{40}$ the Human β Amyloid 1-40 Kit (BioSource, Karlsruhe, Germany) was used, for the detection of

$A\beta_{42}$ the Human β Amyloid 1-42 Kit (BioSource, Karlsruhe, Germany) was used. The ELISA was performed according to manufacturer's instructions.

2.7 Enrichment of subcellular compartments from brain

2.7.1 Enrichment of early endosmes and late endosomes from brain

Early endosomal (EE) and late endosomal (LE) fractions were prepared from post-nuclear supernatants (PNS). PNS were generated from mouse brain by homogenizing brains with an Ultra Turrax and clarifying homogenates by centrifugation (1000 xg; 10 min; 4 °C). The supernatants (PNS) were loaded on a sucrose gradient (from top to bottom): Homogenization buffer (HB; 8.6% sucrose), 25% sucrose solution, 35% sucrose solution and PNS (adjusted to 40.6% sucrose with 62% sucrose stock solution). Same volumes were loaded for all fractions and all steps were carried out on ice. Gradients were centrifuged (14000 xg; 1 h; 4 °C) using a SWTi40 rotor in a Beckman centrifuge (Beckman, Krefeld, Germany). The three interphases were collected with a syringe, whereby LE were enriched between HB and the 25% sucrose solution, EE between 25% and 35% sucrose solutions, and heavy membranes were enriched between 35% sucrose solution and PNS.

Solutions

-Homogenization buffer (HB): 250 mM sucrose; 3 mM imidazole; protease inhibitor cocktail (Complete inhibitor cocktail)
-25% sucrose (0.806 M); 3 mM imidazole, pH 7,4; 1 mM EDTA; aqua dest.
-35% sucrose (1.177 M); 3 mM imidazole, pH 7,4; 1 mM EDTA; aqua dest.
-62% sucrose (2.35 M); 3 mM imidazole, pH 7,4; 1 mM EDTA; aqua dest.

2.7.2 Enrichment of Golgi and endoplasmic reticulum compartments from brain

Mouse brains were homogenized with a Potter S homogenizer in solution A (10 strokes; 900 rpm; on ice). PNS was generated by centrifugation (1000 xg; 10 min; 4 °C). The PNS was loaded on a sucrose gradient (from top to bottom): PNS, then 2.7 ml 0.6 M sucrose solution, 3.4 ml 1 M sucrose solution, 3.4 ml 1.3 M sucrose

solution and 1 ml 2 M sucrose solution.
The sucrose gradients were centrifuged using a SWTi40 at 40000 rpm for 2.5 h at 4 °C. Afterwards, fractions were collected from top to bottom in 23 0.5 ml aliquots.

Solutions

-Solution A (20 mM Tris-HCl, 2 mM $MgCl_2$, 0.25 mM Sucrose, pH 7.5 including Complete and Halt inhibitor cocktails)
-Sucrose solutions were produced by dissolving relevant amounts of sucrose in solution A.

2.7.3 Enrichment of synaptosomes from brain

A minimum of five mouse brains was homogenized in 20 ml HB (10 mM HEPES, pH 7.4; 250 mM sucrose; including Complete inhibitor cocktail) using a Potter S (Sartorius AG, Göttingen, Germany) (10 strokes; 900 rpm; on ice). After centrifugation (1000 xg; 10 min; 4 °C) to generate supernatant 1 (S1), the supernatant was further processed by a second centrifugation (12000 xg; 15 min; 4 °C). S2 was removed and pellet 2 (P2) was washed in 15 ml HB and centrifuged at 13000 xg (15 min; 4 °C). P2' (1.5 ml) was now mixed with 13.5 ml aqua dest. (osmotic shock) and homogenized with the Potter S at maximum speed with 3 strokes. Afterwards, the solution was buffered by adding 75 µl HEPES (10 mM; pH 7.4) and centrifuged again at 21000 xg (20 min; 4 °C). Finally, the supernatant (LS1) was centrifuged at 100000 xg (2 h; 4 °C) and the pellet (LP2) resuspended in 1 ml HB through a 27 G syringe. The post synaptic density (PSD) was prepared from LP1 by dissolving the pellet in 3 ml HB with subsequent discontinuous sucrose gradient centrifugation (gradient from bottom: 1.2 M sucrose; 1 M sucrose; 0.8 M sucrose – each 3 ml). The gradient was centrifuged using a SWTi40 (85000 xg; 2 h; 4 °C). Postsynaptic membranes were enriched (PSM) between layer 1.2 M and 1 M sucrose. Approximately 1 ml PSM was collected and mixed with 7 ml HB and 8 ml HB / triton X-100 (0.5%), incubated on ice (15 min) and centrifuged again (34000 xg; 20 min; 4 °C). The pellet (PSD I) was resuspended in HB/Triton X-100 (1%) and centrifuged at 200000 xg (1 h; 4 °C). The final pellet (PSD II) was resuspended in 200µl of HB/Triton X-100 (1%).

2.8 Messenger RNA expression analysis

2.8.1 Isolation of total RNA from primary neurons

Total RNA was isolated with TRIZOL reagent (Invitrogen, Karlsruhe, Germany). Cells were scraped off in 0,5 ml of TRIZOL reagent per well of a 6-well plate and incubated for 5 min at RT. 0.2 ml of chloroform were added to the sample per 1 ml of TRIZOL reagent. Samples were shaken by hand at RT for 5 min. The phases were separated by centrifugation (12000 xg; 10 min; 4 °C). Following centrifugation, the upper aqueous phase was transferred to a fresh tube and precipitated by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL. The RNA was collected by centrifugation (12000 xg, 10 min, 4 °C) and washed once with 1 ml of 85% ethanol per 1 ml TRIZOL. The pellet was air-dried for 5 min, dissolved in RNase-free water, and stored at -80 °C.

2.8.2 DNA and RNA concentration determination

The concentration of DNA and RNA samples was determined spectrophotometrically at a wavelength of 260 nm (OD_{260}) since the concentration of DNA and RNA is a direct function of the optical density at this wavelength. For DNA, an OD_{260} of 1.0 equals a concentration of 50 µg/ml of double stranded DNA. For RNA an OD_{260} of 1.0 equals a concentration of 40 µg/ml of RNA. DNA quality measurement was performed by measuring the OD_{280}. Pure DNA solutions have an OD_{260}:OD_{280} ratio of 1.8. Lower ratios indicated contamination of the sample with proteins.

2.8.3 DNase treatment, purification and quality control of samples

100 µg RNA were mixed with 20 µl 10x buffer, 2 µl DNAse (2 U/µl Ambion/Applied Biosystems, Darmstadt, Germany), filled to a final volume of 200 µl with aqua dest., and incubated for 20 min at 37 °C. Afterwards, the RNA was purified using the RNEASY-MINI-KIT (Qiagen, Hilden, Germany) according to manufacturer's instructions and RNA content was measured. Quality control of RNA was carried out by electrophoresis on 1.2% formaldehyde gels or by the RNA 6000 Nano Chip Kit (Agilent Technologies, Böblingen, Germany). RNA was stored at -80°C until use.

2.8.4 Reverse transcription

Generation of cDNA from RNA was done using the Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Following components were added to a nuclease-free microcentrifuge tube:

Oligo (dT) 12-18 (500 µg/ml)	1 µl
1 ng to 5 µg total RNA	x µl
1 µl dNTP Mix (10 mM each)	1 µl
Sterile, aqua dest.	to 12 µl

Sample was heated to 65 °C for 5 min and quickly chilled on ice. The content of the tube was collected by brief centrifugation and 4 µl 5x First-Strand Buffer and 2 µl 0.1 M DTT were added, mixed gently, and incubated at 42 °C for 2 min. Afterwards 1 µl (200 U) of SuperScriptTM II RT was added. The content was mixed by gently pipetting up and down and incubated at 42 °C for 50 min. Inactivation of the reaction was carried out by heating to 70 °C for 15 min. The cDNA was now used as a template for amplification by PCR.

2.8.5 TaqMan real-time PCR

TaqMan real-time PCR is one type of quantitative PCR methods. TaqMan uses a fluorogenic probe, which is a single stranded oligonucleotide of 20-26 nucleotides and is designed to bind to the DNA sequence between the two PCR primers. Therefore, only specific PCR products produce fluorescent signal. The RNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end produces fluorescence as soon as the close proximity of the reporter to the quencher is diminished by breakdown of the probe by the 5' to 3' exonuclease activity of the taq polymerase. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter. For TaqMan PCR, primers with a preferred product size of 50-150 bp were designed and a probe with the fluorophore 6-carboxyfluorescein (FAM) and the quencher tetramethylrhodamine (TAMRA) were used. The ABI Prism 7000 Sequence Detection System was used as real-time PCR machine. Samples were measured in triplicates and calculations were carried out using the ABI Prism 7000 SDS software (version 1.2). The TaqMan PCR reaction was carried out as follows.

Reaction mix:

5 µl cDNA

10 µl qPCR MastermixPlus 2x (Eurogentec, Germany)

1 µl primer mix 20x (including probe) (Invitrogen, Karlsruhe, Germany)

0.8 µl BSA (10 µg/µl; Roche, Mannheim, Germany)

3.2 µl water

PCR program:

50 °C 2 min
95 °C 10 min
(40x)
95 °C 15 sec
60 °C 1 min

Primer sequences:

GAPDH mouse 154f: GGC-AAA-TTC-AAC-GGC-ACA-GT

GAPDH mouse 223r: AGA-TGG-TGA-TGG-GCT-TCC-C

GAPDH probe: **FAM**-AAG-GCC-GAG-AAT-GGG-AAG-CTT-GTC-ATC-**TAMRA**

SORTILIN mouse 1376f: GCC-TTC-ATA-TCC-ATG-CTT-CTT-ATA-GC

SORTILIN mouse 1478r: CTA-CCG-TGA-GCG-ATG-ACT-ATG-C

SORTILIN probe: **FAM**-TCT-CCC-AGA-AGC-TAA-ACG-TTC-CAA-TGG-C-**TAMRA**

SORLA mouse 2391F: TGA-ACG-CAA-CTG-CTT-GTA-TTG-G

SORLA mouse 2492R: CCA-GGC-CGG-AAT-TGA-TGA-T

SORLA probe: **FAM**-CCA-GCG-TCT-CTG-CTT-GAA-CGG-GAG-**TAMRA**

2.9 Genotyping of animals

2.9.1 Isolation of genomic DNA from tissue samples

For isolation of genomic DNA, tail tips of mice were used. DNA was isolated by incubating the tissue with Protease K in tail buffer (10 mM Tris-HCl, 0.3 M Na-Acetate, 0.1 mM EDTA, 1% SDS (w/v); pH 7.0) at a final concentration of 0.5 mg/ml

overnight at 52 °C. Proteins were removed by extracting with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) followed by centrifugation (14000 xg; 5 min; RT) to separate the phases. The upper, aqueous DNA containing phase was mixed with 2.5 volumes of 100% ethanol. The precipitate was collected by centrifugation (14000 xg, 10min, 4°C), washed once with 75% ethanol, and dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). Purified DNA was stored at 4 °C.

2.9.2 Polymerase chain reaction (PCR)

If not stated otherwise, PCR was carried out using the following reaction mix.

Reaction mix:

1 µl (50 ng) of template DNA
16 µl water
2.5 µl 10x gitschier buffer (166 mM ammonium sulfate, 670 mM Tris-HCl, pH 8.8, 67 mM $MgCl_2$, 50 mM β-mercaptoethanol, 67µM EDTA)
2.5 µl DMSO (Roche, Mannheim, Germany)
1.25 µl dNTP-mix 10 mM (Roche, Mannheim, Germany)
1.25 µl 0.1% BSA (Roche, Mannheim, Germany)
0.2 µl forward primer (Eurogentec, Berlin, Germany)
0.2 µl reverse primer (Eurogentec, Berlin, Germany)
0.125 µl taq polymerase (Roche, Mannheim, Germany)

PCR program:

93 °C 3 min
59 °C 5 min
65 °C 5 min
(40x)
93 °C 30 sec
59 °C 1 min
65 °C 3 min

65 °C 10 min
(4 °C storage)

Primer sequences:

BPA: GAT-TGG-GAA-GAC-AAT-AGC-AGG-CAT-GC (SORLA KO)
AN15: ACC-GTG-ACA-TGA-CGG-CGG-CAC-ACC (SORLA KO/WT)
SORLA2: CTT-GCT-ATG-GAG-GAG-GAG-ATC (SORLA WT)
2010: ATC-TGG-CCC-TGG-GGA-AAA-AAG (PDAPP)
2011: GAT-GTC-CTT-CCT-CCT-CTG-TTC (PDAPP)
UM42: ATC-ACC-TGG-TTC-TAA-TCA-GAG-GCC-C (APP WT)
UM44: GAG-ACG-AGG-ACG-CTC-AGT-CCT-AGG-G (APP KO/WT)
P3-HYG: CGA-GAT-CAG-CAG-CCT-CTG-TTC-CAC-A (APP KO)

2.9.3 Agarose gel electrophoresis of DNA and RNA

PCR products were separated according to their molecular weight on 0.8% agarose gels. Ethidium bromide was added to the gel at a final concentration 0.5 µg/ml to visualize the DNA fragments after electrophoresis.

2.10 Gait analysis of mice

Analysis of the ambulatory gait of mice was quantified using the DigiGait Imaging System (Mouse Specifics, Inc., Boston, USA). This system enables mice to walk on a motorized transparent treadmill belt. Via a mirror underneath the belt a video camera captures images of the ventral side of the animals. DigiGait automatically pixelates and vectorizes the ventral view of the subject. The software analyzes the resulting digital images and defines the area of each paw corresponding to its movement to generate a set of periodic waveforms that describes the advance and retreat of the four limbs relative to the treadmill belt through consecutive strides. The software automatically identifies the portion of the paw that is in contact with the treadmill belt as the stance phase of the stride and the portions not in contact with the treadmill belt as the swing phase of the stride. Gait dynamics were determined by dissecting the time each limb is spent in various portions of the walking phase, including paw area, paw placement angle during stance, stride length, stepping frequency and stance width. In a pilot experiment the maximal speed that all animals were able to go was determined. In this study the treadmill speed was set at 20 cm/s, at which all of the mice were capable to go for at least 1 min.

2.11 Water maze analysis

Spatial learning and memory were examined in a water maze task with hidden platform, whereby the platform was placed 1 cm below the water surface and remained at fixed position. Water was colored opaque by white paint. Animals were trained on the platform four consecutive days with six trials per day and a 10 s interval between trials. The four starting points were varied daily. Swim path to the platfrom and latency were recorded by an automated video tracking system (San Diego Instruments, San Diego, USA). After a maximum of 40 s the animal was set on the platform, if it did not succeed in finding it. On the fifth day a probe trial was carried out with the platform removed. For the testing period of 60 s the swim path was recorded and later analyzed. On the sixth day four trials were carried out with parameters as before but visible platform.

3 Results

3.1 APP processing in *Sorl1*-deficient mice

Previously Andersen et al. (Andersen, Reiche et al. 2005) have reported the generation of a mouse model with targeted *Sorl1* gene disruption. Loss of receptor activity was shown to increase overall production of sAPP and Aβ, in line with an inverse correlation of receptor activity and APP processing rates in cultured cells. However, the molecular mechanism underlying accelerated APP processing in this mouse model, as well as its effect on neuronal activity and survival remained unanswered.

In the study presented here, the consequences of *Sorl1* deficiency for APP processing *in vivo* were investigated, particularly focussing on the hippocampus, a brain region that is especially vulnerable to AD-related disease processes and that expresses large amounts of SORLA.

When APP was detected in hippocampal extracts from *Sorl1*$^{-/-}$ mice using Western blot analysis, immunoreactive bands corresponding to the full length forms of APP were specifically reduced compared to control animals (Figure 6A, middle panel). Loss of APP protein was more pronounced for the fully glycosylated mature than the immature polypeptide variant. This decrease in mature APP mass in *Sorl1* deficient mice may have been missed in previous studies where the bands corresponding to mature and immature APPs, respectively, were not resolved by SDS-PAGE (Andersen, Reiche et al. 2005).

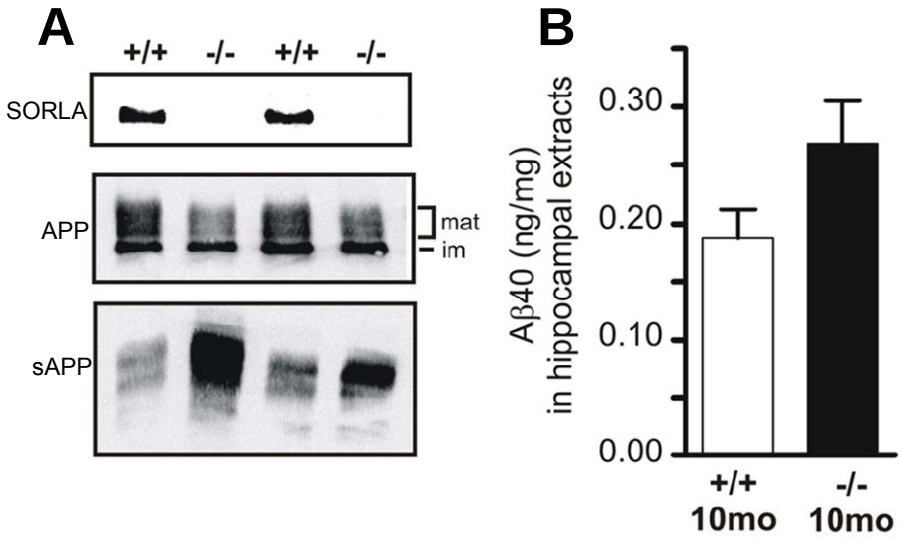


Figure 6: *Sorl1* deficiency accelerates APP processing in mice.

(A) Western blot analysis of hippocampal extracts from 6 months old wild type (+/+) and *Sorl1*-deficient mice (-/-) indicates absence of SORLA expression in knockouts (upper panel), accompanied by a reduction in mature APP (bracket; middle panel), and an increase in soluble (s) APP levels (lower panel). **(B)** Aβ quantification of hippocampal extracts (ELISA) of *Sorl1*$^{+/+}$ mice and *Sorl1*$^{-/-}$ mice (10 months of age)

The reduction of full length APP was likely caused by accelerated proteolytic processing as indicated by a concomitant increase in soluble APP products observed in hippocampal extracts of *Sorl1*$^{-/-}$ mice (Figure 6A, lower panel).

Similar findings were obtained in primary cultures of hippocampal neurons derived from *Sorl1*$^{-/-}$ mice where expression of SORLA can be seen in the perinuclear region of neurons derived from wild type but not from *Sorl1*-deficient newborn mice (Figure 7).

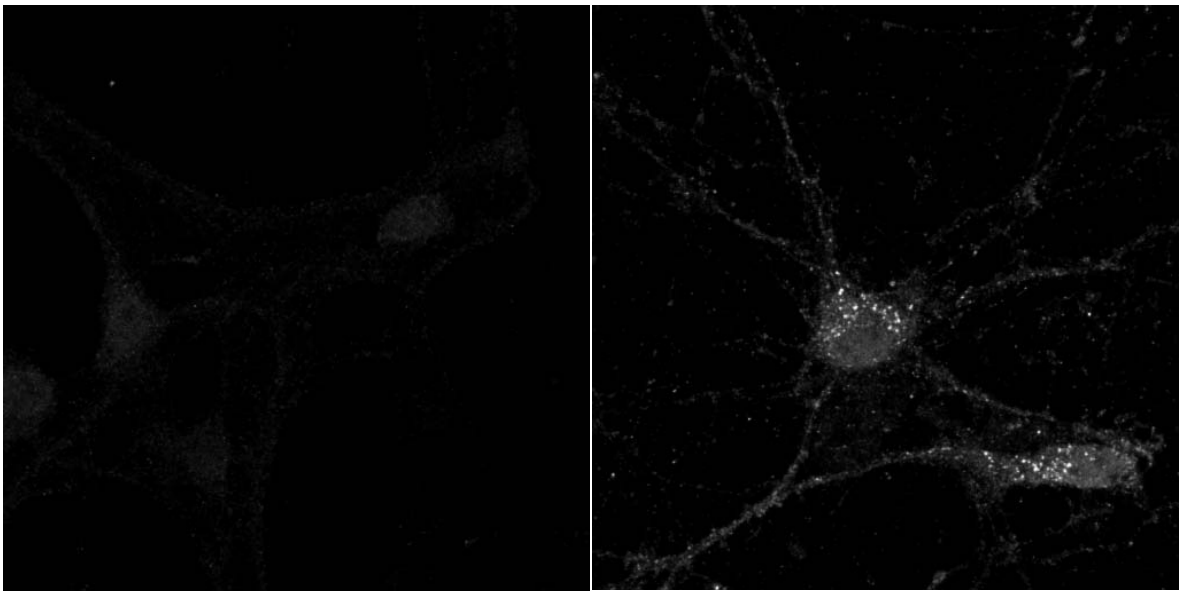


Figure 7: SORLA expression in hippocampal primary neurons

Shown are hippocampal primary neurons derived from *Sorl1*$^{+/+}$ and *Sorl1*$^{-/-}$ mice. The staining for SORLA shows a vesicular pattern in the perinuclear region in *Sorl1*$^{+/+}$ neurons, but no signal in *Sorl1*$^{-/-}$ neurons.

In cultured primary neurons, loss of SORLA receptor activity resulted in a distinct decrease of full length APP as well as a massive increase in soluble APP processing products (Figure 8A). Using densitometric scanning of Western blots exemplified in Figure 8A, the amount of mature APP was calculated to be reduced by approximately 70% whereas sAPP was increased by more than 50% (Figure 8B) as compared to wild type neurons.

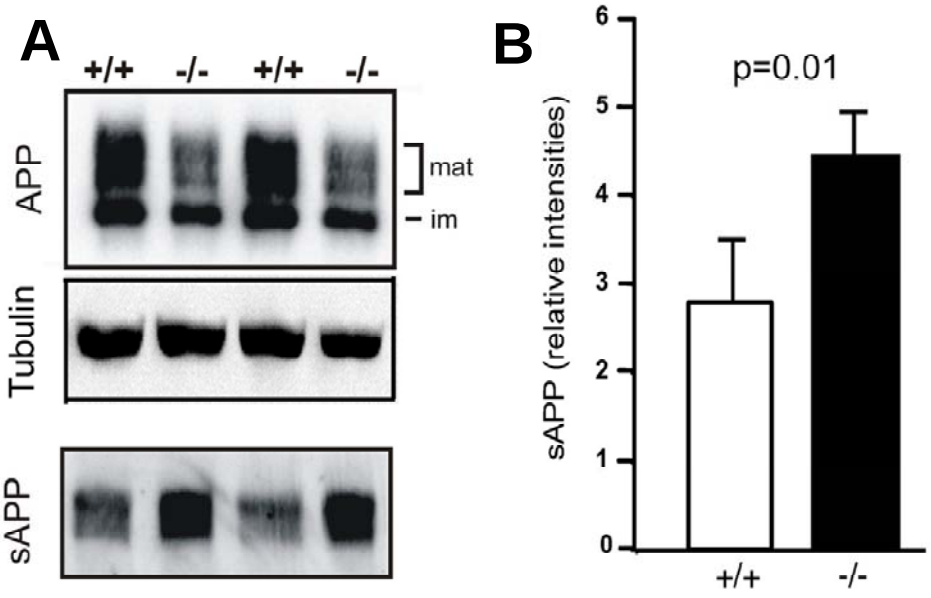


Figure 8: *Sorl1* deficiency accelerates APP processing in primary neurons.

(A) Reduction in mature APP (bracket; upper panel) but increase in sAPP (lower panel) levels in primary hippocampal neurons from *Sorl1*$^{-/-}$ compared with *Sorl1*$^{+/+}$ mice. Immunodetection of tubulin is shown as loading control (middle panel). **(B)** Quantification of sAPP levels in hippocampal neurons of the indicated *Sorl1* genotypes as determined by densitometric scanning of western blots exemplified in A (n=6 independent experiments).

Accelerated conversion of APP into its processing products was also confirmed in an independent mouse model of *Sorl1* deficiency generated by insertion of a β-galactosidase reporter gene into the murine *Sorl1* gene coding region (Figure 9) (sorEX255; kindly provided by W.C. Skarnes).

No changes in *App* gene transcription rates were detected by gene expression profiling comparing *Sorl1*$^{+/+}$ and *Sorl1*$^{-/-}$ deficient brain tissues.

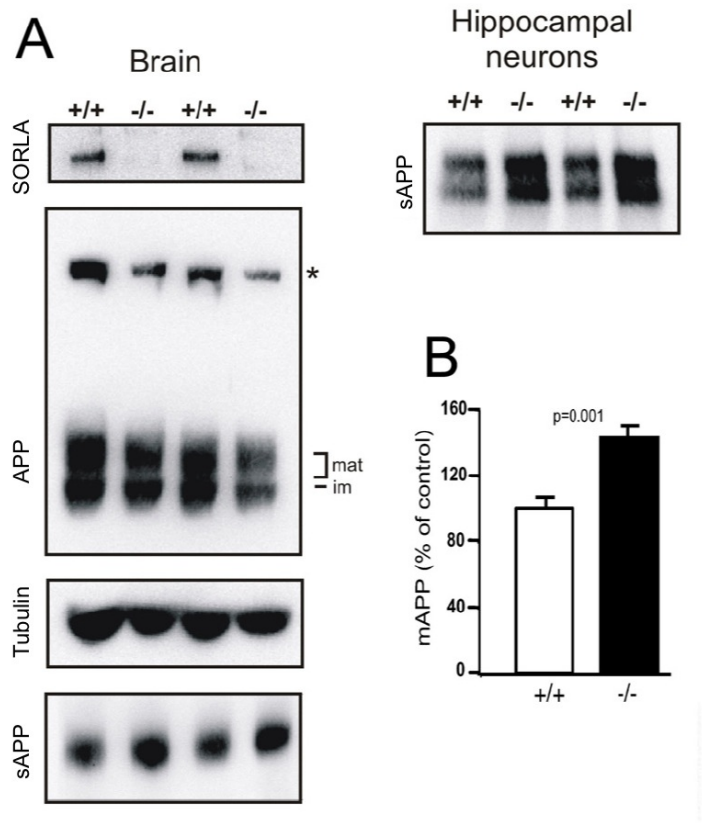


Figure 9: *Sorl1* deficiency enhances APP processing in the sorEX255 line of mice.

(**A**) Western blot analysis of total brain extracts (upper panel) from wild type (+/+) and *Sorl1*-deficient mice (-/-) of the sorEX255 line of mice indicates absence of SORLA expression in knockouts (panel SORLA), accompanied by a reduction in mature APP (bracket; panel APP) and an increase in soluble APP levels (panel sAPP). As loading control, the membranes were re-probed with an antibody directed against neuron-specific β-tubulin. The asterisk in panel APP indicates chondroitin sulfate proteoglycan-modified APP molecules that are also reduced in *Sorl1*$^{-/-}$ animals. A similar reduction in sAPP levels was seen in extracts of primary hippocampal neurons from sorEX255 mice compared to control animals (panel to the right). (**B**) Quantification of mature APP levels in primary hippocampal neurons of wild type (set at 100%) and *Sorl1*-deficient sorEX255 mice as determined by densitometric scanning of Western blots exemplified in A (n=4 independent experiments).

3.2 Amyloidogenic processes in *Sorl1*-deficient mice

To explore the consequences of insufficient SORLA activity for amyloidogenic processes and senile plaque formation, the *Sorl1* gene defect was introduced into the PDAPP line of mice that carries the human *App*^{V717F} transgene. This line represents a well-established mouse model of AD pathology (Games, Adams et al. 1995).

In hippocampal neurons from newborn mice doubly transgenic for human *App*^{V717F} and the *Sorl1 null* allele, levels of full length human APP were reduced (Figure 10A), but concentrations of human sAPPα (Figure 10A, B), sAPPβ (Figure 10A, C) as well as Aβ_{40} (Figure 10D) were significantly increased compared to PDAPP mice expressing SORLA.

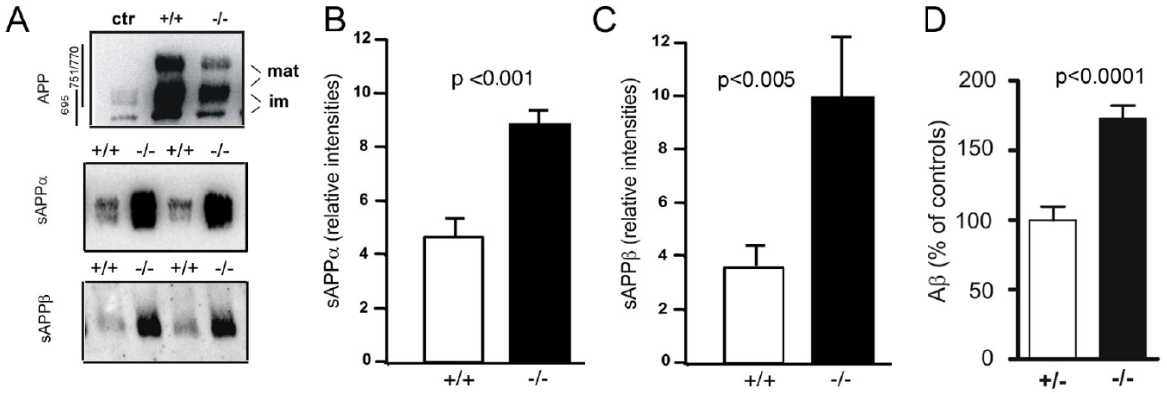


Figure 10: *Sorl1* deficiency accelerates APP processing PDAPP mice.

(**A**) Western blot analysis of hippocampal neurons from PDAPP mice either wild type (+/+) or genetically deficient (-/-) for *Sorl1* indicates decreased levels of mature human APP (mat; upper panel) but increased levels of sAPPα and sAPPβ (lower panels) in mice lacking the receptor. As control, a neuronal extract from a non-APP-transgenic mouse was evaluated in parallel (ctr) to indicate immunoreactive bands corresponding to endogenous murine APP (bracket to the left). Bands corresponding to mature and immature forms of human APP^{695} and to the larger APP variants equally expressed in PDAPP mice are indicated. (**B, C**) Quantification of sAPPα (IgG WO2) and sAPPβ (IgG JP18957) in primary hippocampal neurons of PDAPP mice with the indicated *Sorl1* genotypes as determined by densitometric scanning of western blots exemplified in A (n=5 independent experiments). (**D**) ELISA indicates a 73% increase in human Aβ_{40} levels in hippocampal neurons from PDAPP mice lacking *Sorl1* (-/-) compared to control animals heterozygous for the receptor gene defect (+/-; set at 100%) (n=11 in each genotype).

Taken together, deletion of SORLA equally affected processing of wild type and mutant APP in normal and PDAPP mice, respectively. In *Sorl1*$^{-/-}$ mice, decreased levels of full length APP as well as increased levels of APP processing products sAPP and Aβ were observed.

These findings provide *in vivo* confirmation of previous reports that demonstrated an inhibitory effect of SORLA activity on both α- and β-secretase activities in CHO

cells (Schmidt, Sporbert et al. 2007); an inhibitory activity lost in *Sorl1*-deficient neurons. In PDAPP mice, amyloid deposition starts around six to seven months of age and reaches appreciable levels in ten-months old mice (Johnson-Wood, Lee et al. 1997). In cooperation with the group of T. Bayer it was shown that the extent of human Aβ production in the PDAPP line was significantly accelerated when the *Sorl1* gene defect was introduced into this model (Figure 11A), resulting in a 3-fold increased plaque burden (Figure 11B). Enhanced plaque deposition was seen in all brain areas but was particularly prominent in the hippocampus (Figure 11C).

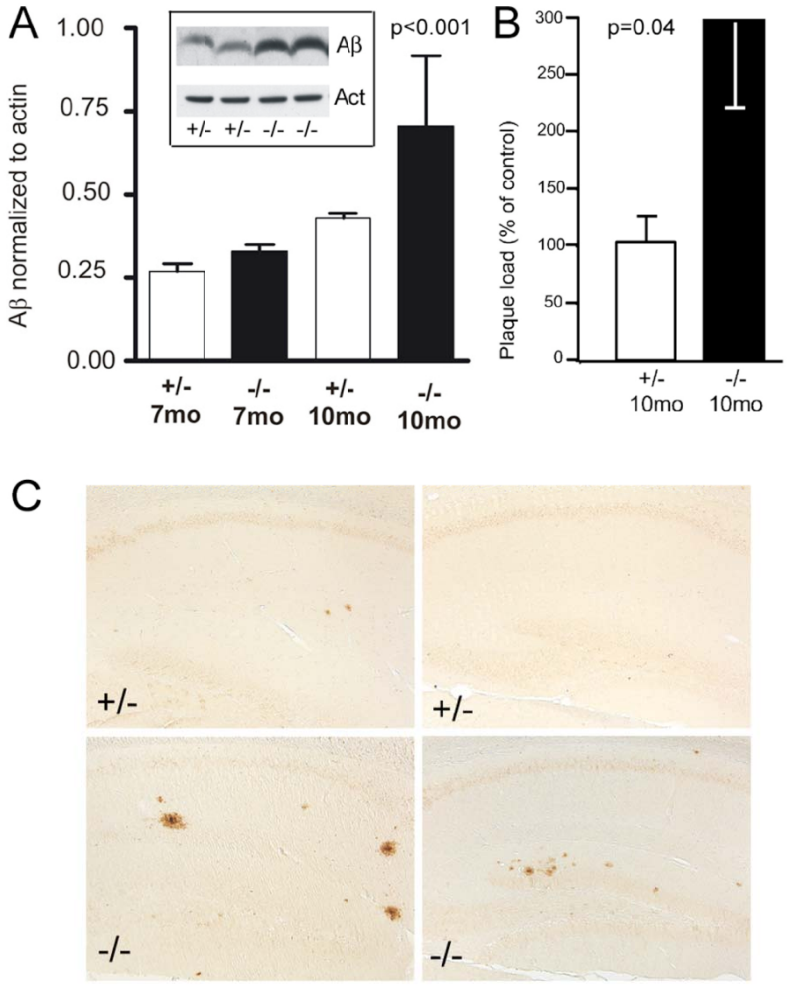


Figure 11: Increased plaque deposition in PDAPP mice lacking *Sorl1*

(**A**) The amount of insoluble Aβ extracted from brains of 7 and 10 months old PDAPP mice either +/- or -/- for *Sorl1* was determined by densitometric scanning of Western blots (inset) and depicted as relative Aβ levels normalized to actin (n=5-10 animals per genotype). (**B**) Amyloid plaque burden in 10 months old PDAPP mice either +/- (n=13) or -/- for *Sorl1* (n=9) was evaluated by immunostaining for human Aβ in the subiculum and hippocampus, and expressed as relative plaque load com-

pared to the control (set at 100%). (**C**) Immunostaining for plaques (IgG 4G8) in the hippocampal region of two wild type and two *Sorl1*-deficient mice.

Similar findings of increased plaque deposition were also obtained when the sorEX255 model of *Sorl1* deficiency was introduced into the PDAPP line (Figure 12). Again, an increase in plaque burden was observed.

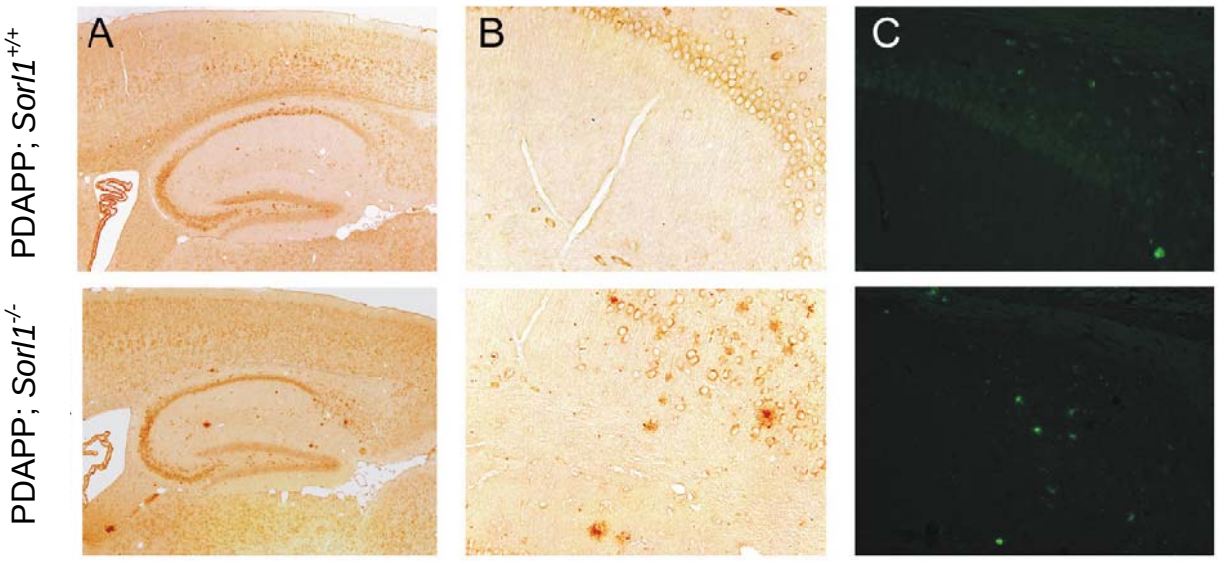


Figure 12: Amyloid plaque burden in *Sorl1*-deficient mice
Histological sections of hippocampus in PDAPP mice either wild type (+/+) or genetically deficient for *Sorl1* (-/-, sorEX255 line) are shown. The sections are stained for amyloid deposits using antiserum 4G8 (A, B) or thioflavin S (C).

Taken together, these data substantiated a role for SORLA in protection of APP from secretase processing *in vivo* and the significance of low receptor activity as a cause of enhanced precursor breakdown and senile plaque formation.

3.3 Neuroanatomy of *Sorl1*-deficient mice

As investigated by Nissl staining of histological brain sections, alterations in APP processing in *Sorl1*$^{-/-}$ animals did neither affect the overall anatomy of the hippocampus, nor other regions of the CNS such as cortex (Figure 13).

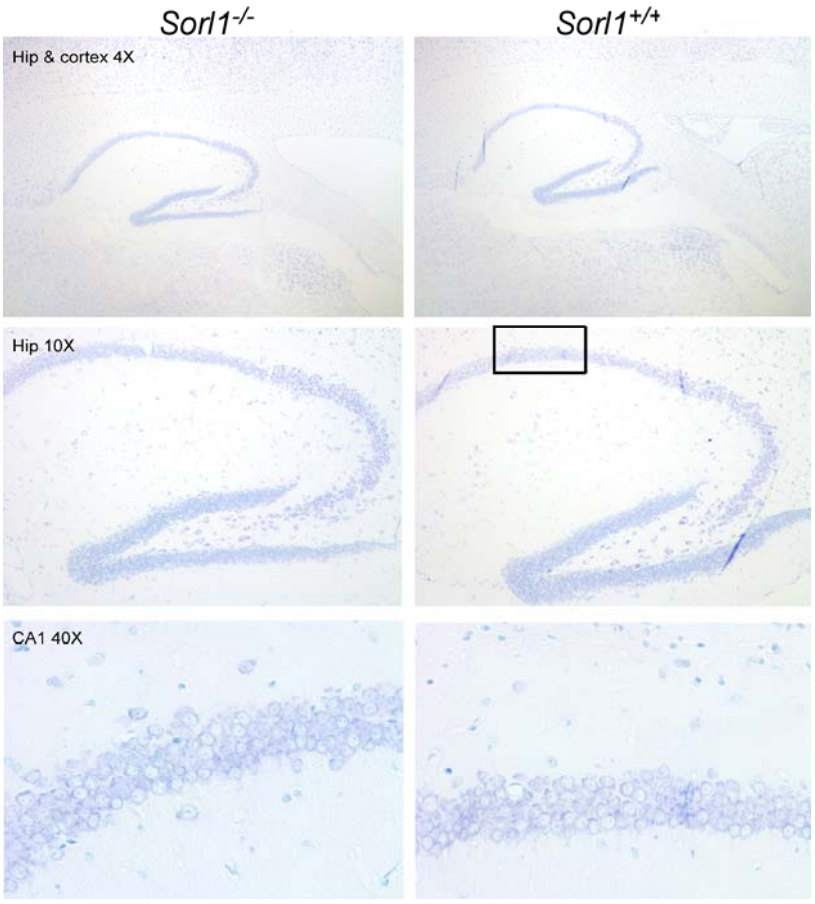


Figure 13: Histological analysis of *Sorl1*-deficient mice
Nissl-stained sections of hippocampus (Hip) and cortex of mice homozygous-deficient for Sorl1 or wild type.

Similar to other mouse models of AD, aggravated plaque load did also not significantly affect overall neuroanatomy in (PDAPP; *Sorl1*$^{-/-}$) mice as determined by immunohistology for markers of cholinergic neurons, activated microglia and astroglia (Figure 14).

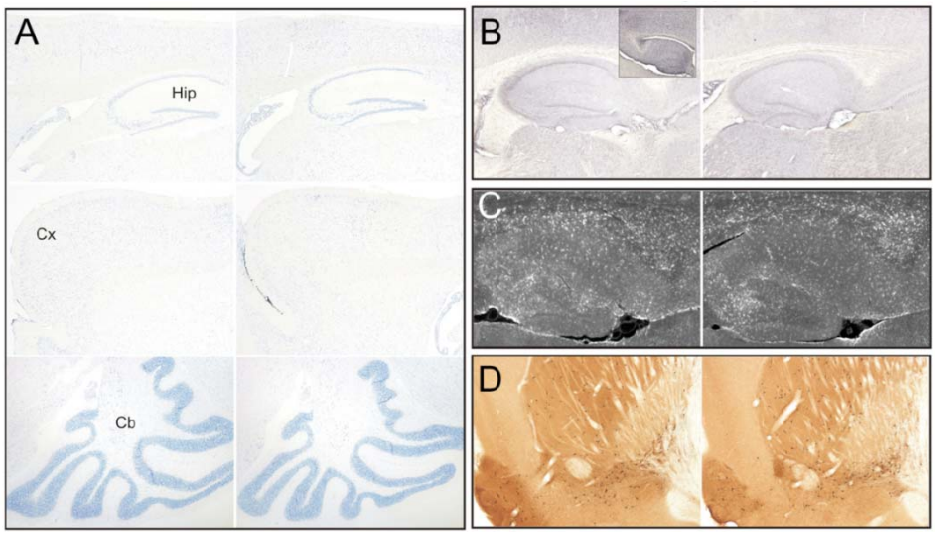


Figure 14: Histological analysis of PDAPPxSORLA mice
(A) Nissl-stained sections of hippocampus (Hip), cortex (Cx) and cerebellum (Cb) of PDAPP mice either heterozygous or homozygous-deficient for *Sorl1*. **(B-D)** Immunohistological detection of microglia in dentate gyrus (B; anti-F480), astrocytes in the hippocampus and corpus callosum (C; anti-GFAP) and cholinergic neurons in the basal forebrain and caudate putamen (D; anti-acetylcholine transferase) in PDAPP mice heterozygous or homozygous-deficient for *Sorl1*. The inset in B represents a control for microglia activation in *ClC7*-deficient mice.

3.4 APP-dependent signaling in *Sorl1*-deficient mice

While formation of neurotoxic Aβ oligomers and senile plaques is firmly established as cause of neuronal dysfunction and ultimate cell death in AD patients, it remains debated how soluble APP products influence normal neuronal function and how they may affect AD disease processes. In particular, sAPP was shown to act as a physiological signaling factor in MAP kinase pathways that are likely to be changed in the context of altered APP processing (Greenberg, Koo et al. 1994).

In this context, the consequences of increased sAPP levels in primary hippocampal neurons from wild type and *Sorl1*-deficient newborn mice were investigated. The focus laid on the activity of extracellular regulated kinase (ERK), a MAP kinase proposed to act downstream of sAPP in neuronal signaling processes (Wallace, Akar et al. 1997).

Intriguingly, a significant increase in the phosphorylated fraction of ERK was detected in *Sorl1*$^{-/-}$ hippocampal neurons compared to controls, while the absolute levels of the kinase remained unchanged (Figure 15).

To test the specificity of this effect, levels of total and phosphorylated forms of AKT (protein kinase B), a kinase activated downstream of sAPPα in neurons (Cheng, Yu et al. 2002) and epithelial cells (Wehner, Siemes et al. 2004) were evaluated. No changes in phospho-AKT levels were detected for this alternative sAPP signaling pathway (Figure 20B).

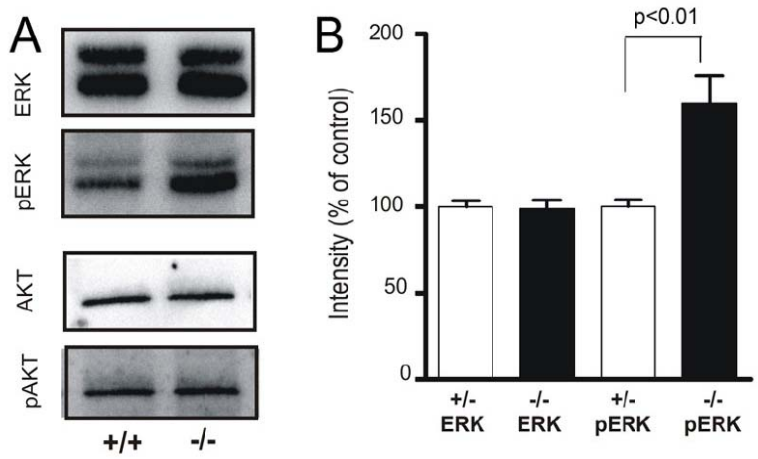


Figure 15: Analysis of mitogen activated protein kinase activation in wild type and *Sorl1*-deficient mice
(A) Western blot analysis of primary hippocampal neurons demonstrates specific increase in phosphorylated ERK (pERK) but not total ERK in mice lacking *Sorl1* (-/-) compared with control animals (+/+; set at 100%). In contrast, levels of AKT and phosphorylated AKT (pAKT) are unchanged. **(B)** Quantification of ERK and pERK levels by densitometric scanning of Western blots (n=6) exemplified in panel A.

Selective modulation of ERK signaling in *Sorl1*$^{-/-}$ neurons was confirmed by global profiling of major neuronal signaling pathways in hippocampal extracts using the Kinetworks™ PhosphoSite neurobiology Screen (KPSS-9.0; Kinexus). This test identified a 44% increase of ERK1 and a 102% increase of ERK2 phosphorylation in *Sorl1*-deficient hippocampi compared to wild type controls. Activity levels of other kinases, such as glycogen synthase-serine kinase 3a, stress-activated protein kinase, c-jun amino terminal kinase, as well as protein kinase C isoforms λ, ε and γ were not altered in this screen.

3.5 Adult hippocampal neurogenesis in *Sorl1*-deficient mice

ERK-dependent signaling pathways are implicated in the stimulation of adult hippocampal neurogenesis (Rueda, Navarro et al. 2002; Persson, Thorlin et al. 2003). To investigate the consequences of elevated sAPP levels and enhanced ERK signaling in the *Sorl1*-deficient brain, hippocampal precursor cell proliferation and survival was determinedin mice 24 hours and 4 weeks after BrdU injection, respectively (Figure 16).

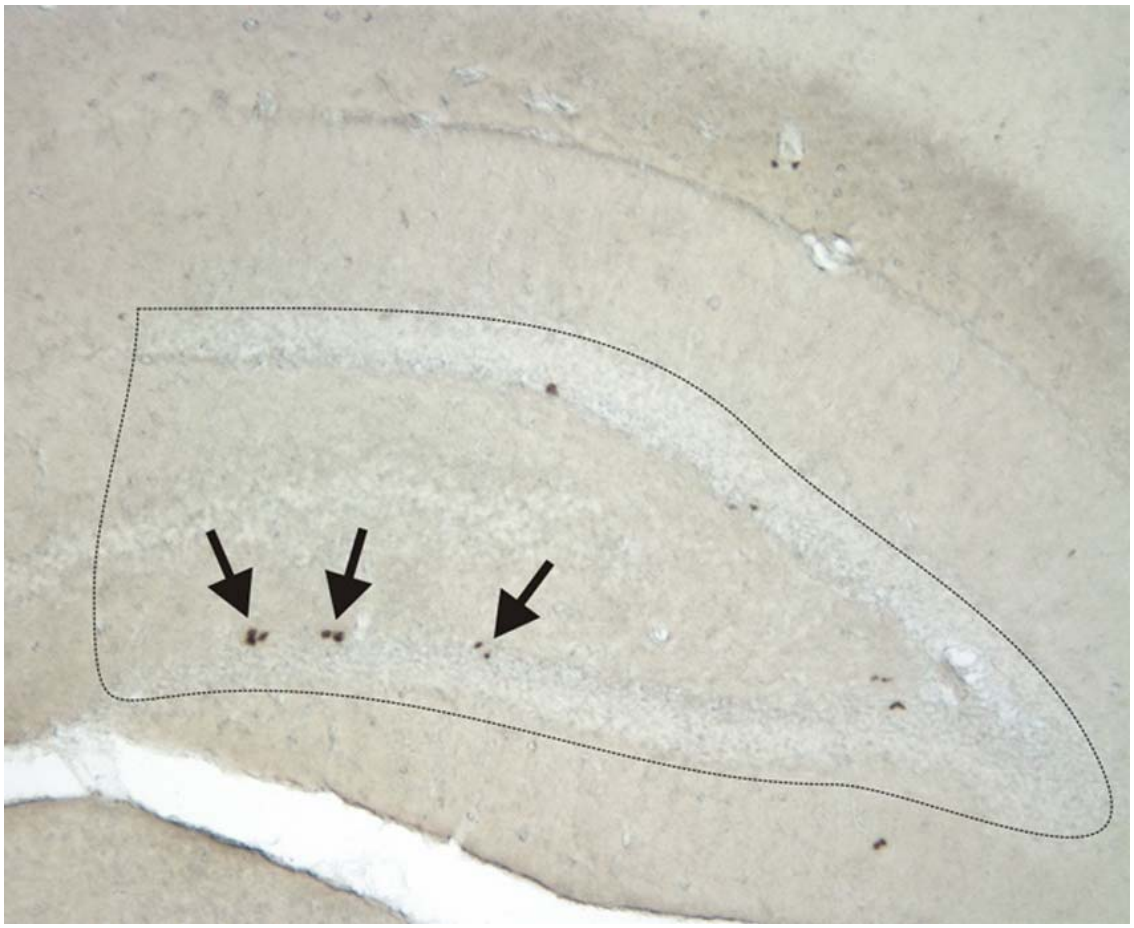


Figure 16: Determination of adult neurogenesis in hippocampus using BrdU incorporation

Example of BrdU positive cells in the dentate gyrus (circled area) of a *Sorl1*$^{-/-}$ mouse used for quantification of neuronal survival in the hippocampus.

Consistent with the observed enhancement of ERK activity, a ~3-fold increase in the number of proliferating cells (Figure 17A) and a ~2-fold increase in the number

of surviving, BrdU-positive cells (Figure 17C) was detected in the dentate gyrus of five-month old *Sorl1*$^{-/-}$ mice compared to control littermates. Increased survival of newborn cells was even seen at 14 months of age, although overall extent of neurogenesis was reduced in both genotypes in an age-related manner (Figure 17C).

Immunofluorescence microscopy for BrdU as well as the neuronal markers doublecortin (DCX) and neuron-specific nuclear protein (NeuN) confirmed that approximately 50% of all proliferating cells detected after 24 hours were newborn immature neurons (positive for DCX) (Figure17B) and more than 90% of surviving cells after four weeks were post-mitotic mature neurons (positive for NeuN) (Figure 17D). No discernable difference in the percent distribution between neuronal and non-neuronal (GFAP positive) cell populations was seen between the *Sorl1* genotypes, indicating a general proliferative effect and no alteration in differentiation.

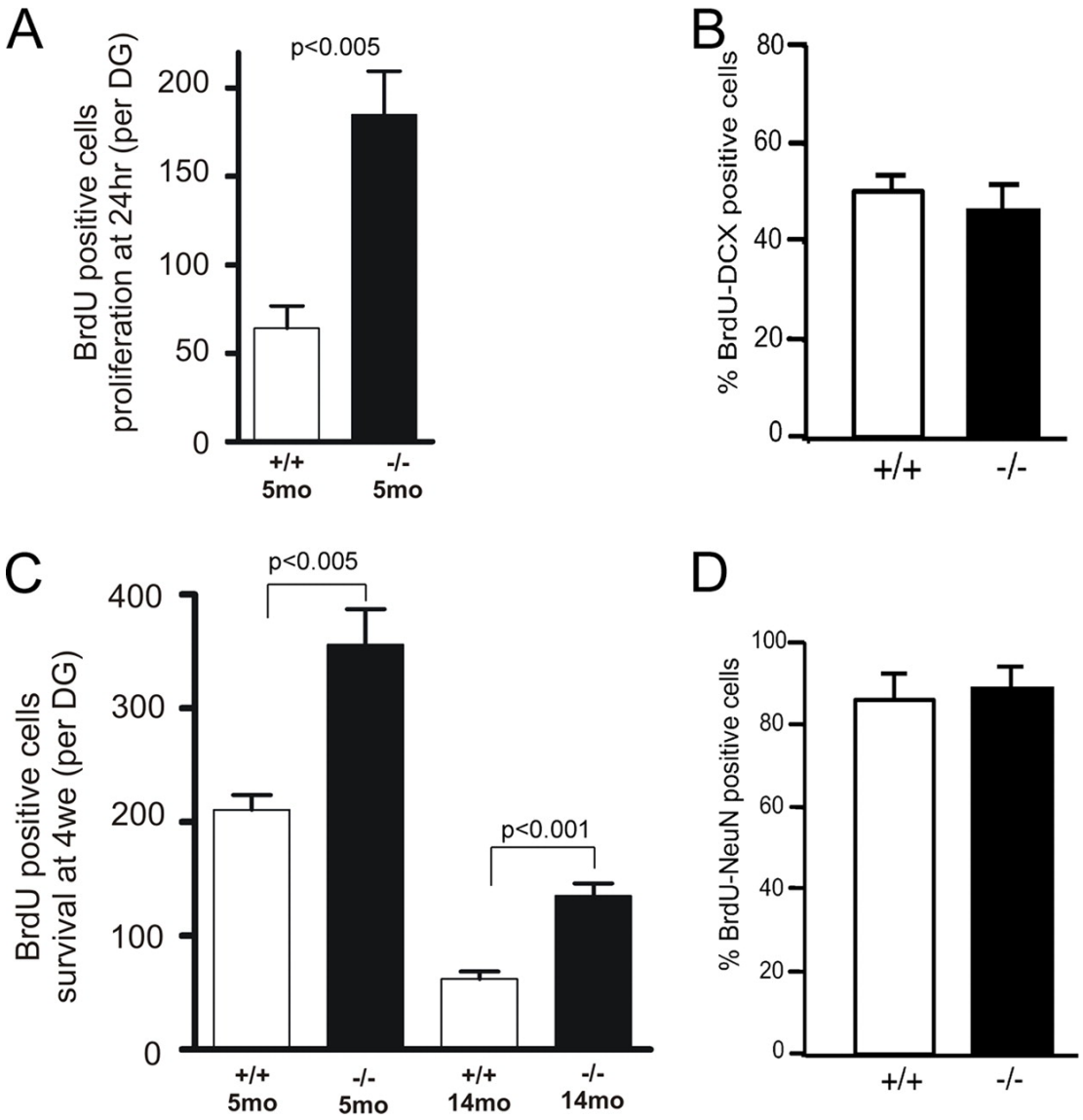


Figure 17: Adult neurogenesis in wild type and *Sorl1*-deficient mice
(A) Quantification of BrdU positive cells in the dentate gyrus 24 hours after BrdU incorporation indicates increased cell proliferation in 5 month-old *Sorl1*-deficient animals compared with wild type controls (n = 6 per genotype). Data are given as total number of BrdU stained cells per dentate gyrus (DG). **(B)** Quantification of cells positive for BrdU and doublecortin (DCX) 24 hours after BrdU incorporation as determined by immunofluorescence microscopy **(C)** Higher numbers of surviving BrdU positive cells were demonstrated 4 weeks after BrdU injection into 5 and 14 month-old mice genetically deficient for *Sorl1* (-/-) compared with controls (+/+). Data indicate total number of positive cells per dentate gyrus (DG). n=6 per genotype. **(D)** Quantification of cells positive for BrdU and NeuN 4 weeks after BrdU incorporation as determined by immunofluorescence microscopy. Data represent the mean ± SEM. Statistical significance was determined by Student's t-test.

In figure 18A, an example (overview of dentate gyrus) of a triple labeling for BrdU, immature neurons (DCX) and glia (GFAP) from a *Sorl1*$^{-/-}$ mouse out of the prolifera-

tion experiment is depicted. Figure 18B exemplifies cells doubly positive for DCX and BrdU as used for the quantification in Figure 17B.

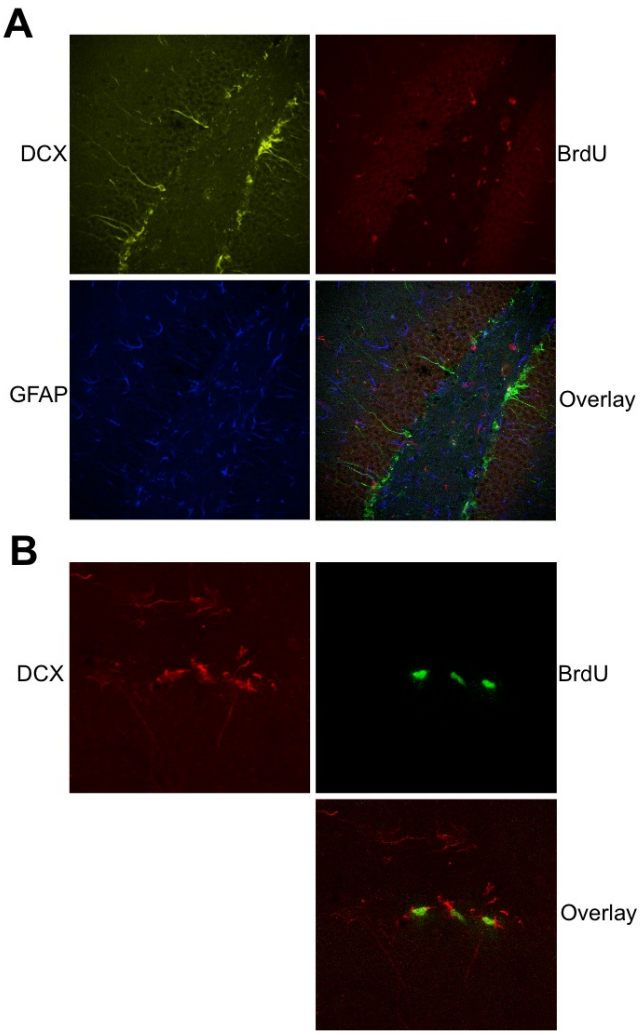


Figure 18: Cells doubly positive for BrdU and DCX
(A) Example of BrdU, DCX and GFAP positive cells in dentate gyrus of a *Sorl1*$^{-/-}$ mouse. **(B)** Cells double positive for DCX and BrdU in dentate gyrus of a *Sorl1*$^{-/-}$ mouse

3.6 Apoptosis in *Sorl1*-deficient mice

Enhanced neurogenesis in *Sorl1*$^{-/-}$ animals was not accompanied by increased neuronal cell death as tested by TUNEL on hippocampal sections (Figure 19A) and Western blot for activated caspase 3 in primary hippocampal neurons (Figure

19B).Therefore, differences found for adult neurogenesis in *Sorl1*$^{-/-}$ animals were not secondary effects due to increased cell loss.

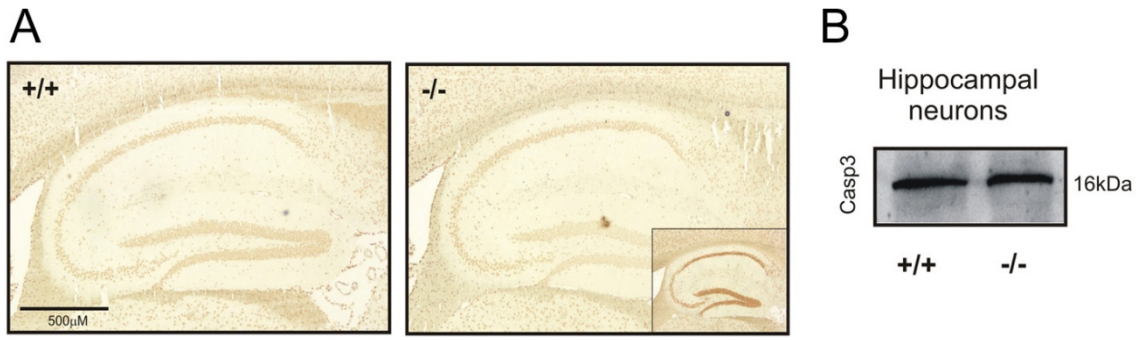


Figure 19: Analysis of cell death in wild type and *Sorl1*-deficient mice
No alteration in cell death is seen in the brain of *Sorl1*-deficient mice compared with controls using TUNEL (A) or Western blot analysis for activated Caspase 3 (B). The inset in A indicates a section treated with DNase to control for accuracy of the assay.

3.7 APP-dependence of hippocampal ERK signaling and adult neurogenesis

One might consider that, stimulation of ERK signaling and enhancement of neurogenesis in *Sorl1*$^{-/-}$ animals may not be linked to altered APP processing, but caused by lack of a yet unknown SORLA activity unrelated to APP metabolism. To exclude this possibility, the receptor gene defect was introduced into an *App*-deficient mouse line (Muller, Cristina et al. 1994) and consequences for neuronal signaling and proliferation were investigated in this new animal model.

In the newly established mouse model, no APP processing products, such as sAPP, were detected (Figure 20A, inset). Furthermore, neuronal survival 4 weeks after BrdU injection was identical in *App*-deficient mice with or without the *Sorl1* gene defect (Figure 20A). In hippocampal extracts, a specific increase in ERK phosphorylation (pERK) was only seen in (*App*$^{+/+}$; *Sorl1*$^{-/-}$) animals (Figure 20B, lane 2), but not in lines that lacked APP expression (Figure 20B, lanes 3 and 4). Similarly, no elevated pERK levels were detected when primary hippocampal neurons from (*App*$^{-/-}$; *Sorl1*$^{-/-}$) were compared to those from (*App*$^{-/-}$; *Sorl1*$^{+/+}$) newborns (Figure 20B, lanes 5 and 6). Thus, activation of the ERK signaling pathway and enhance-

ment of neurogenesis in mice lacking SORLA is dependent on the presence of APP and/or its processing products.

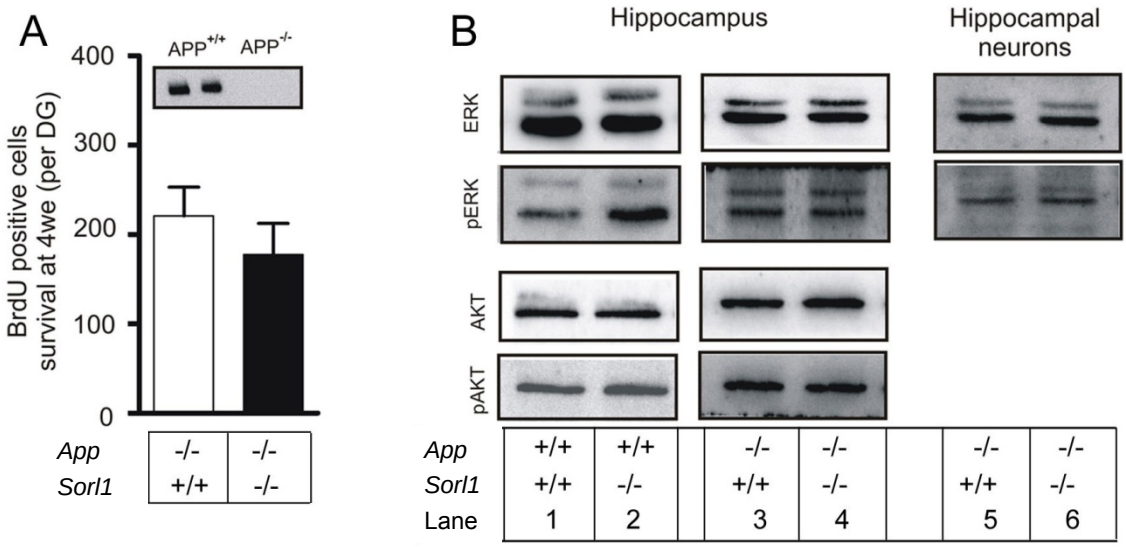


Figure 20: APP-dependent neurogenesis and ERK phosphorylation in mice
(A) Quantification of neuronal survival given as total number of BrdU positive cells in the dentate gyrus four weeks after BrdU injection in *App*-deficient and (*App; Sorl1*)-doubly deficient animals at 5 months of age. The inset depicts Western blot analysis for sAPP in brain extracts of two wild type and two *App*-deficient mice. **(B)** Immunodetection of ERK, phosphorylated ERK (pERK), AKT and phosphorylated AKT (pAKT) in extracts from hippocampus (left panel) or hippocampal primary neurons (right panel) of the indicated *App* and *Sorl1* genotypes. Increase in pERK signal intensity is only seen in mice wild type for *App* but lacking *Sorl1* (lane 2), but not in animals wild type for both proteins (lane 1), deficient for *App* (lanes 3 and 5) or deficient for both (lanes 4 and 6).

Since sAPPα but not Aß caused a concentration-dependent increase in phosphorylation of ERK when applied to primary neuronal cultures, the APP processing product in question is likely to be soluble APP and not Aβ. (Figure 21). Moreover, these findings are in agreement with previous reports, demonstrating stimulation of neurogenesis by sAPPα (Kaltschmidt, Uherek et al. 1997; Lou, Yano et al. 2001).

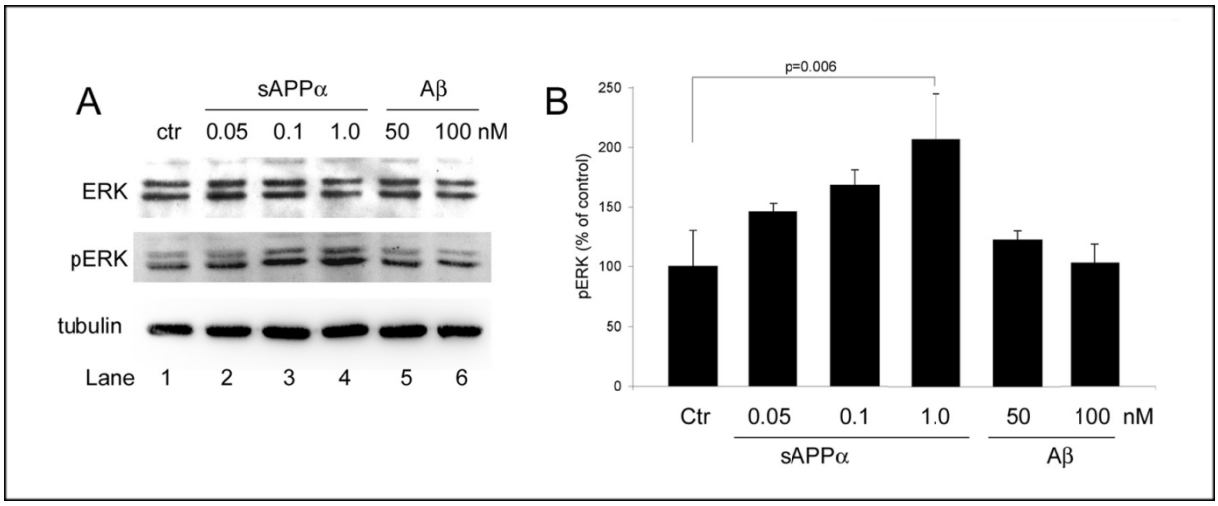


Figure 21: Stimulation of ERK phosphorylation in primary neurons
(A) Primary neuronal cultures of newborn wild type mice were treated with the indicated concentrations of recombinant sAPP (lanes 5 and 6) for 2 days. Thereafter, the amount of total ERK and pERK was determined by Western blot analysis of total cell lysates and compared with cells treated with medium only (ctr, lane 1). Immunodetection of tubulin is shown as a loading control. **(B)** Quantification of pERK levels by densitometric scanning of Western blots (n=2–5) as exemplified in A. Signal intensities are given as % of control (set at 100%).

Taken together, SORLA activity was demonstrated to contribute to neuronal processing of APP *in vivo*; an activity that not only bares relevance for amyloidogenic processing and senile plaque formation, but may also be crucial for controling ERK signaling pathways regulating adult neurogenesis.

3.8 APP localisation in *Sorl1*-deficient mice

Enrichment of Golgi and ER compartments from mouse brain by sucrose gradient centrifugation revealed no obvious difference in APP localisation between *Sorl1*$^{+/+}$ mice and *Sorl1*$^{-/-}$ mice (Figure 22). The experiment was carried out according to Chen et al. (Chen, Yang et al. 2000). In this protocol, fractions 6-9 correspond to Golgi compartmetns, while fractions 13-16 represent smooth ER (sER) and fractions 19-21 rough ER (rER).

In *Sorl1*$^{+/+}$ mice, SORLA as well as APP were especially enriched in Golgi (fractions 7-8) and sER (fractions 13-14). APP localisation was identical in *Sorl1*$^{-/-}$ and *Sorl1*$^{+/+}$ mice. Thus, within the resolution of this method, no alterations in APP localisation could be observed as a consequence of *Sorl1* deficiency. To improve the spatial

resolution, immuno-electronmicroscopy was applied (in cooperation with Bettina Erdmann), focussing on hippocampal neurons, instead of entire brain lysates. This approach seemed sensible based on the high SORLA expression in the hippocampus. The stainings were carried out in (PDAPP; *Sorl1*$^{+/-}$) in comparison to (PDAPP; *Sorl1*$^{-/-}$) mice to have appreciable amounts of APP immunoreactivity for countings.

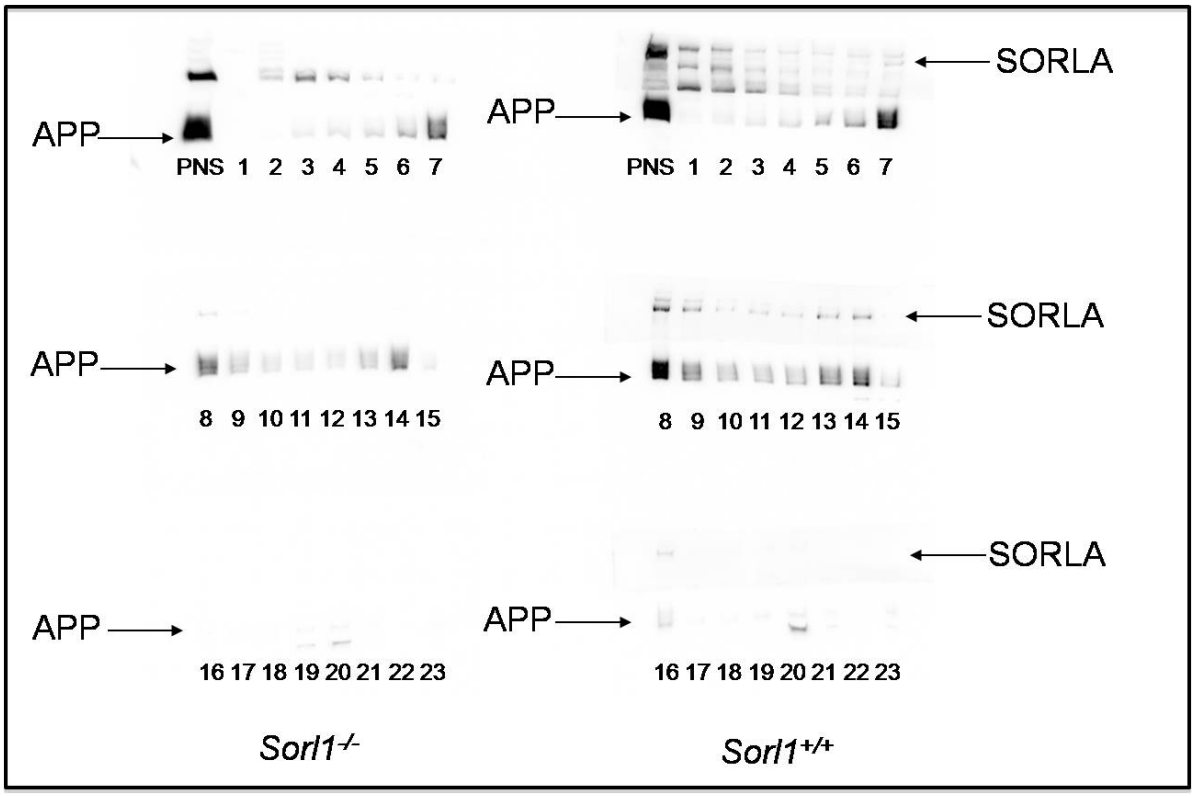


Figure 22: Subcellular fractionation of Golgi and ER membranes from total brain lysates of *Sorl1*$^{-/-}$ and *Sorl1*$^{+/+}$ mice

Total brain lysates of both genotypes have been separated by discontinuous sucrose gradient centrifugation. Fractions 1 to 23 were blotted on 3 membranes for each genotype. All Western blots were developed together for comparison. Membranes of *Sorl1*$^{+/+}$ mice were cut in two pieces to separately incubate them with anti-SORLA and anti-APP (1227) antisera. Membranes of *Sorl1*$^{-/-}$ were incubated with anti-APP only.

Consistent with a proposed role for SORLA in retention of APP in the Golgi (Andersen, Reiche et al. 2005; Schmidt, Sporbert et al. 2007), the total amount of precursor molecules specifically in this organelle was reduced in hippocampal neurons from (PDAPP; *Sorl1*$^{-/-}$) mice compared with (PDAPP; *Sorl1*$^{+/-}$) littermates as shown by immuno-electronmicroscopy (Figure 23A, B). The specificity of the antibody

1227 was confirmed using Western blotting of *App*-deficient brain extracts (Figure 23C).

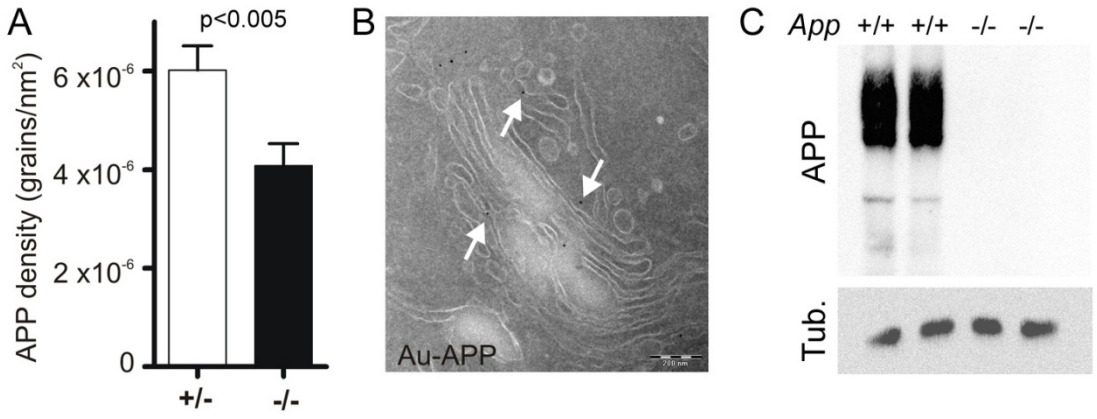


Figure 23: Reduced number of APP molecules in the Golgi of *Sorl1*-deficient neurons
(A) Quantification of APP immunoreactivity in the Golgi region of pyramidal neurons in PDAPP mice of the indicated *Sorl1* genotypes using immuno-electronmicroscopy (IgG1227). **(B)** Micrograph shows one example of a total of 33 Golgi fields from each genotype that were used to determine the number of gold grains (arrows) corresponding to APP per nm^2 Golgi area in A. **(C)** Western blot analysis of APP and tubulin (loading control) in brain extracts from two $App^{+/+}$ and two $App^{-/-}$ mice to document specificity of the anti-APP antiserum 1227 used in B.

In addition, enrichment of early endosomal and late endosomal fractions from total brains of $Sorl1^{+/+}$ and $Sorl1^{-/-}$ mice by discontinuous sucrose gradient centrifugation revealed the trend of increased APP localisation in early endosomal compartments (Figure 24). Only a minor portion of APP was found in late endosomal compartments.

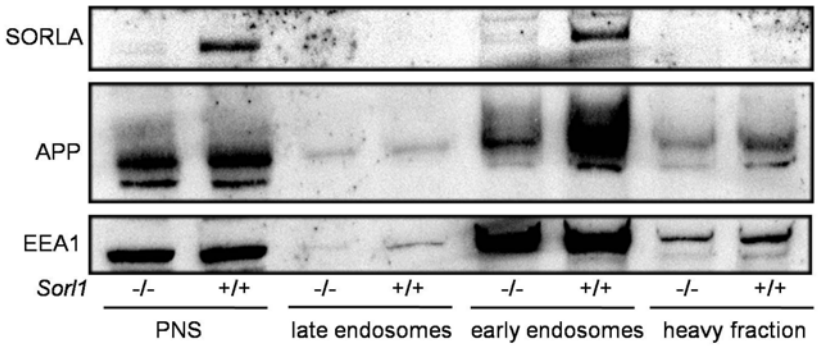


Figure 24: Enrichment of endosomal compartments from total brain lysates

Post nuclear supernatants (PNS) of total brain from *Sorl1*$^{-/-}$ and *Sorl1*$^{+/+}$ animals were separated into late endosomal compartment, early endosomal compartment and the remaining heavy fraction by discontinuous sucrose gradient centrifugation. Fractions were probed for SORLA, APP (1227) and EEA1 (early endosome-associated protein). EEA1 served as a marker for early endosomes and also indicated equal loading for PNS and early endosomes between genotypes. Comparing early endosomes of *Sorl1*$^{-/-}$ and *Sorl1*$^{+/+}$ tissues, an increase in the amount of APP in this compartment was found in *Sorl1*$^{+/+}$ animals.

By enrichment of synaptosomes from brain of *Sorl1*-deficient mice and control littermates, it was also seen that a small portion of mature APP was increased in post synaptic density (PSDII) fractions of *Sorl1*$^{+/+}$ mice compared to *Sorl1*$^{-/-}$ mice (Figure 25), indicating a function for SORLA in trafficking of APP to synaptic compartments. Moreover, enrichment of APP in synaptic vesicles (LP2) is seen in both genotypes, consistent with a role of APP in axonal transport (Simons, Ikonen et al. 1995).

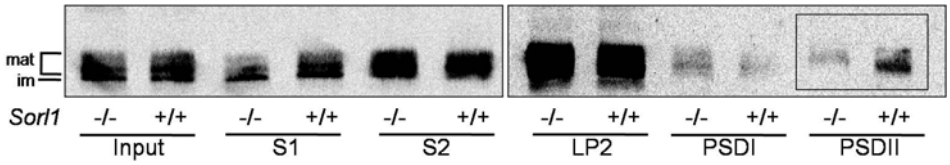


Figure 25: Enrichment of synaptosomes from total brain lysates

Synaptosomes from *Sorl1*$^{-/-}$ and *Sorl1*$^{+/+}$ animals were enriched and separated into synaptic vesicles (LP2) and a final postsynaptic fraction (PSDII). Fractions were probed for APP (1227). Enrichment for APP is seen comparing input to supernatant 2 (S2). Most APP is found in LP2, while a small portion of mature APP is found at the postsynaptic density (PSD). In PSDII an increase of this mature isoform is seen in *Sorl1*$^{+/+}$ animals. This trend was confirmed in 3 independent experiments.

Taken together, effects of SORLA on APP localisation *in vivo* substantiated the role for SORLA as factor retaining APP in early endosomal and Golgi (or trans-Golgi network - TGN) compartments. The degree of SORLA expression in TGN was

evaluated by double immunofluorescence microscopy for SORLA and a marker for TGN (γ-Adaptin) in hippocampal primary neurons, where a partial overlap of both proteins could be seen (Figure 26).

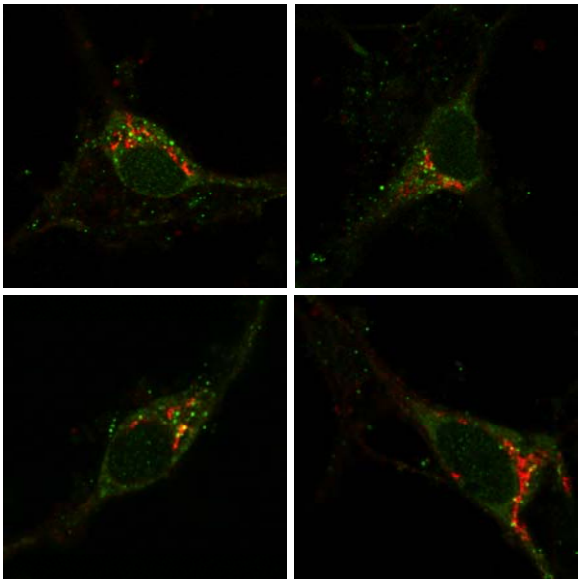


Figure 26: Hippocampal primary neurons showing partial colocalisation of SORLA and γ-Adaptin (TGN marker)

Shown are four representative hippocampal neurons stained for SORLA (green) and γ-Adaptin (red).

3.9 Hippocampal long term potentiation in *Sorl1*-deficient mice

To test whether increased Aβ levels or reduced levels of mature APP located to the postsynaptic density of *Sorl1*-deficient animals may impair neuronal activity, basic-synaptic transmission and long-term potentiation (LTP) in the CA1 area of the hippocampus, were determined in cooperation with the group of D. Schmitz. In agreement with previous reports (Hsia, Masliah et al. 1999), the presence of the App^{V717F} transgene per se resulted in impairment in synaptic transmission as indicated by a decrease in the slope of input-output curves when comparing $Sorl1^{+/-}$ (Figure 27A, open circles) with PDAPP, $Sorl1^{+/-}$ (open squares; $p<0.05$) animals. In contrast, LTP was not altered by expression of human APP (comparing data for $Sorl1^{+/-}$ animals in Figure 27B with PDAPP, $Sorl1^{+/-}$ mice in Figure 27C). This situation was not altered by the presence or absence of SORLA - neither on the murine APP (Figure 27B; comparing $Sorl1^{+/-}$ and $Sorl1^{-/-}$ animals) nor on the human PDAPP background (Figure 27C; comparing *PDAPP*, $Sorl1^{+/-}$ and *PDAPP*, $Sorl1^{-/-}$ animals).

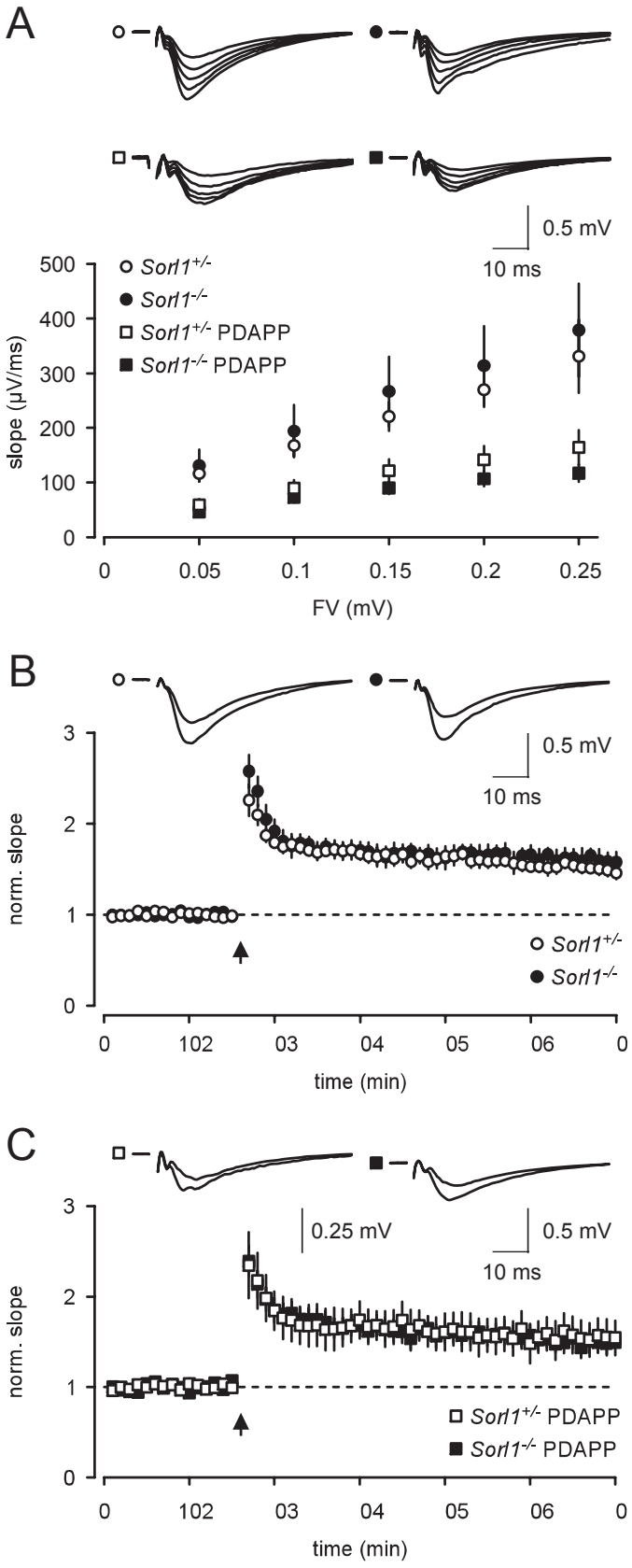


Figure 27: Electrophysiological analysis of *Sorl1*-deficient mice
Input-output curves for basal synaptic transmission (A) and long-term potentiation (BandC) in area CA1 of the hippocampus of mice of the indicated genotypes are shown (age: 10 –12 months, n6 –11 for each genotype). The presence of the hu-

man *App* transgene causes a decrease in the slope of the input-output curves both in PDAPP, *Sorl1*$^{+/-}$ (open squares, p=0.05) and PDAPP, *Sorl1*$^{-/-}$ (closed squares, p=0.001) compared with respective controls (A). Statistical analysis was performed by one-way analysis of variance followed by Tukey' s multiple comparison test. No change in LTP is seen in the presence of the *Sorl1* gene defect on the background of murine (B) and human *App* (C).

3.10 Morris water maze analysis of *Sorl1*-deficient mice

To evaluate the consequence of *Sorl1* deficiency on cognitive performance, *Sorl1*$^{-/-}$ animals were subjected to a spatial learning task, the Morris water maze. Using littermates from heterozygous breeding, a simple learning task of hidden platform acquisition was performed with equal efficiency in *Sorl1*$^{-/-}$ and *Sorl1*$^{+/-}$ genotypes as shown by determination of distance to platform and escape latency time recorded on the first 3 days of the test (Figure 28). Surprisingly, a significant difference was observed in the reversal situation on day 4 and 5, when the hidden platform was moved to the new target quadrant. Under this condition, *Sorl1*$^{-/-}$ animals seemed to perform better than their control littermates with reduced distance to platform and escape latency parameters, as well as less time spent in the old target and more time spent in the new quadrant (Figure 28).

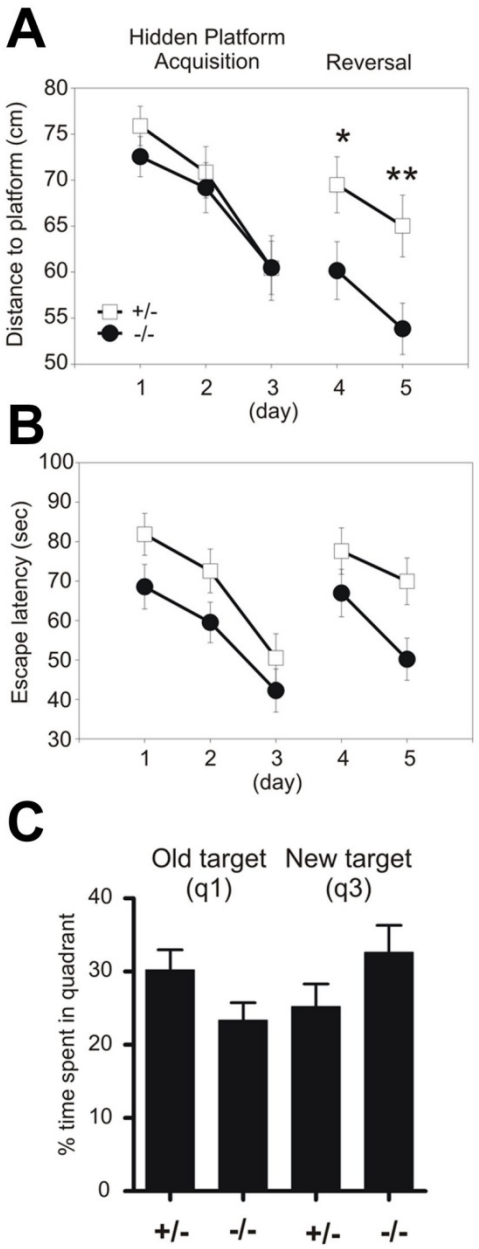


Figure 28: Behavioral analysis of *Sorl1*-deficient mice

Evaluation of learning and memory in male littermates heterozygous (+/-) or homozygous (-/-) deficient for *Sorl1* using Morris water maze test. Data of distance traveled to reach the hidden platform (A), escape latency time (B) and time spent in the indicated quadrants (C) at the probe trials on day 4 are given.

3.11 Gait analysis of *Sorl1*-deficient mice

Because of the unique cerebellar expression pattern of SORLA which is restricted to Purkinje cells in this brain region, it was speculated that lack of SORLA could result in neuromotoric defects. To test this hypothesis, the gait of *Sorl1*-deficient mice and control littermates was analyzed using the DigiGait Imaging System (Mouse Specifics, Inc.). In this test, parameters such as the stance time (the weight-bearing portion of the stride in which the paw remains in contact with the belt) or the running speedwere evaluated.

No differences were observed between *Sorl1*$^{+/+}$ and *Sorl1*$^{-/-}$ mice (Figures 29 and 30), suggesting that no discernible SORLA-dependent differences in the way of walking exist. Since both genotypes also exhibited equal running speed, it was concluded that the physical performance of *Sorl1*$^{-/-}$ mice is not affected. This finding serves as an additional proof that differences found in the water maze task, were not due to altered physical conditions between both genotypes.

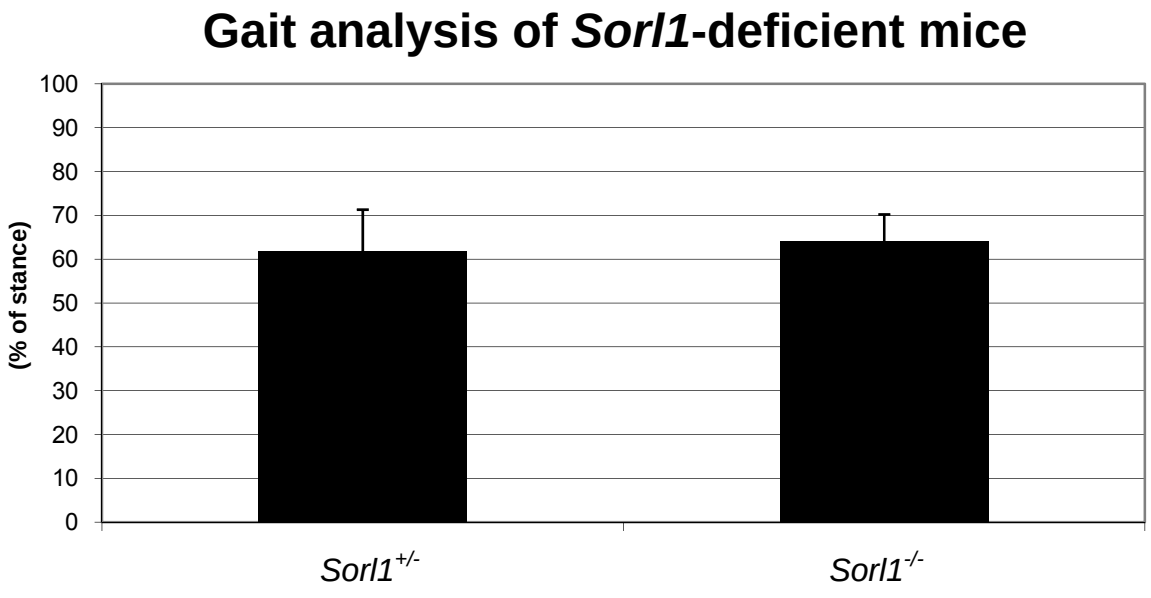


Figure 29: Gait analysis of *Sorl1*$^{+/-}$ and *Sorl1*$^{-/-}$ mice

No difference was seen for percent of stance in gait for both genotypes (shown here, or any other feature monitored). Mice were trained in the DigiGait Imaging System for 3 days. Recordings used for gait analysis were produced on day 4.

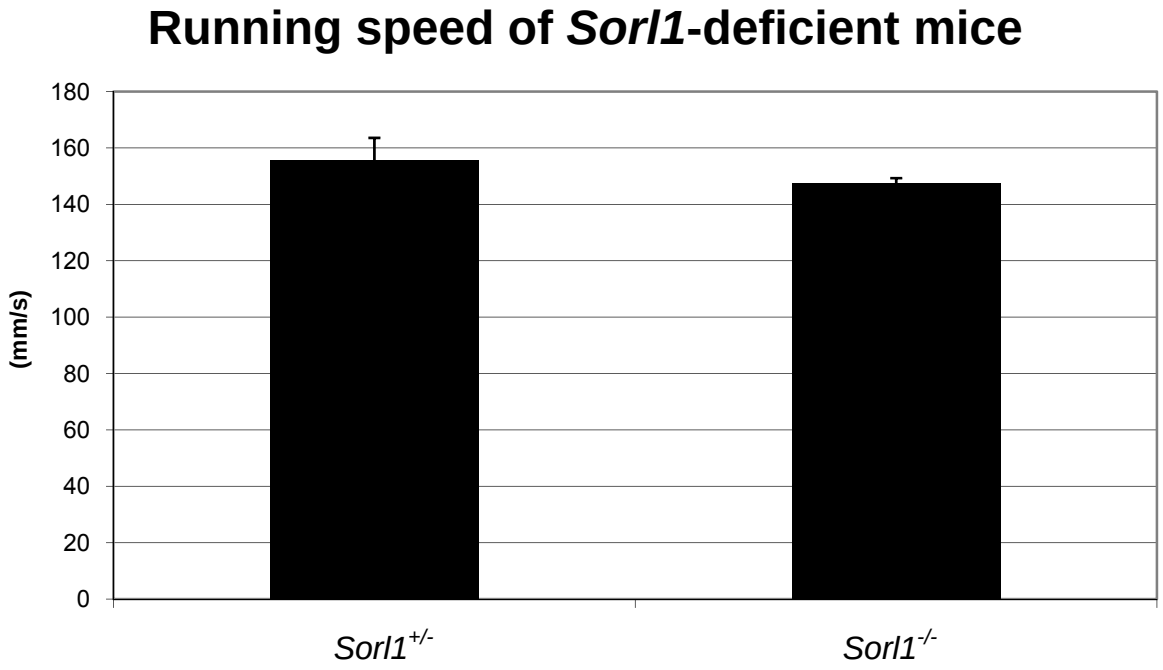


Figure 30: Physical performance of *Sorl1*-deficient mice

Shown is the running speed of mice heterozygous deficient for *Sorl1* or homozygous deficient for *Sorl1*. No difference were seen between genotypes.

3.12 SORLA expression inducing compounds

In order to proof the concept that raising the expression of SORLA could be a therapeutic approach to prevent neurotoxic Aβ production in AD, cytokines were tested whether they induce SORLA expression in primary neurons. Brain derived neurotrophic factor (BDNF), connective tissue growth factor (CTGF), cilliary neurotrophic factor (CNTF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) were applied to hippocampal as well as cortical primary neurons from wild type mice (129Bl6 or Balb/c) with concentrations ranging from 50-150 ng/ml.

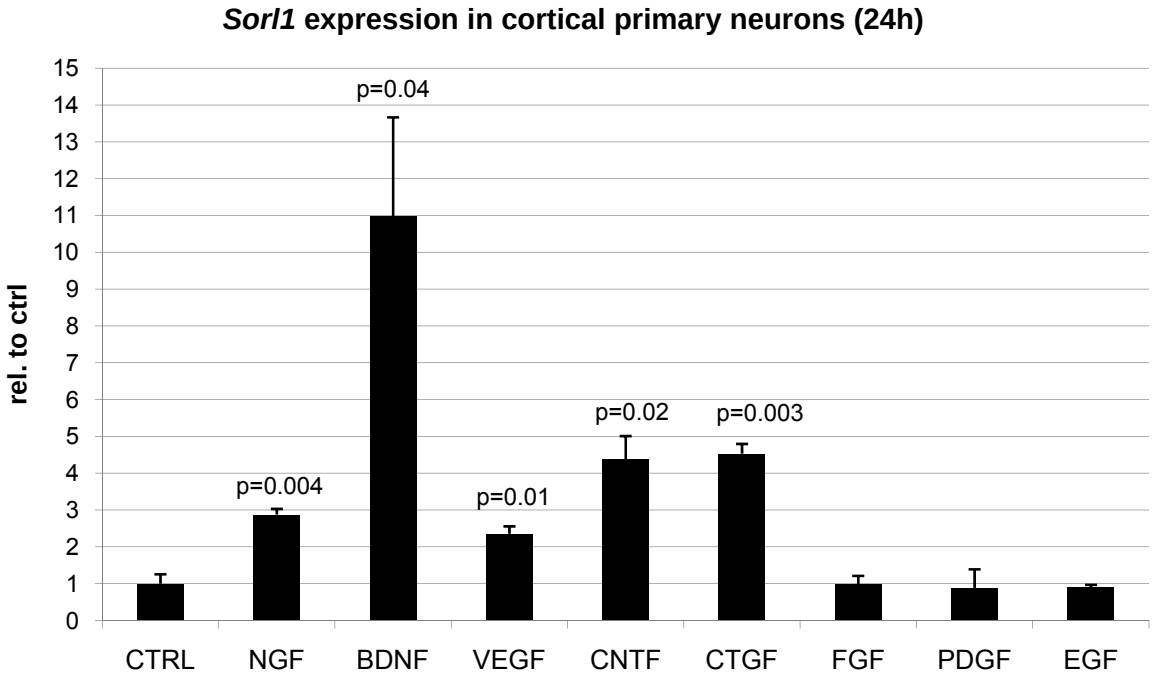
Among the cytokines tested, all compounds known to have a neurotrophic function were found to induce SORLA expression with different intensity as seen in Western blot (Figure 31D) as well as in TaqMan analysis (Figure 31A, C). This observation suggested that SORLA may represent a beneficial factor for neurons. However,

only a few neurotrophins were shown to strongly induce SORLA expression. The strongest effect on *Sorl1* expression was found for BDNF followed by CTGF, CNTF, VEGF and NGF. EGF, PDGF or FGF did not induce *Sorl1* expression (Figure 31A, C).

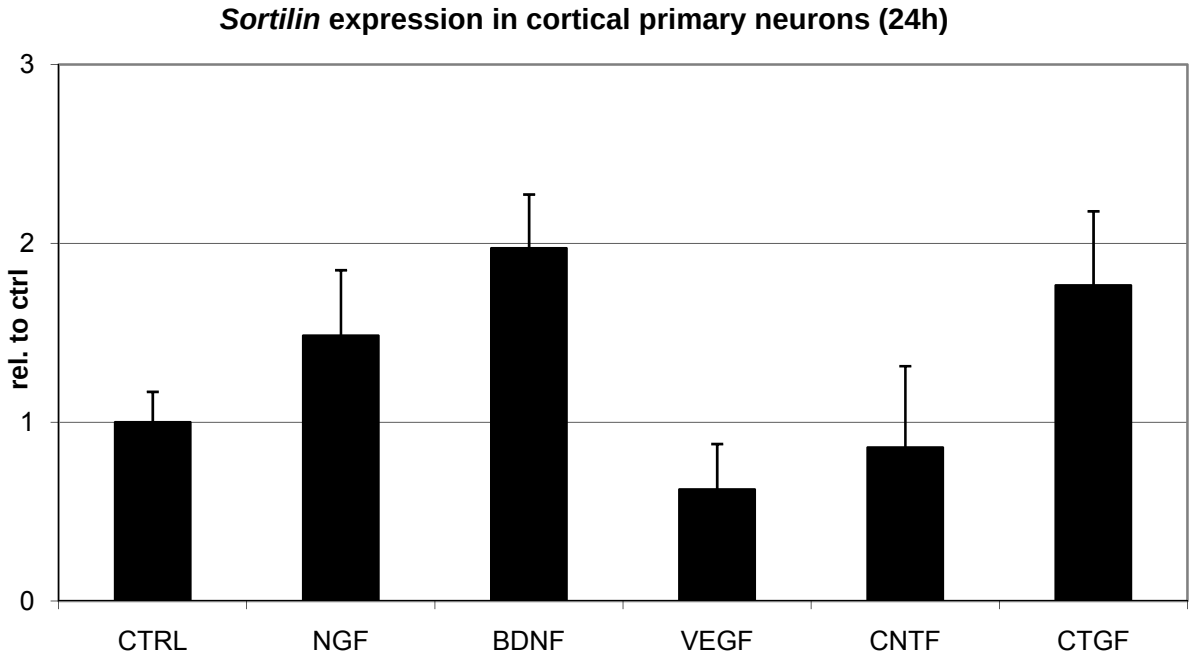
To test the specificity of this effect, BDNF, CTGF, CNTF, VEGF and NGF were also tested for induction of *Sortilin*, another member of the *Vps10* receptor gene family. No induction of *Sortilin* was detected in these experiments (Figure 31B), suggesting a specific effect of the tested factors on *Sorl1*.

At the level of transcription, the strongest induction was seen when primary neurons were incubated with neurotrophins (150 ng/ml) for 24 h (Figure 31A, C), while the induction on protein level was the highest after 2-3 days of incubation (Figure 31D). Since a larger amount of primary neurons can be obtained from cortex rather than hippocampus per animal, cortical primary neurons were selected for further screening purposes. Focussing on BDNF and CTGF four independent experiments were carried out in parallel treating cortical neurons with each of the two compounds for 2 days to densitometrically evaluate all experiments (Figure 32). This densitometric analysis revealed a significant induction of SORLA by treatment with CTGF (ca. +20% compared to control; p=0.02) as well as BDNF (ca. +60% compared to control; p=0,007).

A


Sorl1 expression in cortical primary neurons (24h)
rel. to ctrl
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
p=0.04
p=0.004
p=0.01
p=0.02
p=0.003
CTRL
NGF
BDNF
VEGF
CNTF
CTGF
FGF
PDGF
EGF

B


Sortilin expression in cortical primary neurons (24h)
rel. to ctrl
3
2
1
0
CTRL
NGF
BDNF
VEGF
CNTF
CTGF

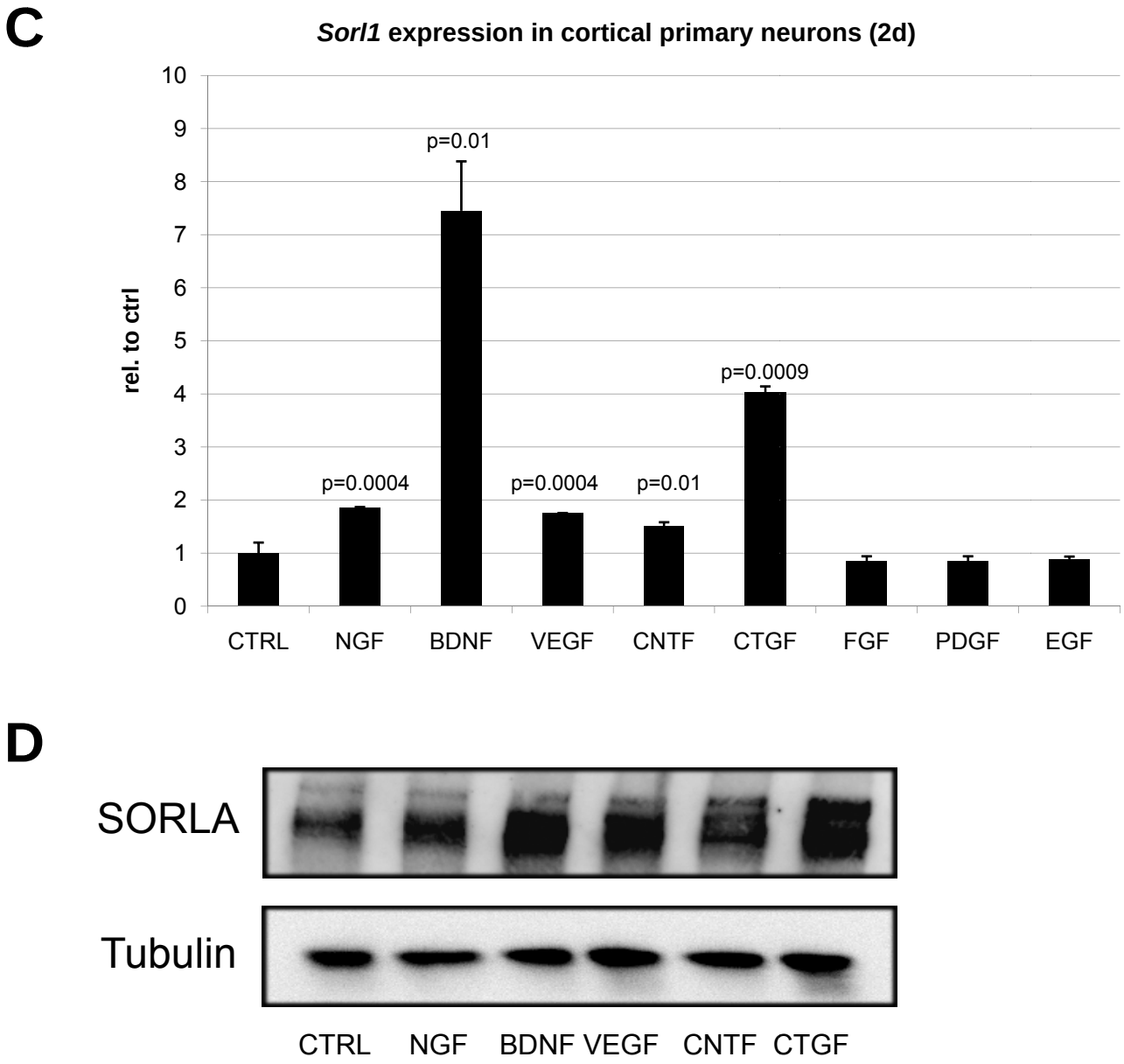
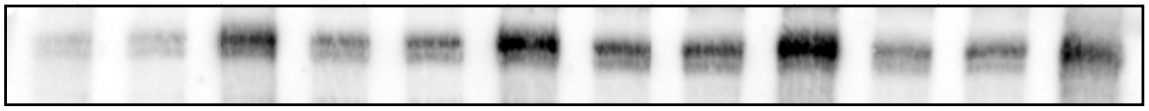


Figure 31: SORLA induction by neurotrophins

(A) TaqMan analysis of cortical primary neurons treated with indicated cytokines for 24 hours. NGF, BDNF, VEGF, CNTF and CTGF showed a significant increase in SORLA expression compared to control (CTRL, medium only). No change was found when treating with FGF, PDGF or EGF.**(B)** TaqMan analysis for sortilin in cortical primary neurons treated for 24 hours with NGF, BDNF, VEGF, CNTF or CTGF showed only minor effects compared to A. **(C)** TaqMan analysis of cortical primary neurons treated with indicated cytokines for 2 days. NGF, BDNF, VEGF, CNTF and CTGF showed a significant increase in SORLA expression compared to control with overall reduced levels compared to A. Again no change was detected when treating cells with FGF, PDGF or EGF. **(D)** Western blot analysis for SORLA of cortical primary neurons treated with NGF, BDNF, VEGF, CNTF or CTGF for 3 days. SORLA protein levels reproduce the findings of TaqMan analysis after 2 days treatment. (n=3, neurons were given a single dosage of 150 ng/ml of tested cytokines)

Positive correlation between SORLA expression and NGF or BDNF treatment was seen in experiments discussed above. To test whether the converse situation was true SORLA levels were investigated in mice genetically deficient for either neurotrophin (kindly provided by Anders Nykjaer). In newborns from heterozygous breedings of *Ngf*$^{+/-}$ mice, no changes in SORLA expression could be found between *Ngf*$^{+/+}$ and *Ngf*$^{-/-}$ mice (Figure 33). Between *Bdnf*$^{+/+}$ mice and *Bdnf*$^{-/-}$ mice a reduction of SORLA was observed, indicating an important function for BDNF in physiological regulation of SORLA expression.

A



CTRL CTGF BDNF CTRL CTGF BDNF CTRL CTGF BDNF CTRL CTGF BDNF

B

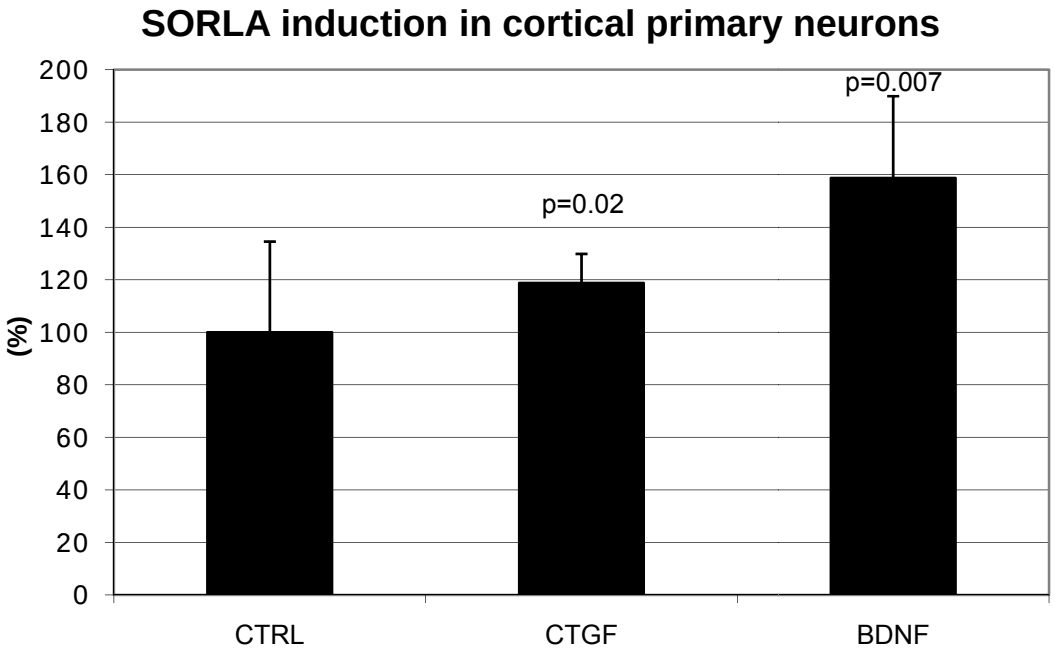


Figure 32: Western blot analysis of four independent experiments in cortical neurons

(A) Western Blot for SORLA in four independent experiments. Neurons were treated with 150 ng/ml of CTGF or BDNF for two days. Tubulin detection served as a load-

ing control. **(B)** Densitometric scanning revealed a significant increase for SORLA when cells were treated with CTGF or BDNF.

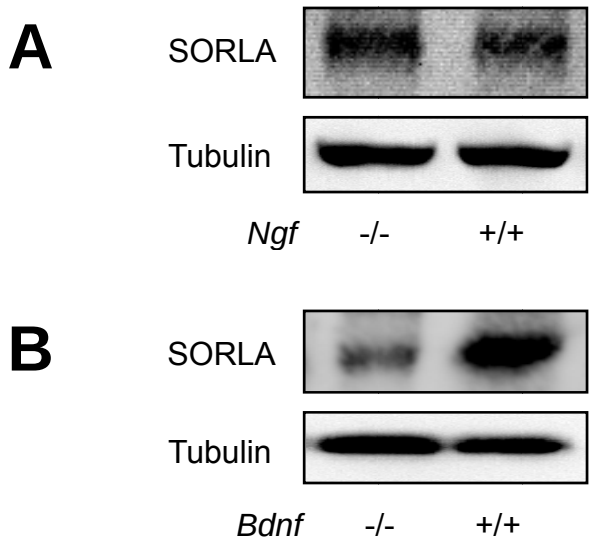


Figure 33: SORLA expression in NGF and BDNF deficient animals

(A) Western blot analysis of animals being *Ngf*$^{-/-}$ or *Ngf*$^{+/+}$. No reduction of SORLA could be seen between groups. Tubulin served as loading control. **(B)** Western blot analysis of animals being *Bdnf*$^{-/-}$ or *Bdnf*$^{+/+}$. A dosage dependent reduction of SORLA could be seen between groups. Detection of tubulin served as loading control.

Treatment with neurotrophins did not affect morphology or cell density of primary neurons as shown by bright field pictures or DAPI staining in figure 34. The SORLA signal was restricted to the cell body of the neuron rather than to peripheral compartments such as the axon. The pattern was vesicularly located in the perinuclear region of the cell. No obvious differences in localisation of SORLA were seen with or without neurotrophin treatment or between the various compounds.

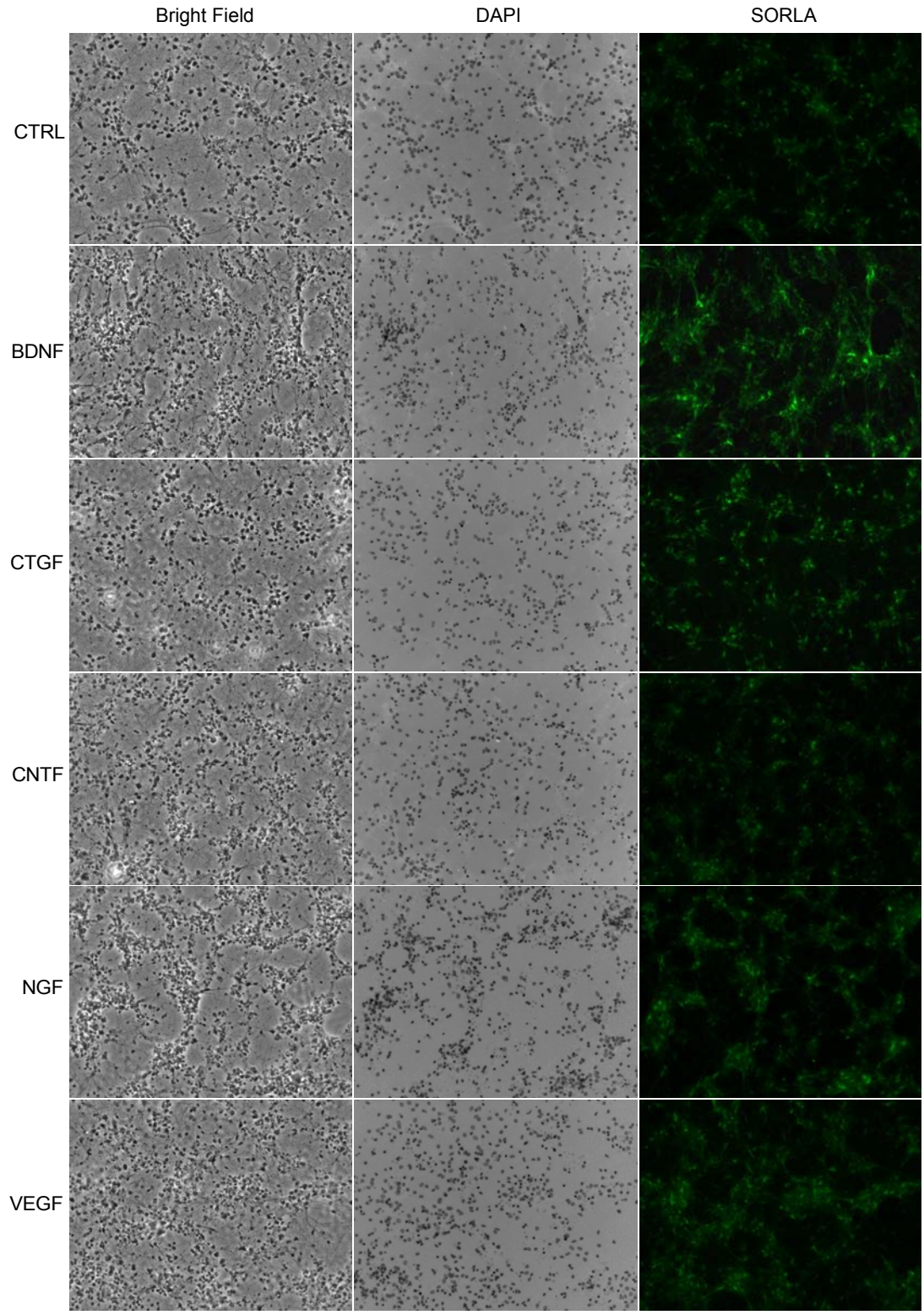


Figure 34: Histological evaluation of cortical primary neurons treated with indicated neurotrophins for 2 days

No obvious differences in morphology can be seen in bright field. Density of cells was comparable between treatments as shown by DAPI staining. SORLA signal seems to be especially pronounced in BDNF treated neurons. Cells were treated with 150 ng/ml of indicated neurotrophins for two days.

3.13 Protective effect of high SORLA expression

To test the hypothesis that high SORLA levels have a protective effect on APP processing *in vivo,* cortical primary neurons were treated for 2 days with a single dosage of 150 ng/ml BDNF or CTGF. The conditioned medium was collected and $A\beta_{40}$ and $A\beta_{42}$ levels were measured by ELISA (Figures 35, 36).

In line with an inhibitory role for SORLA in APP processing, BDNF treatment reduced $A\beta_{40}$ and $A\beta_{42}$ levels significantly (ca. -50% compared to control). To a lower degree, CTGF also reduced $A\beta_{40}$ and $A\beta_{42}$ levels (ca. -25% compared to control). These findings strongly suggested that SORLA induction by BDNF or CTGF could be a therapeutic approach to reduce Aβ production *in vivo*.

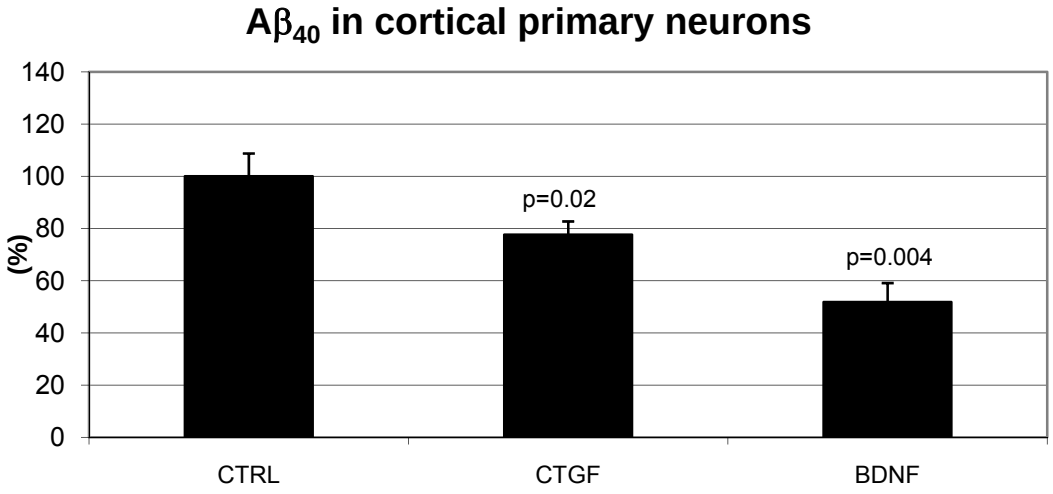


Figure 35: $A\beta_{40}$ levels in conditioned medium of cortical primary neurons treated for 2 days with CTGF or BDNF (150 ng/ml) (n=14)

Neurons treated with CTGF or BDNF secreted less $A\beta_{40}$. The strongest reduction is seen for the neurons treated with BDNF.

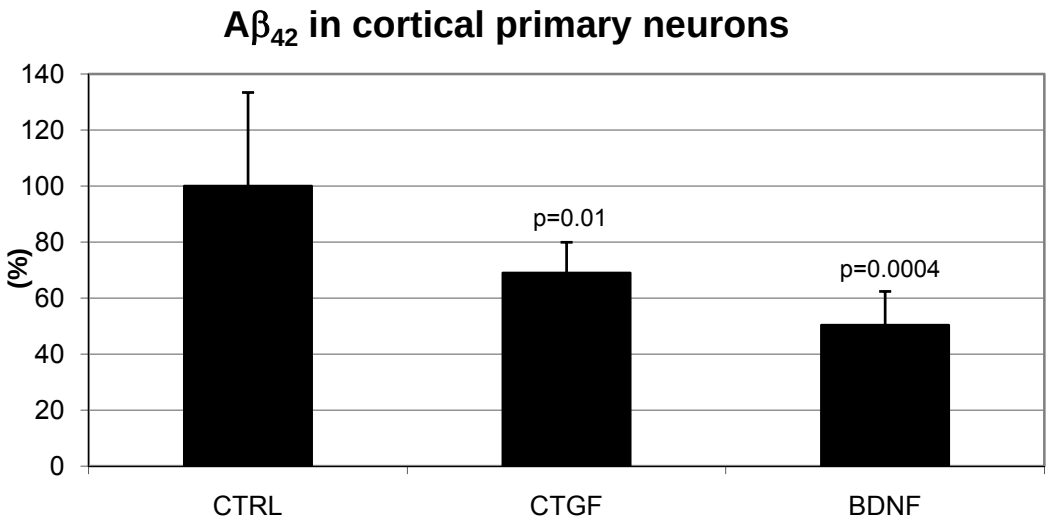


Figure 36: $A\beta_{42}$ levels in conditioned medium of cortical primary neurons treated for 2 days with CTGF or BDNF (150 ng/ml) (n=14)

Neurons treated with CTGF or BDNF secreted less $A\beta_{42}$. The strongest reduction is seen for the BDNF treated neurons.

4 Discussion

4.1 SORLA controls APP processing

So far, SORLA was shown to affect APP processing in cultured cells (Andersen, Reiche et al. 2005; Offe, Dodson et al. 2006; Spoelgen, von Arnim et al. 2006). The pitfall of all studies obtained with overexpression systems in cultured cells is the question of *in vivo* relevance. Because SORLA is highly expressed in the brain, especially in hippocampus and cortex (Hermans-Borgmeyer, Hampe et al. 1998; Kanaki, Bujo et al. 1998) investigations into the physiological function of SORLA should be preferentially carried out in such tissues. This is also of particular interest, since in Alzheimer disease, especially hippocampus is initially and most severely affected. Thus, the major aim of this study was to query the role of SORLA in APP processing and maintainance of neuronal intergrity and function *in vivo*. Such a role for SORLA as regulator of APP processing in the murine brain could be further substantiated. Loss of SORLA receptor activity in different mouse models resulted in accelerated processing of full length APP into its processing products and in a significant increase of amyloid peptide formation. The regulatory function of SORLA on APP processing has now been documented for endogenous murine APP as well as for mutated human APP (Figure 6, 10). These findings provide support for the concept that insufficient SORLA expression in patients may represent a risk factor for human AD (Rogaeva, Meng et al. 2007).

Apart from elevating Aβ levels, lack of SORLA also led to a dramatic rise in soluble APP products with consequences for adult neurogenesis and ERK signaling. Understanding the underlying molecular mechanisms is of great importance since similar changes can be envisioned in AD patients suffering from low levels of receptor expression.

4.2 SORLA regulates adult hippocampal neurogenesis

Sorl1-deficient mice described in this study enabled to evaluate the relevance of SORLA activity for sAPP production and to elucidate the consequences of high

sAPP levels for neuronal function.

There are two major neurogenic regions in the adult brain - the subgranular zone of the dentate gyrus in hippocampus and the subventricular zone (SVZ) of the lateral ventricles. So far evidence for a role of soluble APP products in adult neurogenesis was provided by Caillé et al. who demonstrated an sAPP-dependent increase in proliferation of SVZ progenitor cells when recombinant sAPPα was infused into the lateral ventricle (Caille, Allinquant et al. 2004). Blocking sAPP secretion by infusing batimastat, an inhibitor for α-secretase, or infusing anti-APP antibodies (6E10) reduced the number of proliferating progenitor cells as well as reducing APP synthesis by antisense oligonucleotides. Because no sAPP binding was found for cells of the hippocampus, it was suggested, that soluble APP products could exert their mitogenic stimulus predominantly in the SVZ (Caille, Allinquant et al. 2004). However, the possibility could not be excluded, that binding sites in other brain regions, that were below the detection limit of the applied method, were missed (Caille, Allinquant et al. 2004).

In contrast, in the study presented here, high sAPP levels as a consequence of *Sorl1* deficiency correlated with a strong increase in the number of newborn cells in the adult hippocampus of *Sorl1*$^{-/-}$ animals. Obviously, endogenous sAPP, produced locally in *Sorl1*$^{-/-}$ mice, stimulated proliferation of progenitors in hippocampus of this mice. The explanation for this finding is most likely the local importance of SORLA activity in hippocampus leading to high local sAPP concentrations, if SORLA is lacking.

Since sAPP was previously shown to enhance differentiation of progenitors into astrocytes (Kwak, Choumkina et al. 2006) and to stimulate neural progenitor proliferation (Caille, Allinquant et al. 2004), the portion of proliferating cells being of glial or neuronal origin was determined by colocalisation studies of BrdU and marker proteins for mature neurons (NeuN), immature neurons (DCX) and glial cells (GFAP). The increase in proliferation found was thereby identified to be due to an increased number of newborn neurons in the dentate gyrus. Because both, the rate of cell proliferation at 24h chase and survival rate at 4 weeks chase were altered to a similar extent (Figure 17), SORLA deficiency most likely exerts its primary effect at the level of precursor cell proliferation.

4.3 Aberrant activation of ERK signaling pathway in *Sorl1*-deficient mice

From cell culture experiments it was known that sAPP triggers kinase pathways such as ERK and AKT (Wallace, Akar et al. 1997; Cheng, Yu et al. 2002; Wehner, Siemes et al. 2004). Wallace et al. added purified sAPP to PC12 cells, which resulted in phosphorylation of ERK and neurite outgrowth (Wallace, Akar et al. 1997). Cheng et al. showed that both, the ERK as well as the AKT signalling pathway, are activated by adding recombinant sAPPα to hippocampal primary neurons. In addition, they identified sAPPα as a neurotrophic and excitoprotective factor that protects primary neurons from dying, if they are subjected to trophic factor deprivation or glutamate induced excitotoxic shock (Cheng, Yu et al. 2002).
Results obtained in the study presented here show an aberrant activation of the ERK signaling pathway *in vivo* as the pathophysiological consequence of *Sorl1* deficiency in hippocampal neurons of receptor null mice (Figure 15). Screens performed on brain and primary hippocampal neurons of *Sorl1*$^{-/-}$ animals also revealed that in contrast to ERK, the AKT signaling pathway was not affected by *Sorl1* deficiency (Figure 20).

Interestingly, ERK activation in *Sorl1*-deficient mice was critically dependent on the presence of the wild type *App* gene, indicating that signaling proceeds through APP or its processing products (Figure 20). The most likely candidate for a signaling factor derived from the *APP* gene locus that triggers ERK is sAPP as also proved in hippocampal primary neurons by applying recombinant sAPPα (Figure 21). In this experiment recombinant sAPPα added to the medium of hippocampal primary neurons was capable to induce ERK in a dosage dependent manner. In these hippocampal primary neurons Aβ was not capable to activate ERK. However, it can not be ruled out that another APP processing product like sAPPβ may have similar impact on ERK. It was elevated in the *Sorl1*-deficient neurons derived from PDAPP mice to a similar degree as the sAPPα (Figure 10). In addition, increased APP processing in the *Sorl1*-deficient mice should also lead to elevated levels of the CTF`s (C99, C83) as well as AICD. It cannot be ruled out, that these APP processing products might have impact on ERK as well. Nevertheless, sAPPα was suffi-

cient to induce ERK in primary neurons (Figure 10) which also makes it the most likely candidate for inducing ERK in hippocampus of *Sorl1*$^{-/-}$ mice *in vivo* (Figure 20B). Based on screens performed in brain and primary hippocampal neurons of *Sorl1*$^{-/-}$ animals, here, ERK seems to be the neuronal pathway most relevant in the hippocampus *in vivo*, since the AKT signaling pathway was not affected by *Sorl1* deficiency.

Although a direct correlation between ERK signaling and precursor cell proliferation has not formally been established in this study, the data presented here indicate that enhanced activity of the ERK pathway in *Sorl1*-deficient mice (due to elevated sAPP levels) might be the cause of enhanced adult neurogenesis observed in this mouse model. Intriguingly, several reports have already described increased cell proliferation and even adult neurogenesis in AD (Fitzjohn, Morton et al. 2001; Luo, Wallace et al. 2001). Signs of increased adult hippocampal neurogenesis were seen in patients suffering from sporadic AD (Luo, Wallace et al. 2001), but the specificity of the observation has been disputed (Kwak, Choumkina et al. 2006). However, it was also found that activity of ERK and its direct upstream kinase MEK1 is markedly increased in AD brains (Wen, Shao et al. 2002; Lopez-Toledano and Shelanski 2004; Nagy 2005; Boekhoorn, Joels et al. 2006). In murine models of AD both, reports of up- and down-regulation of adult neurogenesis exist (Luo, Wallace et al. 2001; Caille, Allinquant et al. 2004; Jin, Galvan et al. 2004).

Taken together, the reason for a potentially enhanced neurogenesis in some AD patients might be low levels of SORLA leading to high levels of sAPP and activated ERK.

Since SORLA deficiency is a major risk factor in AD, understanding the role of SORLA in balancing APP processing and subsequent physiological consequences is of major importance to decipher the complex scenario of rather beneficial and destructive functions of APP and its processing products. Since SORLA deficiency is exclusively linked to the sporadic form of AD (Dodson, Gearing et al. 2006), the *Sorl1*-deficient mouse represents an excellent model system for studying the mechanisms underlying sporadic AD-associated APP processing.

4.4 Learning and memory in *Sorl1*-deficient mice

An increase in hippocampal neurogenesis observed in *Sorl1*-deficient mice might have a direct effect on spatiotemporal learning and memory. It is known that learning induces LTP in hippocampus (Whitlock, Heynen et al. 2006). Furthermore, hippocampal learning and memory can be tested by the Morris water maze task.

No SORLA-dependent difference in LTP was measured at Schaffer collaterals in hippocampus in this study. Surprisingly, *Sorl1*-deficient mice performed better in the water maze task. Differences in physical performance between genotypes are likely to be ruled out as cause for better performance, since no differences were found between groups in the gait analysis or for the possible running speed. The better performance observed in the reversal at day 4 and 5 might thus be explained by increased adult neurogenesis in dentate gyrus of *Sorl1*-deficient mice.

The hippocampus was shown to be critical for spatial learning and memory as tested by the Morris water maze task and increased adult neurogenesis can be linked to an improvement of memory formation (Kempermann 2002; Kempermann and Gage 2002). Dupret et al. showed that reduced adult neurogenesis in animals, in which precursor cells are selectively killed, impaired hippocampus-mediated learning tested by the Morris water maze task (Dupret, Revest et al. 2008).

Of course it is very difficult to decipher the complex process of memory formation in hippocampus by a single task, but still *Sorl1*-deficient mice did find the new platform faster in the reversal as the control group, pointing at an effect on especially memory formation in *Sorl1*$^{-/-}$ mice. This effect seems to contrast the memory impairment in AD, but increased adult neurogenesis was also found in AD by some studies (Fitzjohn, Morton et al. 2001; Luo, Wallace et al. 2001). Considering the neuronal loss in AD, one must keep in mind that increased adult neurogenesis and subsequent beneficial effects for memory, will not be able to compensate for the massive loss of neurons found in brains of AD patients. In the *Sorl1*$^{-/-}$ mouse the situation differs from the diseased human brain, since no induction of apoptosis was found. Therefore, only the beneficial effects of increased neurogenesis contribute to the phenotype in *Sorl1*-deficient mice. Since all mouse models of AD only partially resemble human AD it is not possible to directly project data obtained in mice onto the

situation in human AD patients. The severe neurological deficits present in AD patients (not resembled in the mouse model) could not be compensated by elevating hippocampal neurogenesis, here the consequences of strongly elevated Aβ levels would dominate, if SORLA is lacking.

4.5 SORLA expression is regulated by neurotrophins

To answer the question whether high levels of SORLA could be protective against Aβ production *in vivo* various cytokines were tested on primary neurons for SORLA expression inducing effects. BDNF, CTGF, NGF, CNTF, VEGF, PDGF, EGF or FGF were added to the culture medium of the neurons and SORLA expression in the treated cells was determined. PDGF, EGF and FGF were not capable of inducing SORLA while BDNF, CTGF, NGF, CNTF and VEGF all induced SORLA to a different degree. Intriguingly, among the tested cytokines, BDNF was identified as the most potent activator of SORLA expression in neurons. This finding is particulary interesting in the light of the proposed role of this neurotrophin in AD related processes.

BDNF has been in focus of AD research for years. BDNF belongs to the neurotrophin family of growth factors and affects the survival of neurons (Hofer and Barde 1988). BDNF is strongly expressed in various regions of the brain, but the highest levels of expression are seen in hippocampus followed by cerebral cortex (Hofer, Pagliusi et al. 1990). BDNF improves survival of cholinergic neurons in basal forebrain as well as of hippocampal and cortical neurons (Nonomura and Hatanaka 1992; Burke, Mobley et al. 1994; Ghosh, Carnahan et al. 1994; Lindholm, Carroll et al. 1996). It was also observed that BDNF gene activity and protein levels are reduced in AD brains (Murray, Gall et al. 1994; Connor, Young et al. 1997; Hock, Heese et al. 2000). Beyond promoting neuronal survival and recovery after injury, BDNF modulates activity-dependent plasticity which underlies the process of learning and memory. Brain regions of high plasticity are the hippocampus and cortex; both are critical centers for learning and memory. The hippocampus is a central brain region for encoding new information and damage in this area severely impairs learning. The hippocampus is early affected by amyloid plaques in the course of AD and this is considered to cause memory problems that characterize this disease.

The reduction of BDNF found in the brains of human AD patients might impair the hippocampal function in different ways. In terms of plasticity, insufficient BDNF would impair synaptic strength, while in terms of its neurotrophic function, reduced BDNF levels would make hippocampal neurons more vulnerable to insult and degeneration. Neuronal activity during information processing was shown to stimulate BDNF gene transcription, transport of BDNF mRNA into dendritic spines and BDNF protein release into the synaptic cleft (Hartmann, Heumann et al. 2001). BDNF is transported into the dendrite and may also be synthesized locally in the spine. This way BDNF might play a role in tagging active synapses within dendrites. BDNF acts on neurons at presynaptic and postsynaptic sites via binding to its tyrosine kinase receptor TRKB. Internalization of the formed complex does not lead to termination of the BDNF signal, as it occurs for most other growth factor receptors. The internalized TRKB receptor remains activated. It becomes a specialized compartment called a "signaling endosome," which seems to be a critical factor for downstream signaling of BDNF on the cell body (Lu 2003).

Considering increased SORLA expression in hippocampus and cortex as a result of increased BDNF levels, which in turn is a consequence of increased synaptic activity, SORLA expression would be triggered by synaptic activity. In order to test whether lack of BDNF would also affect SORLA expression, *Bdnf*$^{-/-}$ animals were compared to wild type mice. A dosage dependent reduction in SORLA expression was observed. Thus, a direct correlation between BDNF levels and SORLA levels holds true. Since SORLA is not only induced by high levels of BDNF, but also down-regulated at low levels of this neurotrophin, the special importance of BDNF for the regulation of SORLA is highlighted.

Possible synaptic functions for APP have been postulated. APP serves as an adapter between the motor protein kinesin and transport vesicles and is transported axonally (Kamal, Almenar-Queralt et al. 2001; Matsuda, Yasukawa et al. 2001; Scheinfeld, Roncarati et al. 2002; Inomata, Nakamura et al. 2003; Matsuda, Matsuda et al. 2003), thereby probably serving as a cargo receptor for kinesin vesicles and proteins that are transported along the axon (Gunawardena and Goldstein 2001). In addition, in this study, a SORLA-dependent increase in mature APP at the post synaptic density was found in *Sorl1*$^{+/+}$ mice as compared to *Sorl1*$^{-/-}$ mice. The physiological consequence of this finding is unknown, but it is tempting to

speculate that SORLA might have impact on synaptic function by sorting mature APP to synaptic compartments. Since BDNF is strongly involved in synaptic function, especially synaptic tagging, a function of APP in this process would represent an interesting additional physiological function for APP. In this scenario, SORLA would act as mediator between APP and BDNF, being induced by BDNF and delivering APP to synapses as consequence.

By enhancing synaptic transmission and neuronal excitability (Figurov, Pozzo-Miller et al. 1996; Griesbeck, Korte et al. 1996), BDNF modulates hippocampal long-term potentiation (LTP). LTP is a neuronal mechanism associated with learning and adaptive behaviours in adult animals (Poo 2001; Tyler, Alonso et al. 2002). BDNF/TRKB signaling was shown to play an important role in plasticity mechanisms. LTP (and learning) is impaired in BDNF/TRKB-signaling deficient mice and restoring BDNF reverses both the electrophysiological and learning deficits (Levine, Dreyfus et al. 1995; Korte, Griesbeck et al. 1996; Patterson, Abel et al. 1996). *Bdnf*-deficient mice were also shown to have decreased synaptic innervation and reduced levels of synaptic vesicle proteins (Martinez, Alcantara et al. 1998; Pozzo-Miller, Gottschalk et al. 1999), which demonstrates the importance of BDNF for proper synaptic signaling (Martinez, Alcantara et al. 1998).

Nevertheless, no SORLA-dependent differences in hippocampal LTP at the Schaffer collaterals were seen in this study, which might be explained by SORLA being involved in processes occuring after LTP (often also called late-LTP), that require gene transcription and protein synthesis and perhaps APP processing/sorting. Since the extracellular signal-regulated kinase (ERK) subfamily of MAPKs may be the molecular link between early and late processes of LTP, the SORLA-dependent induction of ERK via APP might also point at a function in this system (Kelleher, Govindarajan et al. 2004).

Several polymorphisms in the BDNF gene have been documented so far. For instance, the substitution of a single amino acid in the coding region of the BDNF gene (val/met substitution at codon 66) disturbs trafficking of BDNF within the cell in response to appropriate cellular cues. This mutation correlates with impaired memory and abnormal hippocampal activation (Egan, Kojima et al. 2003). Three different BDNF polymorphisms have been correlated with an enhanced risk for AD (Kunugi, Ueki et al. 2001; Riemenschneider, Schwarz et al. 2002; Ventriglia,

Bocchio Chiavetto et al. 2002). In animal studies it was shown that learning and exercise can increase brain levels of BDNF in hippocampus and other brain regions (Cotman and Berchtold 2002). In humans, regular exercise is associated with benefits to brain health and cognitive function and physically active adults also have a lower risk of developing AD or other dementias (Friedland, Fritsch et al. 2001; Laurin, Verreault et al. 2001). There is also evidence that mental activity/learning may somewhat protect against AD (Valenzuela, Sachdev et al. 2008).

Given all the evidence for BDNF, the linkage between this neurotrophin and SORLA, an additional risk factor for AD, provides novel insight how BDNF may be linked to APP processing. It also further substantiates the role of SORLA in AD and exemplifies that neurotrophins in AD can have impact on APP via SORLA. The knowledge about the impact of BDNF on APP, in particular Aβ, was rather limited so far. In cell culture experiments it was shown that BDNF increases the expression of *App* gene reporter constructs transfected into PC12 cells (Ge and Lahiri 2002) and that in human neuroblastoma cells differentiated by BDNF and retinoic acid (induces expression of TRKB), expression and synthesis of APP is increased while the processing of APP is shifted towards the α-secretase pathway (Holback, Adlerz et al. 2005).

In the study presented here, it was primarily shown that in the close to *in vivo* situation of primary cortical neurons, BDNF reduces $A\beta_{40}$ as well as $A\beta_{42}$ levels significantly by inducing SORLA. With respect to this finding, SORLA could have a key function as survival factor for neurons by modulating APP processing in a neurotrophin-dependent manner. This hypothesis was evidenced by the fact that other neurotrophic factors implicated in AD, such as NGF, VEGF, CNTF and CTGF, also induced SORLA expression even so to a minor degree.

NGF came into focus of Alzheimer research because of the observation that cholinergic basal forebrain neurons (CBF), which provide the major source of cholinergic innervations to the cerebral cortex and hippocampus, undergo selective and severe degeneration in advanced AD and that the survival of CBF neurons depends upon NGF and its receptors, TRKA and p75NTR. Different to *Bdnf*$^{-/-}$ mice, comparing cortical lysates of *Ngf*$^{-/-}$ mice with wild type mice in this study, revealed no correlation of the *Ngf* genotype with SORLA expression.

Similar to BDNF, ciliary neurotrophic factor (CNTF) was found to increase *App* gene expression in cultured rat C6 glioma cells, even so no difference was found in human neuroblastoma cells (Sudoh, Kawakami et al. 1994).
Low levels of VEGF have also been associated with the risk to develop AD (Mateo, Llorca et al. 2007) although a molecular explanation remained elusive.
CTGF was found to be a downstream effector of insulin resistence (IR), which was associated with promotion of Aβ neuropathology in AD patients. In a mouse model of AD (Tg2576), diabetogenic diet that promotes IR resulted in a ~2-fold elevation of CTGF steady-state levels in brain coincident with a commensurate promotion of AD-type amyloid plaque burden. In cell culture experiments using the glioma cell line H4 transfected with APP_{751}, application of recombinant CTGF increased Aβ levels possibly by reducing levels of insulin-degrading enzyme (IDE) (Zhao, Ho et al. 2005). The question that remains unanswered is whether CTGF-dependent elevation of Aβ levels in glioma cells represents a mechanism which can also be found for APP_{695} in neurons *in vivo* or if it is a special case just found in this cell line. As for CNTF, there can be a considerable difference among cell lines from different (glioma or neuroblastoma) origin.

Taken together, the findings that various neurotrophins implicated in AD induce SORLA expression substantiates the major importance of SORLA in this disease and sheds more light on the directed regulation of SORLA expression in neurons. In this respect, SORLA seems to be a nodal point for neurotrophins to modulate neuronal function in AD through regulation of APP processing.

4.6 Outlook

The understanding of SORLA's influence on APP processing is quite significant and substantiated by a number of association studies in humans. Obviously, the next challenge for research will be the evaluation of the interaction partners of this receptor that are required for its function. The important involvement of BDNF in SORLA regulation raises the question, if SORLA might be an important mediator between BDNF's synaptic function and a potential synaptic function of mature APP, transported to post synaptic compartments. The understanding of such a system

would provide insight into mechanisms leading to synaptic loss and subsequently neuronal loss in AD and furthermore could explain the physiological function of APP itself apart from being the origin of Aβ-mediated cell death.

In addition the identification of further downstream targets of SORLA other than APP would provide insight into a greater protein network, regulated by neurotrophins via SORLA. A detailed picture of such a network is necessary to prove the concept of SORLA induction being protective against neurodegeneration and to evaluate the consequences of SORLA overexpression *in vivo*.

5 Summary

SORLA (sorting protein-related receptor with A type repeats) is a type-I membrane receptor belonging to the low-density lipopotein receptor (*Ldlr*) gene family. It is preferentially expressed in neurons of the central nervous system. It is well kown that SORLA binds to the amyloid precursor protein (APP), the key protein in Alzheimer disease (AD), and impairs processing of APP to soluble APP (sAPP) and to amyloid peptide (Aβ). It is also known that SORLA is poorly expressed in the brain of patients with AD, indicating a casual role for the receptor in the pathology of AD. Here, the consequences of *Sorl1* (encoding SORLA) gene defects on brain anatomy as well as neuronal structure and function were evaluated using mouse models of altered receptor activity.

In line with a protective role for SORLA in APP metabolism, lack of the receptor results in increased amyloidogenic processing of endogenous APP and in aggravated plaque deposition when introduced into the PDAPP line of mice expressing mutant human APP. Surprisingly, increased levels of sAPP caused by receptor deficiency correlate with profound stimulation of neuronal ERK signaling and with enhanced adult neurogenesis, providing *in vivo* support for neurotrophic functions of sAPP. The data document a role for SORLA not only in control of plaque burden but also in APP-dependent neuronal signaling and suggest a molecular explanation for increased adult neurogenesis observed in some AD patients.

To test the hypothesis that SORLA induction may be a therapeutical approach for AD, cortical primary neurons were treated with various cytokines to identify agents upregulating neuronal receptor expression. In particular, substances known to have neurotrophic effects induced SORLA expression to a different degree. Brain derived neurotrophic factor (BDNF) and connective tissue growth factor (CTGF) were shown to have the most pronounced effect on SORLA expression. When added to cortical neurons both significantly reduced thereby Aβ production. Therefore both compounds could be of great interest to reduce neurotoxic Aβ production by SORLA induction in AD patients to diminish neuronal loss.

Keywords: SORLA, ERK signaling, adult neurogenesis, APP, BDNF, CTGF

6 Zusammenfassung

SORLA („sorting protein-related receptor with A type repeats“) ist ein Typ-I-Transmembranrezeptor, welcher zur Genfamilie der *Ldl* („low-density lipopotein“)-Rezeptoren gehört und vor allem in Neuronen des Zentralnervensystems exprimiert wird. Es ist bisher bekannt, dass SORLA direkt mit dem „amyloid precursor protein“ (APP), dem Schlüsselprotein der Alzheimerschen Krankheit, interagiert und dessen Prozessierung in amyloidogene Peptide (Aβ) und lösliches APP (sAPP) der α- und β-Form blockiert. Patienten mit Morbus Alzheimer weisen nur eine geringe Expression von SORLA auf, was auf eine kausale Rolle des Rezeptors bei der Entstehung neurodegenerativer Prozesse hindeutet. Der zugrunde liegende Mechanismus ist jedoch noch weitestgehend ungeklärt. Im Rahmen dieser Doktorarbeit wurden die Bedeutung einer Wechselwirkung von SORLA und APP für die Struktur und Funktion von Neuronen und die Konsequenzen eines Rezeptorverlustes für die Entstehung neurodegenerativer Prozesse untersucht. Hierzu wurden sowohl neuronale Zellkulturen als auch Mausmodelle mit genetisch induzierter Veränderung der SORLA Expression verwendet.

Die gezielte Inaktivierung des *Sorl1* (kodiert SORLA) Gens führte zu einer erhöhten Prozessierung von APP in sAPP α und β sowie Aβ. Diese erhöhte Menge an Aβ führte ausserdem zu einer größeren Zahl von Plaques. Bei fehlendem SORLA kam es des Weiteren zur Aktivierung der ERK („extracellular regulated kinase“)-Signalkaskade und zu erhöhter adulter Neurogenese. Der in Folge der SORLA-Inaktivität erhöhte Level an sAPPα war dabei entscheidend für die Aktivierung von ERK.

Außerdem wurden pharmakologische Substanzen identifiziert, welche eine Induktion der SORLA Expression in primären Neuronen bedingen. Von allen getesteten Zytokinen zeigten dabei alle Neurotrophine eine Tendenz zur Induktion von SORLA, wobei Zytokine ohne ausgewiesene Wirkung auf Neurone keine Induktion erzeugten. Die grundsätzlich stärkste Induktion zeigte sich bei der Gabe von BDNF („brain derived neurotrophic factor“) und CTGF („connective tissue growth factor“). Die induzierte SORLA-Expression führte dabei zu der erwarteten reduzierten Aβ-Produktion in primären kortikalen Neuronen. Somit könnte die Induktion der SORLA-Expression durch Neurotrophinbehandlung ein Ansatz sein,

um das Absterben von Neuronen bei Patienten der Alzheimerschen Krankheit zu vermindern.

Schlagwörter: SORLA, ERK-Signalkaskade, Adulte Neurogenese, APP, BDNF, CTGF

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8 Appendix

8.1 Publications

Rohe, M., A.-S. Carlo, et al. (2008). "Sortilin-related Receptor with A-type Repeats (SORLA) Affects the Amyloid Precursor Protein-dependent Stimulation of ERK Signaling and Adult Neurogenesis." J. Biol. Chem. **283**(21): 14826-14834.

Schmidt, V., A. Sporbert, et al. (2007). "SorLA/LR11 Regulates Processing of Amyloid Precursor Protein via Interaction with Adaptors GGA and PACS-1." J Biol Chem **282**(45): 32956-64.

8.2 Abbreviations

d	day
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DNA	desoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
g	gram
HCl	hydrogen chloride
HEPES	hydroxyethylpiperazineethanesulfonic acid
h	hours
K	potassium
KCl	potassium chloride
kDa	kilodalton
KH_2PO_2	potassium dihydrogen phosphate
l	liter
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
M	molar
N	asparagine
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NaOH	sodiumhydroxide
ng	nanogram
P	proline
pH	potential of hydrogen
PMSF	phenylmethanesulfonyl fluoride

RT room temperature
RNA ribonucleic acid
S serine
s second
SDS sodium dodecyl sulfate
Taq thermus aquaticus
Tris trishydroxymethylaminomethane
TUNEL terminal transferase dUTP nick end labeling
U unit
V volt
wt wild type
Y tyrosine
µ micro

8.3 Kurzer Lebenslauf

Mein Lebenslauf wird aus Gründen des Datenschutzes nicht online veröffentlicht.

Bisherige Publikationen insgesamt:

Rohe, M., A.-S. Carlo, et al. (2008). "Sortilin-related Receptor with A-type Repeats (SORLA) Affects the Amyloid Precursor Protein-dependent Stimulation of ERK Signaling and Adult Neurogenesis." J. Biol. Chem. **283**(21): 14826-14834.

Schmidt, V., A. Sporbert, et al. (2007). "SorLA/LR11 Regulates Processing of Amyloid Precursor Protein via Interaction with Adaptors GGA and PACS-1." J Biol Chem **282**(45): 32956-64.

Hartl, D., M. Rohe, et al. (2008). "Impairment of adolescent hippocampal plasticity in a mouse model for Alzheimer's disease precedes disease phenotype." PLoS ONE **3**(7): e2759.

8.4 Danksagung

Ich möchte Prof. Dr. Thomas Willnow für die Möglichkeit danken, dieses spannende Projekt zu bearbeiten und für die gute Betreuung.

Natürlich möchte ich auch Dr. Olav Andersen für die diskussionsfreudige Betreuung meiner Doktorarbeit danken.

Mein besonderer Dank gilt auch Prof. Dr. Fritz Rathjen für die Betreuung meiner Doktorarbeit.

Außerdem danke ich Dr. Anne-Sophie Carlo und Dr. Anje Sporbert für die Hilfe bei der Histologie.

Desweiteren danke ich Bettina Erdmann für die Durchführung der immuno-elektronenmikroskopischen Färbung von APP.

Ich danke Prof. Dr. Thomas Bayer und seiner Arbeitsgruppe für die Bestimmung von Aβ in der Maus.

Dann danke ich außerdem Prof. Dr. Dietmar Schmitz und seiner Arbeitsgruppe für die Messung der LTP in der Maus.

Schließlich danke ich Prof. Dr. Gerd Kempermann für die Möglichkeit in seiner Arbeitsgruppe die adulte Neurogenese bestimmen zu dürfen.

8.5 Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit mit dem Titel “Role of SORLA in the brain and its relevance for Alzheimer disease” selbstständig und ohne Hilfe Dritter angefertigt habe. Sämtliche Hilfsmittel, Hilfen sowie Literaturquellen sind als solche kenntlich gemacht. Ausserdem erkläre ich hiermit, dass ich mich nicht anderweitig um einen entsprechenden Doktorgrad beworben habe. Die Promotionsordnung des Fachbereichs Biologie, Chemie, Pharmazie der Freien Universität Berlin habe ich gelesen und akzeptiert.

Berlin, den 01.09.2008

Michael Stephan Rohe