PCSK9 is not involved in the degradation of LDL receptors and BACE1 in the adult mouse brain

Mali Liu,* Guoxin Wu,* Jennifer Baysarowich,† Michael Kavana,† George H. Addona,† Kathleen K. Bierilo,† John S. Mudgett,§ Guillaume Pavlovic, Ayesha Sitlani,† John J. Renger,* Brian K. Hubbard,† Timothy S. Fisher,[1,†] and Celina V. Zerbinatti[1,*]**

Neurology Department,* Merck Research Laboratories, West Point, PA; Atherosclerosis Department† and Center for Genetically Engineered Models,§ Merck Research Laboratories, Rahway, NJ; and Institut Clinique de la Souris (ICS),** Illkirch, France

Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted protein that regulates hepatic low-density lipoprotein receptor (LDLR) levels in humans. PCSK9 has also been shown to regulate the levels of additional membrane-bound proteins in vitro, including the very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (ApoER2) and the β-site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1), which are all highly expressed in the CNS and have been implicated in Alzheimer's disease. To better understand the role of PCSK9 in regulating these additional target proteins in vivo, their steady-state levels were measured in the brain of wild-type, PCSK9-deficient, and human PCSK9 overexpressing transgenic mice. We found that while PCSK9 directly bound to recombinant LDLR, VLDLR, and apoER2 protein in vitro, changes in PCSK9 expression did not alter the level of these receptors in the mouse brain. In addition, we found no evidence that PCSK9 regulates BACE1 levels or APP processing in the mouse brain. In conclusion, our results suggest that while PCSK9 plays an important role in regulating circulating LDL cholesterol levels by reducing the number of hepatic LDLRs, it does not appear to modulate the levels of LDLR and other membrane-bound proteins in the adult mouse brain.—Liu, M., G. Wu, J. Baysarowich, M. Kavana, G. H. Addona, K. K. Bierilo, J. S. Mudgett, G. Pavlovic, A. Sitlani, J. J. Renger, B. K. Hubbard, T. S. Fisher, and C. V. Zerbinatti. **PCSK9 is not involved in the degradation of LDL receptors and BACE1 in the adult mouse brain. *J. Lipid Res.* 2010.** 51: **2611–2618.**

Supplementary key words atherosclerosis • low-density lipoprotein • proprotein convertase subtilisin/kexin type 9 • β-site amyloid precursor protein-cleaving enzyme 1 • amyloid-β • Alzheimer's disease

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the mammalian subtilisin family of proprotein convertases (1). PCSK9 is a secreted protein predominantly expressed in the liver, small intestine, and kidney, and its role in regulating circulating levels of LDL cholesterol has been demonstrated in several species. In humans, loss-of-function mutations within PCSK9 are associated with a reduction in circulating LDL levels and protection against coronary heart disease (CHD) (2, 3). Conversely, gain-of-function mutations in PCSK9 result in autosomal dominant hypercholesterolemia and premature atherosclerosis (4–6). In support of the human genetics data, deletion of PCSK9 in mice significantly reduces plasma cholesterol levels and enhances LDL clearance, while overexpressing PCSK9 increases plasma LDL cholesterol levels (7–9). Furthermore, reduction of hepatic PCSK9 mRNA transcript levels by anti-sense oligonucleotides or small interfering RNAs (siRNA) significantly reduces circulating LDL cholesterol levels in mice, rats, and cynomolgus monkeys (10, 11). Together, these findings support the development of therapies focusing on PCSK9 inhibition for the treatment of hypercholesterolemia and prevention of CHD.

PCSK9 regulates circulating LDL cholesterol levels primarily through the degradation of hepatic LDL receptor (LDLR) (12). PCSK9 binds directly to LDLR via the first epidermal growth factor-like repeat (EGF-A) in the extracellular domain of LDLR (13). Cocrystal structure analysis shows that the EGF-A domain of LDLR binds to the catalytic domain of PCSK9 (14, 15). In addition, the EGF-A domain of LDLR itself is sufficient for inhibiting the PCSK9 interaction with full-length LDLR and can be used to inhibit PCSK9-mediated degradation of LDLR in cell

Abbreviations: AD, Alzheimer's disease; ApoER2, apolipoprotein E receptor 2; BACE1, β-site amyloid precursor protein (APP)-cleaving enzyme 1; CHD, coronary heart disease; CNS, central nervous system; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9 KO, Pcsk9 knockout mouse line; PCSK9 TG, transgenic mice carrying the human PCSK9 gene; SPR, surface plasmon resonance; VLDLR, very low-density lipoprotein receptor; WT, wild type.

[1]To whom correspondence should be addressed.
e-mail: celina_zerbinatti@merck.com; timothy_fisher@merck.com

Manuscript received 5 March 2010 and in revised form 7 May 2010.

Published, JLR Papers in Press, May 7, 2010
DOI 10.1194/jlr.M006635

culture systems (15, 16). Disruption of the PCSK9-LDLR interaction in cultured human hepatocytes with LDLR subfragments or anti-PCSK9 antibodies results in increased cell surface LDLR levels and cellular LDL uptake (15–18). Consistent with the role of PCSK9 in the degradation of LDLR in vitro, elevation of circulating PCSK9 levels in mice either through parabiosis or intravenous injection reduces the level of hepatic LDLR (9, 19, 20). Injection of PCSK9 into mice also lowers LDLR levels in several extrahepatic tissues, such as the lung, adipose, and kidney (19, 20).

In addition to its role in regulating LDLR levels, there have been recent reports suggesting that PCSK9 regulates cellular levels of two other LDLR family members, the very low density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2). Overexpression of either VLDLR or ApoER2 resulted in increased binding of PCSK9 to CHO-A7 cells (21). Furthermore, cooverexpression of VLDLR or ApoER2 and PCSK9 in cultured cells increased degradation of these receptors. PCSK9 was also shown to directly bind both VLDLR and ApoER2 in cell-free assays (16). These results suggest that PCSK9 is capable of binding receptors other than LDLR and promoting their degradation in vitro.

Because VLDLR and ApoER2 are highly expressed in the central nervous system (CNS) and have been previously implicated in Alzheimer's disease (AD) pathology (22–26), we sought to investigate their potential modulation by PCSK9 in vivo. It has also been recently shown that overexpression of PCSK9 in cells decreased cellular levels of the β-site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1), a membrane protease responsible for the production of toxic β-amyloid peptides (Aβ) that accumulate in neuritic plaques of AD brains (27, 28). In the present study, we investigated whether PCSK9 participates in the regulation of the steady-state levels of LDLR, VLDLR, and ApoER2, as well as BACE1 in the adult mouse brain. We confirmed that PCSK9 directly binds to LDLR, VLDLR, and ApoER2 in vitro. However, steady-state levels of LDLR, VLDLR, and ApoER2 were similar in the brain of wild-type, Pcsk9-knockout and human PCSK9-overexpressing mice. In contrast with previously published data (28), we found that the lack or overexpression of PCSK9 in the mouse CNS did not significantly alter BACE1 levels or APP processing to Aβ. Together, these results demonstrate that while PCSK9 is capable of binding several membrane-bound proteins in vitro, it does not appear to play a significant role in regulating their steady-state levels in the adult mouse brain.

METHODS

Recombinant proteins

Wild-type PCSK9 protein was purified from HEK293 generated media as described (29, 30). Recombinant human LDLR, mouse VLDLR, and human ApoER2 used on surface plasmon resonance (SPR) experiments were purchased from R and D Systems. Recombinant carbonic anhydrase was purchased from Sigma, and streptavidin was from Invitrogen. Recombinant mouse VLDLR (2258-VL, R and D Systems) used as positive control for endogenous brain receptor detected by Western blotting was generated from a murine myeloma cell line and included Thr25-Ala798 with a C-terminal 10-His tag (predicted molecular mass of 86.4 kDa, expected 130–175 kDa band in SDS-PAGE under reducing conditions). Recombinant mouse apoER2 receptor (3520-AR, R and D Systems) was generated from Chinese Hamster Ovary cell line and included Asp35-Lys818 with a C-terminal 10-His tag (predicted molecular mass of 87.5 kDa, expected 140–150 kDa band in SDS-PAGE under reducing conditions).

PCSK9-receptor binding affinity measurements

All SPR experiments were performed using a Biacore T100 instrument at 25°C. Running buffer contained 25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, and 0.005% (v/v) P-20 surfactant. For studies of PCSK9 binding to LDLR, VLDLR, and ApoER2, commercial receptor proteins (rhLDLR, rmVLDLR, and rhApoER2 at 40 μg/ml in 10 mM sodium acetate, pH 4.5) were covalently immobilized by amine coupling to a carboxymethylated dextran sensor surface (CM5 chip, GE Healthcare).

Binding constants were obtained from a series of injections of PCSK9 at a concentration range of 1 nM to 1 μM with a flow rate of 30 μl/min. Following injections of PCSK9, receptor-coupled sensor chip surfaces were regenerated with a 6 s injection of 10 mM HCl and a 60 s stabilization period. Data were analyzed using BIAevaluation software. Base lines were adjusted to zero for all curves, and injection start times were aligned. The reference sensograms were subtracted from the experimental sensograms to yield curves representing specific binding. Steady-state analysis was used to plot equilibrium binding response (R_{eq}) against analyte concentration to obtain thermodynamic dissociation constants (K_D).

Mutant mouse line establishment

The Pcsk9 knockout mouse line (PCSK9 KO) was established at the Institut Clinique de la Souris (ICS; llkirch, France). The targeting vector was constructed by successive cloning of PCR products and contained 3.9 kb 5′ and 4.3 kb 3′ homology arms and a Neomycin selection cassette. Two loxP sequences were located directly at the end of exon 1 and 237 bp before exon 4. The linearized construct was electroporated in C57BL/6N mouse embryonic stem (ES) cells. After selection, targeted clones were identified by PCR using external primers and further confirmed by Southern blot with Neo internal probe and with 5′ and 3′ external probes (data not shown). A positive ES clone was injected into BalbC/J blastocysts, and male chimaeras derived gave germline transmission. The resulting line was verified by PCR using external primers, further confirmed by Southern blot with Neo internal probe (data not shown), and bred with a CMV-Cre deleter model to result in deletion of intron 1–exon2–intron 2–exon 3 and first 1979 bp of intron 3 DNA region. Progeny mice were on C57BL/6 background. RT-PCR was performed with the forward primer (5′ CACCATCACCGACTTCAACA 3′) located on exon 4 and a reverse primer located on exon 5 (GTCACACTTGCTCGCCTGT). The awaited 72 bp was obtained in the wild-type (WT) and $Pcsk9^{+/-}$ liver samples. In the PCSK9 KO liver samples, no product was obtained, suggesting absence of a knockout RNA containing exon 4-5 (data not shown). Transgenic mice carrying the human PCSK9 gene (PCSK9 TG) were obtained from Jay Horton (University of Texas Southwestern Medical Center, Dallas, TX) and were backcrossed onto a C57Bl/6 background (9). Brain and liver tissue samples were obtained following transcardial perfusion with ice-cold PBS containing heparin (3 U/ml) and snap-frozen for further analysis. All animals were handled according to the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals guidelines, and

the study protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

Taqman analysis

Frozen WT and PCSK9 KO liver samples were homogenized in Trizol (Invitrogen, Carlsdbad, CA), and total RNA was extracted according to the manufacturer's instructions. RNA (100 μg) was DNase treated using RNeasy spin columns and RNase-Free DNase set according to the manufacturer's instructions (Qiagen). Total RNA was extracted from frozen WT and PCSK9 KO cerebellum samples using the SV Total RNA Isolation Kit according to the manufacturer's instructions (Promega). The quality and quantity of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies). Total RNA (0.9 μg) was reverse transcribed into cDNA in a total volume of 50 μl using Taqman® reverse transcription reagents according to the manufacturer's instructions (Applied Biosystems). A total of 2 μl, corresponding to approximately 36 ng of input RNA, was used in subsequent Taqman® analysis. The ready-made primer probe sets for PCSK9 (Mm01263610_m1) and GAPDH (Mm99999915_g1) were from Applied Biosystems. Reactions were run and analyzed on the Applied Biosystems 7900 thermocycler under the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 two-step cycles of 95°C for 15 s and 60°C for 60 s.

Immunoblotting

Brain (cortex and hippocampus regions) and liver samples from WT, PCSK9 KO, and PCSK9 TG mice were dounce-homogenized in 10× volume of RIPA lysis buffer (Sigma) containing Complete protease inhibitor cocktail (Roche) and 2 mM phenylmethanesulfonyl fluoride (PMSF). Protein concentration in the supernatants was measured using BCA assay kit (BioRad). Equal amounts of sample protein in Laemmli buffer were separated on 7.5% or 4–15% Tris-HCl polyacrylamide SDS gels (BioRad) and transferred to polyvinylidene fluoride membranes. APP *C*-terminus fragments were separated using 10–20% Tris-Tricine gels (BioRad). Blots were placed in blocking solution with 10% nonfat milk in PBS with 0.05% Tween-20 (PBS-T) for 1 h, followed by incubation with various primary antibodies with 5% nonfat milk in PBS-T for 3 h at room temperature or overnight at 4°C. Primary antibodies include 0.5 μg/ml final concentration of anti-PCSK9 (ab28770, Abcam), 0.2 μg/ml anti-LDLR (AF2255, R and D Systems), 2 μg/ml anti-ApoER2 (H00007804-M01, Abnova), 1 μg/ml anti-VLDLR (MAB2258, R and D Systems), 1 μg/ml anti-BACE1 (ab2077, Abcam), and 0.5 μg/ml anti-APP (51-2700, Invitrogen). Blots were washed with PBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 1:2500 dilution (Amersham) for 1 h at room temperature. Immunoreactive bands were visualized using ECL Plus (Amersham Biosciences) on the Typhoon 9410 (Amersham Biosciences). Data was analyzed using ImageQuant (Molecular Dynamics).

Aβ measurement

Mouse brain samples including the cortex and hippocampus were homogenized with 10× volume to weight of 0.2% diethylamine (DEA) buffer (0.2% DEA, 50 mM NaCl) containing Complete protease inhibitor cocktail (Roche). After homogenization, 1 ml of each sample was boiled for 10 min and spun at 14,000 rpm for 1 h at 4°C. The supernatant was neutralized to pH 7.4 with 10% volume of 0.5M Tris-HCL pH 6.8. Black polystyrene plates (Corning Inc.) were coated overnight with 2.5 μg/ml of rodent *N*-terminal polyclonal antibody (Sig-39153, Covance) as capture antibody, washed, and then blocked with 3% BSA in phosphate-buffered saline. Brain homogenates (100 μl) were added per well in duplicate, followed by 50 μl of Aβ40 *C*-terminal neoepitope antibody G2-10 conjugated with alkaline phosphatase. Synthetic Aβ40 peptide (American Peptide Co., Inc.) was used as standard and prepared in APP KO brain matrix. After overnight incubation at 4°C, plates were washed and developed using alkaline phosphatase substrate (Applied Biosystems). Luminescence counts were measured using the LJL Analyst (Molecular Devices). Standards run in duplicate were fit using a third-order spline fit, coefficients were determined, and unknowns were converted to concentrations.

Statistical analysis

Statistical analysis for BACE1 and Aβ levels between WT and PCSK9 KO mice was performed by Student's *t*-test.

RESULTS

Direct interaction of PCSK9 with VLDLR and ApoER2 in vitro

Previous studies assessing the interaction of PCSK9, VLDLR, and ApoER2 have generated inconsistent results (13, 16, 21). In COS-M cells, PCSK9 binding was only observed when either the LDLR EGF-A domain or a D354L mutation was introduced within the overexpressed VLDLR (13). However, other studies demonstrated an interaction between PCSK9 and both VLDLR and ApoER2 in CHO-A7 cells (21). Since direct interaction between purified PCSK9, VLDLR, and ApoER2 proteins was recently demonstrated using a cell-free binding assay (16), we sought to investigate whether purified PCSK9 bound VLDLR and ApoER2 using SPR (**Fig. 1**). In agreement with previous reports (29, 31), the K_D for PCSK9 binding to LDLR at pH 7.4 was 386 nM (Fig. 1A). PCSK9 also bound VLDLR with a similar affinity (K_D = 379 nM) (Fig. 1B). In addition, PCSK9 was found to directly bind ApoER2 with a relatively weaker affinity (K_D = 516 nM) (Fig. 1C). PCSK9 did not bind either carbonic anhydrase or streptavidin (data not shown), suggesting that the PCSK9 interaction with LDLR, VLDLR, and ApoER2 was specific. Together, these data demonstrate that purified PCSK9 can bind to LDLR, VLDLR, and ApoER2 with comparable affinities in vitro (Fig. 1D).

PCSK9 does not regulate LDLR, VLDLR, or ApoER2 levels in the adult mouse brain

Next, we investigated whether PCSK9 played a role in regulating LDLR, VLDLR, and ApoER2 in the CNS, where these receptors are highly expressed. Detergent extracts obtained from the hippocampus and cortex of four- to six-month-old male WT, PCSK9 KO, and PCSK9 TG mice were analyzed for steady-state receptor expression levels by Western blotting. While overexpression of human PCSK9 in brain tissue was confirmed by Western blotting with an antibody specific to human PCSK9 (**Fig. 2A**), many commercially available antibodies generated against mouse PCSK9 and additional antibodies raised against human PCSK9 were tested in WT and KO mouse tissue without success in detecting endogenous mouse PCSK9 (data not shown). Due to the unavailability of antibodies that could effectively detect mouse PCSK9 by Western blotting, we

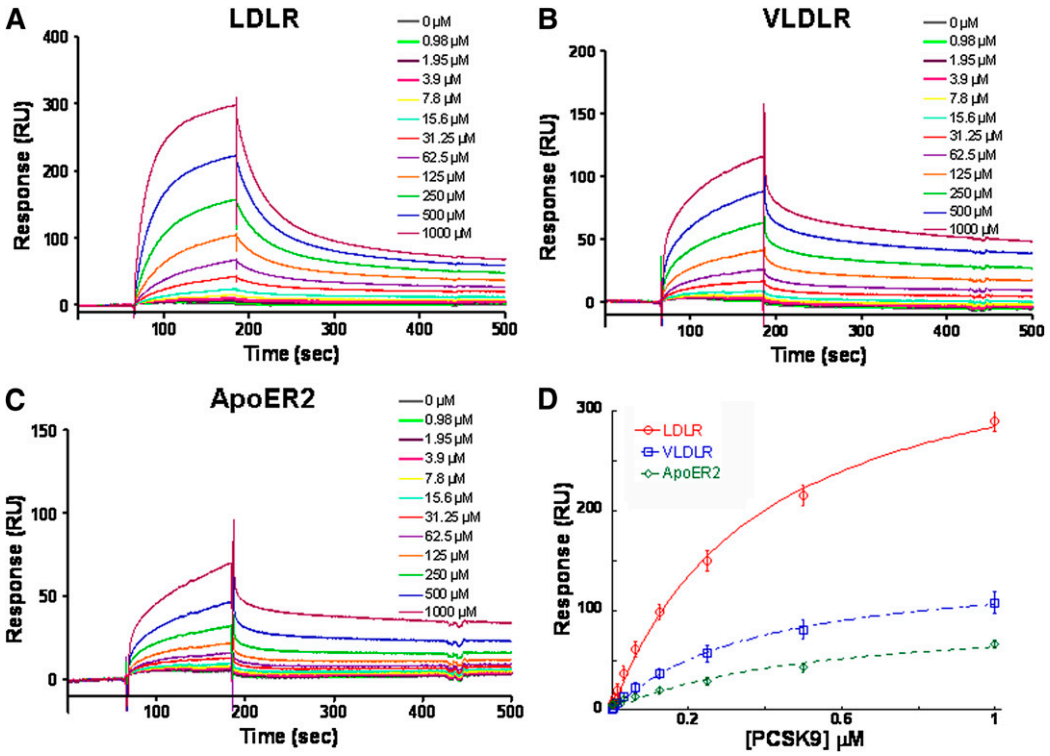

Fig. 1. Binding of PCSK9 to LDLR, VLDLR. and ApoER2. A: PCSK9-LDLR binding measured by surface plasmon resonance (Biacore). Representative sensorgram of response units (RU) versus time upon injection of increasing amounts of purified wild-type human PCSK9 over immobilized LDLR ectodomain. B: PCSK9-VLDLR binding measured by Biacore. C: PCSK9-ApoER2 binding measured by Biacore. D: Replot of RU at the end of the association phase versus concentration for purified PCSK9 injected over LDLR (red circle), VLDLR (blue square), and ApoER2 (green diamond). All data were fit to determine the dissociation constant of the interaction. ApoER2, apolipoprotein E receptor 2; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; VLDLR, very low density lipoprotein receptor.

compared tissue expression levels of PCSK9 in WT and KO mice by determining PCSK9 mRNA levels using RT-PCR. We observed approximately 87% and 100% reduction in PCSK9 mRNA in KO mice compared with WT for the cerebellum and liver, respectively (data not shown).

The deletion of PCSK9 in KO mice was also confirmed by examining LDLR levels in the liver. As expected, while PCSK9 TG mice had markedly reduced hepatic LDLR levels, PCSK9 KO mice showed elevated LDLR protein levels in the liver compared with WT mice (Fig. 2B). However, in contrast to the liver, LDLR steady-state levels in both the hippocampus and cortex were not altered by either deletion or overexpression of PCSK9 (Fig. 2A).

Similar findings were obtained for the other LDLR receptor family members examined (**Fig. 3A**, B). When corrected to the β-actin loading control, steady-state levels of VLDLR and ApoER2 were not altered by PCSK9 deletion or overexpression in the hippocampus and cortex. Recombinant mouse VLDLR and ApoER2 proteins were used to demonstrate the specificity of the antibodies and confirm the immunoreactive bands. In summary, these results suggested that despite indication of the direct interaction between PCSK9 and LDLR receptor family members in vitro, altering PCSK9 levels in vivo did not change the steady-state level of LDLR family members in the adult mouse brain.

PCSK9 does not affect BACE1 levels or APP processing in the adult mouse brain

BACE1 and Aβ levels were previously reported to be significantly increased in the cortex of PCSK9 KO mice (28). However, in contrast to these earlier findings, we found no evidence that overexpression or deletion of PCSK9 affected the steady-state levels of BACE1 in the mouse cortex (**Fig. 4A**). We also examined the steady-state levels of full-length APP and the APP *C*-terminal fragment (β-CTF) produced following β-cleavage by BACE1 and before γ-cleavage to release Aβ using BACE1 KO mouse brain as control. In support of our finding for the BACE1 levels, APP and APP β-CTF were not altered in PCSK9 KO and PCSK9 TG mice compared with WT controls (Fig. 4A).

To further confirm the lack of effect of PCSK9 deletion on BACE1 levels in the mouse brain, we evaluated a larger set of three- to four-month-old male PCSK9 KO and WT C57BL6 mice (n = 10 per group). By Western blotting analysis, BACE1 levels (detected with two different BACE1 antibodies) and LDLR expression levels were again similar in PCSK9 KO and WT mouse cortex (Fig. 4B, C). In the same set of animals, we also found that PCSK9 deletion did not alter the levels of DEA-soluble brain Aβ40, the predominant amyloidogenic fragment released after BACE1 and subsequent γ-secretase cleavage of APP (Fig. 4D). Altogether, the present data suggest that PCSK9 does not

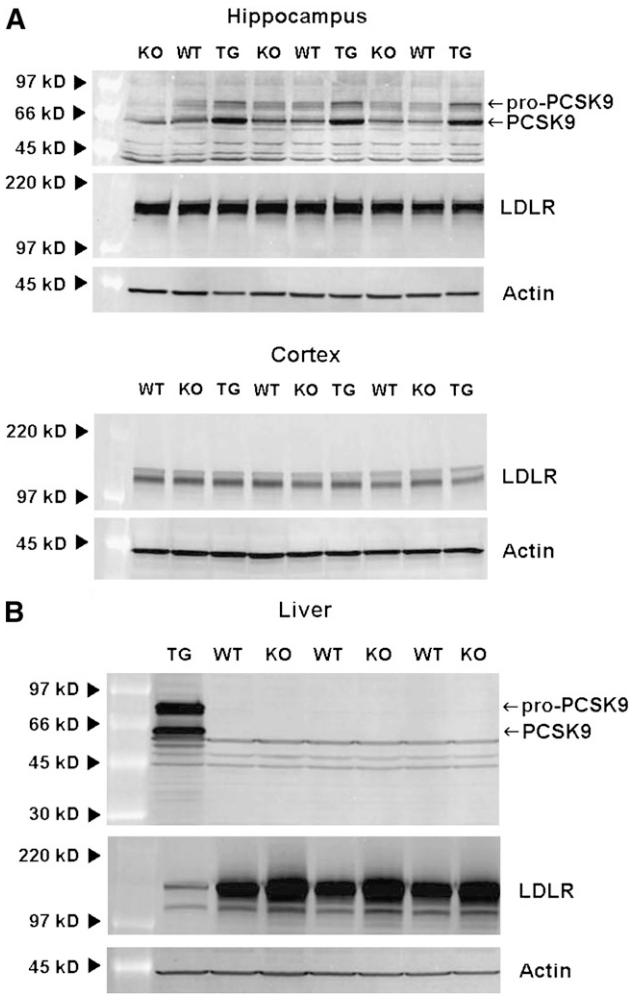

Fig. 2. LDLR protein levels were not regulated by PCSK9 expression in the adult mouse brain. The levels of PCSK9 and LDLR in liver and brain homogenates from WT, PCSK9 KO, and TG mice were evaluated by Western blot analysis as described in "Materials and Methods." Equal amounts of sample protein were loaded per lane and actin was used as an additional loading control for all immunoblots. A: Immunoblots showing PCSK9 and LDLR protein levels in the hippocampus and cortex of WT, PCSK9 KO, and PCSK9 TG mice. B: Immunoblots showing PCSK9 and LDLR protein levels in the liver of WT, PCSK9 KO, and PCSK9 TG mice. KO, knockout; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9 TG, transgenic mice carrying the human PCSK9 gene; VLDLR, very low density lipoprotein receptor; WT, wild type.

appear to play a role in modulating the steady-state levels of BACE1 or the amyloidogenic process of APP in the adult mouse brain.

DISCUSSION

Many studies suggest that inhibition of PCSK9 binding to LDLR and subsequent lowering of circulating LDL-cholesterol can be an effective approach to treat hypercholesterolemia. Gain-of-function mutations in PCSK9 are associated with hypercholesterolemia and autosomal dominant hypercholesterolemia (ADH), whereas loss-of-function mutations in PCSK9 are associated with hypocholesterolemia and decreased incidence of cardiovascular disease (32).

In addition to the well-established role of LDLR in regulating circulating LDL levels and, therefore, the risk of CHD, there is increasing evidence suggesting that LDLR and other LDLR family members may also participate in AD pathology, primarily by regulating the levels of apolipoprotein E (apoE) (33, 34). ApoE is the main lipid transporter in the CNS, and the *ε4* allele of apoE is a well-established genetic risk factor for AD (35). ApoE is known to bind Aβ, and it colocalizes with amyloid plaques in the AD brain (36). Recent findings from LDLR transgenic mice suggest that apoE mediates the clearance of Aβ via binding to CNS LDLR (37). It has been also proposed that altered signaling by apoE receptors at synapses may underlie the AD risk factor of the apoE4 isoform (26, 38).

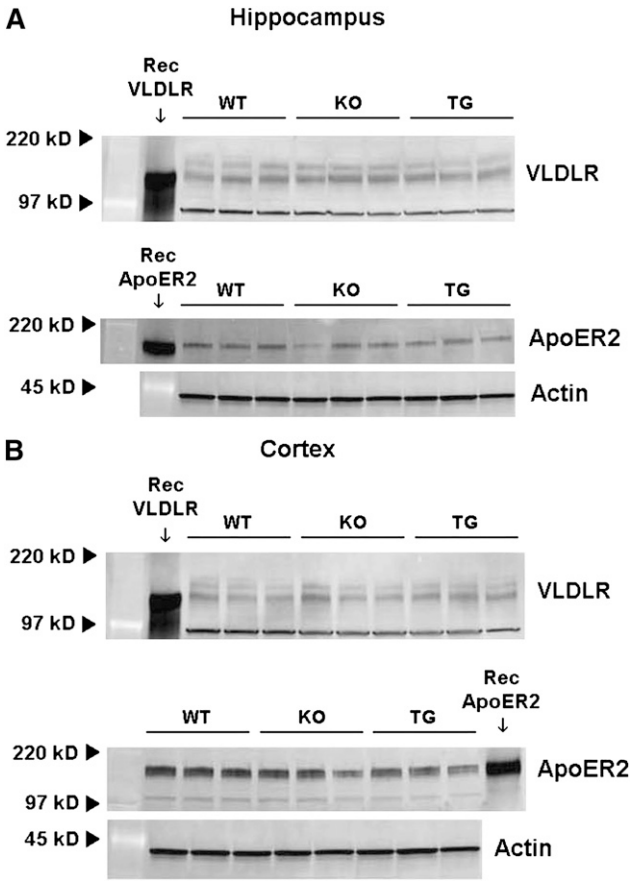

Fig. 3. PCSK9 expression did not alter VLDLR and ApoER2 levels in the adult mouse brain. Protein levels of VLDLR and ApoER2 in the hippocampus and cortex of PCSK9 KO and PCSK9 TG mice were compared with WT mice by Western blot analysis as described in "Materials and Methods." Equal amounts of sample protein were loaded per lane and actin was used as an additional loading control for all immunoblots; recombinant mouse VLDLR and mouse ApoER2 were included as positive controls. A: Immunoblots showing VLDLR and ApoER2 protein levels in the hippocampus of WT, PCSK9 KO, and PCSK9 TG mice. B: Immunoblots showing VLDLR and ApoER2 protein levels in the cortex of WT, PCSK9 KO, and PCSK9 TG mice. KO, knockout; LDLR, low density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9 TG, transgenic mice carrying the human PCSK9 gene; VLDLR, very low density lipoprotein receptor; WT, wild type.

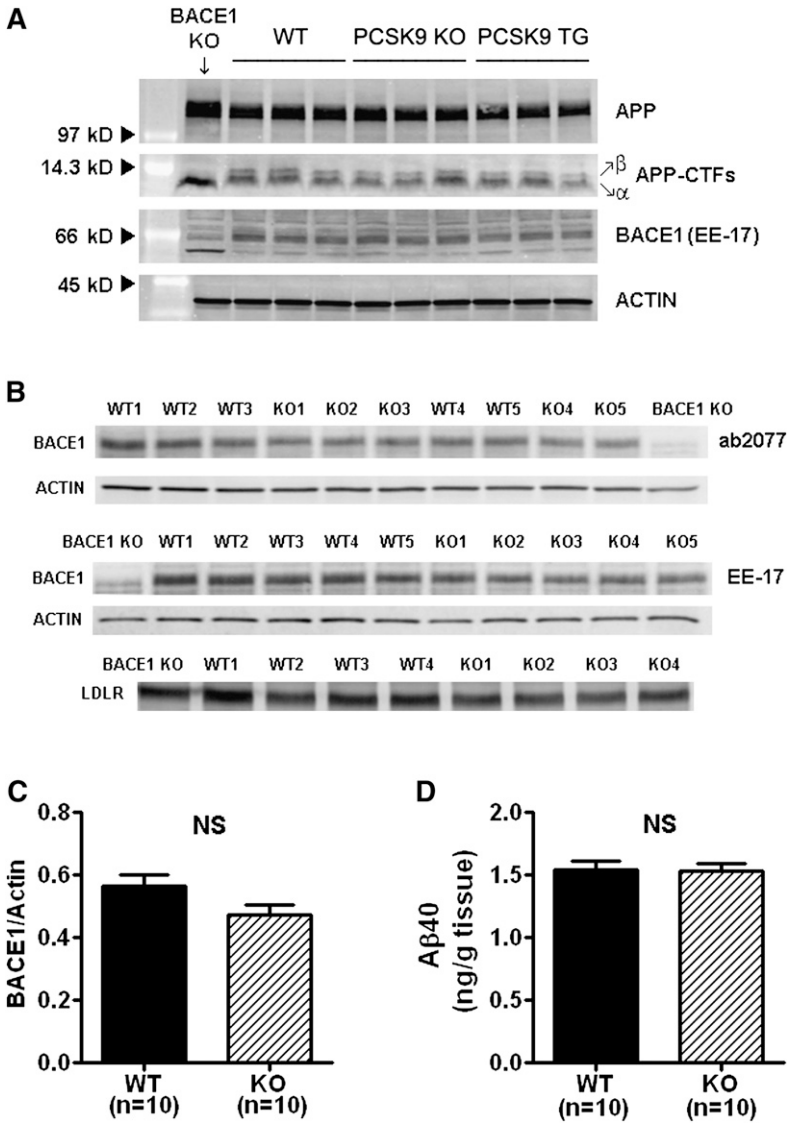

Fig. 4. BACE1, APP, and APP proteolytic fragments were similar in the brains of WT, PCSK9 TG, and PCSK9 KO mice. Brain homogenates from WT, PCSK9 TG, and PCSK9 KO mice were prepared as described in "Materials and Methods." Brain homogenate from BACE1 KO mice was used as control; equal amounts of sample protein were loaded per lane and actin was used as an additional loading control. A: Immunoblots showing full-length APP, APP-CTF fragments, and BACE1 protein levels in the cortex of WT, PCSK9 TG, and PCSK9 KO mice. B: Immunoblots showing BACE1 protein levels determined for additional WT and PCSK9 KO mice using two BACE1 antibodies, ab2077 (Abcam) and EE-17 (Sigma); C: Quantification of BACE1 levels was performed as described in "Materials and Methods" (n = 10 per group). D: Brain (cortex plus hippocampus) Aβ40 levels in WT and PCSK9 KO mice were determined by specific sandwich ELISA as described in "Materials and Methods" (n = 10 per group). BACE1, β-site amyloid precursor protein (APP)-cleaving enzyme 1; KO, knockout; LDLR, low density lipoprotein receptor; NS, no statistical significance by Student's *t*-test; PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9 TG, transgenic mice carrying the human PCSK9 gene; VLDLR, very low density lipoprotein receptor; WT, wild type.

LDLR, LDLR-related protein 1 (LRP1), VLDLR, and ApoER2 are all apoE receptors significantly expressed in the CNS. While LDLR and LRP1 are classic endocytic receptors involved in lipid transport, VLDLR and ApoER2 have been shown to function mainly as coreceptors for the reelin-mediated signaling important for proper neuronal migration to the cortical layers during development (38). Reelin signaling via VLDLR and ApoER2 also plays a role in the adult brain, where it participates in the regulation of N-methyl-D-aspartate (NMDA) receptor function during synapse formation and maintenance (39). Since reelin levels were found to be decreased in the brain of AD patients and in a mouse model of amyloidosis (40), PCSK9 inhibition leading to increased levels of VLDLR and ApoER2 could have the potential to increase reelin signaling and synaptic function in AD patients.

There has been inconsistent data regarding the interaction between PSCK9 and LDLR family members such as VLDLR and ApoER2 (13, 16, 21). We have confirmed by SPR that PCSK9 not only directly binds to LDLR, but also to VLDLR and ApoER2. However, while PCSK9 interacted with LDLR, VLDLR, and ApoER2 in a cell-free assay, there were no differences in the steady-state levels of these receptors in the brain of PCSK9 KO or PCSK9 TG mice when compared with WT mice, suggesting that PCSK9 is not a physiological regulator of these receptors in the CNS. One possible explanation for these differences is that PCSK9 function may be cell- and/or tissue-specific. While PCSK9-mediated degradation of endogenous LDLR was observed in HEK293, NIH 3T3, CHO-A7, CHO-K1, Neuro2A, HuH7, and HepG2 cells, no changes in LDLR degradation were detected in COS-1 cells incubated overnight with exogenous PCSK9 or in human fibroblasts overexpressing PCSK9 (21, 29, 41). In addition, injection of purified PCSK9 protein or adenovirus-mediated overexpression of PCSK9 in mice resulted in lowering of LDLR protein levels in the liver, lung, kidney, and small intestine, but it had no effect on LDLR levels in the adrenal gland (19, 20). Another possible explanation for our findings is that endogenous PCSK9 expression levels in the brain may not be sufficient to alter receptor levels. Indeed, PCSK9 mRNA level in the adult mouse brain is much lower than in the liver (data not shown). An alternative pathway for LDLR degradation was recently reported to be mediated by liver X receptors

(LXR) agonists via Idol, an ubiquitin-ligase that targets LDLR for degradation by the proteosome (42). Interestingly, this LXR-induced mechanism did not alter LDLR levels in the liver, suggesting that tissue-specific mechanisms appear to contribute to the steady-state levels of LDLR and perhaps other LDLR family members.

A recent study suggested that PCSK9 can modulate BACE1 protein levels in the CNS (28). BACE1 is the major β-secretase that cleaves APP to generate a soluble fragment (sAPPβ) and a membrane stub (β-CTF). Subsequent intramembrane cleavage of the β-CTF by the γ-secretase complex releases the amyloidogenic Aβ40 and Aβ42 peptides, which are believed to play a major role in AD pathology. Jonas et al. (28) reported that overexpression of PCSK9 in cell culture decreased BACE1 protein levels, and conversely, siRNA downregulation of PCSK9 increased BACE1 protein levels in vitro. The authors also showed increased BACE1 steady-state protein levels and increased brain Aβ levels with PCSK9 deletion in mice. These findings suggested that PCSK9 inhibition as therapy to reduce CHD could lead to increased risk for AD. However, in contrast to the previously published data, we found no changes in the steady-state levels of BACE1 protein in the brain of PCSK9 KO mice compared with WT controls. Furthermore, there were no changes in the overall levels of APP β-CTF or Aβ40 peptide levels, which would be expected to be elevated with increased BACE1 activity.

CONCLUSION

Our findings suggest that PCSK9 does not have a role in regulating LDLR family members or BACE1 protein levels in the adult mouse brain and that the development of PCSK9 therapies for CHD is likely not to be hampered by potential CNS adverse effects.

REFERENCES

1. Seidah, N. G., S. Benjannet, L. Wickham, J. Marcinkiewicz, S. B. Jasmin, S. Stifani, A. Basak, A. Prat, and M. Chretien. 2003. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. *Proc. Natl. Acad. Sci. USA.* **100:** 928–933.
2. Cohen, J., A. Pertsemlidis, I. K. Kotowski, R. Graham, C. K. Garcia, and H. H. Hobbs. 2005. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat. Genet.* **37:** 161–165.
3. Cohen, J. C., E. Boerwinkle, T. H. Mosley, and H. H. Hobbs. 2006. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N. Engl. J. Med.* **354:** 1264–1272.
4. Abifadel, M., M. Varret, J. P. Rabès, D. Allard, K. Ouguerram, M. Devillers, C. Cruaud, S. Benjannet, L. Wickham, D. Erlich, et al. 2003. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat. Genet.* **34:** 154–156.
5. Leren, T. P. 2004. Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia. *Clin. Genet.* **65:** 419–422.
6. Horton, J. D., J. C. Cohen, and H. H. Hobbs. 2007. Molecular biology of PCSK9: its role in LDL metabolism. *Trends Biochem. Sci.* **32:** 71–77.
7. Maxwell, K. N., and J. L. Breslow. 2004. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. *Proc. Natl. Acad. Sci. USA.* **101:** 7100–7105.
8. Rashid, S., D. E. Curtis, R. Garuti, N. N. Anderson, Y. Bashmakov, Y. K. Ho, R. E. Hammer, Y. A. Moon, and J. D. Horton. 2005. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proc. Natl. Acad. Sci. USA.* **102:** 5374–5379.
9. Lagace, T. A., D. E. Curtis, R. Garuti, M. C. McNutt, S. W. Park, H. B. Prather, N. N. Anderson, Y. K. Ho, R. E. Hammer, and J. D. Horton. 2006. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. *J. Clin. Invest.* **116:** 2995–3005.
10. Frank-Kamenetsky, M., A. Grefhorst, N. N. Anderson, T. S. Racie, B. Bramlage, A. Akinc, D. Butler, K. Charisse, R. Dorkin, Y. Fan, et al. 2008. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. *Proc. Natl. Acad. Sci. USA.* **105:** 11915–11920.
11. Graham, M. J., K. M. Lemonidis, C. P. Whipple, A. Subramaniam, B. P. Monia, S. T. Crooke, and R. M. Crooke. 2007. Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. *J. Lipid Res.* **48:** 763–767.
12. Maxwell, K. N., E. A. Fisher, and J. L. Breslow. 2005. Overexpression of PCSK9 accelerates the degradation of the LDLR in a postendoplasmic reticulum compartment. *Proc. Natl. Acad. Sci. USA.* **102:** 2069–2074.
13. Zhang, D. W., T. A. Lagace, R. Garuti, Z. Zhao, M. McDonald, J. D. Horton, J. C. Cohen, and H. H. Hobbs. 2007. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *J. Biol. Chem.* **282:** 18602–18612.
14. Kwon, H. J., T. A. Lagace, M. C. McNutt, J. D. Horton, and J. Deisenhofer. 2008. Molecular basis for LDL receptor recognition by PCSK9. *Proc. Natl. Acad. Sci. USA.* **105:** 1820–1825.
15. Bottomley, M. J., A. Cirillo, L. Orsatti, L. Ruggeri, T. S. Fisher, J. C. Santoro, R. T. Cummings, R. M. Cubbon, P. Lo Surdo, A. Calzetta, et al. 2009. Structural and biochemical characterization of the wild type PCSK9-EGF(AB) complex and natural familial hypercholesterolemia mutants. *J. Biol. Chem.* **284:** 1313–1323.
16. Shan, L., L. Pang, R. Zhang, N. J. Murgolo, H. Lan, and J. A. Hedrick. 2008. PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. *Biochem. Biophys. Res. Commun.* **375:** 69–73.
17. Duff, C. J., M. J. Scott, I. T. Kirby, S. E. Hutchinson, S. L. Martin, and N. M. Hooper. 2009. Antibody-mediated disruption of the interaction between PCSK9 and the low-density lipoprotein receptor. *Biochem. J.* **419:** 577–584.
18. Chan, J. C., D. E. Piper, Q. Cao, D. Liu, C. King, W. Wang, J. Tang, Q. Liu, J. Higbee, Z. Xia, et al. 2009. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. *Proc. Natl. Acad. Sci. USA.* **106:** 9820–9825.
19. Grefhorst, A., M. C. McNutt, T. A. Lagace, and J. D. Horton. 2008. Plasma PCSK9 preferentially reduces liver LDL receptors in mice. *J. Lipid Res.* **49:** 1303–1311.
20. Schmidt, R. J., T. P. Beyer, W. R. Bensch, Y. W. Qian, A. Lin, M. Kowala, W. E. Alborn, R. J. Konrad, and G. Cao. 2008. Secreted proprotein convertase subtilisin/kexin type 9 reduces both hepatic and extrahepatic low-density lipoprotein receptors in vivo. *Biochem. Biophys. Res. Commun.* **370:** 634–640.
21. Poirier, S., G. Mayer, S. Benjannet, E. Bergeron, J. Marcinkiewicz, N. Nassoury, H. Mayer, J. Nimpf, A. Prat, and N. G. Seidah. 2008. The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. *J. Biol. Chem.* **283:** 2363–2372.
22. Gåfvels, M. E., L. G. Paavola, C. O. Boyd, P. M. Nolan, F. Wittmaack, A. Chawla, M. A. Lazar, M. Bucan, B. O. Angelin, and J. F. Strauss 3rd. 1994. Cloning of a complementary deoxyribonucleic acid encoding the murine homolog of the very low density lipoprotein/apolipoprotein-E receptor: expression pattern and assignment of the gene to mouse chromosome 19. *Endocrinology.* **135:** 387–394.
23. Kim, D. H., H. Iijima, K. Goto, J. Sakai, H. Ishii, H. J. Kim, H. Suzuki, H. Kondo, S. Saeki, and T. Yamamoto. 1996. Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain. *J. Biol. Chem.* **271:** 8373–8380.
24. Weeber, E. J., U. Beffert, C. Jones, J. M. Christian, E. Forster, J. D. Sweatt, and J. Herz. 2002. Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. *J. Biol. Chem.* **277:** 39944–39952.
25. Motoi, Y., M. Itaya, H. Mori, Y. Mizuno, T. Iwasaki, H. Hattori, S. Haga, and K. Ikeda. 2004. Apolipoprotein E receptor 2 is involved

in neuritic plaque formation in APP sw mice. *Neurosci. Lett.* **368:** 144–147.
26. Durakoglugil, M. S., Y. Chen, C. L. White, E. T. Kavalali, and J. Herz. 2009. Reelin signaling antagonizes beta-amyloid at the synapse. *Proc. Natl. Acad. Sci. USA.* **106:** 15938–15943.
27. Vassar, R., B. D. Bennett, S. Babu-Khan, S. Kahn, E. A. Mendiaz, P. Denis, D. B. Teplow, S. Ross, P. Amarante, R. Loeloff, et al. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science.* **286:** 735–741.
28. Jonas, M. C., C. Costantini, and L. Puglielli. 2008. PCSK9 is required for the disposal of non-acetylated intermediates of the nascent membrane protein BACE1. *EMBO Rep.* **9:** 916–922.
29. Fisher, T. S., P. Lo Surdo, S. Pandit, M. Mattu, J. C. Santoro, D. Wisniewski, R. T. Cummings, A. Calzetta, R. M. Cubbon, P. A. Fischer, et al. 2007. Effects of pH and low density lipoprotein (LDL) on PCSK9-dependent LDL receptor regulation. *J. Biol. Chem.* **282:** 20502–20512.
30. Pandit, S., D. Wisniewski, J. C. Santoro, S. Ha, V. Ramakrishnan, R. M. Cubbon, R. T. Cummings, S. D. Wright, C. P. Sparrow, A. Sitlani, et al. 2008. Functional analysis of sites within PCSK9 responsible for hypercholesterolemia. *J. Lipid Res.* **49:** 1333–1343.
31. Cunningham, D., D. E. Danley, K. F. Geoghegan, M. C. Griffor, J. L. Hawkins, T. A. Subashi, A. H. Varghese, M. J. Ammirati, J. S. Culp, L. R. Hoth, et al. 2007. Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. *Nat. Struct. Mol. Biol.* **14:** 413–419.
32. Abifadel, M., J. P. Rabès, M. Devillers, A. Munnich, D. Erlich, C. Junien, M. Varret, and C. Boileau. 2009. Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease. *Hum. Mutat.* **30:** 520–529.
33. Fryer, J. D., R. B. Demattos, L. M. McCormick, M. A. O'Dell, M. L. Spinner, K. R. Bale, S. M. Paul, P. M. Sullivan, M. Parsadanian, G. Bu, et al. 2005. The low density lipoprotein receptor regulates the level of central nervous system human and murine apolipoprotein E but does not modify amyloid plaque pathology in PDAPP mice. *J. Biol. Chem.* **280:** 25754–25759.
34. Liu, Q., C. V. Zerbinatti, J. Zhang, H. S. Hoe, B. Wang, S. L. Cole, J. Herz, L. Muglia, and G. Bu. 2007. Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. *Neuron.* **56:** 66–78.
35. Strittmatter, W. J., A. M. Saunders, D. Schmechel, M. Pericak-Vance, J. Enghild, G. S. Salvesen, and A. D. Roses. 1993. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl. Acad. Sci. USA.* **90:** 1977–1981.
36. Bu, G. 2009. Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. *Nat. Rev. Neurosci.* **10:** 333–344.
37. Kim, J., J. M. Castellano, H. Jiang, J. M. Basak, M. Parsadanian, V. Pham, S. M. Mason, S. M. Paul, and D. M. Holtzman. 2009. Overexpression of low-density lipoprotein receptor in the brain markedly inhibits amyloid deposition and increases extracellular A beta clearance. *Neuron.* **64:** 632–644.
38. Herz, J. 2009. Apolipoprotein E receptors in the nervous system. *Curr. Opin. Lipidol.* **20:** 190–196.
39. Herz, J., and Y. Chen. 2006. Reelin, lipoprotein receptors and synaptic plasticity. *Nat. Rev. Neurosci.* **7:** 850–859.
40. Chin, J., C. M. Massaro, J. J. Palop, M. T. Thwin, G. Q. Yu, N. Bien-Ly, A. Bender, and L. Mucke. 2007. Reelin depletion in the entorhinal cortex of human amyloid precursor protein transgenic mice and humans with Alzheimer's disease. *J. Neurosci.* **27:** 2727–2733.
41. Park, S. W., Y. A. Moon, and J. D. Horton. 2004. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. *J. Biol. Chem.* **279:** 50630–50638.
42. Zelcer, N., C. Hong, R. Boyadjian, and P. Tontonoz. 2009. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science.* **325:** 100–104.