RESEARCH ARTICLE

Telmisartan Modulates Glial Activation: In Vitro and In Vivo Studies

Nofar Torika1, Keren Asraf1, Abraham Danon1, Ron N. Apte2, Sigal Fleisher-Berkovich1*

1 Department of Clinical Biochemistry and Pharmacology, Ben-Gurion University of the Negev, Beer-Sheva P.O.B 653, Israel, 2 Department of Microbiology and Immunology, Ben-Gurion University of the Negev, Beer-Sheva P.O.B 653, Israel

* fleisher@bgu.ac.il

Abstract

The circulating renin-angiotensin system (RAS), including the biologically active angiotensin II, is a fundamental regulatory mechanism of blood pressure conserved through evolution. Angiotensin II components of the RAS have also been identified in the brain. In addition to pro-inflammatory cytokines, neuromodulators, such as angiotensin II can induce (through angiotensin type 1 receptor (AT1R)) some of the inflammatory actions of brain glial cells and influence brain inflammation. Moreover, in Alzheimer’s disease (AD) models, where neuroinflammation occurs, increased levels of cortical AT1Rs have been shown. Still, the precise role of RAS in neuroinflammation is not completely clear. The overall aim of the present study was to elucidate the role of RAS in the modulation of glial functions and AD pathology. To reach this goal, the specific aims of the present study were a. to investigate the long term effect of telmisartan (AT1R blocker) on tumor necrosis factor-α (TNF-α), interleukin 1-β (IL1-β) and nitric oxide (NO) synthesis. Telmisartan effects in vivo were compared to those of perindopril (angiotensin converting enzyme inhibitor). b. to examine the effect of intranasally administered telmisartan on amyloid burden and microglial activation in 5X familial AD (5XFAD) mice. Based on the current view of RAS and our data, showing reduced amyloid burden and glial activation in the brains of 5XFAD transgenic mice, one may envision potential intervention with the progression of glial activation and AD by using AT1R blockers.

Introduction

The pathology of Alzheimer’s disease (AD) is associated with neuroinflammation, which involves activation of central resident immune cells (e.g., microglia, astrocytes) and infiltrating...
peripheral macrophages [1]. Microglia are known as the macrophages of the brain, representing the first immune response defense in the central nervous system (CNS), and, therefore, can be rapidly activated in response to any central pathological changes [2]. Microglial activation plays a dual role in neurodegeneration, since microglia can aid the repair and regeneration of neurons, but they can also instigate neuronal damage [3]. The brain inflammation involved in AD pathology includes the deposition of amyloid β (Aβ) in areas where pronounced microglia and astrocytes cell activation is observed [4,5].

Glia activation by Aβ results in the production of various pro-inflammatory cytokines, prostaglandins and nitric oxide (NO), which contribute to synaptic impairment and neuronal damage [3,4,6]. The cellular injury promotes additional inflammatory cascades, increases cellular injury, and aggravates AD pathology [3,6–10]. Several lines of evidence support the neurotoxic role of glia-secreted NO in neuronal death in vitro [11,12]. Glial inducible nitric oxide synthase (iNOS) isoform has been linked with neurodegeneration [13,14]. Elevated levels of tumor necrosis factor-α (TNF-α) have been also observed in the cerebrospinal fluid (CSF) and serum of AD patients [15]. Interleukin 1-β (IL1-β) levels were reported to increase in early onset AD patients’ serum [16]. Also, both cytokines can aggravate brain inflammation by sustaining microglial activation [17,18].

Accumulating evidence indicate that the renin-angiotensin system (RAS) may contribute to the brain inflammation associated with AD pathology [19]. An intrinsic brain RAS, which is distinct from the peripheral one, was identified [20]. The peripheral hormone and neuropeptide angiotensin II (AngII) is considered the major RAS effector, and acts mainly via stimulation of the angiotensin type 1 receptor (AT1R) [21]. The latter may serve as neuroinflammatory instigator [7,22]. AngII is formed upon the hydrolysis of angiotensin I by the angiotensin converting enzyme (ACE). Angiotensinogen, the angiotensin I precursor, is abundant in the brain’s extracellular and cerebrospinal fluids and is mostly produced in glial cells (mainly by astrocytes and, to a lesser extent, by microglia) [23–25]. Receptors for AngII have been identified in rat, monkey, and human glial cells (microglia and astrocytes) [24,26,27]. Hyperactivation of brain AT1R promotes hypertension and vulnerability to ischemia and to vascular and tissue inflammation, and may enhance neuronal loss and neurodegeneration [7,22,28–30]. There is evidence indicating that RAS blockade by orally administered ACE inhibitors (ACEIs) or AT1R blockers (ARBs), e.g perindopril or telmisartan respectively, may have a beneficial effect on cognitive functions in AD [31–36]. However, improved targeting the brain by these agents is still a major challenge [37]. Intranasal administration may offer a solution, as this noninvasive procedure can bypass the blood brain barrier (BBB) and allow direct entry to the CNS [37].

The overall aim of the present study was to elucidate the role of RAS in the modulation of glial functions and AD pathology. To reach this goal, the specific aims of the present study were a. to investigate the long term effect of telmisartan (ARB) on NO release from BV2 murine microglial cells as well as neonatal primary microglial cells. Regulation of TNF-α, IL1-β release and iNOS expression by telmisartan will be studied in BV2 cells. b. to examine the effect of intranasally administered telmisartan on amyloid burden and microglia/macrophages activation in Alzheimer’s disease (AD) mice. Telmisartan effects in vivo were compared to those of perindopril (ACEI).

The five familial Alzheimer’s disease (5XFAD) transgenic mouse model was used. This model expresses three mutations in the gene for the amyloid precursor protein (APP) and two mutations in the gene for presenilin 1 (PS1). These mutations, in turn, lead to early production of a 42-amino acids Aβ peptide, to brain inflammation, and to glial activation associated with Aβ plaques [38].
Materials and Methods

Cell culture

**BV2 murine microglial cells.** BV2 murine microglial cells were obtained from Professor Rosario Donato (Dep. of Experimental Medicine and Biochemical Sciences, University of Perugia, Italy) [39,40]. Cells were routinely maintained in RPMI-1640 medium with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM) at 37°C in 5% CO2 humidified air. Prior to each experiment, cells were grown for 24 h on 24-well and 6-well plates at a concentration of 3x10^5 and 1x10^6 cells per well, respectively.

**Primary rat neonatal glial cells and microglia.** Whole brains of neonatal Wistar rats, 0–24 h of age were used in order to obtain rat primary glial cultures, according to previous reports [41,42]. Pups were sacrificed by decapitation, the meninges were removed and a steel mesh and nylon sieves of 60 μm pore size were used to harvest brain cells. The cells were seeded in poly-l-lysine-coated 24-well plates and 75 cm² flasks at a concentration of 1x10^6 cells per well and 30–35x10^6 cells per flask, respectively. The medium culture included high glucose DMEM with 10% heat inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (0.2 mM) and insulin (100 U/ml). Immunocytochemistry studies revealed that these cultures contain about 80% astrocytes and about 20% microglia (Levant 2006).

Isolated microglia were obtained from a mixed culture of astrocytes and microglia grown in flasks, at a 37°C humidified atmosphere with 5% CO2, for 12 days. Medium was replaced once a week after culturing. On day 12, flasks were shaken at 200 rpm for 1.5 h at 37°C and medium containing floating microglial cells used for cells reseeding in a 24-well plates, 1x10^6 cells per well. Culture purification immunocytochemistry analysis indicated >95% microglia cells for this enriched microglia procedure [43].

Mixed cultures of glial cells were obtained from cells seeded on poly-l-lysine-coated 24-well plates and grown in a humidified incubator at 37°C with 5% CO2 for 21 days. Culture medium was replaced twice a week prior to experiments.

Serum-free medium (SFM) was added to the cells prior to each experiment. After 4 h of SFM incubation, cells were treated with SFM containing 0.1% BSA and HEPES buffer (10 mM), pH 7.4 in the presence or absence of test agents for the indicated periods of time.

Telmisartan was purchased from Tocris Biosience, Bristol UK and lipopolysaccharides (LPS) was from Sigma-Aldrich.

**Determination of NO levels (Griess reaction)**

Culture supernatant nitrite levels, as an indicator for NO production, were determined by a colorimetric assay using Griess reagent (Sigma—Aldrich). A standard curve of sodium nitrite was used for nitrite concentrations. Equal volume (100 μl) of culture supernatant and Griess reagent were mixed in a 96-well plate and incubated at room temperature for 15 min (light avoided). Then, absorbance at 540 nm was measured using a microplate reader (model 680, Bio-Rad). Meanwhile, cells were harvested with 4°C SFM and counted using Z1 Coulter counter (Coulter Electronics, Miami, FL) [40].

**TNF-α and IL1-β protein assay by ELISA**

TNF-α and IL1-β levels in the medium (supernatant) were assayed using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions.
Western blot analysis

Whole cell lysates were separated on 7.5% polyacrylamide-SDS gels and transferred to nitrocellulose membranes. After blocking (4% BSA, 90 min at room temperature), membranes were incubated overnight at 4°C with specific rabbit anti-iNOS antibody (1:1000, Cayman Chemicals, USA). Following washing, the blots were incubated for 90 min at room temperature in the corresponding-conjugated donkey anti-rabbit antibody (1:10000, GE Healthcare, Buckinghamshire, UK). The position of the individual protein was detected using enhanced chemiluminescence (ECL) solution (according to the manufacturer’s instructions) followed by exposure to X-ray film (Fuji medical X-ray film, FujiFilm). Band intensity analysis was performed using a computerized image analysis system (EZ Quant-Gel 2.2, EZQuant Biology Software Solutions Ltd., Israel). Protein load was normalized by β-actin protein level measurements using mouse anti-β-actin antibody (1:4000, Sigma-Aldrich) followed by exposure to horseradish peroxidase-conjugated goat anti-mouse antibody (1:20,000, Jackson immunoReaserch Inc., USA).

Mice

5XFAD transgenic mice (Tg6799), kindly provided by Professor Robert Vassar (Department of Cell and Molecular Biology, Northwestern University, Chicago, Illinois 60611), were used. These mice exhibit three familial Alzheimer’s disease (FAD) mutations in the human APP695 (Swedish K670N, M671L; Florida I716V and London V717I) and two mutations in the human presenilin-1 (PSEN-1) (M146L, L286V) under the transcriptional control of the neuron-specific mouse Thy-1 promoter [38]. Wild-type (WT) C57BL/6 mice were purchased from Harlan (Jerusalem, Israel) and bred with hemizygous transgenic mice (TG). Both male and female were used. Tail DNA was used for PCR genotyping analysis and detection of the human APP gene. The mice were housed in cages in an air-conditioned room with controlled temperature (22 ± 2°C) and humidity (65 ± 5%) and maintained on a 12h light/dark cycle. Mice were randomly divided into three groups (n = 6–8 animals each, both genders), telmisartan-treated (Tocris Biosience, Bristol UK) (1mg/kg/day) or perindopril-treated (Sigma-Aldrich) (1mg/kg/day) and vehicle-treated mice. The control group included wild-type mice treated with telmisartan or perindopril. Two-month-old mice were treated intranasally (total of 6 μl administered as one 3 μl drops to each nostril) every day for 3.5 to 8 weeks. No significant differences in body weights were observed between different mice groups during and at the end of the experiments.

All primary cell cultures and mice procedures were approved by the Institutional Animal Care and Use Committee of the Ben-Gurion University of the Negev (Beer Sheva, Israel; approval number IL-30-08-2011-15 and IL-54-08-2015-19) [44].

Immunohistochemistry

A mixture of 0.1ml clorketam and 0.1ml sedaxylan was used to anesthetize mice through intra peritoneal (i.p) injection. Then, cardiac perfusion was performed with cold PBS. The brains were removed and one hemisphere of each was incubated in 4% paraformaldehyde (PFA) solution for 24h and transferred to 30% sucrose solution for 48h at 4°C. The tissue was then placed into molds containing OCT compound (Tissue-Tek, Torrance, CA, USA) and frozen at -80°C. Immunohistochemical staining was performed on 40μm sagittal sections (cut by cryostat). First, the sections were rinsed in 0.05% PBS/Tween 20 solution and for further 30 min in 0.5% PBS/Triton X-100 solution. In order to prevent nonspecific binding, blocking was made using primary Ab diluting buffer (GBI labs, Bothell, WA). Immunohistochemical staining was performed for CD11b and Aβ proteins by rabbit anti human Aβ antibody (1:250, gift from Professor Alon Monsonego, The Shraga Segal Department of Microbiology and immunology, faculty
of Health Sciences and the National institute of Biotechnology in the Negev, Ben-Gurion University, Beer-Sheva, Israel) and Rat anti mouse/human CD11b antibody (1:25, Biolegend). An additional rinsing by 0.05% PBS/Tween 20 solution was made, followed by secondary antibody incubation with Cy3-conjugated donkey anti rabbit IgG (1:1000, Jackson ImmunoResearch Laboratories, 711-165-152) or Alexa flour 488-conjugated goat anti rat IgG (1:250, Jackson ImmunoResearch Laboratories, 112-545-003), respectively. Finally, tissues were placed on charged slides, covered with mounting medium containing DAPI (Vector labs) and stored in 4°C [44].

Confocal imaging analysis
Quantification analysis of Aβ plaques and CD11b positive cells in the brain was performed in sections (40 μm thick) per hemisphere stained for Aβ and CD11b. In each experiment five sections from each animal (from the cortex and the hippocampus) were analyzed. Fluorescence baseline intensity was first obtained in sections from control mice (wild type). All images were obtained using an Olympus FluoView FV1000 confocal microscope (Olympus, Hamburg, Germany) at a 1024 X 1024 pixel resolution with X10 objective. Aβ and CD11b staining was quantified using ImageJ software (version 1.40C, NIH, USA) with threshold function. The intensity threshold was set by marking areas stained only with CD11b or Aβ in the hippocampus and cortex. The average florescence stained area was calculated for each treated group [45].

Statistical analysis
Experimental data are represented as the mean±SEM. For significance assessment between groups, one-way analysis of variance (ANOVA) and post hoc multiple comparison test (Tukey—Kramer Multiple Comparison Test) were performed. Statistical significance was considered at P< 0.05.

Results
Telmisartan attenuates the expression of inflammatory markers in LPS-induced glial cells
We examined the production of NO in BV2 cells induced by LPS (7 ng/ml) and treated with telmisartan (Fig 1a). While LPS significantly increased NO production as compared to non-stimulated cells (control), telmisartan attenuated this effect in a concentration-dependent manner. Specifically, a 24h incubation with 1 μM or 5 μM telmisartan decreased LPS-induced NO production by 30% and 60%, respectively (Fig 1a). Telmisartan did not affect NO production in non-stimulated (control) cells (Fig 1ainset). The effect of telmisartan on NO production in BV2 cells was confirmed in primary neonatal rat microglia (Fig 1b) and mixed glial cultures (Fig 1c). In both cultures, LPS (0.5μg/ml) stimulated NO release to the media and telmisartan reduced NO production even more than in BV2 cells. In primary microglia, 1μM and 5μM telmisartan reduced NO production by 30% and 74%, respectively. In mixed glial cells, telmisartan attenuated NO release by 45% and 62%, respectively. In both types of primary glial cell cultures telmisartan did not alter basal levels of NO (Fig 1b and 1c insets).

Telmisartan (5 μM) also substantially attenuated the LPS-induced TNF-α and IL1-β production by approximately 50% and 30%, respectively (Fig 2a and 2b). However, it did not change TNF-α or IL1-β production in non-stimulated BV2 cells (Fig 2a and 2b insets).

As shown in Fig 3, 24h exposure of BV2 cells to LPS (7 ng/ml) resulted in robust increase in iNOS protein levels, namely, by more than 90% as compared with the control. In the LPS-
induced cells, 24h incubation with 1 μM or with 5 μM telmisartan significantly reduced iNOS expression levels by 65% and 85%, respectively.

Intranasal administration of telmisartan decreased Aβ burden and CD11b staining in brains of 5XFAD mice

As expected, the cortex of 3-month-old wild-type (WT) mice treated intranasally with telmisartan did not show any Aβ plaques or CD11b (indicative of microglia and macrophages activation [46]) staining, whereas both these parameters were significantly increased in age-
matched 5XFAD mice treated intranasally with the vehicle (Fig 4). However, as compared with 5XFAD mice treated with the vehicle, age-matched 5XFAD mice treated intranasally for 3.5 weeks with 1 mg/kg/day telmisartan showed a ~52% and ~57% reduction in the cortical area covered by Aβ plaques (Fig 4a and 4b) and CD11b staining, respectively (Fig 4c and 4d). Prolonged (8 weeks) intranasal treatment with telmisartan (Fig 5) also attenuated amyloid plaques and CD11b by ~50% staining, both in the cortex (Fig 5a–5d) and in the hippocampus (~50% for Aβ and ~25% for CD11b; Fig 5e–5h). WT mice treated with telmisartan did not display any plaque deposition or CD11b staining in the hippocampus or in the cortex. The amount of amyloid plaque depositions and CD11b increased with age (compare Fig 4 with Fig 5). Plaque formation and gliosis appear mainly in the cortex but to a lesser extent in the hippocampus in immature stages of 5XFAD mice [38] therefore, the hippocampal section quantification is not shown for the short treatment of 3.5 weeks.

Intranasal administration of perindopril reduces amyloid plaques burden and CD11b staining in the cortex of 5XFAD mice

As compared with the vehicle-treated group, 3.5 weeks intranasal treatment with the ACEI perindopril (1 mg/kg/day) resulted in a reduction of 35% and 55% in amyloid plaques and in CD11b staining, respectively, in the cortex of 3-month-old 5XFAD mice (Fig 6). Neither amyloid plaques nor CD11b staining appeared in the cortex of age-matched WT mice.

Discussion

In the present study we show a long term anti-inflammatory effect of telmisartan manifested by attenuation of LPS-induced NO microglial cell line as well as in primary cultures. TNF-α and IL1-β production as well as iNOS protein expression induced by LPS were decreased by telmisartan in BV2 microglia. In glial cells, LPS is known as a potent stimulator of the production of proinflammatory mediators and reactive oxygen species. As shown by Miyoshi et al, LPS-induced primary rat microglia cultures also displayed elevated expression of AngII protein and increased AT1R mRNA levels [27]. Ang II itself was identified as paracrine mediator of sustaining brain inflammation in neurodegenerative disease models [48]. Taken together, these findings suggest that LPS and AngII may lead to increased production of various proinflammatory factors [7], including TNF-α, IL1-β and NO, which cohesively activate microglia, astrocytes and inflammatory cascades [49,50].
The reduction of LPS-induced NO, IL1-β and TNF-α levels by telmisartan suggests a brain modulatory role for the latter since these pro-inflammatory mediators stimulate the immune response and aggravate the neurodegradative state. [51,52]. The attenuation of TNF-α protein and IL1-β mRNA levels in LPS-stimulated microglia, as reported by Xu Yuan et al., was observed after short term (2 h) pre-treatment with telmisartan [53]. In the present study, a 24 h-inhibitory effect of telmisartan on microglial TNF-α, IL1-β and NO production was shown, suggesting changes in the levels of proteins responsible for the synthesis of NO, TNF-α and IL1-β. Indeed, Western blot analysis for iNOS showed that 24h-treatment with telmisartan decreased iNOS protein levels by 65–85% in LPS-stimulated cells.

Telmisartan and perindopril (1–3 mg/kg/day), upon systemic administration, were shown to penetrate the brain to various extents in different animal models [47,54,55]. Noda A et al [47] also showed blockade of AT1R following telmisartan penetration into the rhesus macaques brain. Oral administration of perindopril (1 mg/kg/day) showed significant inhibitory effect (by 50%) on brain ACE activity [31].

For in vivo studies, the 5XFAD mouse model was applied. This model is unique in that the mice develop rapid and extensive Aβ deposits alongside gliosis, which begins from 2-month of age. Plaques initially accumulate in the cortex, and expand to the hippocampus as the mice age. Moreover, the brains of 5XFAD mice exhibit neuroinflammation and gliosis, which are
Fig 5. Intranasal administration of telmisartan decreases amyloid plaques and CD11b staining in the cortex and hippocampus of 4-month old 5XFAD mice. Mice were treated with telmisartan (Tel) or vehicle (N,N-dimethylformamide/polyethylene glycol 400/saline (2:6:2) [47]) for 8 weeks. The brains of 4-month-old mice were sectioned and immunolabeled with anti-Aβ (red) and anti-CD11b (green) antibodies and counterstained with DAPI (blue). (a, c) Representative cortical sections from WT or 5XFAD mice treated with 1 mg/kg/day telmisartan or with vehicle. (e, g) Representative hippocampal sections of WT or 5XFAD mice treated with 1 mg/kg/day telmisartan or with vehicle. Each experiment included 6 mice per group (n = 18 in total). (b, d, f, h) Quantification of the average sum of Aβ-stained area (b, f) or of CD11b-stained area (d, h), represented as the mean ± SEM percentage of stained area in the corresponding vehicle-treated group in at least 3 determinants. Statistical significance was determined using one-way ANOVA, followed by a Tukey—Kramer Multiple Comparison Test. ***P<0.001 vs. WT+Tel; ^^P<0.01 vs. 5XFAD+vehicle; ^^^P<0.001 vs. 5XFAD+vehicle. Scale bar is 200 μm.

doi:10.1371/journal.pone.0155823.g005
proportional to the levels and production period of the amyloid plaques [38]. As the 5XFAD mouse model robustly develops amyloid plaques compared with other AD mouse models [38], it presents a greater challenge for drugs to modulate plaque formation and gliosis. Our in vivo results also suggest an anti-inflammatory and beneficial role of intranasally administered telmisartan (ARB) and perindopril (ACEI), which attenuated Aβ plaques burden and microglia/macrophages activation in 5XFAD mice [35].

Telmisartan was reported to have a protective effect on cognitive impairment in an AD mouse model injected intracerebrally with the Aβ peptide [33]. Consistent with this, recent human prospective cohort [56] and nested case control [57] analyses reported a lower incidence and a slower rate of progression to dementia and AD in patients prescribed ARBs. Similarly, studies in young Aβ-injected mice [32,33,58] or adult Tg2576 AD mice [36] have shown the ability of ARBs to improve cognition. In contrast, AngII inhibition by ARBs in young or aged triple-transgenic AD mice indicated no benefit on the amyloid, tau or cognitive pathology [59,60].

It is controversial whether ACEI can exert beneficial effects on cognitive decline or AD. Among other studies, the Perindopril Protection Against Recurrent Stroke study [61] has demonstrated that antihypertensive treatment based on perindopril, a brain-penetrating ACEI, can reduce cognitive decline in patients with cerebrovascular disease. Furthermore, it has been suggested that brain penetrating ACEI can reduce the incidence of AD in elderly hypertensive
patients [62–64]. However, the speculated role of ACE as an Aβ-degrading enzyme raised the concern that ACEI treatment may enhance Aβ1–42 levels in the brain of AD patients [65]. As such, the impact of inhibiting ACE on the accumulation of amyloid in AD patients remains unclear. Indeed, several studies have shown that the ACEI captopril promoted Aβ accumulation in the media of cells expressing human APP, and Aβ1–42 depositions in the brain of an AD mouse model [65,66]. Other studies reported that the administration of ACEIs, such as captopril, perindopril, or ramipril, failed to show such an effect and did not elicit changes in brain Aβ levels in APP-treated transgenic mice [31,67] or in a clinical trial [68].

Our findings show a reduction in Aβ deposition in specific brain areas of 5XFAD mice treated intranasally with perindopril. This effect may be attributed to the intranasal delivery, which was not tested previously with perindopril. Intranasal delivery of ARBs and ACEI was indeed expected to increase the direct effects of these compounds in the brain. [35]. As a matter of fact, chronic intranasal treatment with an ARB (losartan) dramatically decreased plaque number in the APP/PS1 AD mouse model [69]. Moreover, in clinical trials with AD patients, nasal application of insulin showed improvement in memory tasks and CSF biomarkers [70].

The removal of Aβ plaques can be achieved by activated microglia, which phagocyte fibrillar Aβ [71], or by an enzymatic degradation of the Aβ peptide, [72]. In vitro and in vivo studies suggest that the neutral endopeptidase neprilysin (NEP), the insulin-degrading enzyme (IDE), and the endothelin-converting enzyme-1 (ECE-1) are, potentially, Aβ-degrading enzymes [73,74]. In AD mouse models, NEP or IDE overexpression prevented the amyloid plaque pathology and premature death [75], and downregulation of NEP mRNA levels was reported in the cerebral cortex and in the hippocampus upon aging and in AD, even in the early stage of the disease [74]. Interestingly, Telmisartan can activate peroxisome-proliferator-activated receptor γ (PPARγ) and the latter serves as receptor-mediated mechanism for pharmacological upregulation of NEP [76].

In light of these findings, the positive effects of telmisartan and perindopril on amyloid plaques burden, as reported in the current study, may be attributed to possible up-regulation of Aβ-degrading enzymes. Moreover, since telmisartan is an ARB with potential strong PPAR-γ agonist properties [7,22,77], it is hypothesized that the effect observed in vitro and in vivo may be attributed not only to its AT1 receptor antagonist characteristics but also to the activation of PPAR-γ. Prevention of cognitive decline due to PPAR-γ activation by telmisartan was shown by Mogi M et al [32] in AD mice. In addition, an interaction between PPAR-γ activation and AT1R expression was suggested by several studies [78,79]. To conclude, we provide evidence that a non-invasive intranasal delivery of telmisartan or perindopril may serve as an efficient alternative for their systemic administration, as it results in the attenuation of microglia/macrophages activation and in the reduction of Aβ plaques. Further studies are required to test whether an up-regulation of Aβ-degrading enzymes or an increased phagocytosis by activated microglia underlie the beneficial effects of telmisartan and perindopril.

Acknowledgments
We thank Bersudsky Marina from the department of Microbiology and Immunology, Ben-Gurion University of the Negev for her technical assistance.

Author Contributions
Conceived and designed the experiments: NT SFB. Performed the experiments: NT KA. Analyzed the data: NT. Contributed reagents/materials/analysis tools: RNA SFB. Wrote the paper: NT SFB AD.
References

1. Tuppo EE, Arias HR The role of inflammation in Alzheimer’s disease. Int J Biochem Cell Biol. 2005; 37: 289–305. PMID: 15474976

2. Rajkowska G, Miguel-Hidalgo JJ Gliogenesis and glial pathology in depression. CNS Neurol Drug Targets 2007; 6: 219–233. PMID: 17511618

3. Amor S, Puentes F, Baker D, van der Valk P Inflammation in neurodegenerative diseases. Immunology 2010; 129: 154–169. doi: 10.1111/j.1365-2567.2009.03225.x PMID: 20561356

4. Batarseh YS, Duong QV, Mousa YM, Al Rihani SB, Elfakhri K, Kadoumi A Amyloid-beta and Astrocytes Interplay in Amyloid-beta Related Disorders. Int J Mol Sci 2016; 4: 17.

5. Prokop S, Miller KR, Heppner FL Microglia actions in Alzheimer’s disease. Acta Neuropathol. 2013; 126: 461–77 PMID: 24224195

6. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH Mechanisms underlying inflammation in neurodegeneration. Cell 2010; 140: 918–934. doi: 10.1016/j.cell.2010.02.016 PMID: 20303880

7. Saavedra JM Angiotensin II AT(1) receptor blockers as treatments for inflammatory brain disorders. Clin Sci (Lond) 2012; 123: 567–590.

8. Aisen PS The potential of anti-inflammatory drugs for the treatment of Alzheimer’s disease. Lancet Neurol 2002; 1: 279–284. PMID: 12849425

9. Tan J, Town T, Paris D, Mori T, Suo Z, Crawford F, et al. Microglial activation resulting from CD40-CD40L interaction after beta-amyloid stimulation. Science 1999; 286: 2352–2355. PMID: 10600748

10. Wyss-Coray T Inflammation in Alzheimer disease: driving force, bystander or beneficial response? Nat Med 2006; 12: 1005–1015. PMID: 16960575

11. Brown GC Mechanisms of inflammatory neurodegeneration: iNOS and NADPH oxidase. Biochem Soc Trans 2007; 35: 1119–1121. PMID: 17962992

12. Gibbons HM, Dragunow M Microglia induce neural cell death via a proximity-dependent mechanism involving nitric oxide. Brain Res 2006; 21: 1–15.

13. Diaz A, Mendieta L, Zenteno E, Guevara J, Limon ID The role of NOS in the impairment of spatial memory and damaged neurons in rats injected with amyloid beta 25–35 into the temporal cortex. Pharmocol Biochem Behav 2011; 98: 67–75. doi: 10.1016/j.pbb.2010.12.005 PMID: 21163295

14. Maezawa I, Zimin PI, Wulff H, Jin LW Amyloid-beta protein oligomer at low nanomolar concentrations activates microglia and induces microglial neurotoxicity. J Biol Chem 2011; 286: 3693–3706.

15. Sriram K, O’Callaghan JP Divergent roles for tumor necrosis factor-alpha in the brain. J Neuroimmune Pharmacol 2007; 2: 140–153. PMID: 18040839

16. Dursun E, Gezen-Ak D, Hanagasi H, Bilgic B, Lohmann E, Ertan S, et al. The interleukin 1 alpha, interleukin 1 beta, interleukin 6 and alpha-2-macroglobulin serum levels in patients with early or late onset Alzheimer’s disease, mild cognitive impairment or Parkinson’s disease. J Neuroimmunol 2015; 15: 283: 50–57.

17. Doens D, Fernandez PL Microglia receptors and their implications in the response to amyloid beta for Alzheimer’s disease pathogenesis. J Neuroinflammation 2014; 11: 48. doi: 10.1186/1742-2094-11-48 PMID: 24625061

18. Clark IA, Alleva LM, Vissel B The roles of TNF in brain dysfunction and disease. Pharmacol Ther 2010; 128: 519–548. doi: 10.1016/j.pharmthera.2010.08.007 PMID: 20813131

19. Villapoli S, Saavedra JM Neuroprotective effects of angiotensin receptor blockers. Am J Hypertens 2015; 28: 289–299. doi: 10.1093/ajh/hpu197 PMID: 25362113

20. McKinley MJ, Albiston AL, Allen AM, Mathai ML, May CN, Mcallen RM, et al. The brain renin-angiotensin system: location and physiological roles. Int J Biochem Cell Biol 2003; 35: 901–918. PMID: 12676175

21. Saavedra JM, Armando I, Bregonzio C, Juorio A, Macova M, Pavel J, et al. A centrally acting, anxiolytic angiotensin II AT1 receptor antagonist prevents the isolation stress-induced decrease in cortical CRF1 receptor and benzodiazepine binding. Neuropsychopharmacology 2006; 31: 1123–1134. PMID: 16205776

22. Saavedra JM, Sanchez-Lemus E, Benicky J Blockade of brain angiotensin II AT1 receptors ameliorates stress, anxiety, brain inflammation and ischemia: Therapeutic implications. Psychoneuroendocrinology 2011; 36: 1–18. doi: 10.1016/j.psyneuen.2010.10.001 PMID: 21035905

23. Intebi AD, Flaxman MS, Ganong WF, Deschepper CF Angiotensinogen production by rat astroglial cells in vitro and in vivo. Neuroscience 1990; 34: 545–554. PMID: 2352643

24. Garrido-Gil P, Valenzuela R, Villar-Cheda B, Lanziego JL, Labandeira-Garcia JL Expression of angiotensinogen and receptors for angiotensin and preproenin in the monkey and human substantia nigra: an
intracellular renin-angiotensin system in the nigra. Brain Struct Funct 2013; 218: 373–388. doi: 10.1007/s00429-012-0402-9 PMID: 22407459

25. Yang G, Gray TS, Sigmund CD, Cassell MD The angiotensinogen gene is expressed in both astrocytes and neurons in murine central nervous system. Brain Res 1999; 817: 123–131. PMID: 9889347

26. Fogarty DJ, Sanchez-Gomez MV, Matute C Multiple angiotensin receptor subtypes in normal and tumor astrocytes in vitro. Glia 2002; 39: 304–313. PMID: 12203396

27. Miyoshi M, Miyano K, Moriyma N, Taniguchi M, Watanabe T Angiotensin type 1 receptor antagonist inhibits lipopolysaccharide-induced stimulation of rat microglial cells by suppressing nuclear factor kappaB and activator protein-1 activation. Eur J Neurosci 2008; 27: 343–351. doi: 10.1111/j.1460-9568.2007.06014.x PMID: 18190523

28. de Gasparo M, Siragy HM The AT2 receptor: fact, fancy and fantasy. Regul Pept 1999; 81: 11–20. PMID: 10395404

29. Phillips MI, de Oliveira EM Brain renin angiotensin in disease. J Mol Med (Berl) 2008; 86: 715–722.

30. Saavedra JM Angiotensin II AT(1) receptor blockers ameliorate inflammatory stress: a beneficial effect for the treatment of brain disorders. Cell Mol Neurobiol 2012; 32: 667–681. doi: 10.1007/s10571-011-9754-6 PMID: 21938488

31. Dong YF, Kataoka K, Tokutomi Y, Nako H, Nakamura T, Toyama K, et al. Perindopril, a centrally active angiotensin-converting enzyme inhibitor, prevents cognitive impairment in mouse models of Alzheimer’s disease. FASEB J 2011; 25: 2911–2920. doi: 10.1096/fj.11-182873 PMID: 21593435

32. Mogi M, Li JM, Tsukuda K, Iwanami J, Min LJ, Sakata A, et al. Telmisartan prevented cognitive decline partly due to PPAR-gamma activation. Biochem Biophys Res Commun 2008; 375: 446–449. doi: 10.1016/j.bbrc.2008.08.032 PMID: 18715543

33. Tsukuda K, Mogi M, Iwanami J, Min LJ, Sakata A, Jing F, et al. Cognitive deficit in amyloid-beta-injected mice was improved by pretreatment with a low dose of telmisartan partly because of peroxisome proliferator-activated receptor-gamma activation. Hypertension 2009; 54: 782–787. doi: 10.1161/ HYPERTENSIONAHA.109.136879 PMID: 19635982

34. Shindo T, Takasaki K, Uchida K, Onimura R, Kubota K, Uchida N, et al. Ameliorative effects of telmisartan on the inflammatory response and impaired spatial memory in a rat model of Alzheimer’s disease incorporating additional cerebrovascular disease factors. Biol Pharm Bull 2012; 35: 2141–2147. PMID: 22307766

35. Hou DR, Wang Y, Zhou L, Chen K, Tian Y, Bao J, et al. Altered angiotensin-converting enzyme and its effects on the brain in a rat model of Alzheimer disease. Chin Med J (Engl) 2008; 121: 2320–2323.

36. Wang J, Ho L, Chen L, Zhao Z, Zhao W, Qian X, et al. Valsartan lowers brain beta-amyloid protein levels and improves spatial learning in a mouse model of Alzheimer disease. J Clin Invest 2007; 117: 3393–3402. PMID: 17965777

37. Dhuria SV, Hanson LR, Frey WH 2nd Intranasal delivery to the central nervous system: mechanisms and experimental considerations. J Pharm Sci 2010; 99: 1654–1673. doi: 10.1002/jps.21924 PMID: 19877171

38. Oakley H, Cole SL, Logan S, Mauz E, Shao P, Craft J, et al. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer’s disease mutations: potential factors in amyloid plaque formation. J Neurosci 2006; 26: 10129–10140. PMID: 17021169

39. Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J Neuroimmunol 1990; 27: 229–237. PMID: 2110186

40. Ben-Shmuel S, Danon A, Fleisher-Berkovich S Bradykinin decreases nitric oxide release from microglia via inhibition of cyclic adenosine monophosphate signaling. Peptides 2013; 40: 133–140. doi: 10.1016/j.peptides.2013.01.006 PMID: 23340021

41. Sarit BS, Lajos G, Abraham D, Ron A, Sigal FB Inhibitory role of kinins on microglial nitric oxide and tumor necrosis factor-alpha production. Peptides 2012; 35: 172–181. doi: 10.1016/j.peptides.2012.03.026 PMID: 22490447

42. Levant A, Levy E, Argaman M, Fleisher-Berkovich S Kinins and neuroinflammation: dual effect on prostaglandin synthesis. Eur J Pharmacol 2006; 546: 197–200. PMID: 16889769

43. Grinshpun J, Tveria L, Fleisher-Berkovich S Differential regulation of prostaglandin synthesis in neonatal rat microglia and astrocytes by somatostatin. Eur J Pharmacol 2008; 584: 312–317. doi: 10.1016/j.ejphar.2008.02.025 PMID: 18325491

44. Asraf K, Torika N, Roasso E, Fleisher-Berkovich S Differential effect of intranasally administered kinin B1 and B2 receptor antagonists in Alzheimer’s disease mice. Biol Chem 2016; 397: 345–351. doi: 10.1515/hsz-2015-0219 PMID: 26556847
45. Kneynsberg A, Collier TJ, Manfredsson FP, Kanaan NM Quantitative and semi-quantitative measurements of axonal degeneration in tissue and primary neuron cultures. J Neurosci Methods 2016; 266: 32–41. doi: 10.1016/j.jneumeth.2016.03.004 PMID: 27031947

46. Badie B, Schartner JM Flow cytometric characterization of tumor-associated macrophages in experimental gliomas. Neurosurgery 2000; 46: 957–961; discussion 961–952. PMID: 10764271

47. Noda A, Fushiki H, Murakami Y, Sasaki H, Miyoshi S, Kakuta H, et al. Brain penetration of telmisartan, a unique centrally acting angiotensin II type 1 receptor blocker, studied by PET in conscious rhesus macaques. Nucl Med Biol 2012; 39: 1232–1235. doi: 10.1016/j.nucmedbio.2012.06.012 PMID: 22890047

48. Lanz TV, Ding Z, Ho PP, Luo J, Agrawal AN, Srinagesh H, et al. Angiotensin II sustains brain inflammation in mice via TGF-beta. J Clin Invest 2010; 120: 2782–2794. doi: 10.1172/JCI41709 PMID: 20626203

49. Smith JA, Das A, Ray SK, Banik NL Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. Brain Res Bull 2012; 87: 10–20. doi: 10.1016/j.brainresbull.2011.10.004 PMID: 22024597

50. Yamakawa H, Jezova M, Ando H, Saavedra JM Normalization of endothelial and inducible nitric oxide synthase expression in brain microvessels of spontaneously hypertensive rats by angiotensin II AT1 receptor inhibition. J Cereb Blood Flow Metab 2003; 23: 371–380. PMID: 12621312

51. Montgomery SL, Bowers WJ Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system. J Neuroimmune Pharmacol 2012; 7: 42–59. doi: 10.1007/s11481-011-9287-2 PMID: 21728035

52. Nakamura T, Lipton SA Redox modulation by S-nitrosylation contributes to protein misfolding, mitochondrial dynamics, and neuronal synaptic damage in neurodegenerative diseases. Cell Death Differ 2011; 18: 1478–1486. doi: 10.1038/cdd.2011.65 PMID: 2197461

53. Xu Y, Wang Y, He L, Jiang Z, Huang Z, Liao H, et al. Effect of a centrally active angiotensin II type 1 receptor antagonist telmisartan administered peripherally inhibits central responses to angiotensin II in conscious rats. J Pharmacol Exp Ther 2001; 298: 62–70. PMID: 11408526

54. Gohike P, Weiss S, Jansen A, Wienen W, Stangier J, Rascher W, et al. AT1 receptor antagonist telmisartan modulates glial activation in vivo: In Vitro and In Vivo Studies. J Neuroimmun 2015; 16: 00410–00419.

55. Li NC, Lee A, Whitmer RA, Kivipelto M, Lawler E, Kazis LE et al. Use of angiotensin receptor blockers and risk of dementia in a predominantly male population: prospective cohort analysis. BMJ 2010; 340: b5465. doi: 10.1136/bmj.b5465 PMID: 20068258

56. Davies NM, Kehoe PG, Ben-Shlomo Y, Martin RM Associations of anti-hypertensive treatments with Alzheimer's disease, vascular dementia, and other dementias. J Alzheimers Dis 2011; 26: 699–708. doi: 10.3233/JAD-2011-110347 PMID: 21709373

57. Takeda S, Sato N, Takeuchi D, Kurinami H, Shinohara M, Niisato K, et al. Angiotensin receptor blocker prevented beta-amyloid-induced cognitive impairment associated with recovery of neuromuscular junctions. J Cereb Blood Flow Metab 2009; 29: 1345–1352. doi: 10.1111/J.CEBF.12011; discussion 961

58. Ferrington L, Miners JS, Palmer LE, Bond SM, Povey JE, Kelly PA, et al. Angiotensin II inhibition drugs have no effect on intraneuronal Abeta or oligomeric Abeta levels in a triple transgenic mouse model of Alzheimer's disease. Am J Transl Res 2011; 3: 197–208. PMID: 21416061

59. Ferrington L, Palmer LE, Love S, Horsburgh KJ, Kelly PA, Kehoe PG Angiotensin II- inhibition: effect on Alzheimer's pathology in the aged triple transgenic mouse. Am J Transl Res 2012; 4: 151–164. PMID: 22611468

60. Tzourio C, Anderson C, Chapman N, Woodward M, Neil B, MacMahon S, et al. Effects of blood pressure lowering with perindopril and indapamide therapy on dementia and cognitive decline in patients with cerebrovascular disease. Arch Intern Med 2003; 163: 1069–1075. PMID: 12742805

61. Ohru T, Tomita N, Sato-Nakagawa T, Matsui T, Muruyama M, Niwa K, et al. Effects of brain-penetrating ACE inhibitors on Alzheimer disease progression. Neurology 2004; 63: 1324–1325. PMID: 15477567

62. Sink KM, Leng X, Williamson J, Kritchevsky SB, Yaffe K, Kuller L, et al. Angiotensin-converting enzyme inhibitors and cognitive decline in older adults with hypertension: results from the Cardiovascular Health Study. Arch Intern Med 2009; 169: 1195–1202. doi: 10.1001/archinternmed.2009.175 PMID: 19597068
64. O’Caoimh R, Healy L, Gao Y, Svendrovski A, Kerins DM, Eustace J, et al. Effects of centrally acting angiotensin converting enzyme inhibitors on functional decline in patients with Alzheimer’s disease. J Alzheimers Dis 2014; 40: 595–603. doi: 10.3233/JAD-131694 PMID: 24496072

65. Hemming ML, Selkoe DJ Amyloid beta-protein is degraded by cellular angiotensin-converting enzyme (ACE) and elevated by an ACE inhibitor. J Biol Chem 2005; 280: 37644–37650. PMID: 16154999

66. Zou K, Yamaguchi H, Akatsu T, Ko M, Mizogushi K, et al. Angiotensin-converting enzyme converts amyloid beta-protein 1–42 (Abeta(1–42)) to Abeta(1–40), and its inhibition enhances brain Abeta deposition. J Neurosci 2007; 27: 8628–8635. PMID: 17687040

67. Hemming ML, Selkoe DJ, Farris W Effects of prolonged angiotensin-converting enzyme inhibitor treatment on amyloid beta-protein metabolism in mouse models of Alzheimer disease. Neurobiol Dis 2007; 26: 273–281. PMID: 17321748

68. Wharton W, Stein JH, Korcarz C, Sachs J, Olson SR, Zetterberg H, et al. The effects of ramipril in individuals at risk for Alzheimer’s disease: results of a pilot clinical trial. J Alzheimers Dis 2012; 32: 147–156. PMID: 22776970

69. Danielyan L, Klein R, Hanson LR, Buadze M, Schwab M, Gleiter CH, et al. Protective effects of intranasal losartan in the APP/PS1 transgenic mouse model of Alzheimer disease. Rejuvenation Res 2010; 13: 195–201. doi: 10.1089/rej.2009.0944 PMID: 20370487

70. Holscher C First clinical data of the neuroprotective effects of nasal insulin application in patients with Alzheimer’s disease. Alzheimers Dement 2014; 10: S33–S37. doi: 10.1016/j.jalz.2013.12.006 PMID: 24529523

71. Fu H, Liu B, Frost JL, Hong S, Jin M, Ostaszewski B, et al. Complement component C3 and complement receptor type 3 contribute to the phagocytosis and clearance of fibrillar Abeta by microglia. Glia 2012; 60: 993–1003. doi: 10.1002/glia.22331 PMID: 22438044

72. Miners JS, Barua N, Kehoe PG, Gill S, Love S Abeta-degrading enzymes: potential for treatment of Alzheimer disease. J Neurochem 2011; 120 Suppl 1: 167–185. doi: 10.1111/j.1471-4159.2011.07510.x PMID: 22122230

73. Nalivaeva NN, Beckett C, Belyaev ND, Turner AJ Are amyloid-degrading enzymes viable therapeutic targets in Alzheimer's disease? J Neurochem 2012; 120 Suppl 1: 167–185. doi: 10.1111/j.1471-4159.2011.07510.x PMID: 22122230

74. Iwata N, Higuchi M, Saito TC Metabolism of amyloid-beta peptide and Alzheimer’s disease. Pharmacol Ther 2005; 108: 129–148. PMID: 16112736

75. Leissring MA, Farris W, Chang AY, Walsh DM, Wu X, Sun X, et al. Enhanced proteolysis of beta-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. Neuron 2003; 40: 1087–1093. PMID: 14687544

76. Nalivaeva NN, Belyaev ND, Zhuravin IA, Turner AJ The Alzheimer’s amyloid-degrading peptidase, neprilysin: can we control it? Int J Alzheimers Dis 2012; 2012: 383796. doi: 10.1155/2012/383796 PMID: 22900228

77. Erbe DV, Gartrell K, Zhang YL, Suri V, Kirincich SJ, Will S, et al. Molecular activation of PPARgamma by angiotensin II type 1-receptor antagonists. Vascul Pharmacol 2006; 45: 154–162. PMID: 16765099

78. Xiao J, Leung JC, Chan LY, Tang SC, Lai KN Crosstalk between peroxisome proliferator-activated receptor-gamma and angiotensin II in renal tubular epithelial cells in IgA nephropathy. Clin Immunol 2009; 132: 266–276. doi: 10.1016/j.clim.2009.04.004 PMID: 19443277

79. Ji Y, Liu J, Wang Z, Liu N, Gow W PPARgamma agonist, rosiglitazone, regulates angiotensin II-induced vascular inflammation through the TLR4-dependent signaling pathway. Lab Invest 2009; 89: 887–902. doi: 10.1038/lab.2009.45 PMID: 19451898