Hypertension Accelerates the Progression of Alzheimer-Like Pathology in a Mouse Model of the Disease

Diana Cifuentes,* Marine Poittevin,* Ekrem Dere, Dong Broquères-You, Philippe Bonnin, Joëlle Benessiano, Marc Pocard, Jean Mariani, Nathalie Kubis, Tatyana Merkulova-Rainon,* Bernard I. Lévy*

Abstract—Cerebrovascular impairment is frequent in patients with Alzheimer disease and is believed to influence clinical manifestation and severity of the disease. Cardiovascular risk factors, especially hypertension, have been associated with higher risk of developing Alzheimer disease. To investigate the mechanisms underlying the hypertension, Alzheimer’s disease cross talk, we established a mouse model of dual pathology by infusing hypertensive doses of angiotensin II into transgenic APPPS1 mice overexpressing mutated human amyloid precursor and presenilin 1 proteins. At 4.5 months, at the early stage of disease progression, only hypertensive APPPS1 mice presented impairment of temporal order memory performance in the episodic-like memory task. This cognitive deficit was associated with an increased number of cortical amyloid deposits (223±5 versus 207±5 plaques/mm²; P<0.05) and a 2-fold increase in soluble amyloid levels in the brain and in plasma. Hypertensive APPPS1 mice presented several cerebrovascular alterations, including a 25% reduction in cerebral microvessel density and a 30% to 40% increase in cerebral vascular amyloid deposits, as well as a decrease in vascular endothelial growth factor A expression in the brain, compared with normotensive APPPS1 mice. Moreover, the brain levels of nitric oxide synthase 1 and 3 and the nitrite/nitrate levels were reduced in hypertensive APPPS1 mice (by 49%, 34%, and 33%, respectively, compared with wild-type mice; P<0.05). Our results indicate that hypertension accelerates the development of Alzheimer disease–related structural and functional alterations, partially through cerebral vasculature impairment and reduced nitric oxide production.

Key Words: Alzheimer Disease ■ hypertension ■ models, animal ■ nitric oxide

Alzheimer disease (AD) accounts for 60% to 80% of dementia cases and represents the most common cause of dementia among the elderly.1 AD is a chronic neurodegenerative disorder characterized by the development of cortical extracellular amyloid plaques, mainly consisting of amyloid β peptide (Aβ) and the intracellular neurofibrillary tangles formed by the aggregated tau protein.2 Aβ is a proteolytic cleavage product of amyloid precursor protein (APP), ubiquitously expressed in the body. APP is processed sequentially by γ- and β-secretases to generate a heterogeneous group of peptides among which Aβ42 and Aβ40 are believed to be particularly important in the pathogenesis of AD.3 Although Aβ remains in the focus of AD research, the pathogenesis of AD is largely undefined and effective treatments are still not available.4 The interest in the role of vascular factors in pathogenesis of neurodegenerative dementias has grown steadily during the past decade. It is now well recognized that cerebrovascular dysfunction could be an essential factor in the pathogenesis of many types of dementia and significantly affects both incidence and course of the disease.5

Cerebrovascular impairment, including cerebral amyloid angiopathy, brain-blood barrier impairment, and small vessel disease, is frequently observed in patients with AD and could influence clinical manifestation and severity of AD and contribute to cognitive decline.6 In addition, cardiovascular risk factors have been associated with higher risk of developing AD.7,8 Cardiovascular disease preventing strategies, like antihypertensive medications, were correlated with better mental health outcomes later in life.9,10 Among cardiovascular risk factors, hypertension has been most strongly associated with AD.1 However, a pathophysiological link between hypertension and the incidence and progression of AD is not established, and the mechanisms involved in the cerebral tissue...
response to hypertension and their contribution to neurodegeneration in AD are not elucidated.

AD animal models represent different lines of transgenic mice carrying AD-linked autosomal-dominant mutations of human APP, presenilins 1 and 2, and tau proteins. Few AD models present the entire spectrum of cerebrovascular dysfunction, close to that found in humans. The aim of this study was to establish an experimental model of AD combined with hypertension, which we expect would recapitulate more fully human AD pathology and may help to provide new insights into the molecular and cellular mechanisms underlying hypertension–AD cross talk. For this purpose, APPPS1 transgenic mice coexpressing human KM670/671NL mutated APP and L166P mutated presenilin 1 and the control C57BL/6J littermates were implanted subcutaneously with osmotic mini-pumps infusing hypertensive doses of angiotensin II (ANGII). The effects of this treatment on vascular patterns, neuropathology, and behavior of mice in the episodic-like memory task have been analyzed.

### Methods

Experimental protocols are available in the online-only Data Supplement. All the experiments were performed in accordance with the European Community and the French National guidelines for the care and use of laboratory animals. All animal procedures were approved by local Ethics Committee in Animal Experimentation (protocol number CEEALV/2012-12-02).

### Results

**ANGII Induces Stable Hypertension in Both Control C57BL/6 and Transgenic APPPS1 Mice**

ANGII treatment was started at the age of 2 months and was continued for 2.5 months. Four experimental groups were analyzed: (1) sham-operated control C57BL/6J mice (wild-type [WT] group); (2) ANGII-treated C57BL/6J mice (hypertensive [HT] group); (3) sham-operated APPPS1 mice (AD group); and (4) ANGII-treated APPPS1 mice (AD&HT group). At 2 months, there was no significant difference of systolic blood pressure between control C57BL/6J and transgenic APPPS1 mice. ANGII treatment resulted in a rapid (2 weeks after implantation), significant, and sustained raise in systolic arterial pressure (≈170 mm Hg against 110 mm Hg in normotensive mice; Figure S1 in the online-only Data Supplement). Blood pressure remains at its higher level in both HT and AD&HT mice until the end of experiment.

**Hypertension Enhances Amyloid Production in AD&HT Mice**

At 4.5 months, we detected deposits positive for Aβ immunostaining in the brain of both AD and AD&HT mice (Figure 1A), in contrast with WT and HT mice being negative for Aβ immunostaining. Quantitative analysis demonstrated that there was a significant increase in the number of amyloid deposits in the cortex of AD&HT mice, compared with AD mice (223±5 versus 207±5 plaques per mm²; *P*<0.05; Figure 1B), whereas amyloid burden in the medial prefrontal cortex (mPFC) and hippocampus was not significantly different between AD and AD&HT mice (data not shown).

The soluble amyloid peptide levels in the brain extracts and plasma were quantified by ELISA (Figure 1C). Although the soluble Aβ40 levels were similar in AD and AD&HT mice, the levels of soluble Aβ42 were doubled in the whole brain lysates (24.6±2.9 versus 12.5±0.2 pmol/g of wet brain weight; *P*<0.05) and in plasma (4.5±0.9 versus 2.5±0.5 pmol/L; *P*<0.05) of AD&HT mice, compared with AD mice; there was also trend for increase in hippocampal soluble Aβ42 levels (21.8±4.5 pmol/g of wet brain weight in AD&HT mice versus 16.5±2.3 pmol/g in AD mice; *P*<0.05).
Hypertension Causes Vascular Damage in AD&HT Mice

To assess the effects of hypertension on cerebral microcirculation, we quantified the collagen IV immunostaining, an established marker of the vessel basal lamina. Cortical microvessels of AD&HT mice were less abundant and less branched than those of animals of the 3 other groups (Figure 2A). Whereas microvessel density (MVD) was increased in the cortex of AD mice compared with WT and HT mice (percentage of microvessel area per field 14.54±0.38% versus 13.15±0.41% and 12.96±0.36%, respectively, P<0.05), AD&HT mice evidenced a significant decrease in MVD (10.89±0.40%). There was also a significant decrease in MVD in mPFC and hippocampus of AD&HT mice, compared with 3 other groups (Figure 2A).

The vascular endothelial growth factor A (VEGF-A) mRNA and protein levels were increased in AD mice; however, this increase was abolished in AD&HT mice (Figure 2B), suggesting that hypertension inhibited the Alzheimer-like pathology-induced increase in VEGF-A.

Figure 2. Vascular impairment is significant already at 4.5 months in Alzheimer disease (AD) & hypertensive (HT) mice. A, Representative micrographs of collagen IV immunostaining in the cortex of wild-type (WT), HT, AD, and AD&HT mice demonstrating between group differences in microvasculature appearance. Scale bar, 100 µm. Microvessel density quantification using an Archimed software demonstrates a significant decrease in the area occupied by microvessel in the cortex, medial prefrontal cortex (mPFC), and hippocampus of AD&HT mice compared with the mice of other groups (*P<0.05; **P<0.01; ***P<0.001; n=6). B, Left, The steady state levels of vascular endothelial growth factor A (VEGF-A) mRNA were measured by quantitative reverse transcriptase polymerase chain reaction and normalized over cyclophilin A in total RNA extracts (*P<0.05; **P<0.01; n=5–6). Right, ELISA of tris-buffered saline–extracted brain homogenates shows a significant decrease in VEGF-A protein in AD&HT mice compared with AD mice (*P<0.05; n=5–6). C, Representative micrographs of amyloid β (green) and collagen IV (red) double immunostaining in the cortex of AD and AD&HT mice showing microvascular cerebral amyloid angiopathy (CAA; arrows). Scale bar, 50 µm. Quantification of amyloid deposits colocalized with microvessels reveals a significantly higher CAA in the cortex and hippocampus of AD&HT mice (*P<0.05; n=6).
Collagen IV and Aβ double immunostaining, used to detect amyloid deposition around the vascular wall, demonstrated that both AD and AD&HT mice develop vascular amyloidosis, involving both larger arteries and microvessels (Figure 2C). Quantification of collagen IV positive vascular area colocalized with Aβ immunostaining demonstrated that a significantly higher percentage of amyloid plaques was associated with microvessels in AD&HT mice, compared with AD mice (cortex, AD: 28.5±2.1%; AD&HT: 37.4±3.1%; P<0.05; hippocampus, AD: 25.7±1.7%; AD&HT: 37.0±2.8%; P<0.05).

Hypertension Contributes to the Impairment of Nitric Oxide Pathway in AD&HT Mice
The nitric oxide synthase 1 (NOS1, neuronal) and 3 (NOS3, endothelial) mRNA (Figure S2) and protein (Figure 3A) levels were decreased in the brain of AD&HT mice, compared with WT mice, whereas the NOS2 (inducible) levels remained unchanged. Accordingly, brain levels of nitrate/nitrite (NOx), marker of NO production, were significantly decreased in AD&HT mice compared with WT mice (90.5±7.6 versus 134.2±10.3 nmol/g of wet brain weight; P<0.05); there was also a trend for decrease compared with AD mice (P=0.09; Figure 3B).

ANGII Infusion Does Not Amplify Inflammatory Response in the Brain of AD&HT Mice
Immunohistochemical analysis of brain sections with anti–induction of brown adipocytes-1 antibody, a marker of microglia, a main cellular player in neuroinflammation, demonstrated that, in both WT and HT mice, microglial cells presented mainly nonactivated phenotype with small cell body and long ramified processes, whereas more activated microglia (adopting a round amoeboid shape) accumulated in AD and AD&HT mice and tightly clustered around the amyloid plaques (Figure 4A). Quantification analysis showed a significant between group difference in the number of induction of brown adipocytes-1–positive activated microglia (P=0.009) with higher levels in both AD and AD&HT mice compared with WT and HT mice. No significant difference was found between WT and HT or AD and AD&HT mice, indicating that microglia activation was stimulated by accumulating Aβ regardless of the ANGII-induced hypertension (Figure 4B).

Moreover, quantitative analysis demonstrated that, in line with the data on the similar levels of proinflammatory NOS2 (Figures 3A and S3), there was no significant difference in the expression of mRNA encoding for proinflammatory cytokines interleukin-1β and monocyte chemoattractant protein-1 between 4 groups (data not shown).

Hypertension Precipitates the Onset of Cognitive Deficit in AD&HT Mice
To investigate whether hypertension would accelerate the development of cognitive deficits in APPPS1 mice, we subjected all animals to an episodic-like memory task evaluating the subject’s memory for objects, location, and recency.13

We first ensured that mice of all 4 groups were equally motivated to approach and explore the objects. There were no significant group differences between either total observation or locomotor activity across 3 trials (2 sample trials plus test trial; data not shown). In the temporal memory component of episodic-like memory, WT and HT mice spent more time exploring the old familiar than recent familiar objects (Figure 5). In contrast, AD&HT mice showed significantly impaired performance, compared with both WT and HT mice (P<0.01 and P<0.05). There was also a trend for a difference between the 2 APPPS1 groups with AD mice outperforming AD&HT mice (P=0.091). No significant difference was obtained for the comparison of WT and HT groups or WT and HT groups and AD group. Thus, AD&HT mice demonstrated impaired temporal order memory performance already at the age of 4.5 months. Comparing the object-place memory discrimination ratios revealed no significant difference between the 4 groups (data not shown).

Discussion
In patients, hypertension is associated with memory, attention, and processing speed deficits, which are cognitive
domains similarly affected in AD.14 In line with these observations, we determined that hypertension accelerated the development of cognitive deficit in AD&HT mice through inducing cognitive impairment as early as at the age of 4.5 months. Spatial learning deficits in the Morris water-maze, Y-maze, and contextual fear task had been reported in APPPS1 mice.12,15 These deficits were detected in 8-month-old but not in younger APPPS1 mice. The episodic memory impairment is a cardinal symptom of AD and precedes a more global cognitive impairment.16,17 Using the episodic-like memory test, we found that AD&HT mice showed impaired temporal order memory compared with both the WT and HT mice. This is the first study in which temporal order memory performance has been investigated in APPPS1 mice.

The temporal order memory deficits in AD&HT mice might be related to a mPFC pathology, believed to be the neuroanatomical substrate of temporal order memory.18 However, we did not found increased levels of amyloid deposits in the mPFC of AD&HT mice, although they evidenced temporal order memory deficit. Instead, we found that AD&HT mice presented a significantly reduced cerebral MVD, compared with AD, HT, and WT mice, and especially in mPFC. Thus, early impairment of temporal order memory in AD&HT mice may be primarily linked to early regional MVD reduction in the mPFC.

It has been suggested that cerebral vasculature in AD could undergo abnormal remodeling19 reminiscent of poststroke angiogenesis,20 associated with upregulation of VEGF accumulating in amyloid plaques.6 Accordingly, we found that at 4.5 months, the brain VEGF-A levels and cerebral MVD were increased in AD mice, possibly representing an adaptive response to cerebrovascular impairment. Interestingly, VEGF-A upregulation was abolished in AD&HT mice, along with the decrease in cerebral MVD. Hypertension likely contributes to this phenomenon through impairment of endothelial cell function and increase in smooth muscle tone resulting in another type of vascular remodeling with arterial wall thickening and microvascular rarefaction.21 Moreover, hypertension has been implicated in reducing the number and function of endothelial progenitor cells.22,23 Our results indicate that hypertension-mediated impairment of vascular repair mechanisms may contribute to a faster development of neuropathology and cognitive deficit in AD&HT mice. This worsening of AD-like pathology in AD&HT mice may not be attributable to the direct proinflammatory action of ANGII because we found that ANGII infusion did not aggravate neuroinflammation in our model that is mainly dependent on accumulated Aβ.

NO deficiency has been previously identified as one of the main mechanisms contributing to the development and progression of both cardiovascular disease21 and AD.24 Our results suggest that the reduced NO bioavailability may be one of the pathophysiological links between hypertension and the earlier manifestation of AD-like pathology in AD&HT mice. In accordance with the link between hypertension and reduced NO bioavailability, we found that the brain levels of NOS1 and NOS3, as well as the levels of nitrite/nitrate, were reduced in AD&HT mice. A recent evidence indicates that NO may protect against increase in Aβ through decreasing the levels of APP and APP-cleaving enzyme, β-secretase.25 The observed increase of the levels of soluble Aβ in AD&HT mice might, therefore, result from the upregulation of APP and β-secretase, consecutive to the reduction of NO production in the brain.
Moreover, Aβ42 levels may be regulated by angiotensin-converting enzyme which has been shown to cleave Aβ42 at several sites, including the resulting formation of Aβ40. Angiotensin-converting enzyme activity was not significantly different in the brains of mice of the 4 experimental groups (data not shown). Our results suggest, therefore, that the increase in soluble Aβ42 levels in AD&HT mice could not be because of the altered processing consequential to the decrease in angiotensin-converting enzyme activity.

There is still no clear answer about the cause of AD. The role of insoluble amyloid deposits, initially considered as the principal trigger of neuronal dysfunction and neurodegeneration, is currently questioned. During the past decade, the idea becoming prevalent that the soluble Aβ oligomers are the principal most toxic products of amyloidogenic cascade that impair synaptic efficacy early in the disease process. In the same vein, we found that the number of amyloid deposits was only moderately increased in AD&HT mice, compared with AD mice. In contrast, the soluble Aβ levels were doubled in the brain and in the plasma of AD&HT mice, associated with the earlier occurrence of temporal order memory impairment. Our study provides, therefore, experimental arguments that mainly soluble Aβ, rather than insoluble amyloid of senile plaques, contributes to neurodegeneration and deterioration of cognitive function in AD.

Perspectives

Our study provides experimental evidence on the interaction between hypertension and AD pathology in provoking brain tissue impairment underlying progressive memory loss and cognitive decline. Our results indicate also that vascular dysfunction is an early event in the disease process mediated by Aβ, pointing out the possible causative role of vascular impairment in AD. Whether vascular dysfunction is a causative factor in AD or only contributes to the disease progression remains to be elucidated. However, clinical-pathological data suggest that vascular lesions aggravate the deleterious effects of AD pathology, reduce the threshold for cognitive impairment, and accelerate disease progression. This could explain why treatments reducing vascular risk factors may delay the development of the disease and improve patient’s condition. Thus, counteracting cerebrovascular impairment becomes a major focus for therapeutic approach in AD and emerges as a promising strategy for prevention of the disease. The present mouse model of AD associated with hypertension is a useful tool for testing and development of new vascular protective therapies particularly designed to delay neurodegeneration and cognitive decline in patients with AD and related dementias.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**
- A novel mouse model of Alzheimer disease associated with arterial hypertension, combining anticipated cerebral amyloid and memory deficit with greater vascular impairment because of one of the major cardiovascular risk factors.

**What Is Relevant?**
- Cognitive deficit is related to aggravated vascular impairment, and only modest increase in the amyloid plaque burden in a mouse model of Alzheimer disease associated with hypertension.
- At the early stage of the disease, Alzheimer pathology stimulates cerebral angiogenesis and increases cortical microvessel density, whereas hypertension impairs cerebral angiogenesis and leads to the microvessel density reduction in a mouse model of Alzheimer disease associated with hypertension.
- Vascular impairment is associated with an increase in soluble amyloid peptide in the brain and plasma and the consecutive earlier onset of temporal order memory deficit in a mouse model of Alzheimer disease associated with hypertension.
- Experimental hypertension contributes to reduced brain NO production in a mouse transgenic model of Alzheimer disease.

**Summary**
Hypertensive Alzheimer disease transgenic mice present signs of both Alzheimer disease–like pathology and early vascular impairment. Given that ≈1/3 to 1/2 patients with diagnosed Alzheimer disease present mixed pathology associating amyloid plaques and vascular disease, this model reproduces more faithfully human pathology and is best suited, therefore, for testing novel vascular protective therapeutic strategies in Alzheimer disease.
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Diana Cifuentes,1,2* Marine Poittevin,1,2* Ekrem Dere,3 Dong Broquères-You,1,2 Philippe Bonnin,2,4,5 Joëlle Benessiano6, Marc Pocard,2,4 Jean Mariani,7 Nathalie Kubis,2,4,5 Tatyana Merkulova-Rainon,1,2* Bernard I. Lévy1,4,8*

1. Institut des Vaisseaux et du Sang, 75475 Paris, France
2. INSERM U965, 75475 Paris, France
3. Max Planck Institute of Experimental Medicine, 37075 Göttingen, Germany
4. Université Paris Diderot, Sorbonne Paris Cité, 75205 Paris, France
5. AP-HP, Hôpital Lariboisière, 75475 Paris, France
6. AP-HP, Hôpital Bichat - Claude-Bernard, 75018 Paris, France
7. CNRS UMR 8256, 75005 Paris, France
8. INSERM, U970, 75015 Paris, France

Corresponding author: Bernard Levy, Institut des Vaisseaux et du Sang, Hôpital Lariboisière, 8 rue Guy Patin, 75475 Paris Cedex 10, France. Phone: +33 1 45 26 21 98; FAX: +33 1 42 82 94 73. e-mail: bernard.levy@inserm.fr

* - These authors contributed equally to this work.
METHODS

Animals
Heterozygous APPPS1 male mice, 7 week-old, were purchase from Einzelhandelsfirma Köslner (Rottenburg, Germany). Mice were housed in a temperature-controlled room (21-22°C) with a 12 h light/dark cycle and had free access to food and water. APPPS1 males were further bred with C57BL6/J female mice purchased from Harlan Laboratories B.V. (Venray, the Netherlands). Tail biopsies were used to determine the genotype of littermates by a standard PCR protocol, as previously described. Heterozygous APPPS1 males and their control C57BL/6J male littermates were used in all experiments. All analyses were performed at 4.5 months. All animal procedures were approved by the Lariboisière – Villemin Ethics Committee in Animal Experimentation (protocol number CEEALV/2012-12-02).

Angiotensin II infusion
Angiotensin II (Sigma-Aldrich, St Lois, MO) was infused at 1000 ng/kg/min, over 2.5 months, using osmotic mini pumps Alzet model 2006 (Charles River Laboratories, L'Arbresle, France). Pumps were implanted subcutaneously in the intrascapular region of isofluorane anesthetized mice aged of 2 months and replaced once at 3.5 months, at the end of the 6 week expiration period of the pump. To control pain, an analgesic (buprenorphine at 0.1 mg/kg) was administered subcutaneously 30 min before the surgical intervention. Systolic blood pressure and heart rate were monitored bimonthly using a tail-cuff plethysmograph connected to a computerized system (BP-2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC) in conscious mice before the treatment and over the entire study period until the sacrifice (from age of 2 months until 4.5 months). Body weight was also recorded bimonthly.

Tissue collection
Mice were anesthetized with an intraperitoneal injection of 100 mg/kg of pentobarbital and transcardially perfused with heparinized saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed overnight in PFA and cryopreserved in 20% sucrose. Coronal 30 μm-thick sections were cut frozen using a cryostat CM 1950 (Leica Biosystems, Nussloch, Germany) and proceeded with immunohistochemical staining. Alternatively, for RNA and proteins extraction, mice were given an overdose of phenobarbital. Blood was collected by heart puncture, plasma was separated by centrifugation for 10 min at 2000 x g and stored at -80°C. Then, mice were killed by decapitation and the brains were rapidly removed and cut sagittally into left and right hemispheres. The hemispheres were snap frozen in liquid nitrogen and stored at -80°C.

Immunohistochemical analysis
Immunostaining was performed on coronal 30 μm-thick free floating sections as previously described. The following primary antibodies were used: polyclonal rabbit β-amyloid antibody, 1:250 (Cell Signaling Technology, Danvers, MA); polyclonal rabbit anti-collagen IV antibody, 1:100 (Abcam, Cambridge, UK). For detection of CAA, immediately after collagen IV immunostaining, sections were washed successively with 70% ethanol (1 min) and 80% ethanol (1 min) and incubated for 15 min with 0.2% thioflavin S solution in 80% ethanol (Sigma-Aldrich) followed by wash in 80% ethanol (1 min), 70% ethanol (1 min) and twice in distilled water. Samples were observed with an Axioobserver.Z1 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Images were taken using a Baumer TXD14
digital monochrome progressive scan camera (Baumer Optronic GmbH, Radeberg, Germany) and Archimed 4.7.0 software (Microvision Instruments, France).

For amyloid load quantification, sections at +1.94 (cortex), +1.32 (cortex and mPFC), +0.74 (cortex), -0.82 (cortex), -1.64 and -2.92 (both cortex and HIP) from Bregma were evaluated. Photos were taken at 5X magnification and quantified with Image J software.

To determine microvessel density (MVD), four sections per animal, located at +1.32 (mPFC and cortex), -0.82 (cortex), -1.64 and -2.92 (both HIP and cortex) from Bregma were analyzed. Photos were taken in both brain hemispheres at 20X magnification; the region of interest (ROI) was 580x440 μm. MVD was calculated as a percentage of collagen IV positive area per ROI. The same ROIs were used to quantify CAA. The percentage of amyloid plaques that co-localized with collagenIV immunostaining was calculated using Histolab software (Microvision Instruments).

**Biochemical analyses**

Total RNA isolation, reverse transcription reaction and quantitative real-time PCR were performed as previously described. The following primer were used in this study (all of the primers are listed in the 5'-3' direction): VEGF-A forward, CTTTAATCCGAAAGCCTGACATG; VEGF-A reverse, AAAGTGCTCCTCGAAGAGTCTCC; NOS1 forward, CTGGCTCAACCGAATACAGG; NOS1 reverse, GTAGCCAGTGTACGCTCTGAAG; NOS2 forward, TGGCTTTGTCGGAAGTGTCA; NOS2 reverse, CGGACCACCTCTCGATTTCT; NOS3 forward, AAGCTGCAGGTATTTGATGC; NOS3 reverse, TATAGCCCGCATAGCTC. The primer set specific for mouse peptidylprolyl isomerase A (cyclophilin A) was purchased from Qiagen (Hilden, Germany).

For protein extraction, brain hemispheres were homogenized at 10% (w/v) in TBS buffer (50 mmol/L tris-HCl, pH 7.6 containing 137 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L sodium vanadate, protease inhibitor cocktail 2 and phosphatase inhibitor cocktail from Sigma-Aldrich) using a Magna Lyser Instrument (Roche, Rotkreuz, Switzerland), 2 x 25 sec at 6500 rpm. Half of the homogenate was centrifuged at 25,000 x g for 60 min, 4°C, at and the supernatant (TBS-extracted fraction) was used for determining the soluble amyloid levels (Chemiluminescent BetaMark x-42 ELISA and BetaMark x-40 ELISA kits from Covance, Princeton, NJ), VEGF-A concentration (Mouse VEGF DuoSet ELISA from R&D Systems, Minneapolis, MN) and the nitrite/nitrate concentration (Nitric Oxide Fluorometric Assay Kit from BioVision, Milpitas, CA). Another half of the homogenate was brought to 1% with triton X-100, incubated for 30 min at 4°C with rotation and centrifuged at 12,000 x g for 15 min, 4°C (triton-soluble fraction, TSF). TSF fraction was used for Western blot analysis. Protein concentration in the supernatants was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Western blot, band visualization and quantification were performed as previously described. The primary antibodies used for Western blot analysis were: polyclonal rabbit anti NOS1 antibody (Abcam), polyclonal rabbit anti NOS2 and anti NOS3 (BD Transduction Laboratories, San Jose, CA), polyclonal rabbit anti ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Episodic-like memory test**

One week before the tests, animals were housed individually in Techniplast® blue label macrolone cages (42.5 x 26.5 x 18 cm) with metal covers and sawdust bedding. Animals were maintained in a closed ventilated cages under constant temperature (22 C°) and humidity conditions (75 %). They had free access to water and standard diet. A 24 h light/dark cycle with lights on between 07h00 am to 07h00 pm was used.
Apparatus and objects. Episodic-like memory was assessed according to the procedure originally described in Dere et al., 2005. The open-field was a rectangular chamber (50 x 50 x 40 cm) made of gray polyvinylchloride. One side wall of the open-field was marked with an array of 7 light-brown circles (2 cm diameter, 2 mm depth) and a distance of 2 cm between 2 circles. The array was positioned in the middle of the side wall at a height of 30 cm from the floor. A CCD infrared video camera was mounted 50 cm above the open-field. The open-field was illuminated by diffuse white light with an intensity of 5 lux at the center of the apparatus. The animal's behavior was recorded and analyzed with a semi-automated tracking device (EthoVision, Noldus, the Netherlands). After each trial, the apparatus was cleaned with an ethanol solution. Two different objects (in quadruplicate) made of glass that differed in terms of height, color, shape, and surface texture were used. Since the objects were made of the same material, they could not be distinguished by olfactory cues during the test trial. The objects had sufficient weight to ensure that the mouse could not displace them. The objects had no known ethological significance for the mice and had never been paired with a reinforcer. Pilot studies ensured that C57BL/6J mice could discriminate the two objects and there was no per se preference for one of these objects.

Procedure. The animals received 3 habituation trials of 5 min duration and an inter-trial-interval of 24 h to familiarize them with the open-field. During the episodic-like memory test each mouse received 2 sample trials followed by a test trial. In the first sample trial the mice were presented with 4 copies of a novel object that were arranged in a triangle-shaped spatial configuration as schematically depicted in Supplementary Figure S3, and were allowed to explore them for 10 min. After a delay of 50 min, the mice received a second sample trial identical to the first, except that 4 novel objects that were arranged in a square spatial configuration were presented. After an additional delay of 50 min, the mice received a test trial identical to the second sample trial except that 2 copies of the object from sample trial 1 ("old familiar" objects) and 2 copies of the object known from sample trial 2 ("recent familiar" objects) were presented. During the test trial, one of the "old familiar" objects was displaced to a position in which it was not encountered during the sample trial 1, while the remaining 3 objects, that is 2 "recent familiar" objects and one "old familiar" object were presented at spatial locations at which they were already encountered during the corresponding sample trials. Thus the animals were required to discriminate between the 2 objects, remember the temporal order of their presentation as well as their original spatial positions during the sample trials.

Data collection. Object exploration was analyzed in a fully automatized experimenter-independent manner using the EthoVision tracking software. The open-field was divided into 9 quadrants of equal size and the time spent in the quadrants where the objects have been placed during the sample and test trial was measured. For the sample and the test trials the total time spent in the 4 object quadrants were analyzed. Additionally, the time spent in the "recent familiar" object quadrants, the time spent in the displaced and stationary "old familiar" quadrants were analyzed separately for the test trial. From these raw data 2 discrimination ratios were calculated that reflected the animal's memory for the temporal order of object presentation (what and when) and for the memory of the spatial positions were objects have been presented (what and where) during the sample trials.

Temporal order memory ratio: \((OD+OS)/[(OD+OS)+(R1+R2)]\).

Object-place memory ratio: \(OD/(OD+OS)\).

(Abbreviations: OD = "old familiar" displaced, OS = "old familiar" stationary, R1 = "recent familiar" object 1, R2 = "recent familiar" object 2).
Two animals (one from the AD&HT and one from the WT group) that exhibited discrimination ratios that differed from the group mean for more than 2 standard deviations were excluded from data analysis.

Statistics
Statistical analyses were performed with Prism 5 software (Prism 5.03, GraphPad, San Diego, CA). Statistical analysis of behavioral tests was performed with the program SigmaStat 3.1 (Systat Software Inc.). Data are expressed as means ± standard error of the mean (SEM). All group comparisons were analyzed by one-way ANOVA test or repeated measures ANOVA and Mann Withney test or Student-Newman-Keuls method (behavioral studies) for pair-wise multiple comparisons if significant main effects of group were obtained. All statistical tests performed were two-tailed and were considered to be significant when P-values lower than 0.05 were obtained.

SUPPLEMENTAL REFERENCES

Figure S1. Evolution of systolic blood pressure and body weight in C657BL/6J and transgenic APPPS1 mice, sham operated or transplanted with ANGII infusing minipumps. Left, starting from the age of 2 months, systolic blood pressure was measured bimonthly by tail-cuff plethysmography (***$P < 0.001$; $n = 12-15$). Right, time-course of body weight gains estimated on a bimonthly basis.
Figure S2. Effects of hypertension and Alzheimer's-like pathology on the NOS isoform expression at transcriptional level. The steady state levels of NOS1, NOS2 and NOS3 mRNA were measured by qRT-PCR and normalized over cyclophilin A in total RNA extracts (*P < 0.05; ***P < 0.001; n = 5-6).
Figure S3. Schematic drawing of the episodic-like memory task. Each mouse receives 2 sample trials with 4 identical copies of a novel object followed by a test trial in which two objects from the first sample trial ("old familiar" objects) are presented together with 2 objects known from the second sample trial ("recent familiar" objects). During the test trial one of the "old familiar" objects had been displaced to a novel location.