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Involvement of the Bradykinin B_1 Receptor in Microglial Activation: *In Vitro* and *In Vivo* Studies

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The importance of brain inflammation to Alzheimer's disease (AD) pathogenesis has been accepted of late, with it currently being held that brain inflammation aggravates AD pathology. One important aspect of brain inflammation is the recruitment and activation of microglia, a process termed microgliosis. Kinins and bradykinin (BK), in particular, are major pro-inflammatory mediators in the periphery, although all of the factors comprising the kinin system have also been described in the brain. Moreover, it was shown that the amyloid β (Aβ) peptide (a component of AD plaques) enhances kinin secretion and activates BK receptors that can, in turn, stimulate Aβ production. Still, the role of bradykinin in modulating brain inflammation and AD is not completely understood. In this study, we aimed to investigate the roles of the bradykinin B_1 receptor (B_1R) and bradykinin B_2 receptor (B_2R) in regulating microglial secretion of pro-inflammatory factors *in vitro*. Furthermore, the effects of intranasal administration of specific B_1R and B_2R antagonists on Aβ burden and microglial accumulation in the brains of transgenic AD mice were studied. The data obtained show that neither R-715 (a B_1R antagonist) nor HOE 140 (a B_2R antagonist) altered microglial cell viability. However, R-715, but not HOE 140, markedly increased lipopolysaccharide-induced nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) release, as well as inducible nitric oxide synthase expression in BV2 microglial cells. Neither antagonist altered NO nor TNF-α production in non-stimulated cells. We also showed that intranasal administration of R-715 but not HOE 140 to 8-week-old 5X familial AD mice enhanced amyloid burden and microglia/macrophage accumulation in the cortex. To conclude, we provide evidence supporting a role of B_1R in brain inflammation and in the regulation of amyloid deposition in AD mice, possibly with microglial/macrophage involvement. Further studies are required to test whether modulation of this receptor can serve as a novel therapeutic strategy for AD.

Keywords: bradykinin, brain inflammation, HOE 140, microglia, R-715

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INTRODUCTION

Alzheimer's disease (AD) is a prevalent neurodegenerative disease (1) that is characterized by two neuropathological hallmarks, namely the deposition of amyloid β plaques and the accumulation of neurofibrillary tangles (2, 3). The role that brain inflammation plays in AD pathogenesis has only recently been appreciated. Currently, brain inflammation is thought to contribute to and to exacerbate AD pathology (4, 5). One important aspect of the central immune response to brain inflammation is microglial activation.

Microglia are the resident phagocytes of the brain. These cells use their processes to scan the brain for pathogens and debris (6). Microglia also maintain brain plasticity and remodel synapses (7, 8). In AD, microglia bind soluble amyloid β (Aβ) oligomers and fibrils and become activated. The activated microglia then start to engulf Aβ fibrils by phagocytosis. Inefficient phagocytosis of Aβ has been identified as a major pathogenic pathway (5). Microglia are likely to exist in a range of phenotypic states during chronic inflammation in AD. Upon binding to Aβ, microglia release pro-inflammatory molecules, such as interleukin-1 β (IL-1β), tumor necrosis factor-alpha (TNF-α), and IL-12, as well as reactive oxygen species, like nitric oxide (NO) (9–11). In turn, pro-inflammatory conditions promote neuronal damage mediated by Aβ and decrease the phagocytosis of these oligomers and their degradation by microglia (12, 13).

Although much is known of the molecular basis of initiating signals and pro-inflammatory chemical mediators in brain inflammation, it has only recently become apparent that endogenous stop signals are critical players at early checkpoints during the various stages of brain inflammation. Some neuropeptides that are produced during the ongoing inflammatory response have emerged as endogenous anti-inflammatory agents that participate in the regulation of processes that ensure self-tolerance and/or inflammation resolution. Neuropeptides, such as kinins, can regulate brain inflammation and affect microglial functions both *in vitro* and *in vivo* (14, 15). Thus, the release of these factors can determine whether microglia assume a neuroprotective phenotype.

Kinins, in particular bradykinin (BK), are pro-inflammatory mediators in the periphery. At peripheral sites, BK can elicit all of the major signs of inflammation, namely pain, hyper-perfusion, and increased vascular permeability (16–19). All kinin system components have also been described in the central nervous system (20). Indeed, high BK levels are found after brain trauma and ischemia (21). Furthermore, it was shown that Aβ upregulates BK receptors and kinin release, followed by BK-induced Aβ synthesis (22). Still, the role that bradykinin plays in AD modulation is not completely understood. BK activates two types of receptors, namely, the B_1 receptor [bradykinin B_1 receptor (B_1R)] and the B_2 receptor [bradykinin B_2 receptor (B_2R)] (23, 24). B_2R is a constitutive receptor and has high affinity for BK, while B_1R is generally upregulated following tissue injury and binds with high affinity to des-Arg9-BK, a kinin metabolite (24). In the brain, microglial cells express both receptors (14, 25).

In the present study, our intent was to investigate the contributions of B_1R and B_2R in mediating microglial inflammation *in vitro*. Moreover, the *in vivo* influence of intranasal administration of specific B_1R and B_2R antagonists on Aβ burden and microglial accumulation in brains of transgenic AD mice was considered.

MATERIALS AND METHODS

Cell Cultures

The BV2 microglial cell line (provided by Prof. Rosario Donato, Department of Experimental Medicine and Biochemical Sciences, University of Perugia) was seeded in 6-well, 24-well, or 96-well plates at densities of 1×10^6, 3×10^5, and 2×10^4 cells per well, respectively. Cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum and 0.4 mM L-glutamine. To create a sterile environment, 100 U/ml of penicillin and 100 µg/ml of streptomycin were added. Cells were grown in humidified atmosphere of 5% CO_2 at 37°C. At the beginning of each experiment, the cells were incubated with serum-free medium (SFM) for 4 h, followed by a 22-h incubation with the indicated test agents in SFM supplemented with 0.1% bovine serum albumin (BSA) and 10 mM HEPES (pH 7.4). BV2 cells were treated with R-715, a B_1R selective antagonist, and HOE 140, a B_2R selective antagonist, both purchased from GL Biochem (Shanghai, China), lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 was purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell Count

At the end of each experiment, cells were harvested after incubation with 1 ml SFM for 1 h at 4°C and counted using a Z1 Coulter counter (Coulter Electronics, Miami, FL, USA).

Cell Viability

Cell viability was determined by a Cell Proliferation Kit (XTT) (Biological Industries, Kibbutz Beit-Haemek, Israel) according to the manufacturer's instructions. The assay was performed using a microplate reader (Bio-Rad model 680).

Determination of NO Levels (Griess Reaction)

Nitrite levels were determined in the culture supernatants using the Griess reaction. Nitrite standard curve samples or supernatants (100 µl each) were mixed with 100 µl Griess reagent (Sigma-Aldrich) in 96-well plates. Thereafter, the plates were incubated for 15 min in the dark at room temperature. Nitrite levels were measured with a microplate reader at 540 nm.

Determination of TNF-α Levels (ELISA)

Tumor necrosis factor-alpha levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

SDS Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The expression levels of inducible nitric oxide synthase (iNOS) protein in BV2 microglial cells were analyzed by Western blot (26). Briefly, cells were harvested using lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM $MgCl_2$, 1% Triton X-100, and 1% deoxycholic acid) containing a protease inhibitor cocktail. Cells lysates were incubated at 4°C for 30 min, followed by a 15 min centrifugation (12,000 *g*) at 4°C. Thereafter, protein levels were determined by Bradford assay (Bio-Rad). Aliquots of whole cell lysates containing 40 µg protein were denatured and separated on 7.5% polyacrylamide-SDS gels and transferred to nitrocellulose membranes. Non-specific sites were blocked by 4% BSA (90 min incubation at room temperature). This was followed by overnight incubation at 4°C with rabbit anti-iNOS antibodies (1:500;

Cayman Chemicals, Ann Arbor, MI, USA). After washing, the membranes were incubated with IgG-horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibodies (1:5,000; GE Healthcare, Buckinghamshire, UK) for 90 min at room temperature. Finally, enhanced chemiluminescence solution was added, and the membranes were exposed to X-ray film (Fuji medical X-ray film, FujiFilm). Protein levels were normalized to β-actin levels using mouse monoclonal anti-β-actin antibodies (1:25,000; MP Biological, Santa Ana, CA, USA) and HRP-conjugated goat anti-mouse antibodies (1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). A computerized image analysis system (EZ Quant-Gel 2.2, EZQuant Biology Software Solutions, Israel) was used for semi-quantitative analysis.

Mice

Wild-type (WT) C57BL/6 mice were purchased from Harlan Israel (Jerusalem, Israel). Transgenic 5X familial AD (5XFAD) mice were provided by Prof. Robert Vassar (Department of Cell and Molecular Biology, Northwestern University). 5XFAD mice include five mutations under the transcriptional control of the neuron-specific mouse Thy-1 promoter, with three mutations in the human APP695 gene (Swedish K670N, M671L, Florida I716V and London V717I) and two mutations in the human presenilin-1 gene (M146L, L286V). At the age of 2 months, 5XFAD mice evolve Aβ accumulation and gliosis (27). The human APP gene was detected by PCR analysis of mice tail tissue DNA. Mice were placed in cages at temperatures of 22 ± 2°C and 65% humidity. Food and water supply was made available, and a 12 h light/dark cycle was maintained. All animal studies were performed according to the recommendations of Institutional Animal Care and Use Committee of Ben-Gurion University of the Negev. The protocol employed was approved by this committee (approval number IL-30-08-2011).

In the first experiment, 8-week-old mice were divided into three groups, namely WT mice treated with R-715, 5XFAD mice treated with saline, and 5XFAD mice treated with R-715. In the second experiment, mice were again divided into three groups, namely WT mice treated with HOE 140, 5XFAD mice treated with saline, and 5XFAD mice treated with HOE 140. The mice received daily intranasal treatment of 1 mg/kg per day for 18 days (5 days/week).

Immunohistochemistry

Mice were anesthetized upon intra-peritoneal injection of 0.2 ml ketamine–xylazine mixer (1:1). Brains were removed following cold PBS cardiac perfusion and were divided into two hemispheres. One hemisphere from each mouse brain was incubated overnight in 4% paraformaldehyde solution at 4°C, followed by incubation in 30% sucrose solution at 4°C for 2 days. The hemispheres were then frozen in molds containing tissue adhesive (O.C.T. compound, Tissue-Tek, Torrance, CA, USA) at −80°C. All hemispheres were cut into sagittal sections (40 μm) using a cryostat and maintained at −20°C in PBS:ethyleneglycol:glycerol (2:1:1) non-freezing solution. Free-floating sections were washed using 0.05% PBS/Tween 20 and permeabilized using 0.5% PBS/Triton X-100. After blocking non-specific binding using antibody diluent solution (GBI Labs, Mukilteo, WA, USA), sections were incubated for 2 h at room temperature with rabbit anti-human Aβ (1:250; a gift from Prof. 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 of the Negev) and rat anti-mouse/human CD11b antibodies (1:25, Biolegend). Primary antibodies were diluted in antibody diluent solution. Thereafter, the sections were rinsed and incubated for 1 h at room temperature with the appropriate secondary antibody, i.e., Cy^3-conjugated donkey anti-rabbit IgG (1:1,000) or Alexa fluor 488-conjugated goat anti-rat IgG (1:250), both from Jackson ImmunoResearch Laboratories. Secondary antibodies were diluted in 0.05% PBS/Tween 20. After washes, the sections were mounted with mounting medium containing DAPI (Vector labs) on charged slides and stored at 4°C. An Olympus FluoView FV1000 confocal microscope (Olympus, Hamburg, Germany) at 1,024 × 1,024 pixel resolution with a ×10 objective was used for imaging. In each experiment, five sections from the cortex of each animal were analyzed. Aβ and CD11b staining was quantified using the ImageJ software version 1.40C (NIH). Average fluorescent Aβ- and CD11b-containing areas were calculated for each treated group.

Statistical Analysis

For each experiment, results are presented as the mean ± SEM. To assess the statistical significance of differences between treatment groups, one-way analysis of variance was performed, followed by a *post hoc* multiple comparison test (Tukey–Kramer Multiple Comparison Test). $P < 0.05$ was considered statistically significant.

RESULTS

Serving as a positive control, actinomycin D, significantly reduced BV2 microglial cell viability (**Figure 1**). By contrast, neither the B_1R antagonist R-715 at concentrations of 10^{-7} and 10^{-6} M (**Figure 1A**) nor the B_2R antagonist HOE 140 at a concentration of 10^{-6} M (**Figure 1B**) altered microglial cell viability, as measured with XTT assay.

The production of NO (**Figure 2A**) and TNF-α (**Figure 2B**) in non-stimulated BV2 cells or in BV2 cells induced by LPS (7 ng/ml) and treated with R-715 (10^{-7} and 10^{-6} M) was next considered. LPS markedly enhanced NO and TNF-α production, as compared with controls. R-715 at concentrations of 10^{-7} and 10^{-6} M significantly increased LPS-induced NO secretion (**Figure 2A**). R-715, at concentrations of 10^{-7} and 10^{-6} M, also increased LPS-induced TNF-α secretion (**Figure 2B**). By contrast, R-715 did not alter NO (**Figure 2A**) or TNF-α (**Figure 2B**) production in non-stimulated cells. However, the selective B_2R antagonist HOE 140 failed to alter NO (**Figure 3A**) or TNF-α (**Figure 3B**) release from either non-stimulated or LPS-stimulated cells.

As shown in **Figure 4**, a 24-h treatment of BV2 cells with LPS (7 ng/ml) significantly increased iNOS expression levels. 10^{-6} M of R-715 increased the LPS-induced iNOS expression by up to 102%.

As anticipated, the cortex of WT mice intranasally administered with R-715 did not show any Aβ plaques (**Figure 5A**). Levels of both Aβ and CD11b (marker for microglial accumulation)

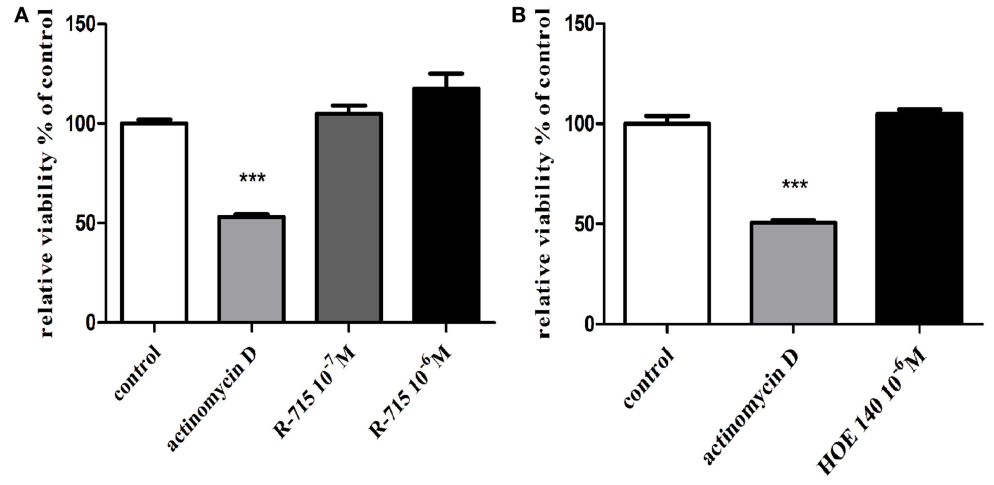

FIGURE 1 | Effects of bradykinin B_1 receptor (B_1R) and bradykinin B_2 receptor (B_2R) antagonists on the viability of BV2 cells. Cells were incubated with **(A)** the B_1R antagonist R-715 (10^{-7} and 10^{-6} M) or **(B)** the B_2R antagonist HOE 140 (10^{-6} M) for 24 h. At the end of the experiment, viability was assessed by the XTT assay. Results are given as mean ± SEM, $n = 4$; a one-way analysis of variance and Tukey–Kramer Multiple Comparison Test were performed to determine statistical significance; $^{***}P < 0.001$ versus the control.

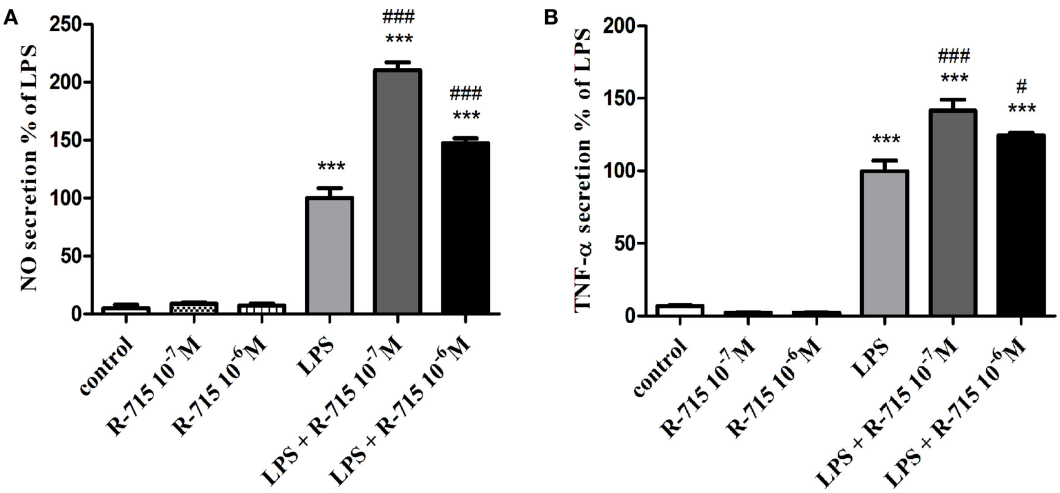

FIGURE 2 | Effects of the bradykinin B_1 receptor (B_1R) antagonist on basal and lipopolysaccharide (LPS)-induced NO and tumor necrosis factor-alpha (TNF-α) release from BV2 cells. Cells were incubated with the B_1R antagonist R-715 (10^{-7} and 10^{-6} M) in the presence or absence of LPS (7 ng/ml) for 24 h. NO **(A)** and TNF-α **(B)** levels were measured in the media, and the cells were counted. Results are representatives of three independent experiments and are presented as mean ± SEM, $n = 3$–6; statistical significance was assessed by one-way analysis of variance followed by a Tukey–Kramer Multiple Comparison Test; $^{***}P < 0.001$ versus the control, $^{###}P < 0.001$ versus LPS, and $^{#}P < 0.05$ versus LPS.

were significantly enhanced in age-matched 5XFAD mice treated intranasally with the vehicle (**Figure 5**). However, as compared to 5XFAD mice treated with the vehicle, age-matched 5XFAD mice treated intranasally with R-715 (1 mg/kg/day) showed close to 100 and 50% increases in Aβ burden (**Figures 5A,D**) and CD11b staining (**Figures 5B,D**), respectively. 5XFAD mice intranasally treated with HOE 140 did not display any differences in plaque burden (**Figures 6A,D**) or CD11b staining (**Figures 6B,D**) in the cortex, as compared to vehicle-treated mice. Modified **Figures 5** and **6** are adapted with permission from Asraf et al. (28).

DISCUSSION

Although BK is generally viewed as a central pro-inflammatory mediator (25, 29, 30), a possible protective role for BK in the brain has been proposed. Recently, we showed a dual effect of kinins on the production of prostaglandin (PG), a pro-inflammatory mediator, in cultured glial cells (i.e., microglia and astrocytes) (31). Specifically, the activation of B_2 receptors increased PG synthesis, whereas B_1R agonists inhibited synthesis of these pro-inflammatory mediators. We have, therefore, suggested that a regulatory loop exists in which B_2 receptors mediate enhanced-glial inflammation.

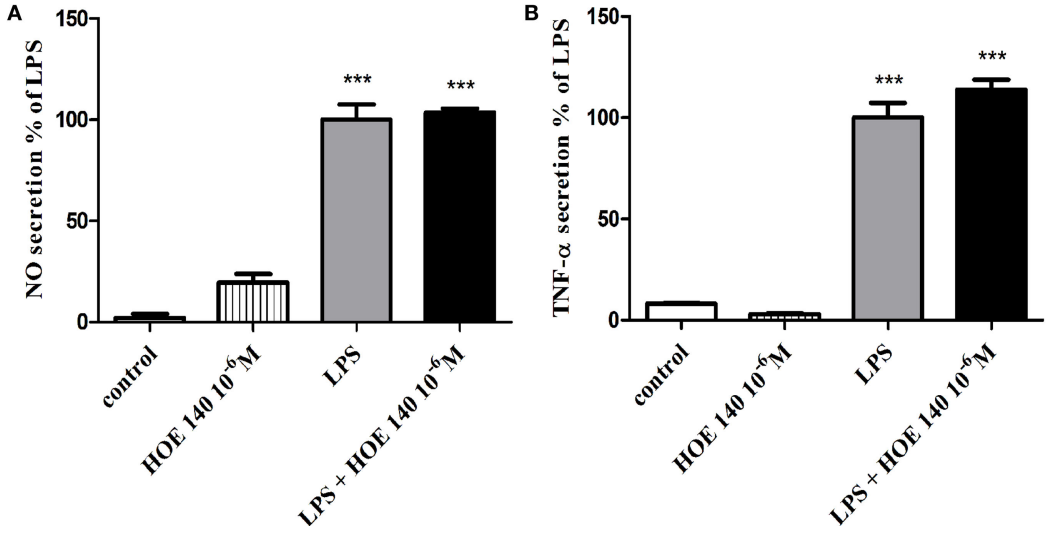

FIGURE 3 | Effect of the bradykinin B_2 receptor (B_2R) antagonist on basal and lipopolysaccharide (LPS)-induced NO and tumor necrosis factor-alpha (TNF-α) release from BV2 cells. Cells were incubated with the B_2R antagonist HOE 140 (10^{-6} M) in the presence or absence of LPS (7 ng/ml) for 24 h. NO **(A)** and TNF-α **(B)** levels were measured in the media, and the cells were counted. Results are representatives of three independent experiments and are presented as mean ± SEM, $n = 3$–6; statistical significance was assessed by one-way analysis of variance followed by a Tukey–Kramer Multiple Comparison Test; $^{***}P < 0.001$ versus control.

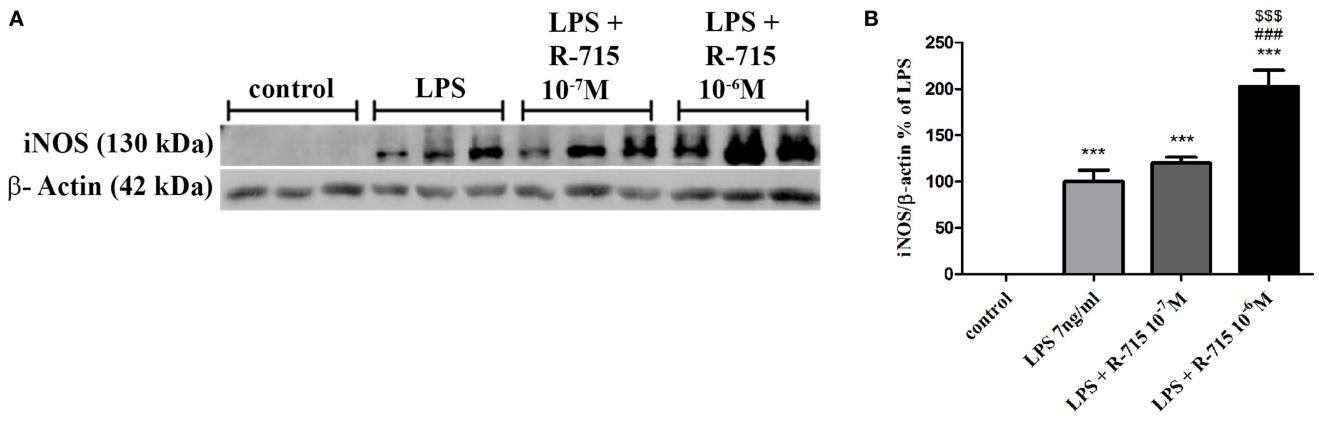

FIGURE 4 | Effect of the bradykinin B_1 receptor (B_1R) antagonist on iNOS expression in lipopolysaccharide (LPS)-stimulated BV2 cells. Cells were incubated with the B_1R antagonist R-715 (10^{-7} and 10^{-6} M) and LPS (7 ng/ml) for 24 h. Whole cell lysates (40 μg) were examined by Western blot analysis using anti-iNOS or β-actin antibodies. The results shown here represent three independent experiments **(A)**. The graph represents the mean ± SEM of three experiments **(B)**, $n = 3$. One-way analysis of variance and a Tukey–Kramer Test were used to determine statistical significance; $^{***}P < 0.001$ versus the control, $^{###}P < 0.001$ versus LPS, and $^{\$\$\$}P < 0.001$ versus LPS + R-715 (10^{-7} M).

B_1 receptors are subsequently upregulated and are involved in the attenuation of glial inflammation (31). These findings were confirmed in part by Noda et al., who documented reduced microglial inflammation upon BK treatment of LPS-induced cells (32, 33). More recently, we also showed that a B_1 receptor agonist abrogated NO and TNF-α production in LPS-treated BV2 microglial cells (26).

In the present study, R-715 significantly enhanced iNOS expression and release of TNF-α and NO from BV2 microglia. Using immunocytochemistry, we previously demonstrated the expression of both B_1 and B_2 receptor sub-types in BV2 microglia (34). In the present study, B_1Rs are blocked by R-715, and no regulatory feedback inhibition exists through B_1 receptors. This suggests that endogenous BK, possibly released from BV2 cells, activates B_2 receptors and contributes to the amplification of inflammation (TNF-α and NO synthesis) induced by LPS. Interestingly, BK and LPS were shown to induce B_2R expression and synergistically enhance nitrosative stress and inflammation in epithelial cells (35). Induction of activated microglial TNF-α and NO release is of particular relevance, as these pro-inflammatory cytokines are both associated with neuronal loss. iNOS expression and NO generation have been described in several brain pathologies, including AD (36). Microglial iNOS is induced by Aβ both *in vitro* and *in vivo* and activated iNOS-expressing microglia were found

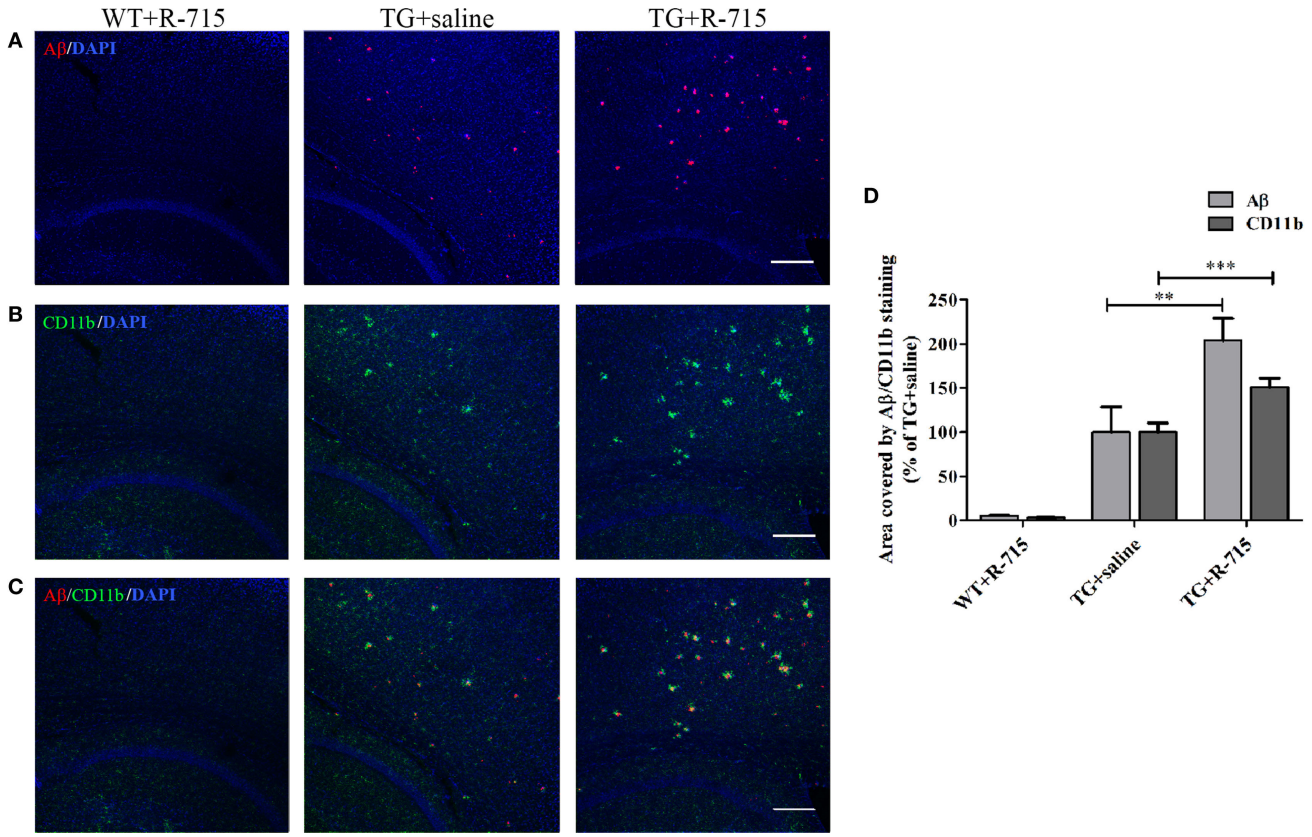

FIGURE 5 | Effects of intranasal delivery of the bradykinin B_1 receptor antagonist on amyloid burden and the accumulation of microglial cells in the cortex of 5X familial AD (5XFAD) mice. Two-month-old mice were treated with R-715 or the vehicle for 3½ weeks. After cardiac perfusion, their brains were cut into sections, stained for Aβ (*red*) **(A)** and CD11b (microglial accumulation) (*green*) **(B)** using appropriate antibodies and counter-stained with DAPI (*blue*). Representative confocal images of cortical sections from wild-type (WT) or 5XFAD mice (TG) are shown. **(C)** Merged images of Aβ and CD11b staining. The dose of intranasally administered R-715 was 1 mg/kg/day. Each group included 4–8 mice (n = 14–23 per experiment). The stained areas were quantified. The mean ± SEM of percentage of the stained areas is shown in the graphs, $n \geq 3$ determinants **(D)**. For statistical comparisons, a one-way analysis of variance and Tukey–Kramer Multiple Comparison Test were conducted. ***$P < 0.001$ versus TG + saline; **$P < 0.01$ versus TG + saline. The scale bar is 200 μm.

in amyloid plaques surrounded by dead and dystrophic neurons. Various modes and mechanisms by which NO can lead to neuronal death have been described (37, 38). TNF-α is also associated with neurodegeneration and furthermore induces the expression of amyloid precursor protein and promotes its cleavage by stimulating secretase activity to release Aβ. Reciprocally, Aβ induces TNF-α synthesis in neurons and glial cells. In addition, Aβ has been shown to physically bind TNFR-1, thereby inducing neuronal death (39).

For *in vivo* studies, the five familial Alzheimer's disease (5XFAD) mouse model was employed. These mice develop rapid Aβ deposits alongside microglial accumulation beginning at 2 months of age, with plaques initially accumulate in the cortex. As shown in **Figure 5**, intranasal treatment of these mice with R-715 for 3.5 weeks significantly enhanced amyloid burden and CD11b expression in the cortex. Intranasal application of this B_1R antagonist was chosen since this mode of delivery likely increases the direct action of the compound by bypassing the blood–brain barrier. Moreover, clinical studies involving nasal application of other compounds, such as insulin, to AD patients showed improvement in memory skills (40). However, peripheral effects of the antagonist cannot be ruled out. Furthermore, the effectiveness of the passage of peptides from nose to brain is controversial (41). Intranasal delivery of B_1R or B_2R antagonists has been tried here, for the first time. Similar results as those reported here were, nonetheless, reported by Passos et al., who showed that an 8 month-long treatment of triple mutant APP (Tg-SwDI) mice with R-715 resulted in enhanced amyloid burden (42). Antagonism of B_1R using R-715 also resulted in significantly greater severity of multiple sclerosis in a mouse model of the disease (43). On the other hand, there is evidence suggesting a role for B_2Rs in regulating brain inflammation and AD. Upregulation of B_1 and B_2 receptors in Aβ-infused rats was observed, mainly in brain regions such as the hippocampus and cortex, suggesting the possible involvement of kinins in AD (44). In a mouse model of AD, Prediger et al. showed improvement of cognitive deficits by genetic deletion or pharmacological antagonism of B_1 or B_2 receptors (45). Blockage of B_2 receptor, as shown by Bicca et al., prevented Aβ-induced cognitive impairment by inhibiting brain inflammation (46). Moreover, differential roles of B_1 and B_2 receptors in memory consolidation were observed during aging in mice.

B_1 and B_2 receptors transduce their signals through similar cellular pathways. They both are generally described as signaling through Gαq and Gαi. However, B_2 receptors also interact with

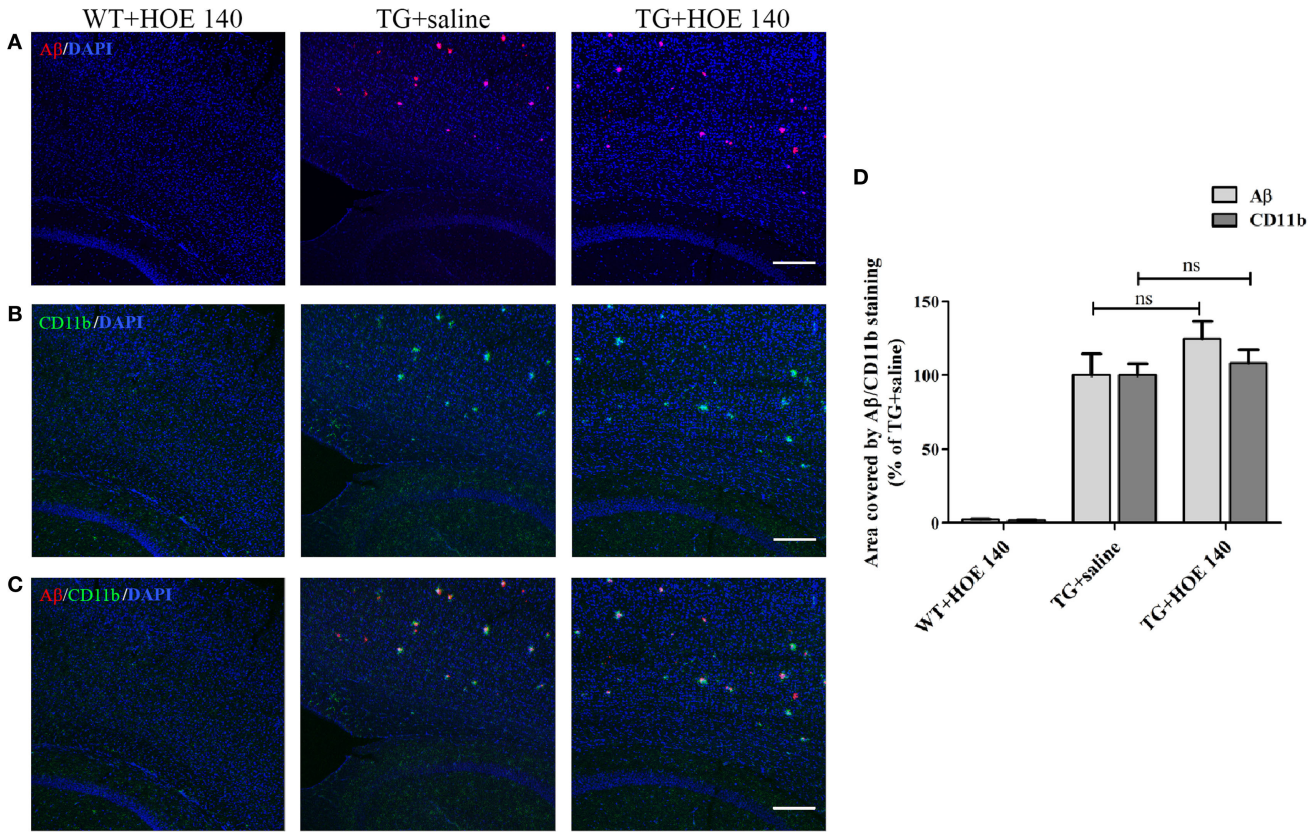

FIGURE 6 | The effects of intranasal delivery of the bradykinin B_2 receptor antagonist on amyloid burden and the accumulation of microglial cells in the cortex of 5X familial AD (5XFAD) mice. Two-month-old mice were treated with HOE 140 or the vehicle for 3½ weeks. After cardiac perfusion, their brains were cut into sections, stained for Aβ (*red*) **(A)** and CD11b (*green*) **(B)** using appropriate antibodies and counter-stained with DAPI (*blue*). Representative confocal images of cortical sections from wild-type (WT) or 5XFAD mice (TG) are shown. **(C)** Merged images of Aβ and CD11b staining. The dose of intranasally administered HOE 140 was 1 mg/kg/day. Each group included 4–7 mice (n = 15–19 per experiment). The stained areas were quantified. The mean ± SEM of percentage of the stained areas is shown in the graphs, $n \geq 3$ determinants **(D)**. For statistical comparisons, a one-way analysis of variance and Tukey–Kramer Multiple Comparison Test were conducted. ns, non-significant. The scale bar is 200 μm.

other G proteins as well, including Gαs and Gα12/13. The signaling patterns are also different for both receptors. For example, in vascular smooth muscle cells, activating B_2R induced transient increase in intracellular Ca^{2+} signaling, whereas B_1 receptor stimulation was sustained (47). The specific way of signaling is possibly the result of different extent of regulation that these receptors are dependent on. Further studies are required to find out whether any of these differences explains differential involvement of BK receptors in modulation of amyloid burden and glial accumulation *in vitro* and *in vivo*.

To achieve better insight into mechanisms by which amyloid deposition is modulated by B_1R, the effect of B_1R antagonism on microglial/macrophage accumulation was investigated. R-715 distinctly augmented microglial/macrophage accumulation in the cortex of 5XFAD mice. Lee et al., similar to us, showed that reduced microglial activation was associated with less amyloid accumulation (48). Specific characterization of the microglia/macrophage phenotype(s) was not done here, although one can envision that a more complete analysis of microglial markers might point to a given functional or activation state that is more favorable for reducing amyloid accumulation.

To conclude, we have presented evidence supporting a role for B_1R in brain inflammation and in the regulation of amyloid deposition in AD mice, possibly with microglial/macrophage involvement. Further studies are required to test whether modulation of this receptor can serve as a novel therapeutic strategy for AD.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee of Ben-Gurion University of the Negev, Beer-Sheva, Israel. The protocol was approved by this committee: approval number IL-30-08-2011.

AUTHOR CONTRIBUTIONS

SF-B and KA designed the experiments. KA performed the experiments. KA and NT analyzed data and prepared figures. SF-B and KA wrote the manuscript.

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