Research article

Angiotensin-converting enzyme overexpression in myelomonocytes prevents Alzheimer’s-like cognitive decline

Kenneth E. Bernstein,1,2 Yosef Koronyo,3 Brenda C. Salumbides,3 Julia Sheyn,3 Lindsey Pelissier,3 Dahabada H.J. Lopes,3 Kandarp H. Shah,1 Ellen A. Bernstein,1 Dieu-Trang Fuchs,3 Jeff J.-Y. Yu,3 Michael Pharm,3 Keith L. Black,3 Xiao Z. Shen,1,3 Sebastien Fuchs,1,2,4 and Maya Koronyo-Hamaoui1,3

1Department of Biomedical Sciences, 2Department of Pathology and Laboratory Medicine, and 3Department of Neurosurgery and the Maxine Dunitz Neurosurgical Research Institute, Cedars-Sinai Medical Center, Los Angeles, California, USA. 4Department of Basic Medical Sciences, College of Osteopathic Medicine, Western University of Health Sciences, Pomona, California, USA.

Cognitive decline in patients with Alzheimer’s disease (AD) is associated with elevated brain levels of amyloid β protein (Aβ), particularly neurotoxic Aβ1–42. Angiotensin-converting enzyme (ACE) can degrade Aβ1–42, and ACE overexpression in myelomonocytic cells enhances their immune function. To examine the effect of targeted ACE overexpression on AD, we crossed ACE10/10 mice, which overexpress ACE in myelomonocytes using the c-fms promoter, with the transgenic APPΔE9 mouse model of AD (ADΔ). Evaluation of brain tissue from these ADΔACE10/10 mice at 7 and 13 months revealed that levels of both soluble and insoluble brain Aβ1–42 were reduced compared with those in ADΔ mice. Furthermore, both plaque burden and astrogliosis were drastically reduced. Administration of the ACE inhibitor ramipril increased Aβ levels in ADΔACE10/10 mice compared with the levels induced by the ACE-independent vasodilator hydralazine. Overall, ADΔACE10/10 mice had less brain-infiltrating cells, consistent with reduced AD-associated pathology, though ACE-overexpressing macrophages were abundant around and engulfing Aβ plaques. At 11 and 12 months of age, the ADΔACE10/WT and ADΔACE10/10 mice were virtually equivalent to non-AD mice in cognitive ability, as assessed by maze-based behavioral tests. Our data demonstrate that an enhanced immune response, coupled with increased myelomonocytic expression of catalytically active ACE, prevents cognitive decline in a murine model of AD.

Introduction

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly and is characterized by a progressive deterioration of cognitive and behavioral functions. Indeed, throughout the world, the incidence of AD is rising as the population ages; it is estimated that over 5 million Americans and well above 35 million people worldwide are currently living with AD (1). AD pathogenesis is intimately associated with elevated brain levels of amyloid β protein (Aβ) in both soluble and extracellular aggregated forms (Aβ plaques) (2, 3). Aβ alloforms are most commonly found as peptides of 38 to 43 amino acids, with Aβ1–42 being most closely associated with AD pathology (4–6). These peptides are the cleavage products of the transmembrane amyloid precursor protein (APP) and are naturally produced within the CNS throughout life. Cerebral Aβ accumulation and aggregation are thought to be one of the main causes of the neuronal loss and cognitive decline seen in AD (7). In addition, persistent neuroinflammation, consisting of reactive astrocytes and activated microglia that become cytotoxic in response to chronically elevated amyloidogenic Aβ, is thought to contribute to disease progression (8–11).

It is still unclear whether abnormalities leading to increased production of Aβ or deficits in its clearance are the primary culprits responsible for cerebral Aβ accumulation in nonfamilial AD (12–14). A recent clinical study suggests that the common sporadic cases are associated with insufficient Aβ clearance rather than increased production (15). While there can be poor correlation between the amount of Aβ plaque deposition and cognitive deterioration, a common view is that any strategy that reduces Aβ, particularly soluble and small oligomeric forms of Aβ1–42, will be advantageous in preventing AD progression (7, 16–18). Aβ removal is regulated by multiple mechanisms, including its degradation by several peptidases and phagocytosis by innate immune cells.

Angiotensin-converting enzyme (ACE) is a carboxy dipeptidase that is best known for the conversion of angiotensin I into the vasoconstrictor angiotensin II (19). However, ACE is a fairly nonspecific peptidase that has been shown to hydrolyze peptides as small as three amino acids and as large as Aβ1–42 (20–22). While large amounts of ACE are made by vascular endothelium, virtually every tissue makes some ACE (23). Because of this and because of the substrate promiscuity of the enzyme, ACE participates in many physiologic processes, though its precise role in clearing Aβ is not well understood.

We previously created a mouse model termed ACE10/10, in which gene targeting was used to inactivate the intrinsic Ace promoter and place Ace expression under the control of the c-fms promoter (24). As a result, ACE was overexpressed in myelomonocytic lineage cells (monocytes, macrophages, microglia, etc.), depending on the degree to which cells normally produce C-FMS. Homozygous ACE10/10 mice lack ACE expression by ECs and other tissues that do not recognize the c-fms promoter, while heterozygous ACE10/WT mice do maintain some endogenous ACE expression.
Using these mouse models, we demonstrated that ACE can also affect immune function (24–26). Specifically, ACE<sup>10/10</sup> mice had an increased immune response and decreased disease after challenge with tumors (24) or bacterial infections (25). This was due to the enhanced immune reactivity of ACE-overexpressing macrophages. The phenotype is transferable by BM transplantation and is directly attributable to ACE catalytic activity.

Numerous studies have suggested that the development of cerebro Aβ plaques elicits both a resident microglial and a blood-borne monocyteic response (27–35). Further, we found that following immunization, increased peripheral monocyte-derived macrophages homed to Aβ plaques and facilitated their clearance (31, 36). These and other studies suggest that BM-derived macrophages are more efficient than microglia in restricting Aβ plaques in mouse models of AD (29–31, 34–41). Once recruited to the brain, such cells appear to attenuate neuropahtology and improve cognitive function.

ACE<sup>10/10</sup> mice have two features that we hypothesized might contribute to reducing the progression of AD. Their macrophages have a markedly enhanced immune response, achieved by overexpression of ACE, an enzyme directly capable of cleaving Aβ<sub>42</sub> into less pathogenic peptides. Thus, we wondered whether the local delivery of an Aβ-cleaving enzyme by inflammatory cells would prove effective at reducing AD progression. Here, we report that the crossing of ACE<sup>10/10</sup> mice with a mouse model of AD results in a marked reduction in AD-like pathology and symptoms. Not only did these mice show a substantial decrease in levels of soluble and insoluble Aβ and diminished astrocytosis, but also a maze-based assessment of cognitive function showed behavior very similar to that of the control non-AD mice. Thus, an approach designed to enhance the peripheral immune response, coupled with increased inflammatory cell expression of an enzyme capable of cleaving Aβ (ACE), resulted in an essentially complete prevention of the cognitive decline observed in a model of AD.

**Results**

**Reduced plaque burden.** To study AD in ACE<sup>10/10</sup> mice, these animals were crossed with double-Tg APP<sub>Swe</sub>/PS<sub>1ΔE9</sub> (ADtg) mice. This is a well-established mouse model for AD, in which animals overproduce human Aβ peptides, develop plaques with age, and show progressive memory and learning deficits mimicking some aspects of human AD (42). The mouse cross-breeding eventually yielded ADtg (AD<sup>+</sup>) mice with WT ACE (AD<sup>ACE<sup>WT</sup>/WT</sup>) mice heterozygous for the ACE 10 allele (AD<sup>ACE<sup>10/WT</sup></sup>) and mice homozygous for this allele (AD<sup>ACE<sup>10/10</sup></sup>), as well as mice lacking the AD Tgs and WT for ACE (AD<sup>ACE<sup>WT/WT</sup></sup>). All mice were on a C57BL/6 background, and the ACE 10 alleles had no effect on blood pressure (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI66541DS1), similar to what was previously reported (24). Assessment of cerebral APP levels (43) showed no differences in the expression of APP among the different ACE genotypes (Supplemental Figure 2). At 3 months of age, brain Aβ levels were equivalent in AD<sup>ACE<sup>10/10</sup></sup> and AD<sup>ACE<sup>WT/WT</sup></sup> mice (Supplemental Figure 3).

An initial evaluation of plaque burden was qualitatively assessed in coronal brain sections from AD<sup>ACE<sup>WT/WT</sup></sup> and age- and gender-matched AD<sup>ACE<sup>10/10</sup></sup> mice that were 255 days (8.5 months) old. Paraffin sections were immunolabeled with anti-human Aβ mAb 6F/3D. This showed a marked reduction of cortical and hippocampal Aβ burden in AD<sup>ACE<sup>10/10</sup></sup> mice as compared with that in AD<sup>ACE<sup>WT/WT</sup></sup> mice (Figure 1A).

To precisely quantitate Aβ plaque pathology, cohorts of 7- to 8-month-old mice were studied, the age at which ADtg mice start to develop cognitive deficits and exhibit substantial neuropathy (44). To measure cerebral Aβ burden, serial coronal brain sections were immunolabeled with anti-human Aβ mAb 6E10. Sections were colabeled with thioflavine-S (Thio-S), which binds to fibrillary Aβ aggregates that are typical of mature plaques. Representative histologic sections showed a substantial reduction in 6E10<sup>+</sup> and Thio-S<sup>+</sup> Aβ plaque burden in both AD<sup>ACE<sup>10/10</sup></sup> and AD<sup>ACE<sup>10/10</sup></sup> mice as compared with that seen in AD<sup>ACE<sup>WT/WT</sup></sup> mice (Figure 1B). Quantitation of cortical and hippocampal plaque areas indicated a significant reduction in both 6E10<sup>+</sup> and Thio-S<sup>+</sup> plaque load in ADtg mice bearing the ACE 10 allele(s) (Figure 1, C and D). Plaque area was reduced by 31% and 56%, respectively, in mice with a single ACE 10 allele (AD<sup>ACE<sup>10/WT</sup></sup>) versus AD<sup>ACE<sup>WT/WT</sup></sup> mice. A greater reduction in plaque area was observed in AD<sup>ACE<sup>10/10</sup></sup> mice, which showed a reduced plaque burden of 69% to 79% compared with AD<sup>ACE<sup>WT/WT</sup></sup> mice (<i>P < 0.0001</i>).

We also assessed brain-insoluble Aβ<sub>1-42</sub> levels by quantitative ELISA analysis (Supplemental Figure 4). We found that 7-month-old AD<sup>ACE<sup>10/WT</sup></sup> and AD<sup>ACE<sup>10/10</sup></sup> mice had a respective average of 33% and 64% less insoluble Aβ<sub>1-42</sub> compared with AD<sup>ACE<sup>WT/WT</sup></sup> mice. At 13 months of age, AD<sup>ACE<sup>10/10</sup></sup> mice averaged 30% less insoluble Aβ<sub>1-42</sub> as compared with AD<sup>ACE<sup>WT/WT</sup></sup> mice. These data substantiate our immunohistochemical analysis.

Analysis of ACE levels in the brain confirmed the expected ACE level increase in AD<sup>ACE<sup>10/WT</sup></sup> and AD<sup>ACE<sup>10/10</sup></sup> mice (Supplemental Figure 5). While there appeared to be an inverse relationship between parenchymal Aβ plaque burden and ACE levels in the brain, differences between AD<sup>ACE<sup>10/WT</sup></sup> and AD<sup>ACE<sup>10/10</sup></sup> mice reached significance only in the Thio-S analysis of cortical and hippocampal plaque areas (Figure 1D).

**Perivascular Aβ deposition.** Deposition of Aβ in the wall of blood vessels has been associated with neuroinflammation and dementia (45). Vascular Aβ was first investigated in Aβ-stained paraffin brain sections from 8.5-month-old AD<sup>ACE<sup>10/10</sup></sup> and age- and gender-matched AD<sup>ACE<sup>WT/WT</sup></sup> mice (Figure 2A). While the AD<sup>ACE<sup>10/10</sup></sup> mice had substantially less overall parenchymal Aβ, they presented with perivascular deposition of Aβ. To more completely characterize vascular Aβ, PFA-fixed hippocampal sections of 7- to 8-month-old AD<sup>ACE<sup>WT/WT</sup></sup>, AD<sup>ACE<sup>10/WT</sup></sup>, and AD<sup>ACE<sup>10/10</sup></sup> mice were stained with Thio-S, anti-CD31 (an endothelial marker), and 6E10. Here, serial sections were studied by fluorescence microscopy, and perivascular Aβ was quantified by Thio-S<sup>+</sup> area (Figure 2, B and C). Separate histologic sections were stained for anti-CD31 and 6E10 (anti-human Aβ), and perivascular Aβ was quantified by 6E10<sup>+</sup> area (Supplemental Figure 6). Both studies produced the same result; whether assessed by Thio-S staining or by staining with an anti-Aβ antibody, AD<sup>ACE<sup>10/WT</sup></sup> mice had substantially less perivascular Aβ than either AD<sup>ACE<sup>WT/WT</sup></sup> or AD<sup>ACE<sup>10/10</sup></sup> mice (Figure 2C, <i>P < 0.0001</i>). In contrast, there was no significant difference in the amount of perivascular Aβ between AD<sup>ACE<sup>WT/WT</sup></sup> and AD<sup>ACE<sup>10/10</sup></sup> mice. One of the major differences between AD<sup>ACE<sup>10/WT</sup></sup> and AD<sup>ACE<sup>10/10</sup></sup> mice is that AD<sup>ACE<sup>10/10</sup></sup> mice lack ACE expression in vascular endothelium.

**Reduced astrogliosis.** Astrocytes are the main type of glial cells in the brain. Associated with AD progression, astrocytes migrate to Aβ plaques and exhibit increased reactivity and cytotoxicity (termed
astrogliosis or astrocytosis) (11, 46, 47). These astrocytes also increase expression of the astrocyte-specific glial fibrillary acidic protein (GFAP). To evaluate astrogliosis, coronal sections from 7- to 8-month-old mice were stained with an anti-GFAP antibody and assessed for both the number of positive cells and the immunoreactive area. Representative histologic sections are shown in Figure 3A, while quantitative analysis is presented in Figure 3B. These data showed a very significant reduction (45%–57%) in both cell numbers and area of astrogliosis in AD+ mice that were either heterozygous or homozygous for the ACE 10 allele (Figure 3B; P values < 0.001). In fact, AD+ACE10/WT and AD+ACE10/10 were not significantly different from age-matched non-Tg control mice (AD–ACEWT/WT).

We also examined astrogliosis in a cohort of 13-month-old mice (Figure 3C). Even at this stage of disease, the area of astrogliosis and the number of GFAP+ cells in AD+ mice that were ACE10/10 demonstrated a very marked reduction in both cell numbers and area of astrogliosis compared with mice WT for ACE. The presence of one or two ACE 10 alleles substantially reduced the plaque area. AD+ACE10/10 mice had fewer plaques than did AD+ACE10/WT mice, but this only reached a significance of P < 0.05 (D, left and right panels) and is not indicated. n = 7–10 mice for 6E10 staining and 5–7 mice for Thio-S staining. *P < 0.05; **P < 0.001; ***P < 0.0001.

Reduced soluble brain Aβ1–42 and Aβ1–40. Soluble Aβ1–42 peptide is thought to be particularly neurotoxic, and elevated cerebral levels of this peptide have been associated with cognitive deficits in AD (5, 7). We measured soluble human Aβ1–42 in the brains of AD+ mice at 5, 7, and 13 months of age (Figure 4, A–C). At 5 months of age, we observed relatively low levels of Aβ1–42 in all groups. However, even at this point, AD+ACE10/10 mice had a 45% reduction in soluble Aβ1–42. By 7 months of age, the trend was clearer, with the AD+ACE10/WT and AD+ACE10/10 mice having 38% and 44% reductions, respectively, in soluble Aβ1–42 levels as compared with those in ADtg mice WT for ACE (AD+ACEWT/WT). These differences are highly statistically significant (P < 0.001). By 13 months of age, the percentage difference between the AD+ACEWT/WT and AD+ACE10/10 mice had contracted (15%), though it remained significant (P < 0.05). At this age, the nature of the aggressive genetic model of AD is such that there is abundant soluble Aβ1–42 that swamps the ability of both groups of animals to clear the peptide. However, the absolute reduction in soluble Aβ1–42 concentrations (i.e., the difference of the means) between AD+ACE10/WT and AD+ACE10/10 mice at 5, 7, and 13 months of age is 23, 109, and 324 pg/mg protein, respectively, and thus increases with age.

Studies have suggested that ACE not only converts Aβ1–42 to Aβ1–40 but can also cleave Aβ1–40 into smaller fragments (20–22). Cerebral levels of Aβ1–40 were measured by ELISA in 7-month-old...
mice than in the AD^ACE^WT/WT mice. However, treatment of AD^ACE^10/10 mice with the ACE inhibitor ramipril resulted in an increase in plaque burden as compared with that observed in untreated AD^ACE^10/10 mice or AD^ACE^10/10 mice treated with hydralazine. We also measured brain-insoluble and soluble levels of Aβ1-42 in the drug-treated mice (Figure 5, C and D) and found that insoluble Aβ1-42 levels were equivalent in mice treated for 28 days with either ramipril or hydralazine. However, by 60 days, we observed that insoluble Aβ1-42 levels were higher in the ramipril-treated AD^ACE^10/10 mice as compared with those in equivalent mice treated with hydralazine (Figure 3D), and we observed this in both the 28-day- and 60-day-treatment cohorts. Thus, in the AD^ACE^10/10 mouse model, inhibiting ACE catalytic activity with ramipril markedly reduced the beneficial effects on plaque burden and on soluble and insoluble levels of Aβ1-42 as compared with the effects seen in the hydralazine-treated mice.

**Blood-borne monocyctic cell infiltration and cellular Aβ uptake.** Cerebral inflammation is a prominent feature in AD. To assess the infiltration of blood-borne monocyctic cells, we isolated and dissociated brains from age-matched 9.9- to 14.5-month-old AD^ACE^WT/WT and AD^ACE^10/10 mice. We then studied resident and peripherally derived inflammatory cells by flow cytometry after antibody labeling for the immune surface markers CD45, CD11b, and Ly6C (49, 50). Initial gating defined two populations: infiltrating hematopoietic cells (CD45^hi^) and microglia (CD11b^+^CD45^lo-intermed^) (Supplemental Figure 8A). The CD45^hi^ cells were further gated for CD11b^+^ and Ly6C^hi^ expression (Supplemental Figure 8B). Several published studies indicate that CD45^hi^Ly6C^hi^CD11b^+^ cells are monocytes originating from peripheral blood (51, 52). Quantification of these cells as a percentage of all CD11b^+^ cells showed a decrease in the percentage of these cells in AD^ACE^10/10 mice as compared with that observed in AD^ACE^WT/WT mice (Supplemental Figure 8C). This is not surprising, given the substantial disease burden we observed in AD^ACE^WT/WT mice and the reduction of this burden in AD^ACE^10/10 mice. Another way to assess infiltrating monocyctic cells is to measure the CD11b^+^Ly6C^hi^ cells as a percentage of total CD11b^+^ cells. This assessment produced the same result of decreased inflammation in the AD^ACE^10/10 mice (Supplemental Figure 8D). Thus, the decreased AD we observed in AD^ACE^10/10 mice (reduced plaque numbers, reduced astrogliosis, etc.) was also associated with a reduction in total cerebral inflammation.

**Figure 2**
Perivascular Aβ deposition in AD^ACE^WT/WT and AD^ACE^10/10 mice. (A) Micrographs of paraffin sections from AD^ACE^WT/WT and AD^ACE^10/10 cerebral cortex (8.5-month-old mice) labeled for human Aβ using rabbit polyclonal antibody AB2539 with standard peroxidase-based techniques. AD^ACE^WT/WT mice showed perivascular deposition of Aβ. (B) Representative images of fluorescence-based immunohistochemistry and Thio-S staining, PFA-fixed hippocampal sections of 7- to 8-month-old mice were colabeled with Thio-S (green), anti-CD31 (red), an EC marker, and 6E10 mAb (blue). The Thio-S signal is also shown in gray scale (lower panels). Scale bars: 10 μm. (C) Quantitation of perivascular Thio-S^+^ area in blood vessels from the hippocampus was performed using serial coronal sections. Data for individual mice as well as for group means and SEM are indicated, along with the percentages of reduction in the mean plaque area. Perivascular Aβ deposition was significantly reduced in AD^ACE^WT/WT mice compared with that in AD^ACE^WT/WT mice or AD^ACE^10/10 mice. There was no statistical difference between the AD^ACE^10/10 and AD^ACE^WT/WT mice. n = 10–12 mice per group. ***P < 0.0001.
To further investigate the local immune changes in AD+ mice, we performed immunohistochemistry on the brains of 7-month-old AD+ACE10/10, AD+ACE10/WT, and AD+ACEWT/WT mice (Figure 6, A–F). To help distinguish between resident microglial and peripheral monocytic cells, we used antibodies against the hematopoietic marker CD45, since infiltrating monocytes and macrophages show intense CD45 staining, while microglia have low-to-intermediate CD45 staining, even when activated (49, 53). We also costained for intracellular Aβ (4G8+), suggesting that these nonresident inflammatory cells were engulfing and perhaps clearing part of the plaque. Figure 6E and F, compares hippocampal plaques from an AD+ACEWT/WT mouse with those from an AD+ACE10/10 mouse. Again, there appeared to be engulfment of Aβ by plaque-associated CD45hiACEhi macrophages in the AD+ACE10/10 model (Figure 6F). While we found that macrophage association with plaques was occasionally present in AD+ACEWT/WT mice (Figure 6E), it was less prominent.

We also performed a quantitation test, in which serial tissue sections were stained with anti-CD45, Aβ (6E10), and IBA1, and we calculated the area of IBA1+CD45hi cells (Figure 6, G and H). Such cells are often considered infiltrating myeloid cells (36). Our data showed fewer such cells in the AD+ACE10/10 mice, but these data did not reach statistical significance. However, when we corrected this number for the total area of 6E10+ plaques to assess the number of infiltrating monocytic cells per plaque area, we found a significant increase in these numbers in the AD+ACE10/10 mice (Figure 6j). In summary, our microscopic analysis suggests that, in AD+ACE10/10 mice, the infiltrating cells are increasingly found to be associated with Aβ plaque and are probably engaged in phagocytosis of Aβ.

Retained cognitive function by the Barnes maze test. While the AD+ACE10/WT and AD+ACE10/10 mice have a reduced plaque burden, less astrogliosis, and lower amounts of soluble amyloidogenic Aβ in the brain, the most crucial measure of resistance to AD progression is cognitive behavior. Spatial learning and memory were assessed in two separate cohorts of mice. One cohort of 11-month-old mice was tested by independent evaluators from the Cedars-Sinai Behavioral Core (Figure 7, A–F). A second cohort of 12-month-old mice was independently evaluated by members of our research group (Figure 8, A–F). All observers were blinded to the mouse genotype. The evaluators of each cohort arrived at the same conclusions. First, an open field test of ambulatory and rearing behavior

Figure 3
Reduced astrogliosis in AD+ACE10/10 and AD+ACE10/WT mice. Coronal brain sections from 7- to 8-month-old mice were stained with a polyclonal antibody against astrocyte-specific glial fibrillary acidic protein (GFAP; yellow). Sections were counterstained for nuclei with DAPI (blue). (A) Fluorescent micrographs of coronal brain sections show reduced staining for cortical GFAP in AD+ mice heterozygous or homozygous for the ACE 10 allele (AD+ACE10/WT and AD+ACE10/10). For simplicity of viewing, the bottom portion of panel A shows the GFAP channel as gray scale. Scale bars: 100 μm. (B) Quantitative analysis was performed to measure the GFAP+ area and cell numbers in 7- to 8-month-old mice. Data for individual mice are shown as well as for the group means and SEM. Arrows indicate the percentage of reduction in the group means as compared with that in AD+ACEWT/WT mice. Mice heterozygous or homozygous for the ACE 10 allele had significantly reduced astrogliosis. (C) A similar analysis of astrogliosis was performed on 13-month-old mice. Even at this age, AD+ACE10/10 mice had significantly fewer GFAP+ reactive astrocytes than did AD+ACEWT/WT mice. n = 5–7 mice per group. *P < 0.05; **P < 0.001; ***P < 0.0001.
The goal of AD treatment is to retard or prevent the cognitive decline that is such a debilitating part of the disease. Thus, the results we obtained when ACE10/10 mice were crossed with ADtg mice were extremely encouraging, in that the spatial learning and memory of 11- and 12-month-old animals were almost identical to those of mice without any genetic predisposition to AD. ACE10/10 mice over-express ACE in myelomonocytic lineage cells; these mice lack ACE expression by ECs. In contrast, ACE10/WT mice maintain some endogenous endothelial ACE expression. Because of the targeted high ACE levels in monocyctic cells and the unusual tissue pattern of ACE expression in ACE10/10 mice, care must be taken in extrapolating to the normal function of ACE. Indeed, the high expression of ACE in ACE10/10 and ACE10/WT in monocyctic cells is best considered as ACE in a pharmacologic fashion.

A number of studies have shown that ACE10/10 mice have a marked enhancement of both their innate and adaptive immune responses. When challenged with a mouse model of melanoma, ACE10/10 mice showed an increased inflammatory response characterized by more aggressive monocyte and macrophage infiltration of the tumor (24). The result was a very substantial reduction in tumor growth. An enhanced innate immune response was also observed when ACE10/10 mice were challenged with L. monocytogenes or methicillin-resistant S. aureus (25). This appeared to be due to an intense monocyctic/macrophagic response, and again, this resulted in reduced disease. While the precise biochemical details of how ACE changes the immune response are not yet known, a substantial feature is that ACE overexpression tilts the development of monocytes and macrophages toward a more inflammatory phenotype (57).

As compared with non-AD mice, we found that AD+ACE10/WT and AD+ACE10/10 mice still had relatively high levels of cerebral-soluble and -insoluble Aβ. Yet, despite this, these mice had near-normal cognitive function, as measured by the Barnes maze test. A striking feature of the AD+ACE10/WT and AD+ACE10/10 mice is that, even at 13 months of age, these animals showed a significant decrease in insoluble and soluble Aβ as compared with the levels seen in the AD+ACEWT/WT mice and that this was accompanied by a marked reduction in astroglia. This may help explain why the cognitive abilities of these genotypes were so well preserved.

Recently, there has been an appreciation that the inflammatory response, including the participation of blood-borne macrophages, can affect the progression of AD. In particular, BM-derived monocyctic cells can infiltrate the brain and reduce Aβ plaque burden (29–32, 34, 36, 38–41, 58). Macrophages readily absorb amyloid fibrils in vitro (59, 60) and seem more efficient than microglia at restricting Aβ plaques. These macrophages may also be able to remove the peptide MOG45D and reduce glial cells in vivo.

Discussion
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Figure 4
Reduced soluble Aβ levels in AD+ACE10/10 and AD+ACE10/WT mice. (A–C) ELISA measurements of soluble human Aβ1–42 in the brains of AD+ and AD– mice with the indicated ACE genotypes. Groups of mice were assessed at 5, 7, and 13 months of age. (D) ELISA measurement of soluble human Aβ1–40 from 7-month-old AD+ACEWT/WT, AD+ACE10/WT, and AD+ACE10/10 mice. Data for individual mice as well as for the group means and SEM are shown. Arrows indicate the percentage of reduction in the group means as compared with that in AD+ACEWT/WT mice. Also shown is the absolute reduction in Aβ1–42 (23 pg, 109 pg, and 324 pg) found in AD+ACE10/10 mice as compared with that seen in equivalently aged AD+ACEWT/WT mice. Soluble levels of both Aβ1–42 and Aβ1–40 were lower in AD+ACE10/10 mice than in AD+ACEWT/WT mice, as evidenced by the absolute reduction in Aβ1–42 levels. The difference between AD+ACE10/10 and AD+ACEWT/WT mice increased with age. n = 5–14 mice per group. *P < 0.05; **P < 0.001.
infiltration of monocytes that resulted in substantial attenuation of AD pathology (31, 36, 61). These mice also had reduced cognitive impairment (62). Importantly, depletion of peripheral mononuclear inflammatory cells or insufficient recruitment of BM-derived myeloid-derived cells to the brain led to accelerated cognitive impairment (62). Importantly, depletion of peripheral mononuclear inflammatory cells or insufficient recruitment of BM-derived myeloid-derived cells to the brain led to accelerated cognitive impairment (62).

In our model, the analysis of perivascular Aβ pathology of Aβ1−42 levels as compared with those in the hydralazine-treated group. Analysis of soluble Aβ1−42 levels by ELISA (D) showed significantly higher brain levels at both 28 and 60 days in the AD+ACE10/10 mice treated with ramipril as compared with Aβ1−42 levels in the equivalently aged mice treated with hydralazine. The mice treated with ramipril or hydralazine for 60 days in C and D were those studied in A and B. **P < 0.01, ***P < 0.001.

Figure 5
Effects of ACE inhibition on brain Aβ levels in AD+ACE10/10 mice. (A) Representative hippocampal micrographs from 13-month-old AD+ACEWT/WT and AD+ACE10/10 mice as well as from AD+ACE10/10 mice treated for 60 days with either ramipril or hydralazine (Hyd). Sections were stained with anti-human Aβ antibody 6E10 (green), anti-GFAP (red), and DAPI (blue). Lower panels show the 6E10 channel in gray scale. Scale bars: 50 μm. (B) Quantitative analysis of 6E10-positive immunoreactive plaque area from cortical and hippocampal regions. Data for individual mice, group means, and SEM are shown. ACE inhibition with ramipril significantly increased the plaque area as compared with that observed in AD+ACE10/10 mice or AD+ACE10/10 mice treated with hydralazine. (C and D) Thirteen-month-old AD+ACE10/10 mice were treated for 28 days (black triangles) or 60 days (black diamonds) with ramipril or hydralazine. Measurement of insoluble human Aβ1−42 by ELISA (C) showed no difference at 28 days between the mice receiving ramipril and those receiving hydralazine. However, after 60 days, the mice treated with ramipril had significantly higher insoluble Aβ1−42 levels as compared with those in the hydralazine-treated group. Analysis of soluble Aβ1−42 levels by ELISA (D) showed significantly higher brain levels at both 28 and 60 days in the AD+ACE10/10 mice treated with ramipril as compared with Aβ1−42 levels in the equivalently aged mice treated with hydralazine. The mice treated with ramipril or hydralazine for 60 days in C and D were those studied in A and B. *P < 0.05; **P < 0.01.

enhanced phagocytosis of amyloid. In the ACE10/10 model, both the IBA1+CD45hi and the ACEE14 phenotypes marked mononuclear cells as originating in the BM. Thus, a more effective immune response of inflammatory cells overexpressing ACE can explain, at least in part, the beneficial phenotype observed in the AD+ACE10/10 and AD+ACE10/10 mice.

There is a second feature of the ACE10/10 mice that should contribute to protection against AD. Several studies have identified ACE as being capable of cleaving a variety of amyloid peptides, including Aβ1−42 and Aβ1−40, into smaller fragments; at least in vitro, ACE can have both carboxypeptidic and endopeptidic activity against these larger amyloidogenic peptides (20–22, 63). In ACE10/10 mice, the serum levels of ACE are no different from WT mice. However, monocytes and macrophages express 16-fold to more than 50-fold greater amounts of normal surface ACE (24). Thus, a second advantage of the ACE10/10 model in AD is the potential for enzymatic elimination of pathogenic peptides. In fact, our findings of reduced cerebral levels of Aβ in 5-, 7-, and 13-month-old AD+ACE10/10 mice are consistent with this hypothesis.

As shown in Figure 5, treatment of AD+ACE10/10 mice with the high-affinity and specific ACE catalytic inhibitor ramipril, known to cross the blood-brain barrier (48), significantly reduced the beneficial effects observed in this model (plaque burden and Aβ1−42 levels) as compared with those seen with hydralazine treatment, a blood pressure-lowering drug that does not affect ACE. This is not surprising, since overexpression of catalytically active ACE is the key mechanistic feature of ACE10/10 mice, and previous studies have shown that ACE10/10 mice treated with ACE inhibitors exhibit the phenotype of WT mice (24, 25). In WT mice and humans, ACE is normally produced by vascular endothelium, but what role this plays in the biology of Aβ, including pathogenic forms such as Aβ1−42, is not known. In our model, the analysis of perivascular Aβ was striking in that the AD+ACE10/10 mice had substantially less perivascular Aβ than either AD+ACEWT/WT or AD+ACE10/10 mice. This was surprising, since by all other measures, AD+ACE10/10 mice showed equal or less AD pathology. As mentioned, endothelium in AD+ACE10/10 mice produces no ACE, and it is possible that this contributes to more perivascular Aβ in this genotype than in AD+ACE10/WT mice, which produce some
endothelial ACE due to the presence of one WT ACE allele. Further, the decreased perivascular Aβ we observed in AD+ACE10/WT mice, together with the substantial reductions in soluble Aβ and astrogliosis, may help explain why the cognitive abilities of this genotype, as measured by the Barnes maze test, were equivalent to those of both AD+ACE10/10 and non-AD mice.

Microglia differentiate from myeloid progenitor cells, and in the ACE10/10 mice, they expressed more ACE than WT microglia, but less ACE than monocytes and macrophages. When microglia from non-AD ACE10/10 brains were studied in vitro following exposure to LPS, they also appeared more inflammatory than similarly treated cells from ACE WT mice (Supplemental Figure 9). Additional experiments, including BM transfer, will be necessary to define the contribution of BM monocytes versus microglia in the AD+ACE10/10 phenotype.

Several groups have studied the in vivo effect of natural levels of ACE on the incidence of AD, and evidence has been presented both for and against the idea that natural ACE levels affect disease progression.
risk (20, 64–67). Several meta-analyses have consistently found that lower serum and tissue ACE levels, as determined by an ACE gene polymorphism, were associated with a small increased risk of developing AD (68–71). On the other hand, recent genomewide association studies have failed to find involvement of ACE genetic variation in AD (64, 72). However, these studies reflect an evaluation of the normal levels of ACE present in humans. As mentioned, we found that ACE expression in the ACE10/10 model was markedly increased, and it is likely that the catalytic activity of ACE, selectively expressed on the surface of macrophages and microglia, benefits the AD-ACE10/10 mice by reducing Aβ levels. This conclusion is supported by the higher brain Aβ1–42 levels seen in mice treated with the ACE inhibitor ramipril as compared with the levels observed in mice treated with hydralazine. At the very least, our studies in ACE 10 mice emphasize the advantage of combining two separate approaches to reduce Aβ burden in AD: an enhanced immune response resulting from ACE overexpression in monocytes and macrophages combined with the direct enzymatic effect of ACE in cleaving Aβ peptides. The effect in the AD-ACE10/WT and AD-ACE10/10 mice was a reduction of Aβ plaques, soluble amyloidogenic Aβ, astrogliosis, and most remarkably, a retention of cognitive abilities that were virtually equivalent to those of non-AD mice.

Our study has limits, in that we did not evaluate the role of local angiotensin II production or angiotensin receptor expression in the mice heterozygous or homozygous for the ACE 10 allele. Also, we did not investigate the effects of this allele on the regulation of angiotensinogen. Several studies have suggested that angiotensin II AT1 receptor antagonists may be beneficial in AD by inhibiting the effects of angiotensin II, while permitting continued ACE cleavage of Aβ (73). The precise role of angiotensin II in the mouse models we studied is not understood.

In ACE10/10 mice, activated macrophages have an enhanced inflammatory response (19). Use of monocytic cells overexpressing ACE in humans could conceivably have side effects. However, our evidence from the AD-ACE10/10 mice showed the opposite, namely, a decrease in overall cerebral inflammation. Also, ACE inhibitors would probably be an effective countermeasure, as ACE10/10 mice treated with such inhibitors have the phenotype of WT mice (24, 25). Further, we have never seen any evidence of autoimmune disease in ACE10/10 mice. While it is possible to envision a strategy for delivering ACE-overexpressing monocytes to patients, perhaps the most informative finding of our studies is the effectiveness of combining an approach to enhance the immune response with that of delivering inflammatory cells to enzymatically destroy Aβ.

Methods

Mice. ACE10/10 mice have been previously described (24). In this strain, targeted homologous recombination was used to insert a c-fms promoter to direct tissue overexpression of ACE in myelomonocytic cells. The ACE genotypes are WT (ACEWT/WT), heterozygous (ACE10/WT), and homozygous for the targeted allele (ACE10/10). These mice were backcrossed at least ten generations to C57BL/6J mice (The Jackson Laboratory).

The AdTg strain was originally purchased from The Jackson Laboratory (B6.Cg-Tg(APPsw,PSEN1DE9)8S5Dbo/J; stock no. 005864), then bred and maintained at Cedars-Sinai Medical Center. The Tgs contain familial AD mutations and are expressed in CNS tissue under the control of the mouse prion protein (PrP) promoter. They contain cDNA sequences for the mutant human/mouse chimeric Aβ (A4) precursor protein (Swedish double mutations in APPK692NQ714I and the exon 9 deletion of human presenilin 1 (PS1L501) (74). ADtg (AD; C57BL/6J background) mice were crossed with ACE10/10 mice to produce the following genotypes: AD-ACEWT/WT, AD-ACE10/WT, AD-ACE10/10, AD-ACEWT/10, and AD-ACE10/10. The mice were genotyped for ACE (24), APP, and PS1 (36) alleles using conditions previously described. Age- and gender-matched mice for each genotype were assessed at various ages, ranging from 3 to 14 months. All mice were maintained in microisolator cages, and all experiments were conducted and recorded by researchers blinded to the mouse genotypes.

Blood pressure. Systolic blood pressure was measured in conscious AD-ACEWT/WT and AD-ACE10/10 mice using a Visitech tail cuff system (75). The individual blood pressure was the average of the blood pressure measured on 4 consecutive days following 4 days of training to accustomed the animals to the procedure. Blood pressure from male and female mice was analyzed separately. The average age was similar for the two genotypes of mice.

Tissue processing. Following perfusion with ice-cold 0.9% saline containing 0.5 mM EDTA, harvested brains were cut in half (36). One hemisphere was used for histology, and the second hemisphere was further cut into two halves and snap-frozen for biochemical and molecular assays.

Immunohistochemistry. For paraffin sections, tissues were fixed and embedded using standard techniques. Sections (5 µm) were cut, deparaffinized, and placed in 88% formic acid for 5 minutes. After rinsing, the tissues were incubated at pH 9 for 20 minutes, then rinsed and incubated for 15 minutes in buffer pH 7.6 (Leica Biosystems, catalog AR9640) with either mouse mAb 6F/3D (1:800; Leica Biosystems) or a rabbit polyclonal anti-human Aβ antibody (1:150; Ab2539, Abcam) targeting amino acids 1–14. The secondary anti-mouse antibody and the DAB chromogen were dispensed using an automated Leica BOND-III slide-staining machine and Leica BOND Polymer Refine Detection reagents (Leica Biosystems, catalog DS9800). The secondary goat anti-rabbit antibody used was the Dako EnVision+ System-HRP Labeled Polymer (K4003; Dako). Standard peroxidase-based and H&E staining techniques were used.

For immunofluorescence, the tissues were fixed in 2.5% PFA following dehydration with 30% sucrose. Coronal cryosections (30 µm) were treated with Target Retrieval solution (Dako) at 95°C for 30 minutes. The tissues were then permeabilized and blocked as previously described (36). The sections were stained overnight at 4°C with combinations of the following primary antibodies in PBS and blocking solution: mouse anti-human Aβ mAb clone 6E10 (1:100, Covance) recognizing amino acids 1–16 or mouse anti-human Aβ mAb clone 4G8 (1:100, Covance) recognizing amino acids 17–24; rabbit anti-CD31 polyclonal antibody (1:50; Ab28364; Abcam); rabbit anti-GFAP polyclonal antibody (1:150; 550539; BD Pharmingen); rabbit anti-IBA1 (1:250; 019-19741; Wako); rabbit anti-ACE polyclonal antibody (1:500; 76); and rat anti-CD45 mAb (1:25; 550539, BD Pharmingen). Secondary polyclonal antibodies donkey anti-mouse, anti-rat, and anti-rabbit (1:200; Jackson ImmunoResearch Laboratories) conjugated with Cy-2, Cy-3, or DyLight 649 were incubated for 1 hour at 37°C. Washing, DAPI nuclei staining, mounting, and microscopic analysis using an ApoTome-equipped Carl Zeiss 21 fluorescence microscope were performed as previously described (36, 62). The specificity of the labeling was established by omitting the primary antibody from parallel brain sections.

Aβ plaque staining by Thio-S. Following secondary antibody staining, brain sections were stained with Thio-S (1% w/v in 70% ethanol; Sigma-Aldrich) as previously described (36).

Quantitative immunohistochemistry. For each genotype, equivalent brain regions were analyzed. Coronal sections (3–6/µm) were taken at 150-µm intervals. For the analysis of Aβ plaque, GFAP-immunoreactive area, manual cell number counts, and IBA1 (CD45R0) area and cell numbers, images were captured encompassing the hippocampal and cortical regions.
and Pacific blue–conjugated anti-Ly6C (1:200; clone HK1.4). The stained samples were analyzed on a Beckman Coulter CyAn ADP, and data were analyzed with FlowJo software (Tree Star, Inc.).

**LPS-induced cytokine expression by microglia.** Eight to 10-week-old AD-ACEWT/WT and AD-ACE10/10 mice were perfused, and the brains were collected. Brains were chopped into small pieces and then digested for 30 minutes with 1.5 mg/ml collagenase IV and DNase I (STEMCELL Technologies) at 37°C. After centrifugation, homogenization, and washing, the pellet was suspended in 70% Percoll and then layered under a 30%/50% Percoll step gradient. After centrifugation for 25 minutes at 700 g, the cells at the interface of the 70% and 50% layers were collected, washed, and incubated in vitro for 6 hours with 5 μg/ml brefeldin A (eBioscience), with or without LPS (Escherichia coli 055:B5; Sigma-Aldrich) stimulation. Cells were then stained for the surface markers CD45 (1:333; clone NJTEN3; eBiosciences), Ly6C (1:200; clone HK1.4), and APC-conjugated anti–IL-1β (1:333; clone NJTEN3; eBiosciences) and APC-conjugated anti–TNF-α (1:100; clone MP6-XT22; eBiosciences). The CD11b+CD45+ microglia were evaluated for cytokine expression by flow cytometry.

**(including entorhinal and cingulate cortex)** using the same exposure times. For perivascular Aβ deposition, areas of Thio-S and 6E10 colabeled with CD31 (a marker for vascular ECs) covering the hippocampal fissure in the outer molecular layer and vessels between the hippocampus and the mediodorsal part of the lateral posterior thalamic nucleus were assessed in 5 to 7 serial brain sections per mouse, and the images were captured using the same exposure times. The images were then converted to gray scale and standardized to baseline using histogram-based thresholds in ImageJ software, versions 1.38x and 1.46r (NIH), as previously described (36, 77).

**Analysis of cerebral inflammation by flow cytometry.** The brains were perfused before harvest, then minced and digested for 30 minutes with 1.5 mg/ml collagenase IV (Worthington Biochemical Corp.) and DNase I (STEMCELL Technologies) at 37°C. After centrifugation, homogenization, and washing, the pellet was suspended in 70% Percoll (GE Healthcare) and then layered under a 30%/50% Percoll step gradient. After centrifugation for 25 minutes at 700 g, the cells at the interface of the 70% and 50% layers were collected and washed. Cells were stained with the following antibodies purchased from eBioscience and BioLegend: FITC-conjugated anti-CD45 (1:200; clone 30-F11), PerCP/Cy5.5-conjugated anti-CD11b (1:100; clone M1/70), and washed. Cells were stained with the following antibodies purchased from eBioscience and BioLegend: FITC-conjugated anti-CD45 (1:200; clone 30-F11), PerCP/Cy5.5-conjugated anti-CD11b (1:100; clone M1/70), and washed. Cells were stained with the following antibodies purchased from eBioscience and BioLegend: FITC-conjugated anti-CD45 (1:200; clone 30-F11), PerCP/Cy5.5-conjugated anti-CD11b (1:100; clone M1/70), and washed.

**Biochemical determination of Aβ1–40 and Aβ1–42 levels by sandwich ELISA.** For brain-soluble and -insoluble ELISA analysis, brain tissues were homogenized in PBS buffer with 0.5% Triton X-100 and a 1X protease inhibitor cocktail set I (Calbiochem). After removal of cell debris, the homogenate was centrifuged at 10,000 g for 20 minutes at 4°C (36, 61). The supernatant was considered the soluble fraction and was used to assess soluble Aβ. The pellet was diluted with the above homogenizing buffer. This was the insoluble fraction and was used to assess insoluble Aβ. After determination of the protein concentration (Thermo Fisher Scientific), soluble and insoluble Tg-derived Aβ1–42 levels were determined using an anti-human Aβ1–42 end-specific sandwich ELISA kit (KHB3442; Invitrogen) according to the manufacturer’s instructions. This kit does not recognize mouse Aβ, nor does it recognize human Aβ1–40 or Aβ1–43. The kit uses two antibodies and is specific for the N- and COOH-termini of the Aβ1–42 sequence; bound rabbit antibody was detected using an HRP-labeled anti-rabbit antibody and was read at 450 nm with a microplate reader (SpectraMax Plus384; Molecular Devices). Soluble Aβ1–40 levels were measured using a similar Invitrogen ELISA kit (KHB3482).
with ACE buffer containing 0.5% Triton X-100. After a second centrifugation at 10,000 g, the supernatant was collected and the protein concentration was measured with a BCA protein assay reagent kit (Pierce Biotechnology) according to the manufacturer’s instructions. ACE activity was measured using the ACE-REA kit from American Laboratory Products Company (ALPCO). ACE activity was defined as that inhabitable by captopril (Sigma-Aldrich). For Western blotting, 20 μg of total protein was separated by SDS-PAGE on a 10% gel and transferred to a 0.45-μm nitrocellulose membrane. The membranes were probed with a 1:250 dilution of a rabbit polyclonal anti-mouse ACE antibody (76) and developed with a goat anti-rabbit IR dye 800CW (LI-COR Biosciences). The blot was scanned using the LI-COR Odyssey digital imaging system.

To inhibit ACE, AD-ACE10/10 mice were treated for 28 days or 60 days with 160 mg/liter of drinking water of either the ACE inhibitor ramipril (NDC 16252-573-01; Watson) or hydralazine (Sigma-Aldrich), which lowers blood pressure independently of ACE.

Open field test. The open field test was performed as previously described (79). Briefly, mice were placed individually in the open field apparatus (San Diego Instruments), with ambulatory and rearing activity measured for 60 minutes after at least 1 hour of habituation. Ambulatory activity was recorded when the mouse broke successive lower beams within a 1-second period. Rearing behavior was counted when an upper beam was broken.

**Western blot analysis of APP.** Snap-frozen forebrain samples (left hemis brains) were homogenized with a hand-held microgrinder (Argos Technologies) in RIPA buffer with a 1X protease inhibitor cocktail (Calbiochem). The homogenate was centrifuged at 10,000 g for 25 minutes at 4°C. The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology). Protein samples (40 μg) were separated on Bis-Tris 4–12% gel (Bio-Rad) with NuPAGE MOPS or MES buffer and transferred to 0.45-μm nitrocellulose or PVDF membranes. The blots were probed with 6E10 mAb (1:1,000; anti-β-actin mAb (1:1,000; Sigma-Aldrich) as a loading control. The relative band intensity was quantified using ImageJ software, version 1.46r.

**Brain ACE expression and activity.** ACE activity and Western blotting were performed as previously described (78). For the preparation of protein extracts, the brains were collected from euthanized animals, weighed, and immediately frozen in liquid nitrogen. Later, they were gently homogenized in ACE buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 25 μM ZnCl2; and 1 mM PMSF). After centrifugation at 10,000 g, the pellets were vigorously rehomogenized with ACE buffer containing 0.5% Triton X-100. After a second centrifugation at 10,000 g, the supernatant was collected and the protein concentration was measured with a BCA protein assay reagent kit (Pierce Biotechnology) according to the manufacturer’s instructions. ACE activity was measured using the ACE-REA kit from American Laboratory Products Company (ALPCO). ACE activity was defined as that inhabitable by captopril (Sigma-Aldrich).

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of the escape box remained the same for a mouse during every trial of the training phase, but was shifted between mice to reduce the potential for unintended intramaze cues. Training was repeated three times per day, with a 15-minute interval separating each trial. Data from each trial were recorded and averaged. The training phase was conducted for 4 days, followed by a 2-day break with no exposure to the maze. On day 7, each animal was re-tested in a three-trial session using the same escape box location and method from the training phase. On day 8 following the retention phase, the reversal phase began, in which the escape box was placed in a different quadrant than the original escape box location. Using the same procedure as above, reversal trials were repeated three times per day over two consecutive days (days 8 and 9). Data were recorded both manually and by a video camera located above the maze.

**Statistics.** Data were analyzed using GraphPad Prism software, version 5.0b (GraphPad Software). Values are presented for the individual mice and/or expressed as the means ± SEM. ANOVA with Tukey’s post-hoc tests were used for multiple comparisons. The behavioral data were analyzed using both repeated-measures 2-way and 1-way ANOVA with Tukey’s post-hoc tests for each day. Two-group comparisons were analyzed by 2-tailed, unpaired Student’s t tests. A P value of less than 0.05 was considered statistically significant.

**Study approval.** All experimental protocols were approved by and conducted according to the instructions of the IACUC of Cedars-Sinai Medical Center.

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Address correspondence to: Maya Koronyo-Hamaoui, Cedars-Sinai Medical Center, 127 S. San Vicente Blvd., Los Angeles, California 90048, USA. Phone: 310.423.7473; Fax: 424.315.4569; E-mail: maya.koronyo@cshs.org.


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