Expression of the plant viral protease NIa in the brain of a mouse model of Alzheimer's disease mitigates Aβ pathology and improves cognitive function

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http://dx.doi.org/10.3858/emm.2012.44.12.082

Abstract

The plant viral protease, NIa, has a strict substrate specificity for the consensus sequence of Val-Xaa-His-Gln, with a scissoring property after Gln. We recently reported that NIa efficiently cleaved the amyloid-β (Aβ) peptide, which contains the sequence Val-His-His-Gln in the vicinity of the cleavage site by α-secretase, and that the expression of NIa using a lentiviral system in the brain of AD mouse model reduced plaque deposition levels. In the present study, we investigated whether exogenous expression of NIa in the brain of AD mouse model is beneficial to the improvement of cognitive deficits. To address this question, Lenti-NIa was intracerebrally injected into the brain of Tg-APPswe/PS1dE9 (Tg-APP/PS1) mice at 7 months of age and behavioral tests were performed 15-30 days afterwards. The results of the water maze test indicated that Tg-APP/PS1 mice which had been injected with Lenti-GFP showed an increased latency in finding the hidden-platform and markedly enhanced navigation near the maze-wall, and that such behavioral deficits were significantly reversed in Tg-APP/PS1 mice injected with Lenti-NIa. In the passive avoidance test, Tg-APP/PS1 mice exhibited a severe deficit in their contextual memory retention, which was reversed by NIa expression. In the marble burying test, Tg-APP/PS1 mice buried marbles fewer than non-transgenic mice, which was also significantly improved by NIa. After behavioral tests, it was verified that the Tg-APP/PS1 mice with Lenti-NIa injection had reduced Aβ levels and plaque deposition when compared to Tg-APP/PS1 mice. These results showed that the plant viral protease, NIa, not only reduces Aβ pathology, but also improves behavioral deficits.

Keywords: Alzheimer disease; amyloid β-peptides; disease models, animal; endopeptidases; maze learning

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease distinguished by extracellular deposition of amyloid-β (Aβ) outside neurons and progressive cognitive dysfunction. Because Aβ is highly toxic to neural cells, accumulation of Aβ has been thought to cause the pathogenesis and cognitive impairment of AD (McLean et al., 1999; Larson and Lesne, 2001; Walsh et al., 2002). Aβ levels in the brain are regulated by the ratio between Aβ production and Aβ clearance (Tanzi and Bertram, 2005). Several endogenous proteases are known to play a role in clearance of Aβ in AD brains. Such endogenous proteases include neprilysin (NEP), insulin degrading enzyme (IDE), endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE), plasmin/tPA/uPA, cathepsin B, and MMP-2/MMP-9 (Wang et al., 2006; Haass and Selkoe, 2007; Miners et al., 2008), among which NEP and IDE have been most extensivelystudied. The pharmacological or genetic
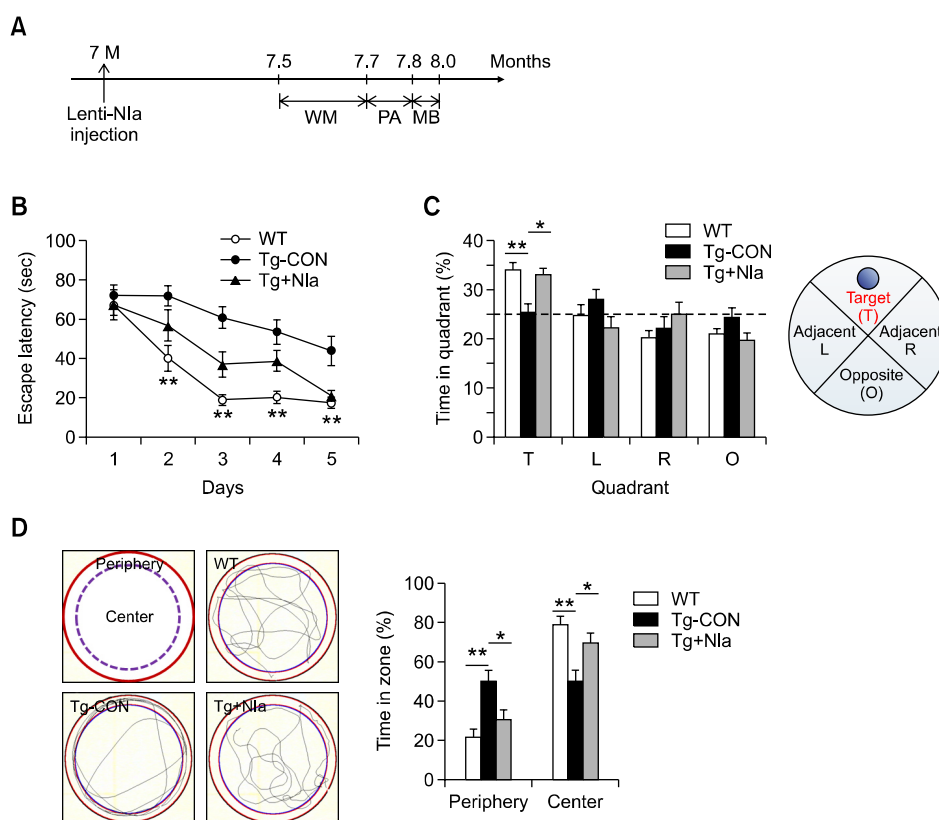
Figure 1. Spatial memory deficits displayed by Tg-APP/PS1 mice were improved by NIa expression. (A) Experimental design of Lenti-NIa injection and behavioral performance. Lenti-NIa was injected in Tg-APP/PS1 mice at 7 months of age and behavioral tests were started 15 days after the injection, for the two-week period, the sequence of which was the Morris water maze test (WM), passive avoidance test (PA), and marble burying test (MB). (B) Escape latency on a hidden platform of Tg-APP/PS1 mouse control (Tg-CON), Tg-APP/PS1 mice infused with Lenti-NIa (Tg+NIa), and their non-transgenic control (WT) in the Morris water maze test. Tg-APP/PS1 mice infused with Lenti-GFP are used as Tg-APP/PS1 mouse control (Tg-CON) throughout this work. Numbers of animals used: WT, 5 males and 5 females; Tg-CON, 5 males and 4 females; Tg+NIa, 5 males and 4 females. Two-way repeated measures ANOVA and *Bonferroni* post-hoc test: significant difference between animal groups [$F_{(2,204)}$ = 19.77, $P < 0.0001$], significant effect of time [$F_{(4,204)}$ = 32.94, $P < 0.0001$] and significant animal group × time interaction [$F_{(8,204)}$ = 2.301, $P < 0.05$]. Data are presented as the means ± SEM. **denotes a difference between WT and Tg-CON at $P < 0.01$ at the indicated time point. Separate two-way repeated measures ANOVA for the data groups between Tg-CON and Tg+NIa showed significant effects of treatment (NIa) [$F_{(1,128)}$ = 10.95, $P < 0.01$] and time [$F_{(4,128)}$ = 14.10, $P < 0.0001$], but no significant treatment × time interaction [$F_{(4,128)}$ = 0.8769, $P = 0.4798$]. Data are presented as the means ± SEM. (C) The percentage of time spent in the target quadrant in the spatial probe trial test. The dashed line represents the chance performance level (25%) at each quadrant. Tg-CON; Tg-APP/PS1 mouse control. Tg+NIa; Tg-APP/PS1 mice infused with Lenti-NIa. WT; non-transgenic control. Target (T), opposite (O), and adjacent quadrants (L, R) are depicted on the right panel. Two-way ANOVA and *Bonferroni* post-hoc test: no difference between animal groups [$F_{(2,96)}$ = 0.0000, $P = 1$], but significant difference between zones [$F_{(3,96)}$ = 14.92, $P < 0.0001$] and significant animal group × zone interaction [$F_{(6,96)}$ = 3.997, $P < 0.01$]. * and ** denote differences between indicated groups in the T zone, at $P < 0.05$ and $P < 0.01$, respectively. Data are presented as the means ± SEM. (D) The percentage of time spent in the center vs. periphery zones in the hidden platform version of the Morris water maze examined on day 5 (right panel). The center and periphery zones are depicted (left panels). The periphery zone is defined as the area between wall and the circle apart by 10-cm from the wall. Representative spatial navigations on the water maze pool for 30-sec period are presented for WT, Tg-CON, and Tg+NIa mice. One-way ANOVA and *Newman-Keuls* post-hoc test; significant difference between animal groups in the periphery zone [$F_{(2,24)}$ = 8.693, $P < 0.01$] and in the center zone [$F_{(2,24)}$ = 8.715, $P < 0.01$]. * and ** denote differences between indicated groups at $P < 0.05$ and $P < 0.01$, respectively. Data are presented as the means ± SEM.

inhibition of NEP in mouse models of AD increased Aβ accumulation and memory impairment (Mouri *et al.*, 2006; Farris *et al.*, 2007), while the overexpression of NEP was shown to reduce Aβ levels, plaque deposition, and plaque-related pathology (Marr *et al.*, 2003). IDE immunoreactivity was shown to be significantly reduced in the brain of those diagnosed with AD (Miners *et al.*, 2008), while mice with a deficiency of the IDE gene exhibited an accumulation of Aβ in the brain (Farris *et al.*, 2003). Therefore, the enzymatic degradation of Aβ has been considered as an attractive strategy for the mitigation of Aβ accumulation in the AD brains (Haass and Selkoe, 2007; Miners *et al.*, 2008).

However, NEP overexpression did not reduce oligomeric Aβ levels and Aβ oligomer-induced

cognitive deficits (Meilandt *et al*., 2009), suggesting that NEP has an ability to degrade monomeric, but not oligomeric, Aβ. Aβ oligomers are more toxic than monomeric or fibrillar Aβ (Dahlgren *et al*., 2002; Ono *et al*., 2009; Benilova *et al*., 2012). IDE degraded the monomeric Aβ peptide, but did not cleave oligomeric Aβ (Qiu *et al*., 1998; Chesneau *et al*., 2000). Because NEP and IDE are endogenous enzymes that have broad substrate specificity and thus, potentially degrade many cellular proteins, these proteases are likely to present many disadvantages through their overexpression in the brain. Therefore, a new strategy that permits the cleavage of both monomeric and oligomeric Aβ, but has a substrate specificity for Aβ, is necessarily sought.

The nuclear inclusion a protease of the Turnip mosaic virus, NIa, has a substrate specificity for the sequence of Val-Xaa-His-Gln with a cleaving property after Gln (Kang *et al*., 2001). We recently reported that Aβ peptides contain the sequence Val-His-His-Gln in the vicinity of the cleavage site by α-secretase, and that NIa degraded Aβ *in vitro*. Moreover, overexpression of NIa using a Lentiviral system reduced plaque levels in the brain of AD mouse model (Han *et al*., 2010). In the present study, we investigated whether NIa expression in the brain of AD mouse model is beneficial towards the improvement of cognitive deficits at the behavioral level in a mouse model of AD.

Results

To investigate whether the exogenous expression of NIa improved cognitive function, Lenti-NIa virus was stereotaxically injected into the lateral ventricles at both hemispheres in the brain of Tg-APP/PS1 mice which were 7 months old, and at this stage where plaque deposition in the brain has been determined to show at low levels in various brain regions (Kim *et al*., 2012). Tg-APP/PS1 mice infused with Lenti-GFP in the same manner were used as a control. Starting 15 days after the injection, a series of behavioral tests were performed for the following 15 days (Figure 1A).

Cognitive impairments displayed by Tg-APP/PS1 mice were improved by NIa expression

In the Morris water maze test, the performance of Tg-APP/PS1 mice (carrying Lenti-GFP) in finding the hidden platform was much reduced when compared to that of the non-transgenic control, indicating that the special recognition learning/memory in Tg-APP/PS1 mice was severely impaired. On the contrary, the behavioral performance of Tg-APP/PS1 mice
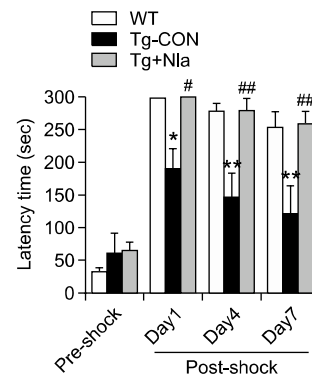

Figure 2. The contextual memory deficit of Tg-APP/PS1 mice was reversed by NIa expression. The entry latency into the dark chamber for Tg-APP/PS1 mouse control (Tg-CON), Tg-APP/PS1 mice infused with Lenti-NIa (Tg+NIa), and their non-transgenic control (WT) in the passive avoidance test. The latency to enter into the dark chamber after shock (post-shock) is used as an indicator of learning and memory. Two-way repeated measures ANOVA and *Bonferroni* post-hoc test for data groups at post-shock days: significant difference between animal groups [$F_{(2,46)}$ = 11.34, $P < 0.001$], time [$F_{(2,46)}$ = 10.08, $P < 0.001$], but no animal group × time interaction [$F_{(4,46)}$ = 0.3358, $P = 0.8524$]; * and ** denote differences between WT and Tg-CON at $P < 0.05$ and $P < 0.01$, respectively; # and ## denote difference between Tg-CON and Tg+NIa at $P < 0.05$ and $P < 0.01$, respectively. Data are presented as the means ± SEM. Numbers of animals used: WT, 5 males and 5 females; Tg-CON, 4 males and 4 females; Tg+NIa, 4 males and 4 females.

injected with Lenti-NIa was significantly improved when compared with that of control mice (Figure 1B). In a probe trial test, Tg-APP/PS1 mice showed reduced dwelling time in the target quadrant compared with the non-transgenic control. Whereas Tg-APP/PS1 mice with Lenti-NIa spent more time in the target quadrant compared with that of Tg-APP/PS1 mice (Figure 1C). These results suggest that the expression of NIa through the Lentivial system in the brain was beneficial for improving cognitive function of Tg-APP/PS1 mice. It was unexpectedly noticed that Tg-APP/PS1 mice spent more time in the periphery of the water maze pool than the non-transgenic control during the 90 sec of the test period. Whereas NIa expression reversed the stereotyped behavior in the periphery to the control level (Figure 1D).

In the passive avoidance test, the post-shock latency for Tg-APP/PS1 mice to enter into the dark chamber was shorter than that displayed by the non-transgenic control (Figure 2). Moreover, this defective memory retention of Tg-APP/PS1 mice was decayed faster than the non-transgenic control in 7 days of repeated tests (Figure 2A). Whereas expression of NIa improved the impaired memory retention to the level of the non-transgenic control (Figure 2). These results suggest that the expression of NIa in the brain of Tg-APP/PS1 mice significantly improved context-dependent memory as well.

In the marble burying test, Tg-APP/PS1 mice
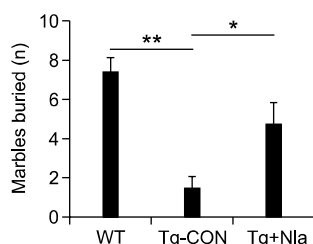
Figure 3. Behavioral changes in the marble burying test were restored by NIa expression. The numbers of glass marbles buried under wood chips by Tg-APP/PS1 mouse control (Tg-CON), Tg-APP/PS1 mice infused with Lenti-NIa (Tg+NIa), and their non-transgenic control (WT) for the 30-min period are presented. NIa expression significantly reverted marble burying behavior. One-way ANOVA and *Newman-Keuls* post-hoc test: [$F_{(2,24)}$ = 13.81, P = 0.0001]. * and ** denote difference at $P < 0.05$ and $P < 0.01$, respectively. Data are presented as the means $\pm$ SEM. Numbers of animals used: WT, 5 males and 5 females; Tg-CON,4 males and 4 females; Tg+NIa, 4 males and 4 females.

buried marbles fewer than the non-transgenic control, while expression of NIa significantly reversed the behavioral change displayed by Tg-APP/PS1 mice (Figure 3).

Suppression of Aβ pathology in the brain with NIa expression

To verify that intracerebral injection of Lenti-NIa reduced Aβ pathology, the plaque pathology in the prefrontal cortex was examined. RT-PCR and Western blot analyses were used to confirm the expression of NIa in the brain of Tg-APP/PS1 mice infused with Lenti-NIa (Figures 4A and 4B). ELISA assay showed that the levels of Aβ (40) in the Tris-buffer soluble faction as well as the formic acid-extractable fraction of male were significantly reduced. The levels of Aβ (40) in the Tris-buffer soluble faction, but not in the formic acid-extractable faction, of female also was significantly reduced (Figures 4C and 4E). The levels of Aβ (42) in both the Tris-buffer soluble, and formic acid-extractable factions of female were significantly reduced, although the reduction of Aβ (42) in male was not statistically significant (Figures 4D and 4F).

Immunohistochemical analysis revealed that plaque deposition levels in the prefrontal cortex, parietal cortex, and piriform cortex tended to decrease in the brain of Tg-APP/PS1 mice infused with Lenti-NIa when compared to the levels in Tg-APP/PS1 mouse control (Figures 4G and 4H). Quantification of plaque deposition levels, using a computer-aid image analysis system, indicated that the number of plaques in the prefrontal cortex was significantly reduced although very subtle reductions were observed for the parietal cortex and piriform cortex (Figure 4I). Quantification of plaque deposition levels

using the photographic plaque deposition reference panel (Kim *et al.*, 2012) also yielded similar results (Figure 4J).

Discussion

Beneficial effects of NIa expression on the cognitive function of Tg-APP/PS1 mice

We recently demonstrated that the plant viral protease NIa effectively degraded Aβ *in vitro*, and that the expression of NIa in the brain of an AD model mice using a Lentiviral system reduced Aβ levels and plaque deposition (Han *et al.*, 2010). Because NIa is a potent exogenous protease, it was questioned whether or not overexpression of NIa produces any detrimental effects on the brain, other than the reduction in the levels of Aβ. After the infusion of Lenti-NIa in the brain, the spatial memory task in the Morris water maze test (Figures 1B and 1C) and the contextual memory task in the passive avoidance test (Figure 2) were significantly improved, suggesting that overexpression of NIa has beneficial effects in alleviating cognitive dysfunction of the AD mouse model. As these beneficial or protective effects were produced by the expression of the Aβ-degrading protease NIa, the results of the present study support also that the cognitive deficits displayed by untreated Tg-APP/PS1 mice were due to the accumulation of Aβ in the brain. We believe that further studies on the effects of a more prolonged expression of NIa over a period of several months to a year, for example, are required in order to more fully elucidate the effects of Aβ regulation through NIa.

Anxiety is yet another of many problematic symptoms endured by patients with AD (Serra *et al.*, 2010; Spalletta *et al.*, 2010). Our results indicated that Tg-APP/PS1 mice had increased anxiety and this emotional change was also reversed by the expression of NIa (Figures 1D and 3), reducing the concern that overexpression of the foreign protease NIa in the brain could have detrimental effects on the emotional behavior.

Repression of Aβ pathology by NIa in the brain of Tg-APP/PS1 mice

It was reported that Tg-APP/PS1 mice showed cognitive deficits at the age of 15 months, but not at 8 months in the Morris water maze test (Hooijmans *et al.*, 2009). A further study reported cognitive deficits in Tg-APP/PS1 mice at the age of 10 months in the Morris water maze test (Timmer *et al.*, 2010). The present study demonstrated that Tg-APP/PS1 mice before reaching to 8 months of age showed
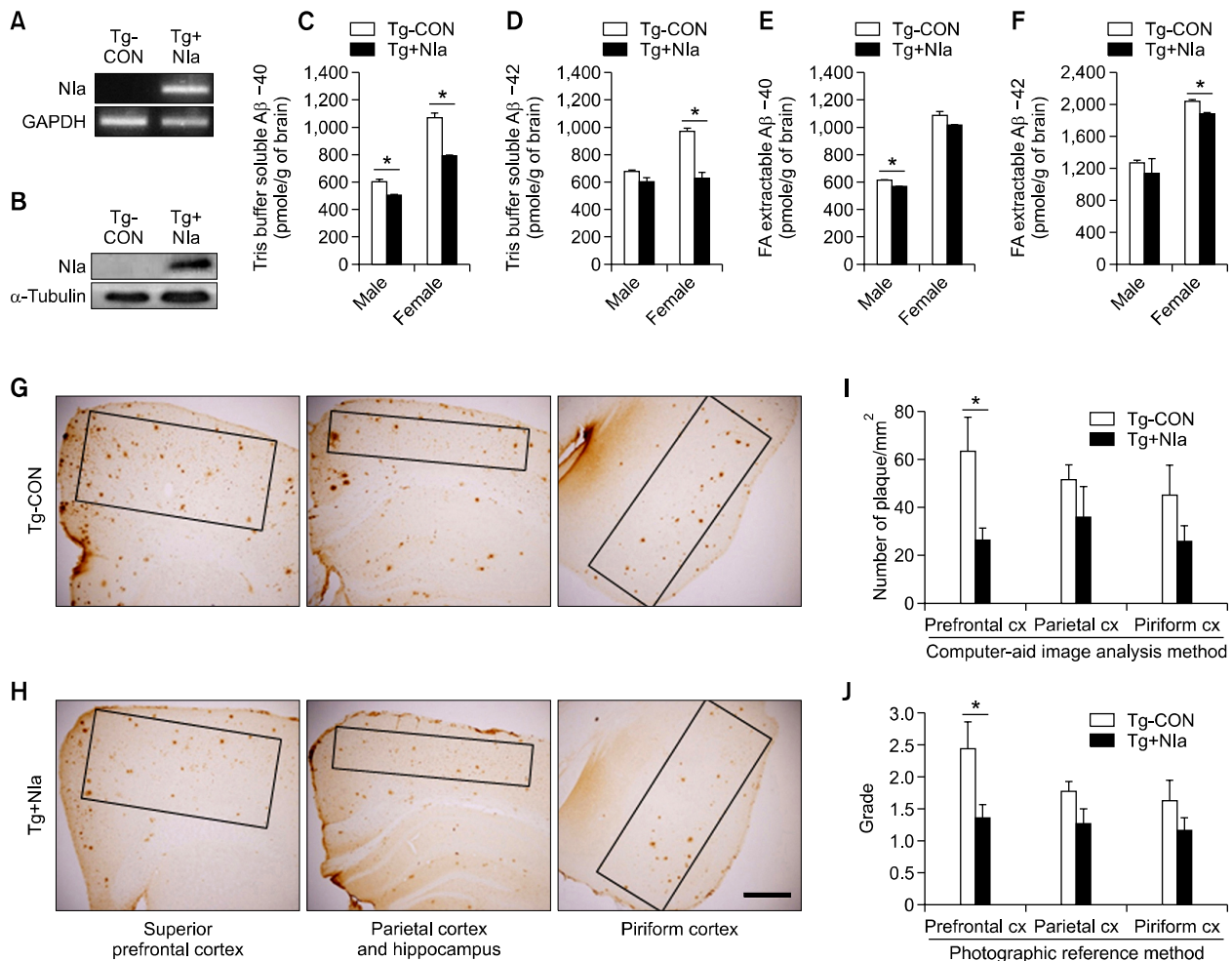
Figure 4. Suppression of Aβ pathology by NIa expression in the brain of Tg-APP/PS1 mice. (A) RT-PCR showing the expression of NIa transcript in the brain of Tg-APP/PS1 mice infused with Lenti-NIa (Tg+NIa).The expression of NIa was analyzed on the parietal cortex of Tg-APP/PS1 mice and their control mice (Tg-CON). GAPDH is a control. (B) Western blots showing the expression of NIa protein in the brain of Tg-APP/PS1 mice infused with Lenti-NIa (Tg+NIa). The expression of NIa (molecular weight: 28 kD) in the prefrontal cortex was examined using anti-NIa. Tg-APP/PS1 mouse control (Tg-CON). α-tubulin was used as a loading control. (C-F) The amounts of Aβ (40) (C, E) and Aβ (42) (D, F) in Tris-buffer soluble (C, D) and formic acid-extractable (E, F) fractions in the brains of Tg-APP/PS1 mouse control (Tg-CON) and Tg-APP/PS1 mice infused with Lenti-NIa (Tg+NIa). (G, H) Photomicrographs showing anti-Aβ (Bam-10)-stained superior prefrontal cortex, parietal cortex and hippocampus, and piriform cortex of Tg-APP/PS1 mouse control (Tg-CON; G) and Tg-APP/PS1 mice infused with Lenti-NIa (Tg+NIa; H). (I, J) Quantification of the levels of plaques (mm^2) in the prefrontal cortex, parietal cortex, and piriform cortex of Tg-APP/PS1 mouse control (Tg-CON) and Tg-APP/PS1 mice infused with Lenti-NIa. Plaque levels presented were counted using computer-aid image analysis program (I) and the 6-grade plaque photographic reference panels (J). All data were from female mice. Scale bar: 500 μm. Data are presented as the means ± SEM. *denotes difference between the indicated groups at $P < 0.05$ (Student t-test).

significant impairments in cognitive function in the Morris water maze test and the passive avoidance test (Figures 1 and 2). This discrepancy between previous result (Hooijmans *et al.*, 2009) and the present study might be attributed to differing conditions in animal rooms or different handing skills in behavioral assays (Wahlsten *et al.*, 2006). Given that Tg-APP/PS1 mice show visible plaque deposition from 6-6.5 months of age (Seo *et al.*, 2011; Kim *et al.*, 2012; unpublished observation), it is not surprising that Tg-APP/PS1 mice exhibited behavioral deficits prior to 8 months of age.

Considering that the cognitive deficits displayed by untreated Tg-APP/PS1 mice were attributed to the accumulation of Aβ in the brain, as discussed above, it would be interesting to specifically examine whether behavioral deficits in Tg-APP/PS1 mice could occur as early as 6.0-7.5 months of age, at the time when plaque deposition has begun to occur, presumably due to enhanced accumulation of Aβ.

In the Morris water maze test, Tg-APP/PS1 mice spent approximately 50% of the time in navigation near the wall of the water maze, and this behavior was in sharp contrast to that of the non-transgenic

mice. This stereotyped behavior is apparently caused by Aβ pathology, as evidenced that the phenotype also was reversed by NIa expression (Figure 1D). We speculate that this stereotyped behavior is likely to reflect the increased anxiety of Tg-APP/PS1 mice, although we do not exclude the possibility of problems associated with their working memory. If the neurobiological feature of such behavior is defined more clearly, this stereotyped behavior might be used as an important behavioral indicator in analysis of transgenic mouse models of AD.

The intracerebral injection of Lenti-NIa in Tg-APP/PS1 mice suppressed the Aβ levels and plaque deposition, the reduction of which was significant in the prefrontal cortex, but not so in the parietal and piriform cortices (Figure 4). However, a similar infusion of Lenti-NIa in Tg-APP/PS1 mice suppressed Aβ pathology within the same brain regions to a somewhat higher degree (Han *et al.*, 2010). Although the present study did not provide further evidence, we speculate that the moderate and differential suppression of plaque pathology by NIa in different brain regions is, in part, due to the uneven penetration of Lenti-NIa in different brain regions. It is also possibly attributed to the fact that the final viral tire used in the present study was 0.8 × 10^8 TU, which is slightly lower than the previous tire of 1 × 10^8 TU (Han *et al.*, 2010). We speculate that it is also related to the partial rescue of behavioral performance in the Morris water maze test, a behavioral test for a hippocampus-dependent spatial learning. Nonetheless, significant or complete rescue of behavioral performance in the Morris water maze test, passive avoidance test, and marble burying test indicated that a partial repression of Aβ levels was sufficient to provide a beneficial effect on behavior. In conjunction with this, it would be worth investigating whether local expression of NIa in specific brain regions produces specific or limited rescue of impaired brain function. Lenti-NIa expression in female Tg-APP/PS1 mice seemed to suppress Aβ contents more effectively than in male (Figures 4C-4F). We examined if there was any sexual difference in the learning and memory retention in relation to this differential effect in Aβ contents, but no evidence for the sexual difference was detected. There was also difference in Tris- and FA-extracted Aβ levels between male and female mice (Figures 4C-4F). Although the mechanisms underlying the differences remain unknown, it may be possible that this was in part due to differential expression of NIa or Aβ availability to given NIa levels in the brains with different genders.

Considering that NEP and IDE do not effectively clear oligomeric Aβ (Haass and Selkoe, 2007; Meilandt *et al.*, 2009) and have a broad substrate specificity for cellular proteins, the results of the present study, together with the previous work (Han *et al.*, 2010), suggest that because NIa cleaves both monomeric and oligomeric Aβ, and has a strict substrate specificity for Aβ, NIa is a good candidate to be used as a strategy for the improvement of Aβ pathology and the cognitive deficits of AD.

Methods

Transgenic AD model mice

Transgenic AD model mice, Tg-APPswe/PS1dE9 (Tg-APP/PS1 for short), overexpressing human mutated APP and PS1 (APPswe/PS1dE9) were maintained in C57BL6 × C3H F1 hybrid mice as described previously (Jankowsky *et al.*, 2001; Seo *et al.*, 2011). The mice were housed in pairs, in normal plastic cages, with free access to food and water in a temperature- and humidity-controlled environment, under a 12 h light/dark cycle. They were fed a diet of lab chow and water *ad libitum*. All animals were handled in accordance with the animal care guidelines of the Ewha Womans University School of Medicine.

Preparation of Lenti-NIa virus

The Lenti-NIa expression vector system was prepared as previously described (Han *et al.*, 2010). In brief, the cDNA for NIa was inserted in the pCD513B-1 lenti viral expression system (System Biosciences, Mountain View, CA). The resulting Lenti-NIa plasmid, together with the pPACK packing plasmid mix (System Biosciences), was transfected in HEK293T cells using a calcium (2.5 M $CaCl_2$)-phosphate method. After 3 days, the supernatant containing Lentiviral particles was collected after filtration with a 0.45-μm filter (Millipore Corporation, Billerica, Massachusetts) and then concentrated by ultra-centrifugation as previously described (Han *et al.*, 2010). The control Lenti-GFP viral vector was prepared similarly. The titers of Lenti-NIa and Lenti-GFP were estimated using a FACS analysis method, and the final tires obtained were 0.8 × 10^8 TU and 0.9 × 10^8 TU, respectively.

Intracerebroventricular injection of Lenti-NIa

Intracerebroventricular (i.c.v.) injection of Lenti-NIa was performed as previously described (Han *et al.*, 2010). Tg-APP/PS1 mice at 7 months of age were randomized into the Lenti-NIa ($n = 8$; 5 males and 3 females), and Lenti-GFP ($n = 9$; 5 males and 4 females), and their non-transgenic control ($n = 10$; 5 males and 5 females) groups. Mice were anesthetized by intraperitoneal (i.p.) injection of 3.5:1 mixture of ketamine (50 mg/ml) and xylazine hydrochloride (23.3 mg/ml) at the dose of 1.0 μl/g body weight and placed on a stereotaxic apparatus (Stoelting Company, Wood Dale, IL) as described in a previous study (Kim *et al.*, 2008; Han *et al.*, 2010). The mice were intra-cerebroventricularly injected, on both sides with a total of 3 μl of Lenti-NIa (0.8 × 10^8 TU in total) or Lenti-GFP with the same titer (0.8 × 10^8 TU) at the speed of 1 μl/min

(stereotaxic coordinates in mm with reference to the bregma: AP,0; ML, ±1.0; DV, -2.6) using a 28-G needle. After 5 min, the needle was removed over three intermediate steps and 3 min, in order to minimize backflow, and mice were kept on a warm pad until they awoke. Surgically manipulated mice awakening from anesthesia were returned to their home cages until use. Behavioral tests were performed15 days of injection.

Immunohistochemistry and quantification of plaque deposition levels

Immunohistochemical works were carried out as previously described (Han *et al.*, 2010; Kim *et al.*, 2012). Mice were sacrificed and perfused with 0.9% saline *via* a trans-cardiac method. One hemisphere of each animal was post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. The other hemisphere of each animal was used for ELISA assay as described below. They were coronally cut into 40 μm-thick sections with a vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). Free-floating sections were blocked with 4% bovine serum albumin in PBS for 1 h, and were reacted with monoclonal anti-β-amyloid protein antibody (1:2,000) (Bam10; # A5213, Sigma, St. Louis, MO) at 4°C overnight. The sections were washed with PBS, reacted with biotinylated secondary antibodies diluted 1:200 in PBS, and visualized using an ABC Elite kit (Vector Laboratories, Burlingame, CA).

Stained images were analyzed using an Olympus BX 51 microscope equipped with a DP71 camera and DP-manager and DP-Controller software (Olympus Co., Tokyo, Japan). Plaque deposition levels in the prefrontal, parietal, and piriform cortices were quantified using the TOMORO ScopeEye 3.6 program (Techsan Community, Seoul, Korea). Plaque deposition levels were also quantified using the 6-grade plaque photographic reference panels (Kim *et al.*, 2012). The 6-grade plaque photographic reference panels consist of a series of anti-Aβ (Bam-10)-stained brain sections images at the levels of the prefrontal cortex, parietal cortex, piriform cortex and hippocampus. The photographic reference panels represent plaque deposition levels assigned with the numerical grades of 0, +1, +2, +3, +4, +5, and +6, where plaque deposition levels increase "sigmoidally" over grading scales. Such "sigmoidal" increase allows the referenced plaque grades to be used to for the semi-quantitative assessment of plaque levels. When necessary, increments of 0.5 were applied.

Assessment of Aβ levels

ELISA assays for Aβ42 and Aβ40 levels were described previously (Lee *et al.*, 2009; Seo *et al.*, 2011). Briefly, the prefrontal cortex of each animal was homogenized in Tris-buffered saline (20 mM Tris and 137 mM NaCl, [pH 7.6]) containing protease inhibitor mixtures (Complete Mini; Roche). Homogenates were centrifuged at 100,000 *g* for 1 h at 4°C, and the supernatant was used to measure the levels of Tris buffer-soluble forms of Aβ. The pellet was sonicated in 70% formic acid and further centrifuged as above, and the resulting supernatant was used to measure the levels of the formic acid-extractable Aβ. The formic

acid-extract was neutralized with 1 M Tris-Cl buffer (pH 11) in a dilution ratio of 1:20 before its use. The final assays were performed using Human Aβ42 or Aβ40 ELISA kits (BioSource, Invitrogen) by following the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed as described previously (Han *et al.*, 2010; Seo *et al.*, 2011). The lateral prefrontal cortices from 5 Tg-APP/PS1 control mice and 5 Tg-APP/PS1 mice carrying Lenti-NIa were analyzed. Anti-NIa was produced in rabbits by repeated injections of NIa which was expressed in bacteria and purified. The detailed procedure for the production of anti-NIa will be described elsewhere. Anti-NIa antibody (1:1,000) and monoclonal α-tubulin antibody (Calbiochem; 1:2,000) were used in Western blot analysis.

RT-PCR analysis

RT-PCR was carried out as described previously (Han *et al.*, 2010) using the following primer sets: NIa: 5'-ACG AAA GAC GGC CAA TGC GGA-3' and 5'-ACC CGA CGG TTG CGA TGC TT-3'; GAPDH, 5'-TCC GTG TTC CTA CCC CCA ATG-3' and 5'-GGG AGT TGC TGT TGA AGT CGC-3'. The parietal cortices from five Tg-APP/PS1 control and five Tg-APP/PS1 mice carrying Lenti-NIa were used.

Behavioral assessments

Mice were brought to the testing room 30 min prior to the start of each behavioral test. Behavioral tests were performed with a computerized video-tracking system (SMART; Panlab S.I., Barcelona, Spain). At all times throughout the test, sound was masked with 60-65dB white noise.

Water maze test

The water maze test was performed as described previously (Lee *et al.*, 2004, 2006). Briefly, the water maze consisted of a 90-cm-diameter cylinder pool filled with opaque milky water (22°C). A hidden platform (10 cm in diameter) was placed in a quadrant 2 cm below the opaque water surface. The pool was placed in the center of a room with environmental and artificial cues including a door, a chair, and two marked posters. The room was illuminated at 35-40 lux. In each daily testing, mice were allowed to swim for a maximum of 90 s. Whether succeeded to locate the platform or failed, the animals were permitted to stand on it for 30 s before the session was terminated. The animals were tested twice a day, with a 6-h interval between trials. The latency of time and distance traveled prior to finding the platform for each trial were recorded for each trial. A spatial probe trial was conducted on day 6 after the 5-daytraining session, in the absence of the hidden platform. Animals were given a single 60-s probe trial to examine their spatial memory retention.

Passive avoidance test

The passive avoidance test was performed as described

previously (Lee *et al.*, 2004, 2006). Briefly, the test apparatus consisted of a brightly lit (1,700 Lux) white chamber, a darkened black chamber equipped with a shock-grid floor(each 15 × 15 × 15 cm), and a door between the two. On the first day of testing, each mouse was placed in the lighted chamber and left to freely explore the light and dark chambers for 5 min. On the second day, the mice were placed in the lighted chamber, after 30 sec, the middle door was opened, and the latency for the mouse to enter the dark chamber was recorded as pre-shock values. When a mouse entered the dark room, the door was closed and two successive electric foot-shocks (100 V, 0.3 mA, 2 sec for each shock and 5 sec for the interval) were delivered through the grid-floor. After this conditioning, mice were then returned to their home cages. One day (24 h), 4 days, and 7 days later, each mouse was replaced in the lighted chamber and the latency for the mouse to enter the dark chamber was measured with cut-off time of 300 sec, which was recorded as post-shock values.

Marble burying test

The marbles burying test was performed as described previously (Deacon, 2006), but with a minor modification. In brief, empty cages were filled with bedding up to 5 cm from the cage floor, and 12 grass marbles were placed evenly throughout the cage. Mice were individually allowed to freely explore the cage with marbles for 30 min, and afterwards, the number of successfully buried marbles was counted. Marble "burying" was defined when less than 25% of a marble was visible.

Statistical analysis

Two-sample comparisons were carried out using the Student's *t*-test, while multiple comparisons were made using one-way or two-way ANOVA followed by post-hoc tests to compare selected pairs of data. GraphPad PRISM software 4.0 (GraphPad Software Inc., San Diego, CA was used to perform statistical analyses. All data are presented as the means ± S.E.M., and statistical differences were accepted at the 5% level.

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