

CC Chemokine Receptor 2 Deficiency Aggravates Cognitive Impairments and Amyloid Pathology in a Transgenic Mouse Model of Alzheimer's Disease

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Circulating monocytoïd cells have the ability to infiltrate nervous tissue, differentiate into microglia, and clear amyloid- β ($A\beta$) from the brain of mouse models of Alzheimer's disease. Interaction between the chemokine CCL2 and its CC chemokine receptor 2 (CCR2) plays a critical role in the recruitment of inflammatory monocytes into the injured/diseased brain. Here, we show that CCR2 deficiency aggravates mnemonic deficits and amyloid pathology in transgenic mice expressing the chimeric mouse/human β -amyloid precursor protein and presenilin 1 ($APP_{Swe}/PS1$). Indeed, memory impairment was accelerated and enhanced in $APP_{Swe}/PS1/CCR2^{-/-}$ mice. Apparition of cognitive decline occurred earlier (i.e., at 3 months of age before plaque formation) and correlated with intracellular accumulation of soluble oligomeric forms of $A\beta$. Memory deficits worsened with age and were aggravated in $APP_{Swe}/PS1/CCR2^{-/-}$ mice compared with their respective control groups. Soluble $A\beta$ assemblies increased significantly in $APP_{Swe}/PS1$ mice in a context of CCR2 deficiency, whereas the plaque load remained relatively similar in the brain of aging $APP_{Swe}/PS1$ and $APP_{Swe}/PS1/CCR2^{-/-}$ mice. However, CCR2 deficiency stimulated the expression of TGF- β 1, TGF- β receptors, and CX₃CR1 transcripts in plaque-associated microglia, a pattern that is characteristic of an antiinflammatory subset of myeloid cells. A decreased expression of CCR2 could play a potential role in the etiology of Alzheimer's disease, a neurodegenerative pathology that could be treated by a genetic upregulation of the transgene in monocytoïd cells.

Introduction

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized by a progressive decline in memory. The pathological hallmarks of AD are senile plaques, intracellular neurofibrillary tangles, accumulation of activated microglia and astrocytes around β -amyloid ($A\beta$) plaques, and degenerative neurons (Selkoe, 2002). The $A\beta$ peptide is produced by amyloid precursor protein (APP) cleavage, and two main $A\beta$ isoforms exist (i.e., $A\beta_{1-40}$ and $A\beta_{1-42}$). $A\beta$ can form different oligomeric assemblies, such as monomers, dimers, or $A\beta^{*56}$, to finally generate amyloid plaques (Lesné et al., 2006; Haass and Selkoe, 2007). These senile plaques contain densely packed $A\beta$ fibrils and a large number of molecules and cellular elements. In particular, microglia, the mononuclear phagocytes of the brain (Perry and Gordon, 1988), are attracted around amyloid plaques and originate in part from the periphery (Malm et al., 2005; Stalder et al., 2005; Simard et al., 2006).

Monocytes constitutively express several chemokine receptors, which induce leukocyte migration and attraction by binding to their

specific chemotactic cytokines, or chemokines (Luster, 1998; Charo and Ransohoff, 2006; Auffray et al., 2009). CC chemokine receptor 2 (CCR2) is mainly expressed on the surface of monocytes (Mack et al., 2001). In mice, two principal subsets of monocytes are defined by CCR2 expression, CX₃CR1^{lo}CCR2⁺GR1⁺ inflammatory and CX₃CR1^{hi}CCR2⁻GR1⁻ resident monocytes (Geissmann et al., 2003). CCR2 is involved in the brain infiltration of monocytes in a mouse model for multiple sclerosis (Izikhon et al., 2000), at sites of axonal injury in the hippocampus (Babcock et al., 2003) and in systemic organs during inflammation (D'Mello et al., 2009). The main CCR2 chemokine ligand is monocyte chemotactic protein-1 (MCP-1) [CCL2 (CC chemokine ligand 2)] and is expressed in many cells, including endothelial cells (Cushing et al., 1990; Rollins et al., 1990), astrocytes (Glabinski et al., 1996; Smits et al., 2002), and microglia (Meda et al., 1995; El Khoury et al., 2003; Deshmane et al., 2009).

MCP-1 is upregulated in the brain of AD patients in mature senile plaques, microglia (Ishizuka et al., 1997), and microvessels (Grammas and Ovase, 2001). MCP-1 expression is also increased in the brain of AD transgenic mice (Simard et al., 2006; El Khoury et al., 2007) and $A\beta$ stimulates MCP-1 production in cultures of microglia (Meda et al., 1995; El Khoury et al., 2003) and astrocytes (Smits et al., 2002). Together, these data strongly suggest a predominant role of CCR2 in AD, since CCR2 is the key signaling receptor for MCP-1 (Deshmane et al., 2009). This was recently evidenced by a study using APP Tg2576 mice, a mouse model of AD developing the hallmarks of the disease tardily (9–10 months

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Table 1. Plasmids and enzymes used for the synthesis of the cRNA probes

Plasmid	Vector	Insert (bp)	Antisense probe	Sense probe	Source
Mouse CX3CR1	PCR II Topo	1132	BamHI/T7	XhoI/Sp6	Cloned by PCR
Mouse MCP-1	pGEM-1	578	BamHI/T7	SacI/Sp6	Dr. S. C. Williams (Texas Tech University, Lubbock, TX)
Mouse TGF- β 1 R1	PCR II Topo	1093	NotI/Sp6	SpeI/T7	Cloned by PCR
Mouse TGF- β 1 R2	PCR II Topo	933	XhoI/Sp6	BamHI/T7	Cloned by PCR
Mouse TGF- β 1	pBSKS+	1173	EcoRI/T3	XhoI/T7	Dr. S. Lacroix (Laval University, Quebec City, Quebec, Canada)

of age) (Hsiao et al., 1996). Unfortunately, their breeding with CCR2^{-/-} mice produced APP-CCR2^{-/-} mice dying prematurely (i.e., before A β plaque formation or microglia recruitment around A β deposits) (El Khoury et al., 2007), severely limiting the *in vivo* study of disease development. To overcome this limitation, we used another APP transgenic mouse model, the APP_{Swe}/PS1 mice, a well established model of AD (Borchelt et al., 1997; Simard et al., 2006; Richard et al., 2008; Boissonneault et al., 2009).

The main objective of this study was to follow progression of the disease in a context of CCR2 gene deficiency. All our results converge into a very important beneficial role of CCR2-expressing cells in AD.

Materials and Methods

Transgenic mice. We used adult male C57BL/6 mice [wild type (WT)], CCR2^{-/-} (B6.129S4-Ccr2tm1Ifc/J), and APP transgenic mice (APP_{Swe}/PS1) harboring the chimeric mouse/human β -amyloid precursor protein (APP695swe) and the human presenilin 1 (A246E variant) under the control of independent mouse prion promoter elements [B6C3-Tg(APP695)3Dbo Tg(PSEN1)5Dbo/J]. APP_{Swe}/PS1 were bred with the CCR2^{-/-} mouse strain for three or more generations to generate APP_{Swe}/PS1/CCR2^{-/-} triple transgenic animals. All mouse strains were purchased from The Jackson Laboratory and maintained in a C57BL/6J background. All newborn pups were genotyped as described in The Jackson Laboratory protocol. A total of 201 mice were used to study behavioral and biochemical modifications from 3 to 12 months of age. Mice were housed at three to five per cage and acclimated to standard laboratory conditions (12 h light/dark cycle; lights on at 7:00 A.M. and off at 7:00 P.M.) with *ad libitum* access to mouse chow and water. Animal breeding and experiments were conducted according to Canadian Council on Animal Care guidelines, as managed by the Laval University Animal Care Committee.

Water T-maze. Mice were tested during the “lights-on” phase of the day. The behavioral experimenter was blinded to the genetic and treatment status of the animals. To assess hippocampal-dependent spatial learning and memory, mice were trained in the water T-maze task. In this paradigm, we evaluate the mouse's ability to remember the spatial location of a submerged platform. The T-maze apparatus (length of stem, 64 cm; length of arms, 30 cm; width, 12 cm; height of walls, 16 cm) was made of clear fiberglass and filled with water (23 \pm 1°C) at a height of 12 cm. A platform (11 \times 11 cm) was placed at the end of the target arm and was submerged 1 cm below the surface. The acquisition phase allows to evaluate animals for left–right spatial learning. During the first two trials, platforms were placed on each arms of the maze to test the spontaneous turning preference of the mouse. After these two trials, the least chosen arm was reinforced by the escape platform. The mice were placed in the stem of the T-maze and chose to swim either left or right until they found the submerged platform and escaped to it, to a maximum of 60 s. After reaching the platform, the mice remained on it for 20 s and then were immediately put back in the maze. If the animals did not find the platform within this time limit, they were gently guided onto it. Repeated trials were presented on the same day up to a maximum of 48 trials. A rest period of at least 10–15 min was allowed between each block of 10 trials. A mouse was considered to have learned the task when it made no errors in a block of five consecutive trials. The reversal learning phase was then conducted 48 h later. During this phase, the same protocol was repeated, except that the mice were trained to find the escape platform on the side

opposite to that on which they had learned during the acquisition phase. The number of trials needed to reach the criterion (five of five correct choices made on consecutive trials) was noted as well as the latency for finding the escape platform.

Passive avoidance test. Based on the animal's natural tendency to prefer the dark environment, mice were also evaluated on their retention of nonspatial memory for one-trial passive avoidance task. The passive avoidance apparatus (Ugo Basile) was divided into two sections, one illuminated (start compartment) and the other dark (escape compartment). The floor of each compartment contained a grid, with only the dark compartment being electrified by a generator. On the training day, mice were placed into the lighted (start) compartment for a 60 s acclimation period. The guillotine door was then opened, and the latency to enter the dark side was recorded. Immediately after entering the dark compartment, the door was closed and an electric shock (0.5 mA for 2 s) was delivered. The mouse was kept in the dark compartment for 10 s before being returned to its home cage. On the next day, the mice were again placed in the lighted compartment, and the step-through latency time to enter the dark side was measured for up to 300 s.

Tissue analyses. Transgenic mice were anesthetized under isoflurane and blood was drawn via cardiac puncture before decapitation. Brains were rapidly removed from the skulls and placed in cold PBS solution. Then hemibrains were separated and olfactory bulbs and cerebellum were removed. One hemibrain was rapidly frozen in liquid nitrogen and stored at -80°C for protein analysis. The other one was postfixed for 2–4 d in 4% paraformaldehyde, pH 9.5, at 4°C , and then placed in a paraformaldehyde solution containing 10% sucrose overnight at 4°C . The frozen brains were mounted on a microtome (Reichert-Jung) and cut into 25 μm coronal sections. The slices were collected in cold cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, and 20% glycerol) and stored at -20°C until immunocytochemistry or *in situ* hybridization histochemistry.

In situ hybridization and immunohistochemistry. Every 12th section of brain slices, starting from the end of the olfactory bulb to the end of the cerebral cortex, was mounted on Colorfrost/Plus microscope slides (Thermo Fisher Scientific) for APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice (3–12 months of age; $n = 5$ –12 per group). Histochemical localization of CX₃CR1, TGF- β 1, and TGF- β receptors R1 and R2 by *in situ* hybridization was performed using ³⁵S-labeled cRNA probes. Plasmids were linearized, and sense and antisense cRNA probes were synthesized with the appropriate RNA polymerase, as described in Table 1. Riboprobe synthesis and preparation, as well as *in situ* hybridization were performed according to a protocol described previously (Laflamme et al., 1999; Nadeau and Rivest, 2000; Laflamme and Rivest, 2001; Naert et al., 2009).

Dual labeling combining immunocytochemistry and *in situ* hybridization was performed as described previously (Nadeau and Rivest, 2000; Laflamme and Rivest, 2001) to detect CX₃CR1, TGF- β 1, TGF- β R1, and TGF- β R2 transcripts in microglia (iba-1⁺-staining). We used polyclonal rabbit anti-ionized calcium binding adaptor molecule 1 (iba-1) (1:3000; Wako Chemicals).

The expression level of each transcript was estimated by qualitative analysis using a Nikon Eclipse 80i microscope over the whole-brain sections for 5–10 animals/group. Quantitative analysis of hybridization signal for CX₃CR1 mRNA was performed on images captured using a Nikon Eclipse 80i microscope equipped with a digital camera (QImaging) and the ImageJ software. Three coronal sections were chosen for prefrontal cortex at +2.58, +2.34, and +2.10 mm from the bregma according to a stereotaxic atlas (Paxinos and Franklin, 2001) and four

sections for hippocampus/cortex at -1.70 , -1.94 , -2.46 , and -2.92 mm. For each structure, labeling area was measured for three to four animals and means were used for statistical analysis (mean \pm SEM).

All images were captured using a Nikon Eclipse 80i microscope equipped with a digital camera (QImaging), processed to enhance contrast and sharpness using Adobe Photoshop 7 (Adobe Systems), and then assembled using Adobe Illustrator (Adobe Systems). The images depicted by the different panels are representative of the signal detected on the slides for each group of mice.

Stereological analysis. An observer who was blind to the treatment status of the material did all quantitative histological analyses. To count $A\beta$ plaques and plaque-associated microglia, sections of APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice (3–12 months of age, $n = 7$ –15 per group) were immunostained for $A\beta$ (polyclonal mouse anti- $A\beta$ 6E10; 1:3000; Covance) and iba-1 proteins with DAPI (4',6'-diamidino-2-phenylindole), as previously reported (Simard et al., 2006; Richard et al., 2008). Two sections were chosen for prefrontal cortex at $+2.34$ and $+2.10$ mm from the bregma according to a stereotaxic atlas (Paxinos and Franklin, 2001) and four sections for hippocampus/cerebral cortex at -1.70 , -1.94 , -2.46 , and -2.92 mm. Unbiased stereological analysis was performed as described previously (Simard et al., 2006; Richard et al., 2008; Boissonneault et al., 2009). Briefly, the contours of the prefrontal cortex, the hippocampus, and the cortex areas were traced as virtual overlay on the streamed images and areas were calculated. For 3- and 6-month-old APP_{Swe}/PS1 mice, the area occupied by all $A\beta$ -labeled plaques was determined as well as the number of plaque-associated microglia in each structure. For 9- and 12-month-old APP_{Swe}/PS1 mice, the Stereo Investigator software (MicroBrightField) sequentially chose counting frames (670×500 mm) at every $2000 \mu\text{m}$ along the x -axis and every $1000 \mu\text{m}$ along the y -axis while automatically moving the motorized stage into the previously delimited zones in the prefrontal cortex, the hippocampus, and the cortex. The areas of analyzed structures and areas occupied by plaques were calculated, as well as the number of plaque-associated microglia in these counting frames. All parameters analyzed were reported to the whole section.

Protein extraction and detection of total $A\beta$ levels by Western blot. Proteins from hemi-forebrains were extracted using a modification of the procedure described by Lesné et al. (2006). All manipulations were done on ice to minimize protein degradation. One hemi-forebrain was placed in a 1 ml syringe with a 20 G needle. Five hundred microliters of buffer A [50 mM Tris-HCl, pH 7.6, 0.01% NP-40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor mixture] were added and 10 up-and-down passages were made to homogenize the tissue, followed by a 5 min centrifugation at 3000 rpm at 4°C. The supernatant (extracellular protein-enriched fraction) was then collected and frozen at -80°C . The insoluble pellet was suspended in 500 μl of TNT buffer (buffer B; 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, protease inhibitor mixture), followed by a 90 min centrifugation at 13,000 rpm at 4°C. The supernatant (cytoplasmic protein-enriched fraction) was then collected and frozen at -80°C . The pellet was suspended in 500 μl of buffer C (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EGTA, 3% SDS, 1% deoxycholate, 1

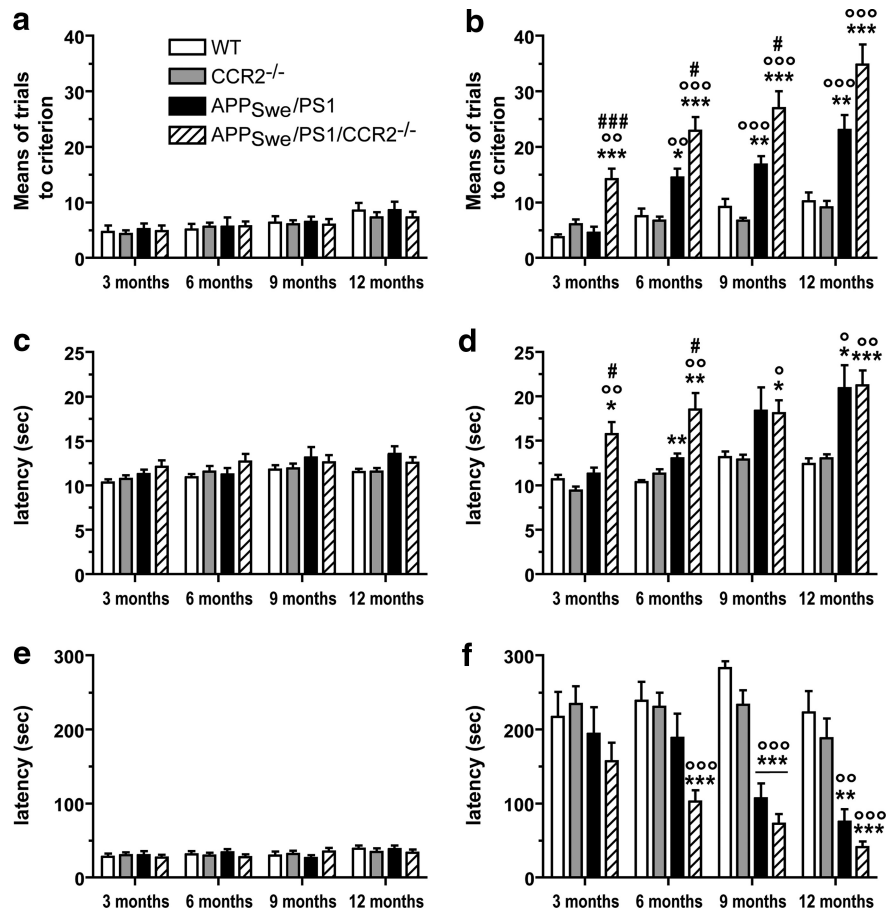


Figure 1. CCR2 deficiency accelerates the onset of spatial and contextual memory deficits and aggravates cognitive impairment in APP_{Swe}/PS1 mice. Spatial memory was assessed using the water T-maze test (**a–d**) in wild-type, CCR2^{-/-}, APP_{Swe}/PS1, and APP_{Swe}/PS1/CCR2^{-/-} mice aged from 3 to 12 months. The numbers of trials (**a, b**) and the latency (**c, d**) to accomplish the task were determined during the acquisition (**a, c**) and reversal learning phases (**b, d**). APP_{Swe}/PS1/CCR2^{-/-} mice exhibited an earlier and greater spatial memory decline than APP_{Swe}/PS1 mice. The passive avoidance test was used to establish contextual memory deficit (**e, f**). The latency was determined at 3–12 months of age during the acquisition phase (**e**) and 24 h after the conditioning test (**f**) for each group. The latency to enter the dark compartment was already decreased at 6 months of age in APP_{Swe}/PS1/CCR2^{-/-} mice compared with the control groups of mice. Results are expressed as mean \pm SEM; $n = 8$ –16 per group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. °Versus WT at same age; °°Versus CCR2^{-/-} at same age; °°°Versus APP_{Swe}/PS1 at same age. (Two-way ANOVA was performed and, for each parameter analysis, revealed a significant interaction between the factors of age and genotype. Then, one-way ANOVA at each level of age was performed using Bonferroni's or Tamhane's *post hoc* test.)

mM PMSF, protease inhibitor mixture) and incubated at 4°C, 50 rpm for 1 h. The samples were centrifuged for 90 min at 13,000 rpm and 4°C, and the supernatant (membrane protein-enriched fraction) was collected and frozen at -80°C . The protein concentration in each fraction was determined using the Quantipro bicinchoninic acid assay kit (Sigma-Aldrich) according to the manufacturer's protocol.

For total $A\beta$ detection, 10–30 μg of extracellular, cytoplasmic, and membrane protein fractions were separated on a precast 10–20% SDS polyacrylamide Tris-Tricine gel (Bio-Rad). Separated proteins were then transferred onto polyvinylidene fluoride membranes (PerkinElmer Life and Analytical Sciences) and detected by Western blotting. Blots were probed with a mouse anti- $A\beta$ protein monoclonal antibody clone 6E10 (1:1000; Covance) in a buffer containing 1 M Tris-HCl, pH 8.0, 5 M NaCl, 5% skim milk, and 0.05% Tween 20. Blots were visualized using an anti-mouse secondary antibody tagged with horseradish peroxidase (1:1000; Jackson ImmunoResearch) and enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences). Membranes were stripped in 25 mM glycine-HCl, pH 2.0, containing 1% SDS to detect β -actin using first a mouse β -actin antibody (MAB1501; 1:5000; Millipore Bioscience Research Reagents) and then a goat anti-mouse peroxidase-conjugated secondary antibody (1:1000; Jackson ImmunoResearch).

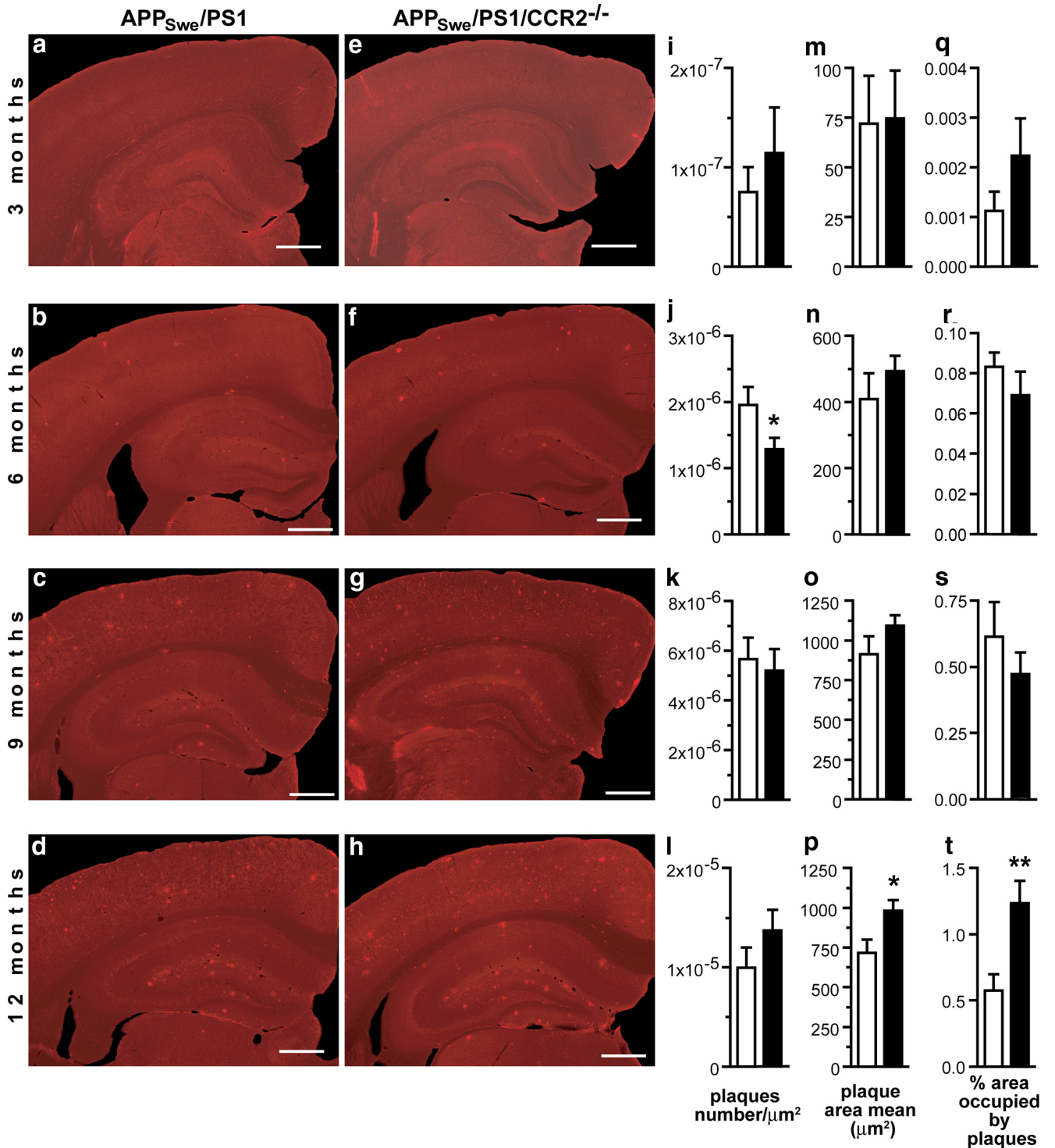


Figure 2. Amyloid plaque burden in the hippocampus of APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice. Brain sections of 3- to 12-month-old APP_{Swe}/PS1 (a–d) and APP_{Swe}/PS1/CCR2^{-/-} mice (e–h) were immunostained for Aβ. Unbiased stereological analysis of plaques was performed in hippocampus to determine plaque density (i–l), plaque size (m–p), and the area percentage occupied by plaques (q–t) of 3- to 12-month-old APP_{Swe}/PS1 (open bar) and APP_{Swe}/PS1/CCR2^{-/-} mice (solid bar). APP_{Swe}/PS1/CCR2^{-/-} and APP_{Swe}/PS1 mice had similar amyloid plaque formation at 3, 6, and 9 months of age. However, a significant higher Aβ load was found in the hippocampus of 12-month-old APP_{Swe}/PS1/CCR2^{-/-} mice. Results are expressed as mean ± SEM; n = 7–15 per group; Student's *t* test; **p* < 0.05 and ***p* < 0.01 versus APP_{Swe}/PS1 at same age. Scale bar, 500 μm.

Quantification was done by determining integrated density of the bands using a gel imaging system (scanner Agfa Arcus II; NIH ImageJ software, version 1.32j), and background values were subtracted. Optical values were normalized relatively to the actin loading control. Results are expressed as mean ± SEM.

Statistical analysis. Results are expressed as mean ± SEM. Statistical analysis was performed by one- or two-way ANOVA, followed by either

Bonferroni's or Tamhane's tests as appropriate, as *post hoc* comparisons (SPSS software). Data were analyzed using standard two-tailed unpaired *t* test for comparisons between two groups. Correlations were estimated by linear regression analysis and the Spearman's correlation coefficient (GraphPad Software) with *p* and *r* values and 95% confidence intervals included in the graph. A value of *p* < 0.05 was considered statistically significant.

Results

CCR2 deficiency aggravates cognitive impairment in APP_{Swe}/PS1 mice

To determine whether CCR2 deficiency affects disease progression, we bred APP_{Swe}/PS1 mice with CCR2-deficient animals. APP_{Swe}/PS1/CCR2^{-/-} mice appeared healthy and had a survival curve comparable with APP_{Swe}/PS1 mice, reaching >12 months of age (Fig. 1). Mice of each genotype from 3 to 12 months of age were submitted to hippocampus-based spatial learning in a water T-maze (Fig. 1*a–d*) and displayed similar swimming capacity and motivation to escape from water. During this acquisition phase, the means of trials (Fig. 1*a*) and the latency (Fig. 1*c*) to reach the criterion were similar between mice independently of genotype and age. During the reversal phase, 3- to 12-month-old WT and CCR2^{-/-} mice were able to learn the novel platform location. APP_{Swe}/PS1 mice exhibited memory impairment starting at 6 months of age and this deficit worsened with age (Fig. 1*b,d*). The spatial memory was affected earlier and to a greater extent in APP_{Swe}/PS1/CCR2^{-/-} mice, which exhibited a significant increase in the number of trials made (14.2 ± 1.8 vs 4.6 ± 1.1 for APP_{Swe}/PS1; $p < 0.001$) starting at 3 months of age, and the time spent to reach the platform (15.7 ± 1.4 vs 11.3 ± 0.7 for APP_{Swe}/PS1; $p < 0.05$). The larger decline of spatial memory seen in APP_{Swe}/PS1/CCR2^{-/-} mice progressed gradually with age, as evidenced by the number of trials at 6 and 9 months of age (22.9 ± 2.4 vs 14.5 ± 1.6 for APP_{Swe}/PS1; $p < 0.05$ and 26.9 ± 3.1 vs 16.8 ± 1.5 for APP_{Swe}/PS1; $p < 0.05$, respectively) and by the increased latency at 6 months (18.5 ± 1.8 vs 13.0 ± 0.5 for APP_{Swe}/PS1; $p < 0.05$). At 12 months of age, no difference in spatial memory was shown with the water T-maze task, suggesting that memory impairment had reached a plateau at that age in both groups of APP_{Swe}/PS1 mice.

To assess contextual memory, mice were submitted to the passive avoidance test. All animals were trained to avoid a dark compartment and presented similar latency to enter the dark side from 3 to 12 months of age during the acquisition phase (Fig. 1*e*). Twenty-four hours after the conditioning test, both APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice exhibited a decreased latency, although it appeared earlier in APP_{Swe}/PS1/CCR2^{-/-} mice (Fig. 1*f*). At 6 months, a significantly decreased latency was observed only in APP_{Swe}/PS1/CCR2^{-/-} mice (103 ± 15 vs 239 ± 26 for WT; $p < 0.001$). This contextual memory impairment reached a significant level at 9 months in APP_{Swe}/PS1 mice (107 ± 20 vs 283 ± 9 for WT; $p < 0.001$). This cognitive decline was maintained with age and was similar at 9 and 12 months between the two groups of APP_{Swe}/PS1 mice.

These results demonstrate a greater cognitive impairment in APP_{Swe}/PS1/CCR2^{-/-} mice, which appeared earlier than in APP_{Swe}/PS1 mice, suggesting that CCR2 deficiency has deleterious effects on memory and learning processes.

CCR2 deficiency increases levels of soluble A β oligomers without changing A β deposition

The major hallmark of AD is A β accumulation in brain, which correlates strongly with mnemonic impairment (Selkoe, 2002). We first quantified the plaque load by stereological analyses on coronal sections stained with the 6E10 antibody specific for the 1–17 fragment of the human A β protein. Similar A β plaque burden was found in APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice from 3 to 9 months of age in hippocampus (Fig. 2), prefrontal cortex (Fig. 3, left column), and cerebral cortex (Fig. 3, right column). Plaque number, plaque size, and area occupied by A β plaques increased with age in both groups. At 12 months of age, the area

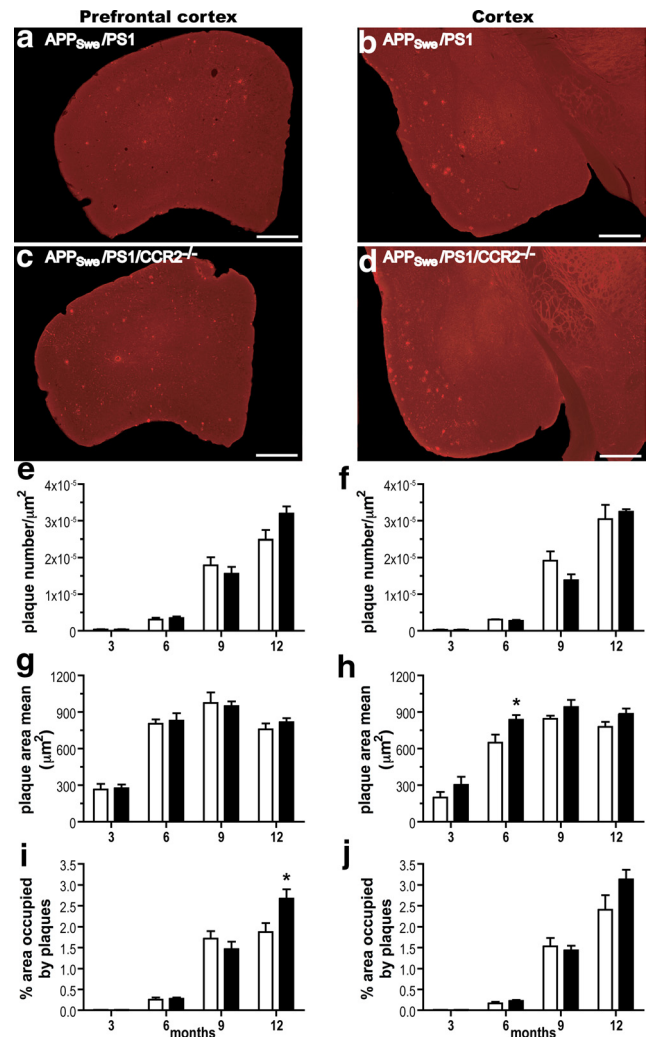


Figure 3. Amyloid plaque formation in the prefrontal cortex and cerebral cortex of APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice. Anti-A β immunoreactivity is depicted in the prefrontal cortex (left column) and cerebral cortex (right column) of APP_{Swe}/PS1 (*a, b*) and APP_{Swe}/PS1/CCR2^{-/-} mice (*c, d*) at 9 months of age. A detailed analysis of plaque quantification was performed to determine plaque density (*e, f*), plaque size (*g, h*), and the area percentage occupied by plaques (*i, j*) of 3- to 12-month-old APP_{Swe}/PS1 (open bar) and APP_{Swe}/PS1/CCR2^{-/-} mice (solid bar). Amyloid plaque formation was similar in both groups at 3, 6, and 9 months of age, although A β plaque load significantly increased in the prefrontal cortex of 12-month-old APP_{Swe}/PS1/CCR2^{-/-} mice. Results are expressed as mean \pm SEM; $n = 7–15$; Student's *t* test; * $p < 0.05$ vs APP_{Swe}/PS1 at the same age. Scale bar, 500 μ m.

occupied by plaques in prefrontal cortex (Fig. 3*i*) and hippocampus (Fig. 2*t*) was larger in APP_{Swe}/PS1/CCR2^{-/-} mice ($2.67 \pm 0.22\%$, $p < 0.05$, and $1.24 \pm 0.17\%$, $p < 0.001$, respectively) than APP_{Swe}/PS1 littermates (1.87 ± 0.21 and $0.57 \pm 0.12\%$, respectively).

These results do not support a significant correlation between plaque formation and the occurrence of cognitive impairment in a context of CCR2 deficiency. It is important to mention that the severity of dementia in AD patients correlates more strongly with cortical levels of soluble A β species than with insoluble A β plaque burden (Lue et al., 1999; McLean et al., 1999). Moreover, memory deficits also correlate with soluble A β oligomers instead of A β plaque in mouse models of AD (Lesné et al., 2006; Cheng et al., 2007). Therefore, we next determined levels of soluble A β in the extracellular- and intracellular-enriched, and membrane-associated protein fractions by Western blot, using the method published by Lesné et al. (2006).

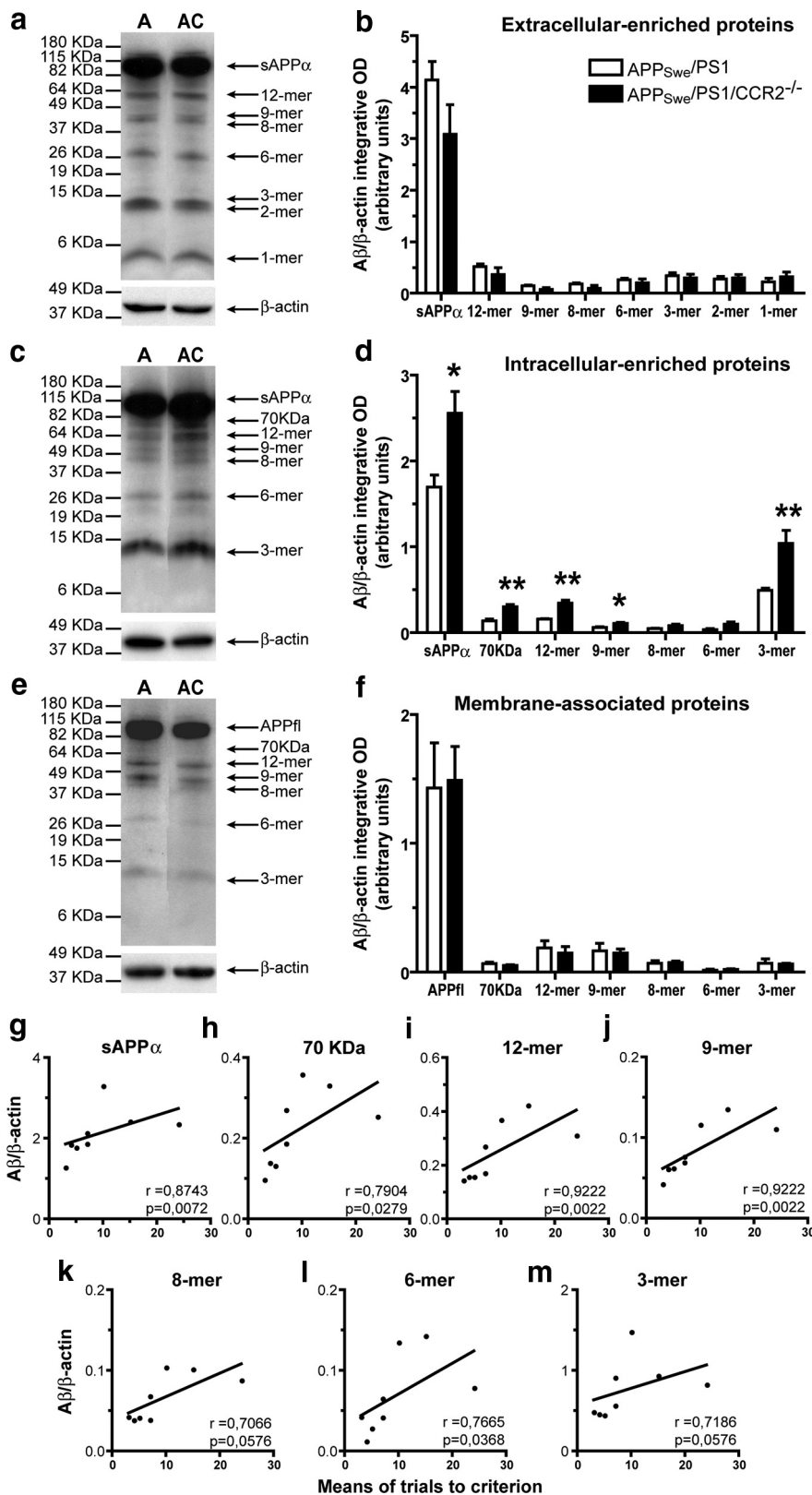


Figure 4. Soluble intracellular Aβ species are increased in 3-month-old APP_{Swe}/PS1/CCR2^{-/-} mice and are strongly correlated with spatial memory decline. Hemibrain extracts from soluble extracellular (a), intracellular (c), and membrane-associated proteins (e) of 3-month-old APP_{Swe}/PS1 (A) and APP_{Swe}/PS1/CCR2^{-/-} (AC) mice were assessed by Western blot on a 10–20% Tris-Tricine denaturing polyacrylamide gel to separate Aβ species using anti-Aβ antibody 6E10. The intensity of each band was quantified by densitometric analysis and normalized per β-actin values. Ratios of Aβ species (Aβ/β-actin) are represented for extracellular (b), intracellular (d), and membrane-associated proteins (f). Please note the increase of soluble intracellular Aβ species in the brain of APP_{Swe}/PS1/CCR2^{-/-} mice. Positive significant correlations exist between spatial memory decline (water

T-maze test) and soluble intracellular Aβ species (Fig. 4g–m). We noted a significant positive correlation between the levels of most Aβ species and impairment of spatial memory, with a better correlation for high-molecular-mass assemblies (Fig. 4g–j).

Although Aβ levels in extracellular-enriched and membrane-associated fractions remained comparable in 6-month-old APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice (Fig. 5a,b; e,f, respectively), higher intracellular trimer Aβ levels were found in the brain of APP_{Swe}/PS1/CCR2^{-/-} (Fig. 5c,d). At 9 months of age, levels of soluble intracellular Aβ species were significantly increased in extracellular-, intracellular-enriched, and membrane-associated protein fractions of APP_{Swe}/PS1/CCR2^{-/-} (Fig. 5). These changes in extracellular and intracellular soluble Aβ vanished at 12 months of age, but APP_{Swe}/PS1/CCR2^{-/-} brains still had significantly higher 9-, 6-, and 5-mer levels in membrane-associated fractions (Fig. 5f).

These data provide evidence that levels of soluble Aβ oligomers in extracellular-, intracellular-enriched, and membrane-associated fractions increased in the brains of APP_{Swe}/PS1/CCR2^{-/-} mice and intracellular Aβ correlated strongly with early cognitive deficits in a context of CCR2 deficiency.

Enhanced microglial expression of TGF-β1 and TGF-β receptors in APP_{Swe}/PS1/CCR2^{-/-} mice

In addition to Aβ production and accumulation, microglia are attracted to amyloid deposits, are activated by Aβ, and express inflammatory molecules. In particular, TGF-β1, a pleiotropic cytokine

(water T-maze test) and soluble intracellular Aβ species (g–m) and, more importantly, for high-molecular mass products (g–j). Results are expressed as mean ± SEM; n = 4; Student's t test; *p < 0.05 and **p < 0.01, versus APP_{Swe}/PS1. Correlation test was performed using the Spearman's correlation coefficient.

with a central role in immune suppression (Li et al., 2006), was shown to contribute to A β deposition in a transgenic mouse model of AD (Wyss-Coray et al., 1997). We found a robust signal for TGF- β 1 and its receptors from 6 to 12 months of age in APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice (Fig. 6). The signal was mainly associated to senile plaques in prefrontal cortex, hippocampus, and cerebral cortex and all mRNAs are expressed in iba1-positive microglial cells (Fig. 6*c,f,i*). Qualitative analysis demonstrated an increase in the number of positive cells and the signal intensity for TGF- β 1 and TGF- β -R2 mRNA in APP_{Swe}/PS1/CCR2^{-/-} mice, whereas TGF- β -R1 mRNA levels were lower in 9- and 12-month-old APP_{Swe}/PS1/CCR2^{-/-} mice (Fig. 6*j*).

Enhanced recruitment of CX₃CR1 microglia surrounding A β plaques in APP_{Swe}/PS1/CCR2^{-/-} mice

Stereological analyses were performed on coronal brain sections immunostained for iba1 and A β to count plaque-associated microglia. At 3 months of age, APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice had a similar number of microglia around the few A β plaques (Fig. 7*f-h*). Microglia recruitment in the plaque vicinity reached a plateau at 6 months in APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice and more plaque-associated microglia were found in the cortex of APP_{Swe}/PS1/CCR2^{-/-} mice (0.0045 ± 0.0002 vs 0.0019 ± 0.0002 microglia/ μm^2 for APP_{Swe}/PS1; $p < 0.05$) (Fig. 7*h*). This enhanced recruitment of microglia became more pronounced in 9-month-old APP_{Swe}/PS1/CCR2^{-/-} mice and was significantly different from APP_{Swe}/PS1 mice for the prefrontal cortex (0.0027 ± 0.0003 vs 0.0038 ± 0.0002 microglia/ μm^2 ; $p < 0.001$), hippocampus (0.0034 ± 0.0003 vs 0.0024 ± 0.0002 microglia/ μm^2 ; $p < 0.01$), and cerebral cortex (0.0026 ± 0.0001 vs 0.0017 ± 0.0002 microglia/ μm^2 ; $p < 0.001$) (Fig. 7*f-h*). At 12 months of age, both groups of mice exhibited a similar recruitment in all regions studied. This increased recruitment was concomitantly observed with an enhanced expression of MCP-1. In fact, *in situ* hybridization was performed for MCP-1 mRNA (Fig. 7*a-d*) and showed a strong increase of MCP-1 signal in APP_{Swe}/PS1/CCR2^{-/-} mice from 3 to 12 months of age (Fig. 7*e*). In APP_{Swe}/PS1 mice, MCP-1 expression was induced later at 6 months and also increased with age (Fig. 7*e*). In addition, this increased recruitment correlated with a strong expression of CX₃CR1 mRNA in microglia (Fig. 8). Although the transcript was constitutively expressed in microglia in both groups of mice, APP_{Swe}/PS1/CCR2^{-/-} mice exhibited higher CX₃CR1 mRNA levels than APP_{Swe}/PS1 mice in the prefrontal cortex (data not shown) and cerebral cortex at 9

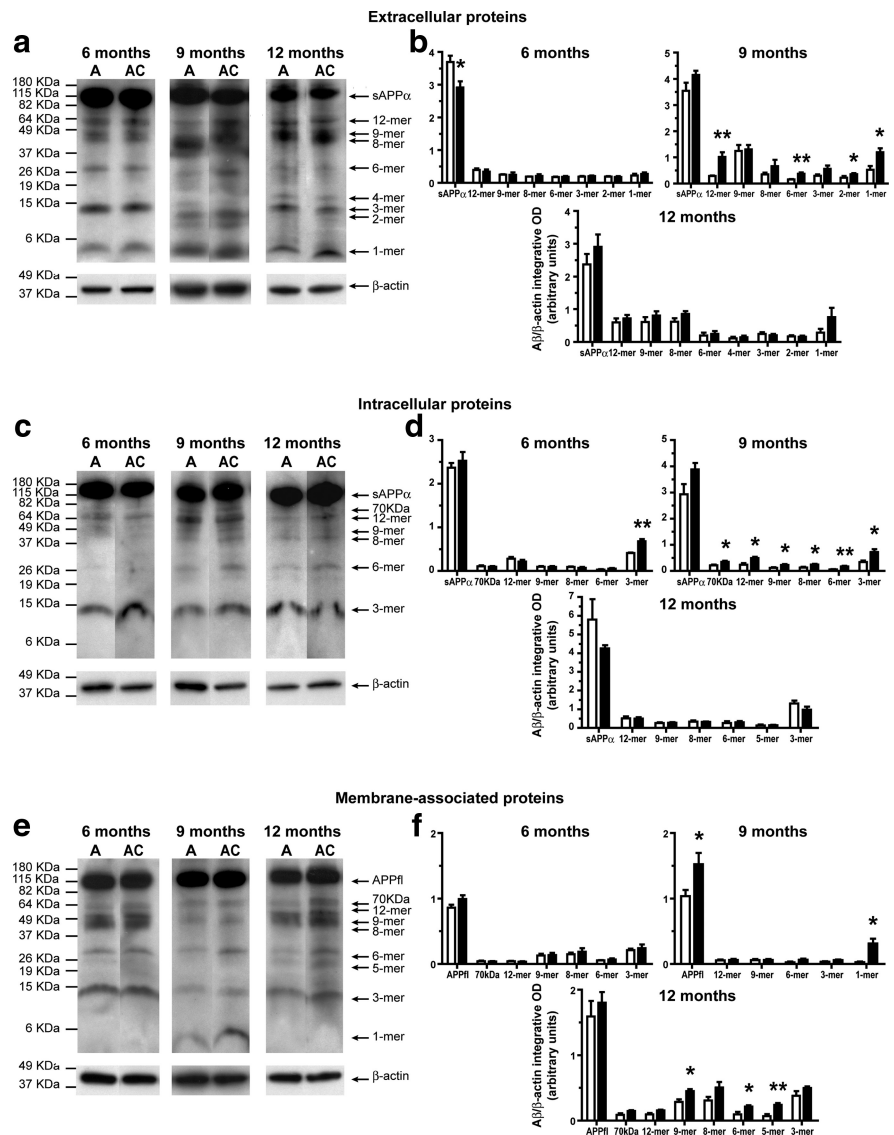


Figure 5. Age-dependent increase of soluble A β species in extracellular, intracellular, and membrane-associated fractions extracted from the brain of APP_{Swe}/PS1/CCR2^{-/-} mice. Hemibrain extracts from soluble extracellular (*a*), intracellular (*c*), and membrane-associated proteins (*e*) of 6- to 12-month-old APP_{Swe}/PS1 (A) and APP_{Swe}/PS1/CCR2^{-/-} (AC) mice were assessed by Western blot. The intensity of each band was quantified by densitometric analysis and normalized per β -actin values. A β species ratios (A β / β -actin) are represented for extracellular (*b*), intracellular (*d*), and membrane-associated proteins (*f*). Soluble A β species levels were generally higher in the brains of APP_{Swe}/PS1/CCR2^{-/-} mice than those of APP_{Swe}/PS1 mice. Results are expressed as mean \pm SEM; $n = 4-5$; Student's *t* test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. *Versus APP_{Swe}/PS1 at the same age.

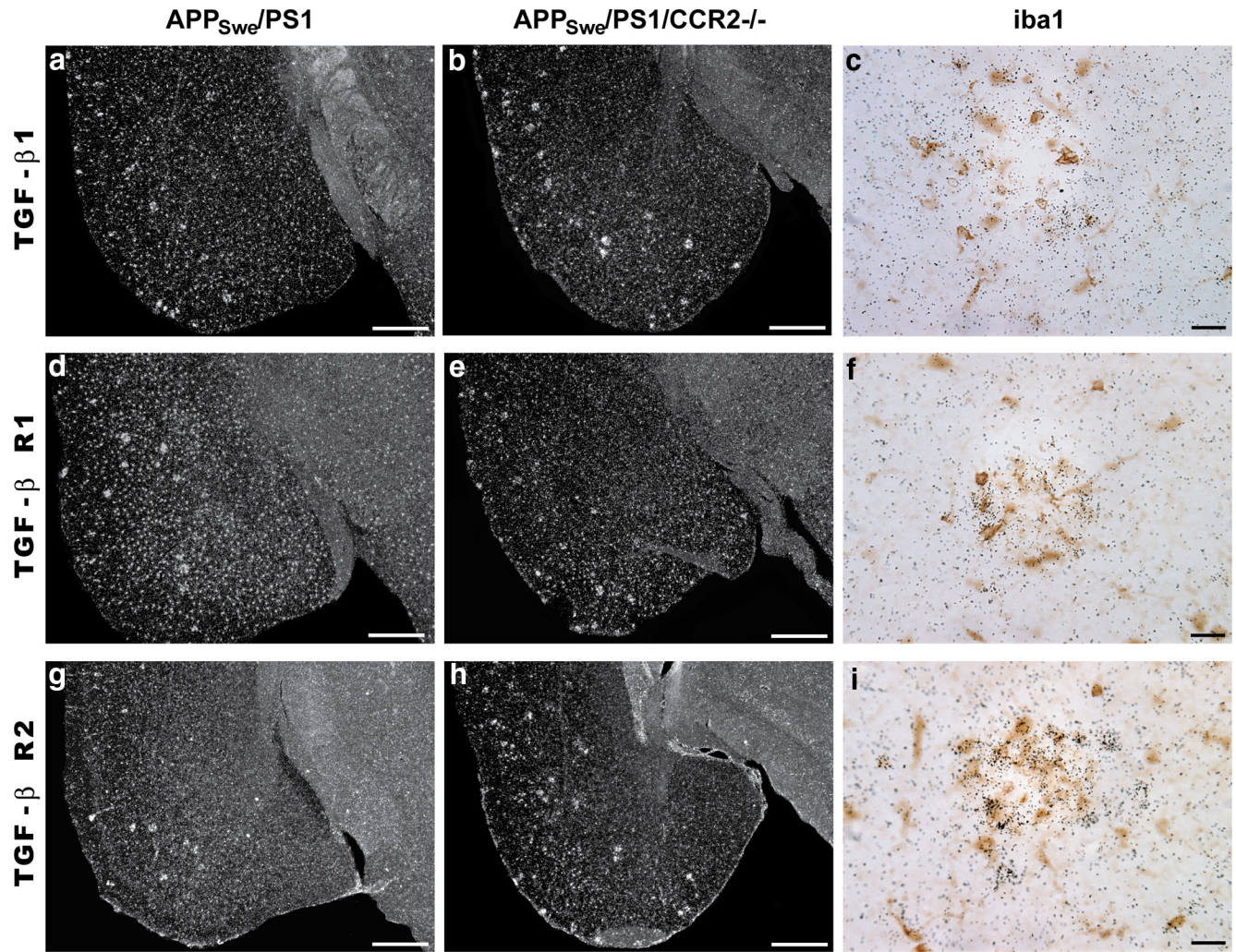
months of age (1.15 ± 0.10 vs $0.65 \pm 0.17\%$ in APP_{Swe}/PS1; $p < 0.05$) (Fig. 8*f*).

We have shown that, surprisingly, microglia recruitment in and around senile plaque is increased, strongly suggesting that a compensatory process takes place in the brain to counteract CCR2 deficiency.

Discussion

Our data offer new insight on the importance and role of microglia in the etiology of AD. We demonstrate that CCR2 deficiency in a mouse model of AD accelerated the onset of disease and aggravated mnemonic deficits and A β pathology.

AD brain contains soluble and insoluble assemblies of A β , both of which have been hypothesized to underlie dementia. However, the correlation between parenchymal amyloid deposi-



j age (months)	APP _{Swe} /PS1	APP _{Swe} /PS1/CCR2 ^{-/-}	APP _{Swe} /PS1	APP _{Swe} /PS1/CCR2 ^{-/-}	APP _{Swe} /PS1	APP _{Swe} /PS1/CCR2 ^{-/-}
	TGF-β1 mRNA		TGF-β R1 mRNA		TGF-β R2 mRNA	
3	∅	∅	∅	∅	∅	∅
6	+	++	+	++	+	++
9	+++	++++	++++	+++	++	+++
12	++++	+++++	+++++	++++	+++	++++

Figure 6. TGF-β1 and TGF-β receptor 1 and 2 mRNA levels are strongly increased in microglia surrounding plaques of CCR2-deficient APP_{Swe}/PS1 mice. Representative dark-field photomicrographs of *in situ* hybridization showing the cortical expression of TGF-β1 (a, b), TGF-β-R1 (d, e), and TGF-β-R2 mRNA (g, h) in the brain of 9-month-old APP_{Swe}/PS1 (left column) and APP_{Swe}/PS1/CCR2^{-/-} mice (middle column). Brain sections of 12-month-old APP_{Swe}/PS1/CCR2^{-/-} mice were stained with an anti-iba1 antibody and peroxidase-conjugated secondary antibody, and thereafter hybridized with a mouse TGF-β1 (c), TGF-β-R1 (f), or TGF-β-R2 (i) cRNA probe. Each transcript (agglomeration of silver grains) is clearly expressed in microglia associated to plaques (brown cells). Qualitative quantification was performed for each transcript in the whole brain of 3- to 12-month-old APP_{Swe}/PS1 and APP_{Swe}/PS1/CCR2^{-/-} mice (n = 5–10 per group). CCR2 deficiency clearly increased microglial expression of TGF-β1 and TGF-β-R2 around Aβ plaques of APP_{Swe}/PS1 mice. Scale bars: Left and middle panels, 500 μm; right panel, 20 μm.

tion and cognitive decline remains controversial. The amyloid hypothesis has been revised recently to include the notions that smaller soluble Aβ oligomers may play an early and significant role in AD and correlate with the impairment in cognitive function, learning, and memory (Lue et al., 1999; McLean et al., 1999; Lesné et al., 2006, 2008; Cheng et al., 2007; Selkoe, 2008). In this regard, we found no significant variation in Aβ deposits despite an early aggravation of memory impairment in APP_{Swe}/PS1/CCR2^{-/-}. Cognitive impairment occurs at 3 months of age when there are only few scattered plaques. By contrast, brain levels of soluble intracellular Aβ species are drastically increased at this age,

suggesting a rapid accumulation of Aβ species within neurons. The significant correlation between cognitive deficit and intracellular Aβ levels supports the hypothesis that intracellular Aβ impairs neuronal functions (Wirhns et al., 2004; LaFerla et al., 2007). These data confirm previous studies showing intraneuronal Aβ accumulation in different mouse models of AD (Billings et al., 2005; Cruz et al., 2006; Oakley et al., 2006; Knobloch et al., 2007) and in postmortem brain tissues from AD patients (Gouras et al., 2000, 2005). Intracellular Aβ species may therefore be responsible for the early AD-related cognitive deficits before Aβ plaque formation.

APP_{Swe}/PS1/CCR2^{-/-} mice exhibited increased intracellular levels of all A β species, suggesting the increase of A β production in their brain. In addition, inhibition of A β degradation could also participate in A β accumulation in the brain, since the expression of neprilysin/Mme, a major A β -degrading enzyme, is decreased in a context of CCR2 deficiency (El Khoury et al., 2007). Moreover, synaptic activity can reduce intraneuronal A β by Mme action and protect against A β -related synaptic alterations (Tampellini et al., 2009). In this regard, the expression of synaptic genes (e.g., EGR1, PSD95, and NR2A) is downregulated in the brain of 3-month-old APP_{Swe}/PS1/CCR2^{-/-} mice (data not shown). Since A β can be phagocytosed by microglia (Mandrekar et al., 2009) and astrocytes (Matsunaga et al., 2003; Nielsen et al., 2009), CCR2 deficiency can also affect A β clearance by preventing the recruitment of CCR2⁺ monocytes and contributing therefore to soluble A β accumulation. Such a mechanism is supported by the soluble extracellular A β levels (including 12-, 6-, 2-, and 1-mers), which were significantly higher in the brain of APP_{Swe}/PS1/CCR2^{-/-} mice.

Many soluble A β assemblies have been shown to induce cognitive impairment, such as extracellular A β *56 (Lesné et al., 2006) or soluble A β dimer and trimer (Cleary et al., 2005). Moreover, Mme overexpression fails to prevent learning and memory deficits despite restricting A β deposition in cortex and hippocampus. Interestingly, soluble A β trimers and A β *56 levels were not reduced in the brain of these APP mice (Meilandt et al., 2009). In the Tg2576 model, memory impairment begins when A β dimers start to accumulate (i.e., ~6 months before plaque formation) (Kawarabayashi et al., 2004). In AD patients, soluble intracellular and membrane-associated A β in temporal neocortex is more closely related to AD symptoms than other A β species (Steinerman et al., 2008). A β binding to cell membranes is therefore not negligible and could disrupt their fluidity and, therefore, synaptic activity and neurotransmission. Another important aspect of the amyloid cascade is that extracellular-, intracellular-, and membrane-associated pools of A β are tightly interconnected. For example, extracellular A β can increase its own production and then induce A β accumulation within neurons (Peters et al., 2009; Tampellini et al., 2009). Moreover, animals with amyloid plaques have normal memory function throughout an episode of reduced extracellular A β oligomers (including A β *56), which

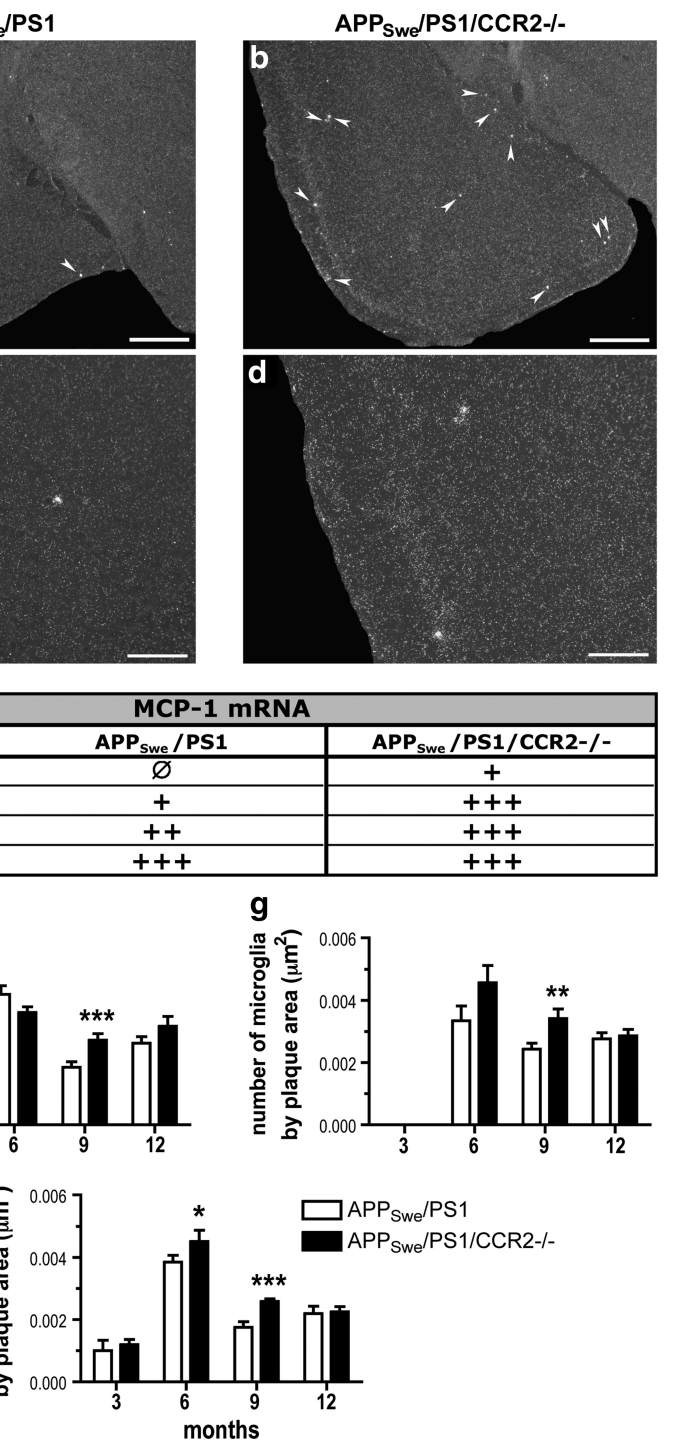


Figure 7. CCR2 deficiency increases MCP-1 expression and microglial recruitment around A β plaque formation in APP_{Swe}/PS1 mice. Representative dark-field photomicrographs of *in situ* hybridization showing MCP-1 expression in brains of 9-month-old APP_{Swe}/PS1 (left column) and APP_{Swe}/PS1/CCR2^{-/-} mice (right column). Qualitative analysis of brains hybridized with a MCP-1 probe from 3- to 12-month-old mice ($n = 5-10$ per group) shows an enhanced expression in APP_{Swe}/PS1/CCR2^{-/-} mice (e). Increased expression of MCP-1 is concomitant with an increase recruitment of microglia in the plaque vicinity in prefrontal cortex (f), hippocampus (g), and cortex (h). Results are expressed as mean \pm SEM; $n = 7-15$; Student's *t* test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. *Versus APP_{Swe}/PS1 at same age. Arrowheads, MCP-1 positive signal. Scale bars: a, b, 500 μ m; c, d, 200 μ m.

occurs during a period of accelerated amyloid plaque formation (Lesné et al., 2008). These data strongly suggest that the A β entities responsible for memory decline and disruption of synaptic plasticity in AD pathology are variable, but always associated to soluble fractions. Our study further reinforces this concept, be-

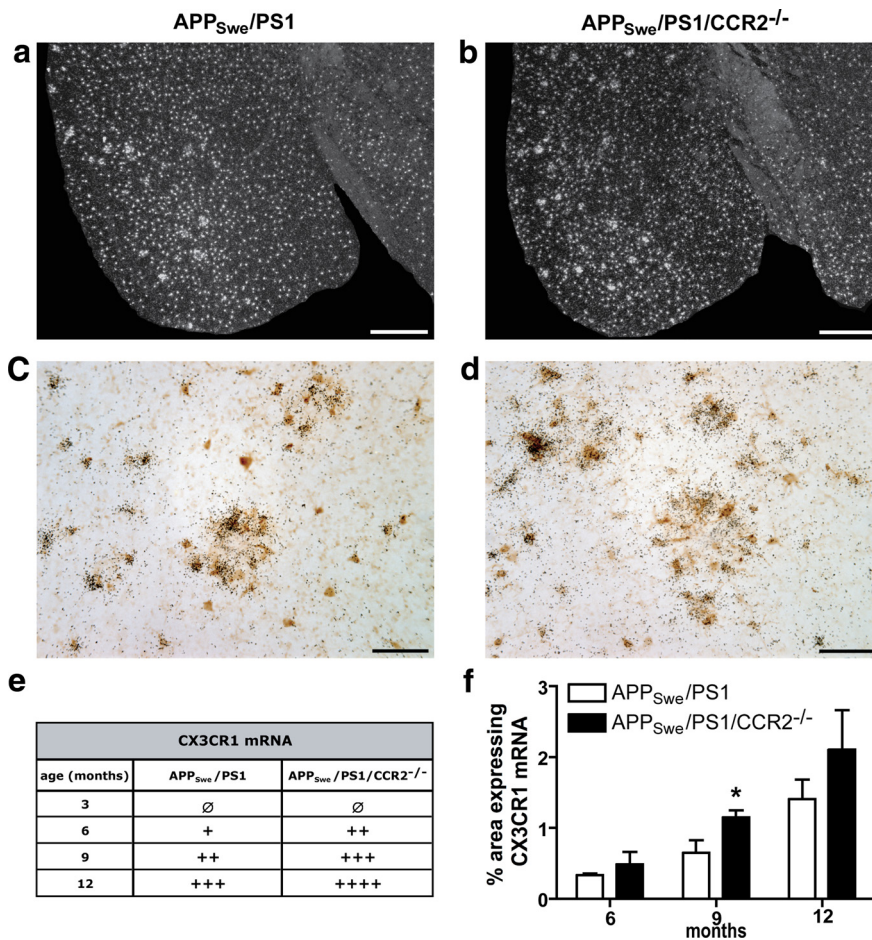


Figure 8. Increased expression of CX₃CR1 mRNA in microglia surrounding Aβ plaques of APP_{Swe}/PS1/CCR2^{-/-} mice. Representative dark-field photomicrographs of cortical brain sections hybridized with radioactive cRNA probe for CX₃CR1 mRNA of 9-month-old APP_{Swe}/PS1 (a) and APP_{Swe}/PS1/CCR2^{-/-} mice (b). CX₃CR1 mRNA signal always colocalized with iba1-immunoreactive cells (brown cells), as shown by double-labeled cells in 12-month-old APP_{Swe}/PS1 (c) and APP_{Swe}/PS1/CCR2^{-/-} mice (d). Note the particular ring formation of microglia strongly expressing CX₃CR1 mRNA. Qualitative analysis of CX₃CR1 indicated an increased expression signal from 3 to 12 months of age in APP_{Swe}/PS1/CCR2^{-/-} mice (e) (n = 3–5). Such a tendency was validated by measuring the area containing CX₃CR1-positive message in cortex on dark-field photomicrographs (f). Results are expressed as mean ± SEM; n = 3–4; Student's t test; *p < 0.05 versus APP_{Swe}/PS1 at same age. Scale bars: a, b, 500 μm; c, d, 50 μm.

cause soluble Aβ accumulation occurs before plaque formation in cognitively impaired young CCR2-deficient mice.

We expected to find a lower number of plaque-associated microglia in CCR2-deficient mice because of the inability of CX₃CR1^{lo}CCR2⁺GR1⁺ monocytes to infiltrate the brain despite enhanced MCP-1 gene expression in AD mouse models (Simard et al., 2006; El Khoury et al., 2007). Surprisingly, we found more microglia recruited around Aβ plaques in APP_{Swe}/PS1/CCR2^{-/-} mice than APP_{Swe}/PS1. Microglia are known to react quickly to Aβ production and subsequently migrate toward plaques (Bolmont et al., 2008). Despite similar Aβ deposits, microglia recruitment was enhanced in APP_{Swe}/PS1/CCR2^{-/-} mice from 6 months of age, suggesting a compensatory mechanism induced by the lack of CCR2-expressing cells. As CCR2^{-/-} cells cannot migrate in response to MCP-1 (El Khoury et al., 2007), the chemokine responsible for this strengthened attraction has yet to be identified. These plaque-associated microglia expressed strongly TGF-β1 and TGF-β1 receptor-R1 and -R2 transcripts, which were increased in the brain of APP_{Swe}/PS1/CCR2^{-/-} mice. Enhanced TGF-β1 levels have already been described in AD patients (van der Wal et al., 1993; Wyss-Coray et al., 1997; Rota et

al., 2006) and in our mouse model of AD (Richard et al., 2008). Although the role of TGF-β1 in AD pathology remains controversial, this cytokine binds Aβ with high affinity and enhances its oligomerization *in vitro* (Mousseau et al., 2003). Moreover, TGF-β1 overexpression accelerates and promotes amyloidogenesis in AD mice (Wyss-Coray et al., 1997, 2000, 2001). Interruption of TGF-β1 signaling in bone marrow-derived microglia/macrophages mitigates cognitive impairment and attenuates amyloidosis in AD mice (Town et al., 2008). Indeed, macrophage lacking TGF-β1 signaling phagocyte Aβ monomers and oligomers. By contrast, TGF-β1 in brain dampens microglial activation (Brionne et al., 2003). This effect of TGF-β1 could suppress the ability of macrophage/microglia to eliminate or restrict soluble Aβ oligomers and consequently aggravate memory deficits. These results further support previous studies suggesting a detrimental role of TGF-β system on cognitive impairment and Aβ accumulation.

Microglia surrounding Aβ plaques also strongly expressed CX₃CR1 transcripts. CX₃CR1 expression is enhanced in APP_{Swe}/PS1/CCR2^{-/-} mice, correlating with the degree of microglial recruitment. CX₃CR1 is primarily produced by neurons and signals via CX₃CR1 within microglia and macrophages (Harrison et al., 1998; Cardona et al., 2006). CX₃CR1 deficiency is neurotoxic in mouse models of Parkinson's disease, amyotrophic lateral sclerosis, and systemic lipopolysaccharide administration, suggesting that CX₃CR1–CX₃CR1 signaling in microglia is beneficial to neurons (Cardona et al., 2006). By contrast, memory impairment

and Aβ pathology are worsened in APP_{Swe}/PS1/CCR2^{-/-} mice, a phenomenon associated with increased recruitment of CX₃CR1-positive microglia and upregulation of CX₃CR1 transcript levels. These data point toward a detrimental role of CX₃CR1 in APP_{Swe}/PS1 mice. Indeed, a recent study demonstrated that CX₃CR1 deficiency prevents neuronal loss in a mouse model of AD (Fuhrmann et al., 2010). This discrepancy might be explained by a phenotypic change of microglia in a context of CCR2 deficiency. Indeed, the robust expression of TGF-β1, TGF-β1 receptors, and CX₃CR1 in microglia associated to plaques supports the idea that CCR2 deficiency gives rise to a phenotype of alternatively activated microglia (Mosser and Edwards, 2008), which are apparently unable to counteract AD pathology.

CCR2 is required for monocytes to emigrate from bone marrow (Serbina and Pamer, 2006; Tsou et al., 2007; Engel et al., 2008; Jia et al., 2008). Adoptive transfer studies with Ly-6C^{high} monocytes have shown that they rapidly return to bone marrow in the absence of active recruitment to inflammatory sites (Varol et al., 2007, 2009). Therefore, CCR2 deficiency drastically decreases the frequency of monocytes in blood, which could contribute to the detrimental effects of CCR2 deficiency in APP_{Swe}/

PS1. A “peripheral sink hypothesis” exists, based on the findings of active A β transport from the CNS into the periphery across the blood–brain barrier via the low-density lipoprotein receptor (Deane et al., 2004), and from the periphery into CNS via the receptor for advanced glycation end products (Shibata et al., 2000; Deane et al., 2003). The peripheral sink hypothesis proposes that even low levels of free A β in the blood may alter the dynamics of transport and thereby contribute to a net efflux of A β from the brain (DeMattos et al., 2001; Holtzman et al., 2002; Lemere et al., 2003). It would then be possible that CCR2-competent mononuclear cells participate in the clearance of blood A β and are part of such a “sink hypothesis” by carrying soluble A β species out of the brain.

In conclusion, we demonstrated that CCR2 deficiency in APP_{Swe}/PS1 mice provokes a rapid cognitive decline closely correlated with brain accumulation of soluble A β oligomers and a robust mRNA expression of TGF- β 1, TGF- β -R2, and CX₃CR1 in microglia. Our study supports a neuroprotective role of CCR2-competent cells in the pathophysiology of AD. We propose that such innate immune mechanisms may be deficient or suboptimal in AD patients and, therefore, that improving the availability of CCR2⁺ monocytes has considerable therapeutic potential.

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