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Abstract

In Alzheimer's disease (AD) amyloid beta (Aβ) plaques accumulate in the brain parenchyma, or cortex as well as in the cerebral vasculature. This increase in Aβ accumulation in blood vessels causes cerebral amyloid angiopathy (CAA) and, possibly, hemorrhagic stroke. Changes in cerebral blood pressure and blood flow, due to aging or hypertension, likely lead to the cognitive dysfunction (dementia) that is associated with AD. This study aimed to determine if prolonged hypertension in transgenic (Tg) AD model mice causes cerebral hypoperfusion, or insufficient blood flow through the brain, which in turn leads to cognitive impairment and, possibly, increased Aβ deposition. Tg AD model mice were made hypertensive and compared to non-hypertensive Tg AD mice. Blood pressure and cerebral blood flow were measured, and behavioral tests were performed to analyze the cognitive changes in the animals. The amount of amyloid deposition, glial activation, and changes in blood vessel density/integrity were measured. The results show that prolonged mid-life hypertension in Tg AD model mice significantly increased behavioral deficits, elevated Aβ deposition, and increased glial activation compared to non-hypertensive Tg AD mice. Furthermore, there was an increase in dysfunctional vessels in the plaque-deposit rich areas in the brain of hypertensive mice compared to non-hypertensive animals. Together the data indicate that early treatment of hypertension could reduce the incidence and severity of Alzheimer's disease.

Introduction

In the elderly, Alzheimer's disease (AD) is the most common form of dementia. The two pathologies that characterize the disease are the presence of large numbers of intracellular neurofibrillary tangles (NFTs) and extracellular neuritic plaques in the brain (e.g., Braak and Braak, 1991; 1998; Selkoe, 2001). Neurofibrillary tangles consist of hyperphosphorylated, twisted filaments of the cytoskeletal protein tau, whereas plaques are primarily made up of amyloid β (Aβ [Selkoe, 2001; Dickson and Vickers, 2002]), a 39-43 amino acid long peptide derived from the proteolytic processing of the amyloid precursor protein (APP [Selkoe, 2001). When APP is sequentially cleaved by the β-secretase and γ-secretase, one of the resulting breakdown products is Aβ, in contrast, initial cleavage by α-secretase (in the middle of the Aβ sequence) leads to production of APPs-α and the C83 peptide (Selkoe, 2001).

Most cases of AD are sporadic, however approximately 5 % of AD cases are familial (Selkoe, 2001), these cases are related to mutations in the genes for APP, and presenilin 1 and 2 (PS1 and PS2 [Selkoe, 2001]). Transgenic mice expressing mutated human AD genes offer a powerful model to study the role of Aβ in the development of pathology (e.g., Duff and Suleman, 2004; McGowan et al, 2006).

Hypertension is a risk factor for stroke and vascular dementia, and the incidence of these diseases grows with increasing blood pressure. Recent studies have shown that hypertension is also a risk factor for Alzheimer's disease (AD). AD is associated with the accumulation of amyloid beta (Aβ) in plaques in the brain parenchyma but also with substantial amyloid beta deposition in the cerebral vasculature leading to cerebral amyloid angiopathy and hemorrhagic stroke. Furthermore, it has been demonstrated that AD patients have cerebral hypoperfusion and cerebral hypoactivity. Clearly, cerebrovascular disease is not the sole cause of AD, but mounting evidence indicates that changes in cerebral blood flow do contribute to the cognitive dysfunction associated with AD. The brain controls cerebral blood pressure and blood flow, but with long-standing hypertension and increasing age this regulation is altered. This change results in modifications in the cerebral circulation as sustained hypoperfusion impacts the aging process to induce augmented pathology. The relation between blood pressure and cognitive function and dementia has, in recent years, been researched extensively (e.g., De la Torre, 2004). Some studies have shown an inverse association between blood pressure and the prevalence of dementia and Alzheimer's disease, whereas other studies have yielded mixed results that largely depend on the age at which blood pressure is measured and the time interval between blood pressure and outcome assessments (Qiu et al, 2005). Some studies suggest that midlife high blood pressure is a risk factor for late-life cognitive impairment and dementia, and that low diastolic pressure and very high systolic pressure in older adults may be associated with subsequent development of dementia and Alzheimer's disease (Qiu et al, 2005; Skoog and Gustafson, 2006). Our preliminary studies indicate that hypertensive Tg AD model mice exhibit increased amyloid depositions compared to normotensive Tg AD mice. These results have led to the hypothesis that sustained hypertension will lead to decreased functional hyperemia in the brain which in turn contributes to brain hypoperfusion and adds to the cognitive impairments of AD. Further, decreased blood flow will concurrently lead to increased amyloid beta deposition in the brain due to decreased clearance of Aβ. Thus, the following studies are designed to test the hypothesis that long-term untreated hypertension will lead to perturbed vascular function, and increased cognitive dysfunction and pathology in AD model mice and that antihypertensive treatment of hypertensive Tg AD model mice will attenuate cerebral hypoperfusion

and significantly decrease cognitive deficits and amyloid beta deposition in the brain.

Materials and Methods

Animals:

In our study we used APP-DI (Davis et al., 2004) transgenic AD female mice (10/group). At six months of age Alzet minipumps containing human Angiotensin II (130 μg/day) or saline were implanted s.c. under anesthesia with isoflurane. The mice were treated (and, thus, were hypertensive) for two months. All mice were maintained in accordance with Institutional Animal Care and Use Committee (IACUC) regulations at the University of Alabama at Birmingham.

Behavioral analysis:

After seven weeks of hypertension, the mice were subjected to a battery of behavioral tests for hippocampus-related spatial memory. To rule out differences in anxiety or stress levels between animals, the open field and elevated plus maze were used as basic measures; to analyze cognitive deficits, we tested the mice in the water maze task (Liu et al, 2002) and the Barnes maze (Barnes, 1979) these tests were performed in the UAB Behavioral Core).

The *water maze apparatus* and procedure previously described in detail, uses a blue plastic pool, 120 cm in diameter and a see-through round platform, 10 cm in diameter, located 1.0 cm below the water surface (Liu et al, 2002). During day 1 through 5 of the testing period, the mice are trained to find the hidden platform that is kept in a constant position throughout these 5 days. Four trials are run per mouse each day so that all starting positions are equally used (in a random order). The mice are given 90 s to find the platform and 10 s to stay on the platform. The inter-trial interval is approximately 10 min. Learning of the task is evaluated by recording the swimming speed, latency to find the platform, path length, and percentage of trials each animal finds the platform.

The *Barnes maze* that is used has a design similar to the one developed by Barnes (1979) for rats but it has been adapted for mice testing. The raised (120 cm above the floor), round platform has a diameter of 140cm, and the escape holes are distributed along the rim of the platform. Only the correct escape hole, one of the many holes around the rim, has the escape cage attached under it. The mice are trained to escape from the open platform (the start position is in the middle of the platform) to the by finding this specific cage. An entry into an incorrect hole is deemed a reference memory error, and an entry into a hole the mouse has previously examined, is a working memory error. The mice are removed from the maze after escaping or after 4 minutes has passed, whichever comes first. An entry into the cage is defined as the head entering the escape hole. Learning of the task is evaluated by recording the latency to find the correct escape hole, the path length, and percentage of trials each animal finds the correct hole. Four trials are run per day per mouse; the learning of the maze takes an average of 5 days.

Blood pressure measurements:

Blood pressure was measured every two weeks starting from 1 week prior to the infusion, using indirect, tail-cuff blood pressure measurements supported by the Hatteras blood pressure measurement apparatus.

Blood flow measurements:

The head of the animals were fixed into a stereotaxic frame, the apex of the skull being exposed, to allow laser-Doppler flowmetry (LDF) of the cerebral blood flow (CBF). A hole was drilled in the skull above the middle cerebral artery. The laser Doppler probe (BIOPAC; LDF100c unit with TSD144 probe) was placed stereotaxically above this location (which corresponds closely to the expected region of maximal change in the flow response). The LDF signal, systemic arterial blood pressure, and heart rate were recorded continuously while simultaneously being processed on a PC (see figure; changes following phenylephrine injection). We recorded CBF before, during, and after a 5 sec whisker stimulation to measure the functional hyperemia response (i.e., the cerebral autoregulation; Rosengarten et al, 2005).

Histological analysis:

Aβ deposition, inflammation and blood vessel density and size were quantified in the mice. At 8 months of age (following the behavioral, blood pressure and blood flow analyses described above), the animals were sacrificed. The mice were transcardially perfused with 0.9 % saline followed by 4% paraformaldehyde in 0.1 M Na-phosphate buffer. The brains were removed and placed in the fixative for 2 hours, then transferred to a 30% sucrose solution; the brains are kept in this solution overnight on a shaker table. Six series of (1 in 6) coronal sections (35 μm) were cut through the brain using a sliding, freezing microtome. One half of the first series of sections were stained with cresyl violet, the other half - with an antibody against human Aβ using the W0-2 antibody (mouse anti-humanA□β4-10). One half of the second series were immunohistochemically stained for GFAP (mouse anti-GFAP; Sigma), whereas the other half were stained for CD11b (rat anti-mouseCD11b; Serotec), a marker of microglia. One half of the fourth series is stained for glucose transporter1 (Glut-1, rabbit anti-Glut1; Chemicon).

In short, the sections were rinsed overnight in Tris-buffered saline (TBS), then the series of sections were transferred to a solution containing the primary antibody, the solution consisting of TBS with 0.5 % Triton X-100 added (TBS-T). Following incubation in this solution for 18 h on a shaker table at room temperature (20oC) in the dark, the sections

are rinsed in TBS-T and transferred to the solution containing the appropriate secondary antibody (at 1:500). After two hours, the sections are rinsed with TBS-T and transferred to a solution containing mouse ExtrAvidin® (Sigma), following another rinse the sections are incubated for approximately 3 min with Ni-enhanced DAB. Following rinsing in 0.1 M phosphate buffer (pH 7.4), all sections are mounted on slides and coverslipped with DPX and inspected on a microscope using brightfield illumination.

Quantification of Aβ deposits:
The appropriate areas of the brain were digitized using a Olympus DP70 digital camera, and the images were converted to grey scale. To avoid changes in lighting, which could affect measurements, all images were acquired in one session. The area covered by Aβ (in the W0-2 stained material) was measured using the ScionImage (NIH; Frederick, MD, USA) program (Kadish et al. 2002). These measurements were done in triplicate (i.e., at three rostrocaudal levels of the appropriate cortical areas and arteries; Kadish et al., 2002) to minimize variability. The percentage of area covered by Aβ (i.e., the Aβ load) is the area occupied by Aβ divided by the total area of brain measured. Data were analyzed by ANOVA (SPSS version 10.0), and post-hoc tests (Tukey and Scheffe) were carried out to determine the source of a significant main effect or interaction.

Results

Angiotensin II treatment:
The treatment with Ang II for two months using Alzet minipumps resulted in a significant increase in blood pressure (Figure 1), sham treatment (i.e., saline) did not affect blood pressure. General health and body weight was not affected by Ang II treatment. The blood pressure of Ang II treated mice was significantly higher by twelve days after implantation of the Alzet mini-pumps, and stayed significantly higher during the full infusion period.

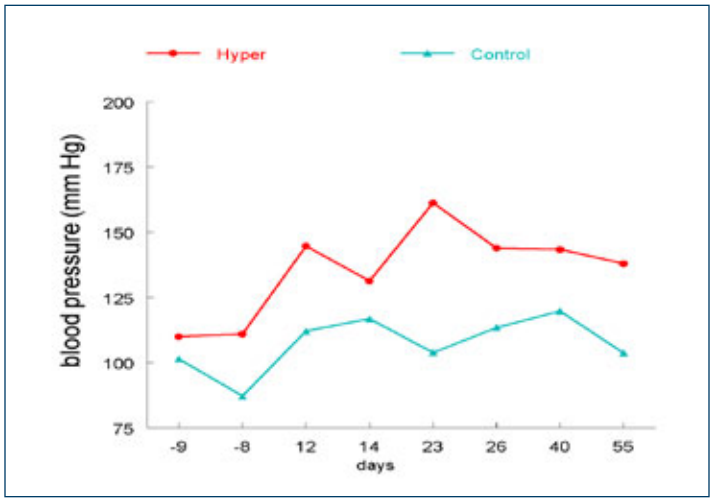

Fig. 1 Graph showing the difference in blood pressure between the Angiotensin II treated and control mice. Notice the significant increase in blood pressure in the Angiotensin II treated mice after implantation of the Alzet mini-pumps.

Behavioral test analysis:
Ang II treated mice had a longer escape latency in the Barnes maze (Figure 2), compared to the timings of the saline-treated, control animals. Similarly, in the water maze the Ang II treated mice had a longer escape latency than the control mice (Figure 2).

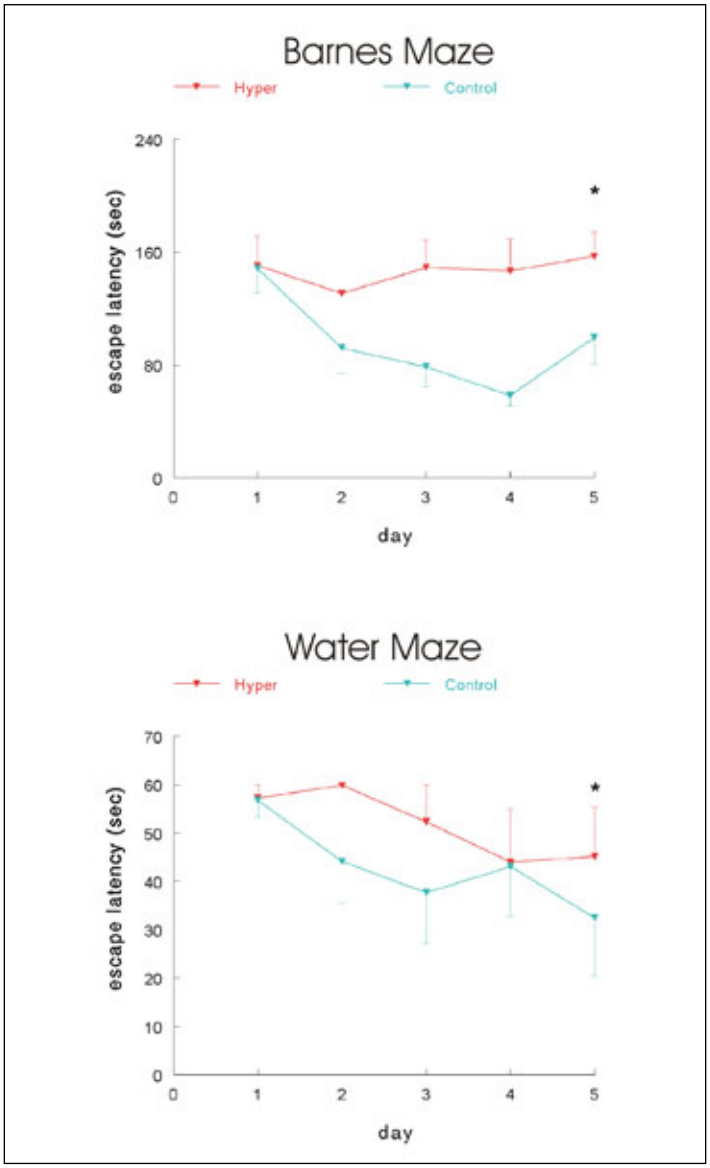

Fig. 2 Graphs showing the learning curves in the water maze (A) and the Barnes maze (B) of Angiotensin II treated and control mice over a five day testing period. Note the significant learning deficit in Angiotensin II treated mice.

Laser Doppler blood flow measurement:
The analysis of laser Doppler blood flow graphs shows that Ang II treated animals took a longer time to respond to the stimulus and also had significantly slower recovery time to return to the baseline blood flow level (Figure 3), i.e., they had

a significantly impaired hyperemia response, in comparison to the response of the control animals.

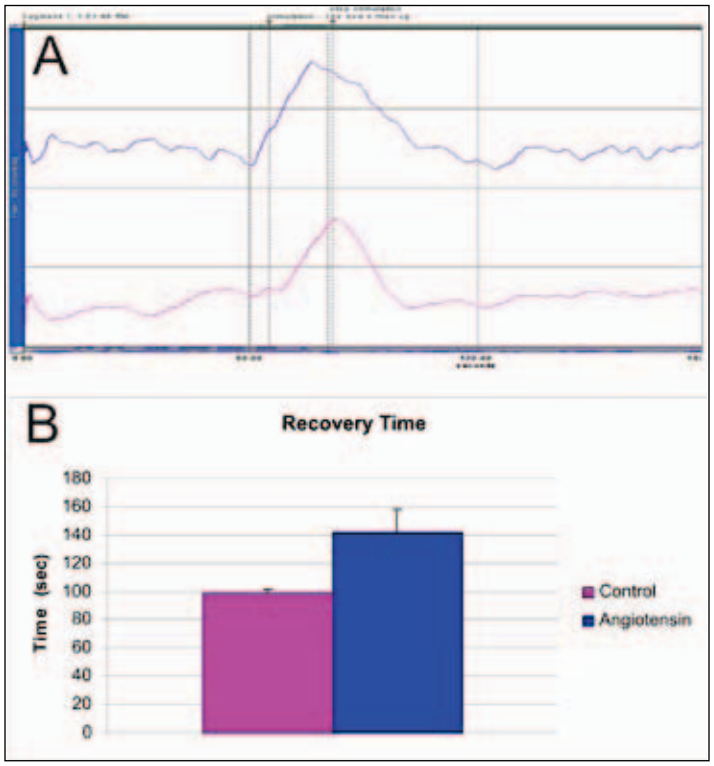

Fig. 3 **A:** graph showing the laser Doppler measurement of blood flow in somatosensory cortex of Angiotensin II treated and control mice. **B**: comparison between recovery times after whisker stimulation for Angiotensin II treated and control mice.

Pathology:
In contrast to the sham treatment, the Ang II treatment increased the amount of Aβ deposition significantly, 0.89±0.10 and 2.92±0.33 ($P<0.05$) respectively (Figure 4), especially in the hippocampus. Further, the number of blood vessels in the thalamus that show CAA significantly increased however, no changes are present in cortical arterioles.

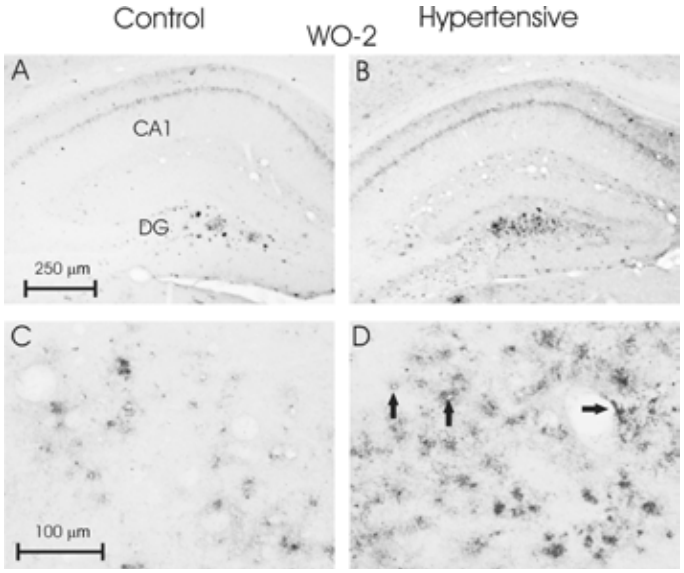

Fig. 4 (bottom of last column) Four photomicrographs showing Aβ stained sections of the dorsal hippocampus (A, B) and the ventrobasal complex of the thalamus (C, D), A and C from Angiotensin II treated mice, B and D from Control mice. Arrows in panel D shows amyloid beta deposition in blood vessel walls. CA1 – CA1 field of the hippocampus, DG – dentate gyrus.

There was significant increase in density of staining for GFAP (Figure 5). In the astrocytes there are increased amyloid depositions in the Ang II treated mice in comparison to the depositions in the control mice, indicating an elevated inflammation state in hypertensive mice. Blood vessel density did change slightly in the hippocampus (Figure 5), and there are similar changes in the thalamus (results not shown). More interestingly, both the hippocampus and thalamus show an increase in dysfunctional vessels in the plaque-deposit areas (Figure 5).

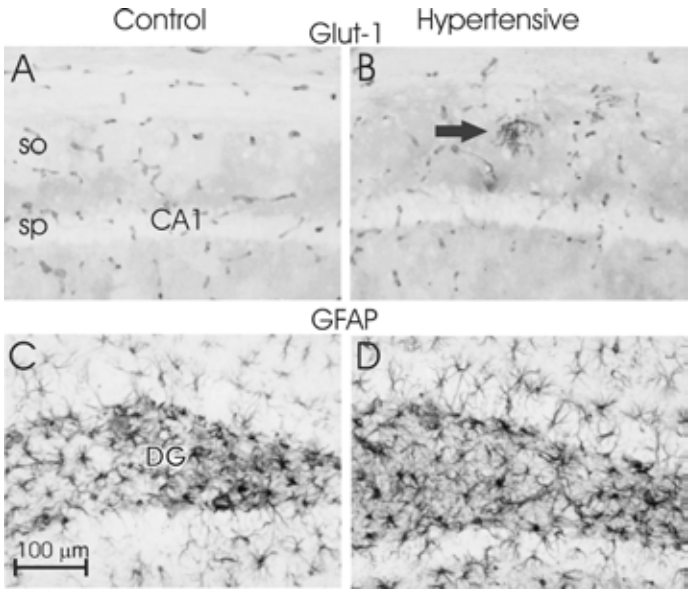

Fig. 5 Four photomicrographs showing Glut1 (blood vessels) and GFAP (astrocytes) stained sections of the dorsal hippocampus left side from Angiotensin II treated mice, right side from Control mice. Arrow in panel B shows abnormal blood vessel in the brain of hypertensive mouse. so – stratum oriens, sp – stratum pyramidale.

Discussion
The results of these studies demonstrate that mid-term hypertension (6 – 8 months of age) in AD model mice significantly increased Aβ deposition in the hippocampal formation. Furthermore, the hypertensive animals showed a significant decrease in learning abilities and memory capacity. Due to the impaired cerebral hyperemia there is decreased cerebral blood flow which leads to increased amyloid deposition and greater glial activation; in turn, these events lead to cognitive deficits. Therefore, maintenance of normal blood pressure is critically important for brain neuronal function.

The relationship between blood pressure, cognitive function, and dementia has, in recent years, been researched extensively (e.g., De la Torre, 2004). Most studies suggest that midlife high blood pressure is a risk factor for late-life cognitive impairment and dementia, and that low diastolic pressure and very high systolic pressure in older adults may be associated with subsequent development of dementia and Alzheimer's disease (Qiu et al, 2005; Skoog and Gustafson, 2006). Observational studies and randomised clinical trials have provided evidence for a protective effect of antihypertensive therapy against dementia and stroke-related cognitive decline (Savaskan, 2005). Atherosclerosis resulting from long-standing hypertension, and cerebral hypoperfusion secondary to severe atherosclerosis (and to low blood pressure) may be major biological pathways linking both high blood pressure in midlife and low blood pressure in late-life to cognitive decline and dementia. Our data confirm that hypertension increases learning and memory deficits in AD model mice.

In our current studies, we have used the APPSwDI Tg mouse line (Davis et al., 2004), these mice develop pathology relatively rapidly, the first parenchymal plaques appear approximately at 3 months of age, furthermore at that age several arteries are already loaded with Aβ. With increasing age both the cortical and the blood vessel Aβ load significantly rise. Our data indicate that midlife hypertension (i.e., from two months of age) significantly increases amyloid deposition in both the parenchyma and in blood vessel walls. One of the two characteristic pathological hallmarks of human AD is the presence of neuritic plaques (Braak and Braak, 1991). Neuritic plaques have a dense core of aggregated Aβ peptides (Braak and Braak, 1991; 1998; Selkoe, 2001). It has been suggested that these deposits of extracellular Aβ originate from direct secretion of Aβ followed by extracellular accumulation. Alternatively, this protein would first accumulate intracellularly, followed by cell-death and subsequent release of the accumulated Aβ (e.g., Gouras et al., 2000; Selkoe, 2001). Presently, it is still unclear how the extracellular deposition of Aβ and the progression in AD neuropathology are related to the increased production of APP and Aβ.

Most neuritic plaques are surrounded by activated glial cells (e.g., Akiyama et al., 2000; Rogers and Lue, 2001). Similarly, plaques in AD-model mice are quite often accompanied by activated glial cells, both astrocytes and microglia (e.g., Bondolfi et al., 2002). However, the role of activated microglia is unclear; on one hand they could protect the brain by removing Aβ , on the other hand they secrete inflammatory cytokines and generate NO, and can thus damage and kill bystander neurons (Akiyama et al., 2000). Their role in the uptake of Aβ is disputed, however, with some groups seeming to show clearance of Aβ by microglia (e.g., Rogers and Lue, 2001), while others show that microglia do not seem to take up Aβ (Stalder et al., 2000). Most of the plaques in our animals are surrounded by activated glial cells and likewise, most CAA deposits are associated with both activated microglia and activated astrocytes.

Together the data indicate that early treatment of hypertension could reduce the incidence and severity of Alzheimer's disease.

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