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Intranasal Delivery of NEMO-Binding Domain Peptide Prevents Memory Loss in a Mouse Model of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is the most common form of dementia. Despite intense investigations, no effective therapy is available to halt its progression. We found that NF- κ B was activated within the hippocampus and cortex of AD subjects and that activated forms of NF- κ B negatively correlated with cognitive function monitored by Mini-Mental State Examination and global cognitive z score. Accordingly, NF- κ B activation was also observed in the hippocampus of a transgenic (5XFAD) mouse model of AD. It has been shown that peptides corresponding to the NF- κ B essential modifier (NEMO)-binding domain (NBD) of I κ B kinase α (IKK α) or I κ B kinase β (IKK β) specifically inhibit the induction of NF- κ B activation without inhibiting the basal NF- κ B activity. Interestingly, after intranasal administration, wild-type NBD peptide entered into the hippocampus, reduced hippocampal activation of NF- κ B, suppressed hippocampal microglial activation, lowered the burden of A β in the hippocampus, attenuated apoptosis of hippocampal neurons, protected plasticity-related molecules, and improved memory and learning in 5XFAD mice. Mutated NBD peptide had no such protective effect, indicating the specificity of our finding. These results suggest that selective targeting of NF- κ B activation by intranasal administration of NBD peptide may be of therapeutic benefit for AD patients.

Keywords

Alzheimer's disease; memory; NBD peptide; neuroinflammation; NF- κ B; plasticity

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SUPPLEMENTARY MATERIAL

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INTRODUCTION

Alzheimer's disease (AD) is the most common human neurodegenerative disorder that gradually destroys cognitive abilities with its first clinical sign appearing after age 60. Although the etiology of AD remains unknown, it is now accepted that AD is a multifactorial disorder that is affected by a mix of genetic, environmental, and lifestyle factors [1–3]. The classic pathologic marks of the disease are the presence of senile plaques and neurofibrillary tangles as well as neuronal loss. Neurofibrillary tangles and neuritic plaques are composed of aggregates of misfolded proteins consisting of amyloid- β (A β), a 40–43 amino acid long proteolytic fragment of the amyloid- β protein precursor (A β PP), and phosphorylated tau, respectively [4–6].

Although mechanisms that cause AD are poorly understood, recent studies support the role of inflammation in hippocampal degeneration in AD. First, long-term use of nonsteroidal anti-inflammatory drugs has been shown to be protective for AD [7]. Second, activated astrocytes and microglia are seen in close association with amyloid plaques in the human condition and in mouse models of AD [8, 9]. Third, A β peptides induce inflammation and glial activation in cultured glial cells and *in vivo* in the brain [10, 11]. Fourth, AD lesions display biochemical and histochemical hallmarks of oxidative and nitrosative injury, including nitration of protein tyrosine residues [12], suggesting the vicinal production of peroxynitrite from nitric oxide and superoxide. Consistently, deficiency of inducible nitric oxide synthase (iNOS) substantially protected from AD-like disease pathogenesis in transgenic mice expressing mutant human A β PP and presenilin-1 (hPS1) [13]. Fifth, a variety of proinflammatory cytokines including TNF- α , IL-1 β , and IL-6 are found in affected AD brain regions. These cytokines are also able to promote the accumulation of A β peptide [14]. Together, these findings suggest that regulation of glial inflammation is of therapeutic interest in mitigating neurodegeneration in AD.

NF- κ B is an important regulator of inflammation [15, 16]. Activation of NF- κ B requires the activity of I κ B kinase (IKK) complex containing IKK α and IKK β and the regulatory protein NF- κ B essential modifier (NEMO) [15, 16]. Ghosh and colleagues [17] have shown that peptides corresponding to the NEMO-binding domain (NBD) of IKK α or IKK β specifically inhibit the induction of NF- κ B activation without inhibiting basal NF- κ B activity. Here, we demonstrated that NF- κ B activation was induced *in vivo* in the cortex and hippocampus of AD patients and that activated forms of NF- κ B negatively correlated with cognitive function. Interestingly, intranasal administration of NBD peptide resulted in reduction in hippocampal activation of NF- κ B, suppression of hippocampal microglial activation, lowering of A β load, protection of hippocampal plasticity, and improvement of memory and learning in 5XFAD mice.

MATERIALS AND METHODS

human subjects

Thirty-three cases with antemortem clinical diagnosis of no cognitive impairment (NCI; $n = 12$, 8 women/4 men), mild cognitive impairment (MCI; $n = 11$, 4 women/7 men), and AD ($n = 10$; 5 women/5 men) obtained from the Rush Religious Order Study [18, 19] were

analyzed (Table 1). All participants agreed to a detailed annual clinical evaluation and brain donation upon death. Human Investigations Committees of the Rush University Medical Center approved the study.

Clinical and neuropathologic evaluations

Clinical criteria for diagnosis of NCI, MCI, and AD have been reported elsewhere [18, 20–22]. Of the 11 MCI cases included in this study, 7 were diagnosed as amnesic MCI. Final clinical and neuropsychological testing, which included the Mini-Mental State Examination (MMSE) and a battery of 19 cognitive tests, was performed within 2 years of death. A global cognitive z score (GCS) comprising the 19 tests was available for all cases. Neuropathological diagnosis, including Braak staging of neurofibrillary tangles [20], NIA-Reagan criteria [21], and recommendations of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [22], was performed as previously described [18, 23]. Subjects with pathological findings other than AD (e.g., stroke, Parkinson disease, Lewy body dementia) were excluded from the study. Clinical, demographic, and neuropathological details of all cases are presented in Table 1. Tissue and clinical information is under the protection of the Health Information Privacy Administration rules.

Tissue samples and western blotting

Superior frontal cortex (Brodmann area 9) was dissected free of white matter at autopsy on dry ice to prevent thawing and was maintained at -80°C until assay. Tissue was homogenized (150 mg/ml) on ice in homogenization buffer (250 mM sucrose, 20 mM Tris base) containing protease and phosphatase inhibitors (Sigma), further diluted in homogenization buffer free of detergents or surfactants and analyzed for protein concentration with a NanoDrop (Thermo). For western blotting, 30 μg lysate was resolved on 8 or 10% Bis-Tris SDS polyacrylamide gels in a continuous buffer system and electrophoretically transferred to nitrocellulose membranes (BioRad) with a semi-dry blotter (Pierce) as described earlier [24–26]. Membranes were blocked for 1 h with blocking buffer (LI-COR Biosciences) and incubated in primary antibodies (Supplementary Table 1) overnight at 4°C . Membranes were then washed and incubated with IR-Dye-labeled secondary antibodies (1:18,000; LI-COR Biosciences) for 45 min at room temperature, washed again and visualized with the Odyssey infrared imaging system (LI-COR Biosciences). Blots were converted to binary, analyzed using ImageJ (NIH), and normalized to loading control (β -actin).

Animals and intranasal delivery of NBD peptides

B6SJL-Tg(APP^{sw}FILon,PSEN1*^{M146L}*^{L286V}) 6799Vas/J transgenic (5XFAD) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained, and experiments were conducted in accordance with National Institutes of Health guidelines and were approved by the Rush University Medical Center Institutional Animal Care and Use Committee. Five month old male 5XFAD mice were treated intranasally with wtNBD or mNBD peptides (0.1 mg/Kg body wt/2d) for 30d. Briefly, NBD peptides were dissolved in 5 μl normal saline, mice were held in supine position, and saline was delivered into one nostril using a pipetman.

NBD peptides (>99% pure) were synthesized in the custom peptide synthesis facility of Peptide 2.0 (Chantilly, VA). Wild type (wt) and mutated (m) NBD peptides contain the Antennapedia homeodomain (lower case) and IKK β (upper case) segments. Positions of W→A mutations are underlined [17, 27].

wtNBD: drqikiwfnrrmkwkkLDWSWL; mNBD: drqikiwfnrrmkwkkLDASAL

Electrospray ionization (ESI)-MS analysis of wtNBD peptide in hippocampal extracts

5XFAD mice were treated intranasally once with wtNBD peptide (0.1 mg/Kg body wt). After 30 min, mice were perfused with sterile saline and hippocampi were homogenized in 10 mM HEPES (pH 7.9) containing 1.5 mM MgCl₂, 10 mM KCl, 100 mM DTT, protease and phosphatase inhibitor cocktail. The homogenate was centrifuged and the supernatant was transferred to methanol:chloroform:water (4:3:1) mixture followed by centrifugation at 14,000 rpm for 90 s. The aqueous phase was analyzed for wtNBD by ESI-MS.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from hippocampus using Ultraspec-II RNA reagent (Biotecx Laboratories, Inc., Houston, TX) following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. RT-PCR was carried out as described earlier [11, 28, 29] using a RT-PCR kit (Clontech, Mountain View, CA) and primers (Supplementary Table 2).

Real-time PCR analysis

DNase-digested RNA was analyzed by real-time PCR in the ABI-Prism7700 sequence detection system (Applied Biosystems, Foster City, CA) as described earlier [11, 28–30]. Data were processed using the ABI Sequence Detection System 1.6 software.

Barnes maze and T maze

Maze experiments were performed as described [31, 32]. Briefly, for Barnes maze, mice were trained for 2 consecutive days followed by examination on day 3. During training, the overnight food-deprived mouse was placed in the middle of the maze in a 10 cm high cylindrical black start chamber. After 10 s, the start chamber was removed to allow the mouse to move around the maze to find out the color food chips in the baited tunnel. The session was ended when the mouse entered the baited tunnel. The tunnel was always located underneath the same hole (stable within the spatial environment), which was randomly determined for each mouse. After each training session, maze and escape tunnel were thoroughly cleaned with a mild detergent to avoid instinctive odor avoidance due to mouse's odor from the familiar object. On day 3, the maze was illuminated with high wattage light that generated enough light and heat to motivate animals to enter into the escape tunnel [33], allowing us to measure latency (duration before all four paws were on the floor of the escape box) and errors (incorrect responses before all four paws were on the floor of the escape box).

For T maze, mice were also habituated in the T-maze for two days under food-deprived conditions so that animals can eat food rewards at least five times during a 10-min period of

training. During each trial, mice were placed in the start point for 30 s and then forced to make a right arm turn which was always baited with color food chips. On entering the right arm, they were allowed to stay there for 30–45 s, then returned to the start point, held for 30 s, and then allowed to make right turn again. As described above, after each training session, T maze was thoroughly cleaned with a mild detergent. On day 3, mice were tested for making positive turns and negative turns. The reward side is always associated with a visual cue. Number of times the animal eats the food reward would be considered as a positive turn.

Novel object recognition task

Novel object recognition task was performed to monitor the short term memory as described by others [34] and us [32]. Briefly, during training, mice were placed in a square novel box (20 inches long by 8 inches high) surrounded with infrared sensor. Two plastic toys (between 2.5 and 3 inches) that varied in color, shape, and texture were placed in specific locations in the environment 18 inches away from each other. The mice were able to explore freely the environment and objects for 15 min and then were placed back into their individual home cages. After 30 min, mice were placed back into the environment with two objects in the same locations, but now one of the familiar objects was replaced with a third novel object. The mice were then again allowed to explore freely both objects for 15 min. The objects were thoroughly cleaned with a mild detergent.

Immunohistochemistry

After treatment, mice were anesthetized and perfused with PBS (pH 7.4) and then with 4% (w/v) paraformaldehyde solution in PBS followed by dissection of the brain from each mouse for immunofluorescence microscopy [27, 35]. Briefly, samples were incubated in PBS containing 0.05% Tween 20 (PBST) and 10% sucrose for 3 h and then 30% sucrose overnight at 4°C. Brain was then embedded in O.C.T (Tissue Tech) at –80°C, and processed for conventional cryosectioning. Frozen sections (30 µm) were treated with cold ethanol (–20°C) followed by two rinses in PBS, blocking with 3% bovine serum albumin in PBST and double-labeling with two antibodies (Supplementary Table 1). After three washes in PBST, sections were further incubated with Cy2 and Cy5 (Jackson ImmunoResearch Laboratories, Inc.). The samples were mounted and observed under a Bio-Rad (Hercules, CA) MRC1024ES confocal laser-scanning microscope.

Counting of A β plaques

Amyloid plaques in hippocampus and cortex were counted using the touch counting module of the Olympus Microsuite 5™ imaging software. Briefly, captured images were opened in the infinity image viewer window and the area of the entire image was measured by drawing a rectangular object around the image. After that, plaques were counted by touch counting. Both area of the image and counted signals were exported in the excel sheet and calculated as a unit of number of signals per square millimeter area.

Fragment end labeling of DNA

Fragmented DNA was detected *in situ* by the terminal deoxynucleotidyltransferase-mediated binding of 3'-OH ends of DNA fragments generated in response to fibrillar A β ₁₋₄₂, using a commercially available kit (TdT FragEL™, Calbiochem) as described before [10]. Briefly, cover slips were treated with 20 μ g/ml proteinase K for 15 min at room temperature and washed prior to terminal deoxynucleotidyltransferase staining.

ELISA

For A β ₁₋₄₂ ELISA, hippocampal or cortical tissues were homogenized in TBS, pelleted for 30 min $\times$ 150,000 *g*. The pellet was resuspended in 3 volumes (wt/vol original tissue weight) of TBS+1% Triton X-100, pelleted for 30 min $\times$ 150,000 *g* and the supernatant recovered and stored. Samples were assayed for protein concentration and diluted 10-fold prior to performing ELISA according to manufacturer's instruction (BioLegend, SIG-38956).

Statistical analysis

Clinical and biochemical data of human tissues were compared across diagnoses using nonparametric tests (i.e., Kruskal-Wallis test or Fisher's exact test, with Dunn's correction for multiple comparisons), which are more robust to outliers, non-normality and unequal sample sizes. Two-tailed Spearman Rank-Order correlations assessed variable associations between cognitive test scores and protein optical densities. Correlations were unadjusted for demographic information (i.e., age, gender, etc.) as these metrics were not significantly different between clinical groups. Statistical tests were performed using SPSS 19 (IBM), and significance was set at $\alpha = 0.05$ (two-sided).

Mice behavioral measures were examined by an independent one-way ANOVA using SPSS. Homogeneity of variance between test groups was examined using Levene's test. *Post-hoc* analyses were conducted using Tukey's or Games-Howell tests, where appropriate. $p < 0.05$ was considered statistically significant. Other data were expressed as means $\pm$ SD of three independent experiments. Statistical differences between means were calculated by the Student's *t*-test. A *p*-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

Activation of NF- κ B in AD

Activation of classical NF- κ B heterodimer (p65:p50) is required for a successful inflammatory response [15]. In addition to stimulus-induced nuclear translocation of NF- κ B, it has been shown that stimulus-induced phosphorylation and acetylation of p65 are also important for transcriptional activation [15, 36]. Therefore, to investigate the role of induced activation of NF- κ B in the pathogenesis of AD, we measured levels of phospho-p65 and acetylated-p65 by immunoblot analysis in extracts of prefrontal cortex (Brodmann area 9) from 33 subjects who died with a premortem clinical diagnosis of AD ($n = 12$), MCI ($n = 11$), and age-matched NCI ($n = 10$) (Table 1). No significant difference was found for age, gender, postmortem interval, brain weight, Braak staging, NIA-Reagan criteria, and recommendations of CERAD across groups (Table 1). However, as evident from Fig. 1A, E,

& F, levels of both phospho-p65 and acetylated-p65 were higher in AD than NCI, indicating activation of NF- κ B in prefrontal cortex of AD. On the other hand, level of acetylated-p65 in MCI was almost similar to that found in NCI (Fig. 1A & F). We also measured levels of p65, p50, and p105, the precursor of p50) in prefrontal cortex extracts. However, we did not see any significant difference in p65, p50, or p105 among NCI, MCI, and AD (Fig. 1A–D), suggesting the specificity of the effect.

To confirm the activation of NF- κ B in the CNS of AD subjects, we performed a double-label immunofluorescence analysis of acetylated-p65 and Iba-1 in cortical and hippocampal sections. The level of acetylated-p65 was greater in cortex (Fig. 1K–M) and hippocampus (Fig. 1K, L, N, & O) of AD brain compared with NCI. We also noticed greater Iba-1 expression (microglial activation) in cortex and hippocampus of AD compared to NCI (Fig. 1K–L). Iba-1-positive cells were also positive for acetylated-p65 in hippocampus and cortex of AD subjects (Fig. 1L).

Although NF- κ B may be required for memory [37], upon activation, NF- κ B drives the transcription of many proinflammatory molecules from glial cells [15], leading to neuroinflammation. Therefore, an important question remains whether activation of NF- κ B in AD contributes to cognitive decline. We addressed this issue by using measures of cognitive function derived from neuropsychiatric assessments performed longitudinally in the Religious Orders Study and the Rush Memory and Ageing Project. The Spearman rank-order correlation showed that both acetylated p65 and phospho-p65 levels in prefrontal cortex are negatively correlated with MMSE and GCS with high levels of statistical significance (Fig. 1G–J & Table 2).

Activation of NF- κ B in 5XFAD transgenic (Tg) mice

Next, to investigate if NF- κ B is also activated in the CNS of an animal model of AD, we examined the status of acetylated p65 and phospho-p65 in the hippocampus and cortex of 5XFAD (B6SJL-Tg(APP^{SwFlon},PSEN1^{*M146L*L286V})6799Vas/J) Tg mice. Similar to that found in the CNS of AD subjects, we observed higher levels of acetylated-p65 and phospho-p65 in cortex and hippocampus of Tg mice as compared to age-matched non-Tg mice (Supplementary Figure 1A–D). Accordingly, we found marked increase in microglial activation as evidenced by Iba-1 immunoreactivity and many Iba-1-positive cells also co-localized with acetylated-p65 (Supplementary Figure 1B) and phospho-p65 (Supplementary Figure 1D).

To further monitor the induced activity of NF- κ B, we performed EMSA on nuclear extracts isolated from hippocampal tissues harvested from non-Tg and Tg mice. Consistent with an increase in phospho-p65 and acetylated-p65 in the CNS of Tg mice, the DNA-binding activity of NF- κ B was greater in the hippocampus of Tg mice than non-Tg mice (Supplementary Figure 1E). These results show the activation of NF- κ B *in vivo* in the hippocampus of Tg mice.

Intranasal administration of wtNBD peptide inhibits the induction of NF- κ B activation in vivo in the hippocampus of 5XFAD Tg mice

Because activated NF- κ B is negatively correlated with cognitive decline in AD and NF- κ B is also activated in the hippocampus and the cortex of Tg mice, we examined whether targeting NF- κ B had any beneficial effect in Tg mice. Earlier we have demonstrated that after intraperitoneal administration, NBD peptide enters into different parts of the brain of mice [27]. Recently we have provided evidence that NBD peptide can also ingress into the brain of hemiparkinsonian monkeys after intramuscular injection [38]. For better therapeutic application, here, we tested intranasal administration of NBD peptides in 5XFAD Tg mice. However, before testing clinical efficacy of intranasal NBD peptides in 5XFAD Tg mice, it was important to determine whether these peptides entered into the hippocampus and were capable of inhibiting the induction of NF- κ B activation *in vivo* in the hippocampus of Tg mice. Thirty minutes after intranasal administration of one dose of wtNBD peptide (0.1 mg/kg body wt), we detected wtNBD peptide in the hippocampus of Tg mice by ESI-MS (Supplementary Figure 2C). In contrast, we did not find any peak associated with wtNBD peptide in the hippocampus of untreated Tg mice (Supplementary Figure 2A-B). Similarly, after intranasal administration, we also detected this peptide in different parts of the brain by infra-red scanning (Supplementary Figure 2D). Since in subsequent studies, mice were treated with wtNBD (0.1 mg/kg body wt/2d) for 30 d, we tried to monitor the concentration of wtNBD peptide in the hippocampus under similar treatment condition. By HPLC, we found the peak for wtNBD peptide in the hippocampus of wtNBD-treated, but not untreated, Tg mice (Fig. 2E-H). Upon quantification, the level of wtNBD peptide was found to be 0.312 μ g/mg hippocampal tissue in comparison to nil in saline-treated Tg mice. These results demonstrate that after intranasal administration, NBD peptide enters into the hippocampus.

Next, we examined if NBD peptides were capable of modulating the activation of NF- κ B in the hippocampus of Tg mice. As seen by western blots in Supplementary Figure 3A-B, wtNBD, but not mNBD, peptides inhibited the expression of p65 and suppressed the phosphorylation of p65 *in vivo* in the hippocampus of Tg mice. This was confirmed by double-label immunofluorescence of hippocampal and cortical sections. The level of phospho-p65 was inhibited in Tg mice by treatment with wtNBD, but not mNBD, peptide (Supplementary Figure 3C). To further monitor the effect of wtNBD peptide on induced activity of NF- κ B, we performed EMSA on nuclear extracts isolated from hippocampal tissues. Consistent to decreased expression of p65 and phospho-p65, wtNBD treatment suppressed the DNA-binding activity of NF- κ B in the hippocampus of Tg mice (Supplementary Figure 3D). These results suggest that intranasal administration of wtNBD peptide is capable of suppressing activation of NF- κ B in the hippocampus of 5XFAD Tg mice.

Intranasal administration of wtNBD peptide inhibits inflammation in vivo in the hippocampus of 5XFAD Tg mice

Inflammation plays a role in the loss of neurons in AD and other neurodegenerative disorders [13, 27, 38]. Because wtNBD peptides inhibited the activation of NF- κ B *in vivo* in the hippocampus of Tg mice, we examined whether these peptides were able to suppress the

expression of various proinflammatory molecules in the hippocampus. As shown by semi-quantitative RT-PCR (Fig. 2A) and quantitative real-time PCR (Fig. 2B) experiments, the mRNA expression of iNOS and IL-1 β was higher in the hippocampus of Tg mice than non-Tg mice. This was also confirmed by western blot analysis of iNOS in hippocampal tissues (Fig. 2C-D). However, wtNBD, but not mNBD, peptide strongly inhibited the expression of these proinflammatory molecules *in vivo* in the hippocampus of Tg mice (Fig. 2A, B). Western blot analysis of iNOS in hippocampal tissues (Fig. 2C, D) and double-label immunofluorescence analysis for Iba-1 and iNOS in hippocampal and cortical sections (Fig. 2E) also show that the expression of iNOS protein was higher in Tg mice than non-Tg mice and that treatment of Tg mice with wtNBD, but not mNBD, peptides led to the suppression of iNOS protein.

Intranasal administration of wtNBD peptide reduces plaque formation in the hippocampus of 5XFAD Tg mice

A β peptides are the main component of the amyloid plaques found in the brain of AD patients. A β is formed after sequential cleavage of A β 1PP by α -, β -, and γ -secretases. The γ -secretase that produces the C-terminal end of the A β peptide cleaves within the transmembrane domain of A β PP, generating a number of isoforms of 36–43 amino acid residues in length [39]. The most common isoforms are A β ₄₀ and A β ₄₂, which are recognized by the 82E1 and 6E10 monoclonal antibodies (mAb). We examined if wtNBD treatment was capable of reducing the load of A β in the hippocampus of 5XFAD mice. Immunostaining of hippocampal and cortical sections with 82E1 mAb (Fig. 3A, B), immunoblot analysis of hippocampal homogenates with 6E10 mAb (Fig. 3C, D) and ELISA of hippocampal and cortical extracts for A β ₁₋₄₂ (Fig. 3E) demonstrate that the level of A β peptides is markedly higher in the CNS of Tg mice as compared to non-Tg mice. Interestingly, treatment of Tg mice with wtNBD, but not mNBD, led to significant decrease in A β (Fig. 3A-E), indicating that intranasal administration of wtNBD is capable of reducing the burden of A β in the hippocampus of 5XFAD mice.

Intranasal administration of wtNBD peptide suppresses neuronal degeneration *in vivo* in the hippocampus of 5XFAD Tg mice

Neurodegeneration is a hallmark of AD and accordingly, postmortem AD brains exhibit increased fluoro-Jade B (FJB) and neuronal TUNEL staining, suggesting that AD neurons undergo apoptosis [40] and that its reversal may have beneficial effects in AD. Therefore, we tested the effect of intranasal wtNBD peptide on neuronal degeneration and in the hippocampus of 5XFAD Tg mice. After 1 month of treatment, neuronal degeneration was detected by double-labeling of hippocampal sections for NeuN and FJB. As expected, a number of FJB-positive bodies co-localized with NeuN in the CA1 region of the hippocampus of Tg mice as compared to age-matched non-Tg mice (Fig. 4A). However, treatment of Tg mice with wtNBD peptide led to marked suppression of neuronal degeneration in the hippocampus (Fig. 4A). Similarly, a number of TUNEL-positive bodies co-localized with NeuN in the hippocampus of Tg mice as compared to non-Tg mice (Fig. 4B). However, intranasal administration of wtNBD, but not mNBD, peptide attenuated neuronal apoptosis in the hippocampus (Fig. 4A). This result was confirmed by detection of several other apoptosis-related molecules. As shown in Fig. 4C-D, treatment of Tg mice

with wtNBD, but not mNBD, peptides reduced the elevated level of cleaved caspase 3 in the hippocampus. Since dephosphorylation of BAD is also required for apoptosis, we examined the status of phospho-Bad in the hippocampus of NBD-treated and untreated Tg mice. As expected, the level of phospho-Bad decreased in the hippocampus of Tg mice (Fig. 4E, F). However, treatment of Tg mice with wtNBD led to significant increase in phospho-Bad in the hippocampus of Tg mice (Fig. 4E, F). On the other hand, mNBD peptide had no effect on the level of phospho-Bad (Fig. 4E, F). Together, these results suggest that intranasal wtNBD peptide treatment is capable of reducing neuronal degeneration *in vivo* in the hippocampus of Tg mice.

Intranasal administration of wtNBD peptide restores plasticity-related molecules in vivo in the hippocampus of 5XFAD Tg mice

Strong down-regulation of NMDA and AMPA receptor proteins and loss of calcium excitability in hippocampal neurons are often observed in AD brain, and a reversal of these cellular events may have functional implications in AD and other forms of dementia. Therefore, we next examined the effect of intranasal NBD peptide treatment on mRNA expression of these plasticity-related molecules in Tg mice. We observed strong down-regulation of NR2A and GluR1 mRNAs in the hippocampus of Tg as compared to non-Tg mice (Supplementary Figure 4A,B). CREB is regarded as the master regulator of memory and learning, which is known to control the transcription of most of the plasticity-related molecules [41]. Accordingly, we also found decrease in CREB mRNA in the hippocampus of Tg mice as compared to non-Tg mice (Supplementary Figure 4A,B). However, this deficit of CREB, NR2A and GluR1 in Tg mice was markedly restored by intranasal administration of wtNBD, but not mNBD, peptide (Supplementary Figure 4A, B).

As evident from Fig. 5A-B, the expression of CREB, NR2A, GluR1, and PSD-95 proteins also markedly decreased in the hippocampus of Tg mice as compared to non-Tg mice. However, consistent to mRNA analysis, treatment of Tg mice with wtNBD, but not mNBD, peptide led to significant restoration of CREB, NR2A, GluR1, and PSD-95 proteins *in vivo* in the hippocampus (Fig. 5A, B), suggesting that intranasal delivery of wtNBD, but not mNBD, peptides is capable of protecting plasticity-related molecules in the hippocampus.

Kv1.1, a K-channel subfamily member, is known to be involved in short-term memory. Similar to the regulation of NR2A and GluR1, the mRNA expression of Kv1.1 also decreased in the hippocampus of Tg as compared to non-Tg mice (Supplementary Figure 4A, C). However, treatment of Tg mice with wtNBD, but not mNBD, peptide led to the restoration of Kv1.1 (Supplementary Figure 4A, C). In contrast to NR2A, GluR1, and Kv1.1, we found increased mRNA expression of Gabra5 (protein encoded by this gene is known to support long-term depression or LTD) in the hippocampus of Tg mice as compared to non-Tg mice (Supplementary Figure 5A, B). Interestingly, treatment of Tg mice with wtNBD, but not mNBD, peptide led to the suppression of Gabra5 mRNA expression in the hippocampus of Tg mice (Supplementary Figure 5A, B). Taken together, these results suggest that intranasal administration of wtNBD peptide increases the expression of plasticity-related molecules, while suppressing the expression of LTD-related

molecule *in vivo* in the hippocampus of Tg mice, highlighting the specificity of wtNBD peptide treatment.

Effect of intranasal administration of NBD peptides on NMDA- and AMPA-mediated calcium current *in vivo* in the hippocampus of 5XFAD Tg mice

Next, we investigated if intranasal administration of NBD peptides improved the synaptic function in the hippocampus of Tg animals. Since the calcium influx through NMDA- and AMPA-sensitive receptors is directly implicated in postsynaptic activity, we measured NMDA- and AMPA-dependent calcium influx in the hippocampal slices of Tg mice that were treated with wtNBD and mNBD peptides intranasally for one month. Interestingly, mice that received wtNBD peptide showed significant calcium influx in their hippocampus once stimulated with NMDA (Fig. 5C), whereas untreated Tg animals and mNBD-treated Tg animals did not show any calcium influx (Fig. 5C). Similarly, Tg animals that received wtNBD, but not mNBD, displayed a strong AMPA-dependent calcium influx; the effect was even comparable to non-transgenic animals (Fig. 5D). We have confirmed our analyses from five different animals per group. The recording was performed from three hippocampal slices of each animal. These results suggest that intranasal administration of wtNBD peptide is capable of augmenting calcium influx *in vivo* in the hippocampus of Tg mice.

Intranasal administration of wtNBD peptide protects spatial learning and memory in 5XFAD Tg mice

The ultimate therapeutic goal of neuroprotection in AD is to improve and/or protect memory. The hippocampus regulates the generation of long term memory and spatial learning. Therefore, to examine whether intranasal wtNBD peptide protects only against structural damage or also against functional deficits seen in the 5XFAD Tg mice, we evaluated Barnes maze and T maze activities. Barnes circular maze test, a hippocampus-dependent cognitive task, requires spatial reference memory. Either wtNBD or mNBD peptide did not significantly alter total distance travel (Supplementary Figure 6A), number of movements (Supplementary Figure 6B), movement time (Supplementary Figure 6C), rest time (Supplementary Figure 6D), horizontal activity (Supplementary Figure 6E), and stereotypy (Supplementary Figure 6F) in Tg mice, suggesting that NBD peptide does not modulate gross motor activities in this model. On the other hand, wtNBD-treated mice significantly improved memory performance on Barnes maze test as shown by track plot (Fig. 6A), latency [$F_{3,28} = 55.014$, $p < 0.001(=0.00005)$] (Fig. 6B) and number of errors [$F_{3,28} = 5.550$, $p < 0.05(=0.027)$] (Fig. 6C). *Post hoc* tests of multiple comparisons using Games-Howell analyses showed that Tg mice took longer time to find the reward hole and exhibited more latency [$F_{3,28} = 0.027$, $p < 0.05(=0.00016)$] and higher errors [$F_{3,28} = 0.211$, $p < 0.05(=0.040)$] in the Barnes maze as compared to non-Tg mice. However, wtNBD-treated Tg mice were as capable as healthy non-Tg in finding the target hole (Fig. 6A) and exhibited significantly less latency [$F_{3,28} = 0.011$, $p < 0.001(=0.000015)$] and fewer errors [$F_{3,28} = 0.018$, $p < 0.001(=0.000061)$].

Next, we performed T maze tests to determine whether intranasal wtNBD treatment improved spatial memory in Tg mice. In this case as well, wtNBD treatment displayed significant effect on successful positive turns [$F_{3,28} = 1.222$, $p < 0.05(=0.406)$] (Fig. 6D) and

number of errors [$F_{3,28} = 0.888, p < 0.05(=0.445)$] (Fig. 6E). Untreated Tg mice exhibited less number of positive turns [$F_{3,28} = 0.363, p < 0.05(=0.121)$] and more number of negative turns [$F_{3,28} = 0.500, p < 0.05(=0.209)$] than age-matched non-Tg mice in T maze apparatus (Fig. 6D, E). On the other hand, wtNBD significantly improved the hippocampus dependent memory performances as wtNBD-treated Tg mice exhibited higher number of positive turns [$F_{3,28} = 1.125, p < 0.05(=0.445)$] and less negative turns [$F_{3,28} = 1.000, p < 0.05(=0.500)$] compared to mNBD-treated Tg mice in T maze (Fig. 6D, E).

We also monitored short-term memory by novel object recognition task. This task is particularly attractive as it requires no external motivation, reward, or punishment, and it can be completed in a relatively short time with minimal stress. Tg mice showed profound impairment [$F_{3,28} = 13.433, p < 0.01(=0.002)$] in short-term memory as evidenced by discrimination index (i.e., the difference between time spent exploring novel and familiar objects during test phase) as compared to age-matched non-Tg mice (Fig. 6F). However, we noticed markedly significant improvement [$F_{3,28} = 0.039, p < 0.01(=0.00051)$] or [$F_{3,28} = 0.018, p < 0.001(=0.000062)$] in short-term memory in wtNBD-treated Tg mice as compared to untreated or mNBD-treated mice (Fig. 6F).

DISCUSSION

Increasing longevity indicates the prevalence of AD will rise even further, which is typically characterized by accumulation, oligomerization, and fibrillation of $A\beta_{1-42}$, resulting in the formation of amyloid plaques. The amyloid plaques eventually trigger a cascade of neurodegenerative events associated with inflammatory responses, synaptic dysfunction, neuronal death, and ultimately clinical dementia. At present, no effective therapy is available to halt the progression of AD. Therefore, understanding the mechanism of the disease process of AD and development of effective therapeutic approach to halt the disease progression are of paramount importance.

Here, we delineate that activation of NF- κ B, a proinflammatory transcription factor, is induced in the CNS of AD patients. Although NF- κ B functions in synaptic signaling [42], activated NF- κ B negatively correlates with cognitive function in AD monitored by clinical and neuropsychological testing, which was performed within 2 years of death (Table 2 & Fig. 1). Therefore, NF- κ B activation could be a target to protect memory and learning in AD, and we tested the validity of this target in 5XFAD Tg mouse model using cell-permeable NBD peptide, which is capable of inhibiting the induction of NF- κ B activation without altering the basal activity of NF- κ B. NF- κ B was induced *in vivo* in the CNS of 5XFAD Tg mice and NBD peptide protected hippocampal neurons and cognitive function from AD toxicity. Our conclusions are based on the following. First, the activation of NF- κ B is induced in hippocampus and cortex of 5XFAD Tg mice. NBD peptide entered into the hippocampus after intranasal administration. WtNBD, but not mNBD, peptide inhibited hippocampal activation of NF- κ B in 5XFAD Tg mice. Second, microglia were activated and the expression of various proinflammatory molecules was induced in the hippocampus of 5XFAD Tg mice. However, treatment of mice with wtNBD, but not mNBD, peptide resulted in attenuation of microglial activation and expression of proinflammatory molecules. Third, inflammation contributes to plaque formation and vice versa. Interestingly, wtNBD

treatment reduced hippocampal plaque load in 5XFAD Tg mice. Fourth, as observed in AD, hippocampal neurons underwent apoptosis in 5XFAD mice. But treatment with wtNBD, but not mNBD, peptide protected hippocampal neurons from AD toxicity in 5XFAD Tg mice. Fifth, decrease in NMDA and AMPA receptor proteins and loss of calcium excitability in hippocampal neurons are often observed in AD brain. However, treatment with wtNBD, but not mNBD, peptide protected these plasticity-related molecules and restored calcium current *in vivo* in the hippocampus. Sixth, wtNBD, but not mNBD, peptide also protected spatial learning and memory in 5XFAD Tg mice. We did not notice any drug related side effect (e.g., hair loss, weight loss, untoward infection, etc.) in any of the mice used during the course of the study. Since activated NF- κ B in the CNS of AD is directly correlated with cognitive impairment, NBD peptide may reduce plaque formation, slow down the loss of hippocampal neurons and protect memory and learning in AD.

Various trophic factors including BDNF, NT-3, and TGF- β have been shown to protect memory and learning in animal models of AD [43–45]. However, clinical application of those molecules has been limited because of difficulties in delivery and side-effects. These peptides do not readily diffuse across the blood-brain barrier and have limited or unstable bioavailability and some toxicity. Peptides usually do not enter into the CNS and therefore, sending peptides across the blood-brain barrier is an important area of research. Earlier we [27] have demonstrated that antennapedia homeodomain present in NBD peptide helps this peptide to enter into the CNS. Therefore, after intraperitoneal injection, NBD peptide entered into the CNS, reduced nigral activation of NF- κ B, suppressed nigral expression of proinflammatory molecules, and attenuated nigrostriatal degeneration in MPTP-intoxicated mice. Similarly, after intramuscular injection, NBD peptide entered into the CNS and protected the nigrostriatum in hemiparkinsonian monkeys [38]. Peptides are generally costly. Therefore, to reduce the cost by one-tenth and to help in clinical translation, here, we tried intranasal administration. Interestingly, after intranasal administration, NBD peptide also entered into the hippocampus and protected memory in 5XFAD Tg mice.

In summary, we have demonstrated that activated NF- κ B negatively correlates with cognitive function in AD and that after intranasal administration, NBD peptide enters into the hippocampus, blocks the activation of NF- κ B in the hippocampus, inhibits the expression of proinflammatory molecules and the activation microglia in the hippocampus, reduces A β load, attenuates the apoptosis of hippocampal neurons, and improves spatial learning and memory in 5XFAD Tg mice. Although experiments with the 5XFAD mouse model certainly have limitations with respect to direct comparisons with the situation of neurons in the brain of AD patients, our results provide evidence that intranasal administration of these peptides may be used for therapeutic intervention in AD and other neurodegenerative disorders where inflammation within the CNS plays an important role in disease pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Monitoring activation of NF-κB *in vivo* in the CNS of cases clinically diagnosed as no cognitive impairment (NCI), mild cognitive impairment (MCI), and Alzheimer's disease (AD). A) Pre-frontal cortex homogenates (25 μg) from NCI (light blue), MCI (dark blue), and AD (grey) were subjected to western blotting with antibodies against p65, p105, p50, p65 phosphorylated at serine 536 ($p\text{-p65}^{\text{S536}}$), and p65 acetylated at lysine 310 ($\text{ac-p65}^{\text{K310}}$). β-actin was used to normalize the immunoreactive signal obtained by densitometric measurement (ImageJ) in the blots and Coomassie staining was used to verify equal protein loading. Twelve NCI, eleven MCI, and ten AD cases were included in the analysis. Expression of p65 (B), p105 (C), and p50 (D) in three clinical groups. Phosphorylation of $p65^{\text{S536}}$ (E) was significantly higher in AD cases compared with NCI subjects ($p = 0.043$) whereas acetylation of $p65^{\text{K310}}$ (F) was significantly elevated in AD relative to both NCI ($p < 0.001$) and MCI ($p < 0.001$) subjects. Phosphorylation of $p65^{\text{S536}}$ negatively correlated with Mini-Mental State Examination (MMSE) test scores (G; -0.370 , $p = 0.034$) but not with global cognitive z score (GCS) index (H; -0.267 , $p = 0.133$), whereas acetylation of $p65^{\text{K310}}$ negatively correlated with MMSE test scores (I; -0.574 , $p < 0.001$) and GCS (J; -0.533 , $p = 0.001$). NCI, light blue diamonds; MCI, dark blue diamonds; AD, grey diamonds. kDa, kilodalton; OD, optical density. Cortical and hippocampal sections of NCI (K) and AD (L) brains were double-labeled with Iba-1 (microglia) and acetyl-p65. Results represent analysis of two sections from each of four different brains. Acetyl-p65-positive cells were counted in two sections (two images per slide) of each of four different brains per

group in an Olympus IX81 fluorescence microscope using the MicroSuite imaging software (M, cortex; N, CA1; O, CA3). $*p < 0.001$ versus NCI.

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Fig. 2.

Intranasal delivery of wild type (wt) NBD peptides suppresses the expression of proinflammatory molecules *in vivo* in the hippocampus of Tg 5XFAD mice. Tg mice (5 month old) were treated with wtNBD and mutated (m) NBD peptides (0.1 mg/kg body wt/2d) via intranasal route. After 30 d of treatment, mice were sacrificed followed by monitoring the mRNA expression of iNOS and IL-1 β by RT-PCR (A) and real-time PCR (B). Results are mean $\pm$ SEM of four mice per group. ^a p < 0.001 versus non-Tg; ^b p < 0.001 versus Tg. Protein level of iNOS was monitored in the hippocampus by western blot (C). Bands were scanned using the NIH Image J software and results are represented as relative to the non-transgenic (non-Tg) group (D). Results are mean $\pm$ SEM of four mice per group. ^a p < 0.001 versus non-Tg; ^b p < 0.001 versus Tg. In a parallel experiment, after treatment, hippocampal and cortical sections were analyzed by double-label immunofluorescence for iNOS and Iba-1 (E). Results represent analysis of four sections of each of five mice per group.

Fig. 3.

Intranasal delivery of wild type (wt) NBD peptides reduces the burden of amyloid beta from the hippocampus of Tg 5XFAD mice. Tg mice (5 months old) were treated wtNBD and mNBD peptides. After 30 d of treatment, hippocampal sections were immunolabeled with 82E1 mAb (A). Aβ-positive plaques were counted in areas outlining the hippocampus and the cortex by using the touch counting module of the Olympus Microsuite 5TM imaging software (B). Six sections of each of six mice per group were used for counting. Therefore, results represent mean ± SEM of six mice per group. ^a*p* < 0.001 versus non-Tg; ^b*p* < 0.001 versus Tg. Hippocampal homogenates were also analyzed for protein levels of Aβ by western blot using 6E10 mAb (C). Arrowhead indicates 4 kDa Aβ band. Bands were scanned and results presented as relative to control (non-Tg) (D). Results represent mean ± SEM of four mice per group. ^a*p* < 0.001 versus non-Tg; ^b*p* < 0.001 versus Tg. ELISA quantification of Aβ₁₋₄₂ was performed in hippocampal and cortical homogenates (E). Results represent mean ± SEM of three mice per group. ^a*p* < 0.001 versus non-Tg; ^b*p* < 0.001 versus Tg.

Fig. 4.

Intranasal delivery of wild type (wt) NBD peptides inhibits neuronal degeneration and apoptosis *in vivo* in the hippocampus of Tg 5XFAD mice. Tg mice (5 month old) were treated with wtNBD and mNBD peptides. After 30 d of treatment, mice were sacrificed and perfused followed by double-label immunofluorescence analysis of hippocampal sections for fluoro-J B (degenerating neurons) & NeuN (A) and TUNEL & NeuN (B). Results represent analysis of four sections of each of five mice per group. In parallel experiments, apoptosis was also monitored by western blot analysis of cleaved caspase 3 (C&D) and phospho-Bad (E&F). Bands were scanned using the NIH Image J software and results are represented as relative to the non-transgenic (non-Tg) group (D). Results are mean $\pm$ SEM of four mice per group. ^a $p < 0.001$ versus non-Tg; ^b $p < 0.001$ versus Tg.

Fig. 5.

Intranasal delivery of wild type (wt) NBD peptides improves the protein level of plasticity-related molecules and restores NMDA- and AMPA-dependent calcium influx *in vivo* in the hippocampus of Tg 5XFAD mice. Tg mice (5 month old) were treated with wtNBD and mNBD peptides. After 30 d of treatment, protein level of plasticity-related molecules (CREB, NR2A, GluR1, and PSD-95) by western blot (A). Bands were scanned using the NIH Image J software and results are represented as relative to the non-transgenic (non-Tg) group (B). Results are mean $\pm$ SEM of four mice per group. ^a $p < 0.001$ versus non-Tg; ^b $p < 0.001$ versus Tg. In parallel experiments, hippocampal slices were recorded for NMDA-dependent (C) and AMPA-dependent (D) calcium influx. Results were confirmed after recording from hippocampal slices of four different animals per group.

Fig. 6.

Intranasal delivery of NBD peptide improves memory and learning in Tg 5XFAD mice. Tg mice (5 month old) were treated with wtNBD and mutated (m) NBD peptides. After 30d of treatment, different groups of mice were tested for Barnes maze (A, Track plot; B, Time taken; C, Number of errors made) and T maze (D, Number of positive turns; E, Errors). Short-term memory was also monitored by novel object recognition test, which is represented by discrimination index (F). Eight mice ($n = 8$) were used in each group.

Table 1

Clinical, demographic, and neuropathologic characteristics by clinical diagnosis category

	NCI (n = 12)	MCI (n = 11) ^a	AD (n = 10)	Total (n = 33)	p	Pairwise
Age (y) at Death, Mean ±SD (Range)	82.18±5.13 (67.40–86.90)	84.87±6.23 (75.00–97.50)	88.73±5.89 (80.10–95.90)	85.06±6.19 (67.40–97.50)	0.106 ^b	–
Number (%) of Males	4 (33.33%)	7 (63.63%)	5 (50.00%)	16 (48.48%)	0.357 ^b	–
Number (%) with ApoE ε4 allele	4 (33.33%)	2 (18.18%)	5 (50.00%)	11 (33.33%)	0.295 ^c	–
MMSE, Mean±SD (Range)	27.25±2.77 (20–30)	25.91±1.92 (22–29)	13.30±5.27 (6–21)	22.58±7.11 (6–30)	<0.001 ^b	NCI, MCI >AD
Global Cognitive z Score, Mean±SD (Range)	0.44±0.32 (–0.12–1.15)	0.09±.27 (–0.23–0.64)	–1.13±0.39 (–1.63 – –0.43)	–0.15±0.74 (–0.163–1.15)	<0.001 ^b	NCI, MCI >AD
PMI (h), Mean±SD (Range)	7.45±6.36 (2.20–24.00)	5.15±3.12 (2.50–13.90)	6.57±3.33 (3.50–12.40)	6.42±4.58 (2.20–24.00)	0.457 ^b	–
Brain Weight (g), Mean±SD (Range)	1247.0±146.2 (1000–1510)	1303.0±184.5 (990–1500)	1158.6±155.3 (980–1400)	1238.9±168.0 (980–1510)	0.155 ^b	–
Distribution of Braak Scores					0.094 ^b	–
No AD	0	0	0	0		
I/II	2	2	1	5		
III/IV	10	7	5	22		
V/VI	0	2	4	6		
NIA Reagan Criteria Diagnosis					0.074 ^b	–
No AD	0	0	0	0		
Low	5	3	1	9		
Intermediate	7	7	6	20		
High	0	1	3	4		
CERAD Diagnosis					0.087 ^b	–
No AD	5	3	0	8		
Possible	0	0	0	0		
Probable	5	6	6	17		
Definite	2	2	4	8		

AD, Alzheimer's disease; ApoE, apolipoprotein E; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; MCI, mildly cognitively impaired; MMSE, Mini-Mental State Examination; NCI, non-cognitively impaired; NIA, National Institute on Aging.

^a n = 7 MCI cases were amnestic.

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^b Kruskal-Wallis test corrected for multiple comparisons.

^c Fisher's Exact test.

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Table 2

Summary of superior frontal cortex protein levels by clinical diagnosis and correlations with cognitive test scores

	NCI (<i>n</i> = 12)	MCI (<i>n</i> = 11)	AD (<i>n</i> = 10)	<i>p</i> ^{<i>a</i>}	Pairwise	<i>r_s</i> ^{<i>b</i>}	
						MMSE	GCS
p65	39.25±17.47 (15.36–73.48)	40.81±22.98 (15.36–73.48)	45.49±20.94 (21.31–83.87)	0.088	–	0.014, <i>p</i> = 0.940	–0.045, <i>p</i> = 0.802
p105	25.05±9.06 (12.85–43.19)	27.08±13.47 (13.18–49.74)	39.55±33.10 (13.74–109.29)	0.849	–	0.060, <i>p</i> = 0.741	0.004, <i>p</i> = 0.983
p50	93.19±15.11 (69.58–115.21)	92.80±24.11 (38.30–129.10)	119.95±34.88 (76.96–199.32)	0.713	–	–0.368, <i>p</i> = 0.035	–0.366, <i>p</i> = 0.741
phospho p65 ^{S563}	20.46±9.91 (9.57–42.16)	38.54±36.48 (8.16–110.97)	36.80±16.49 (20.00–69.85)	0.043	NCI<AD	–0.370, <i>p</i> = 0.034	–0.267, <i>p</i> = 0.133
acetyl p65 ^{K310}	74.21±78.24 (38.06–106.08)	78.25±40.32 (27.13–147.45)	159.45±53.94 (105.01–281.70)	<0.001	NCI, MCI <AD	–0.574, <i>p</i> < 0.001	–0.533, <i>p</i> = 0.001
MyD88	9.35±11.02 (0.82–41.96)	14.69±14.65 (0.75–46.01)	46.04±26.97 (7.28–94.94)	0.001	NCI, MCI <AD	–0.538, <i>p</i> = 0.001	–0.475, <i>p</i> = 0.005
TLR-2	38.25±22.65 (4.92–84.60)	29.98±37.94 (2.50–106.75)	68.63±40.04 (23.41–123.16)	0.027	MCI <AD	–0.278, <i>p</i> = 0.117	–0.177, <i>p</i> = 0.326
TLR-4	14.67±12.31 (4.93–41.96)	14.62±16.32 (1.09–47.97)	10.90±5.50 (3.72–21.81)	0.895	–	–0.173, <i>p</i> = 0.336	0.047, <i>p</i> = 0.794

Values represent mean±SD (range). AD, Alzheimer's disease; MCI, mild cognitive impairment; NCI, no cognitive impairment; MMSE, Mini-Mental State Examination; GCS, Global Cognitive z Score.

^{*a*}Kruskal-Wallis test corrected for multiple comparisons.

^{*b*}Spearman's Rank-Order correlation (2-tailed), unadjusted.