

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

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Abbreviations: APP, amyloid precursor protein; Nla, nuclear inclusion a; PS1, presenilin 1; TuMV, Turnip mosaic virus

Abstract

The plant viral protease, Nla, has a strict substrate specificity for the consensus sequence of Val-Xaa-His-Gln, with a scissoring property after Gln. We recently reported that Nla efficiently cleaved the amyloid- β (A β) peptide, which contains the sequence Val-His-His-Gln in the vicinity of the cleavage site by α -secretase, and that the expression of Nla 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 Nla in the brain of AD mouse model is beneficial to the improvement of cognitive deficits. To address this question, Lenti-Nla 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-Nla. In the passive avoidance test, Tg-APP/PS1 mice exhibited a severe deficit in their contextual memory retention, which was reversed by Nla expression. In the marble burying test, Tg-APP/PS1 mice buried marbles fewer than non-transgenic mice, which was also significantly improved by Nla. After behavioral tests, it was verified that the Tg-APP/PS1 mice with Lenti-Nla injection had reduced A β levels and plaque deposition when compared to Tg-APP/PS1 mice. These results showed that the plant viral protease, Nla, not only reduces A β 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 (A β) outside neurons and progressive cognitive dysfunction. Because A β is highly toxic to neural cells, accumulation of A β has been thought to cause the pathogenesis and cognitive impairment of AD (McLean *et al.*, 1999; Larson and Lesne, 2001; Walsh *et al.*, 2002). A β levels in the brain are regulated by the ratio between A β production and A β clearance (Tanzi and Bertram, 2005). Several endogenous proteases are known to play a role in clearance of A β 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 extensively studied. The pharmacological or genetic

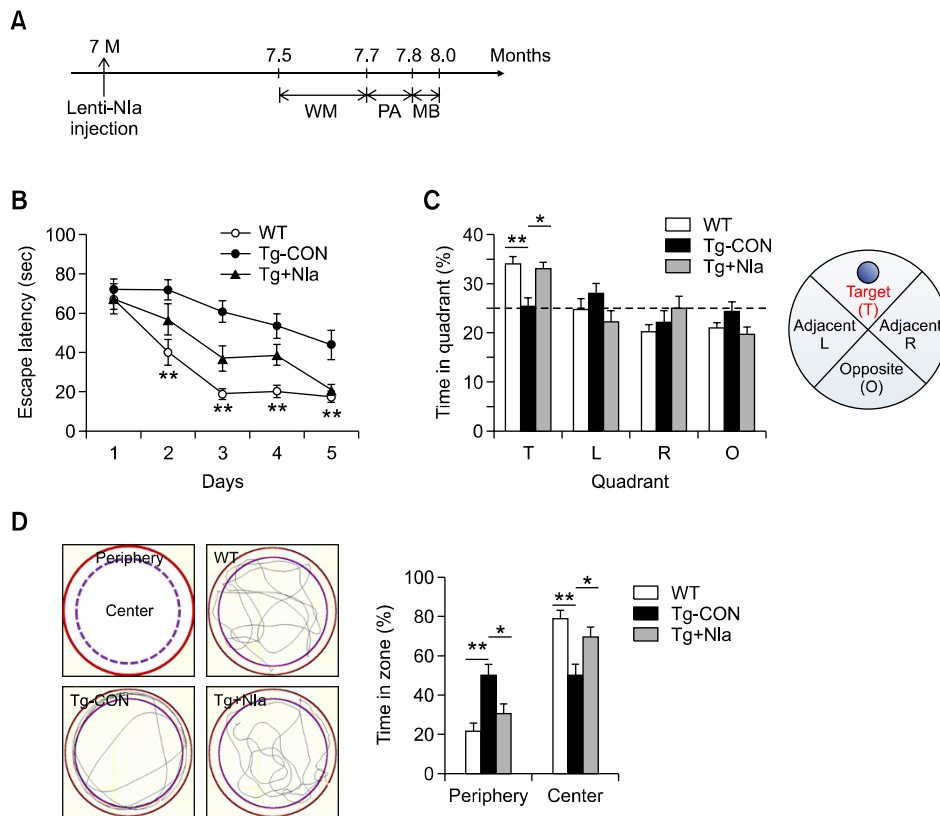


Figure 1. Spatial memory deficits displayed by Tg-APP/PS1 mice were improved by Nla expression. (A) Experimental design of Lenti-Nla injection and behavioral performance. Lenti-Nla was injected in Tg-APP/PS1 mice at 7 months of age and behavioral tests were started 15 days after the injection, for the 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-Nla (Tg+Nla), 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+Nla, 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 \times time interaction [$F_{(8,204)} = 2.301, P < 0.05$]. Data are presented as the means \pm 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+Nla showed significant effects of treatment (Nla) [$F_{(1,128)} = 10.95, P < 0.01$] and time [$F_{(4,128)} = 14.10, P < 0.0001$], but no significant treatment \times time interaction [$F_{(4,128)} = 0.8769, P = 0.4798$]. Data are presented as the means \pm 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+Nla; Tg-APP/PS1 mice infused with Lenti-Nla. 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 \times 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 \pm 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+Nla 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 \pm SEM.

inhibition of NEP in mouse models of AD increased A β accumulation and memory impairment (Mouri *et al.*, 2006; Farris *et al.*, 2007), while the over-expression of NEP was shown to reduce A β 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 A β in the brain (Farris *et al.*, 2003). Therefore, the enzymatic degradation of A β has been considered as an attractive strategy for the mitigation of A β accumulation in the AD brains (Haass and Selkoe, 2007; Miners *et al.*, 2008).

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

cognitive deficits (Meilandt *et al.*, 2009), suggesting that NEP has an ability to degrade monomeric, but not oligomeric, A β . A β oligomers are more toxic than monomeric or fibrillar A β (Dahlgren *et al.*, 2002; Ono *et al.*, 2009; Benilova *et al.*, 2012). IDE degraded the monomeric A β peptide, but did not cleave oligomeric A β (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 A β , but has a substrate specificity for A β , is necessarily sought.

The nuclear inclusion a protease of the Turnip mosaic virus, Nla, 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 A β peptides contain the sequence Val-His-His-Gln in the vicinity of the cleavage site by α -secretase, and that Nla degraded A β *in vitro*. Moreover, overexpression of Nla 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 Nla 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 Nla improved cognitive function, Lenti-Nla 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 Nla 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

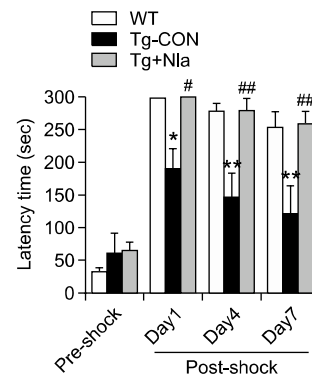


Figure 2. The contextual memory deficit of Tg-APP/PS1 mice was reversed by Nla expression. The entry latency into the dark chamber for Tg-APP/PS1 mouse control (Tg-CON), Tg-APP/PS1 mice infused with Lenti-Nla (Tg+Nla), and their non-transgenic control (WT) in the passive avoidance test. The latency to 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 \times 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+Nla 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+Nla, 4 males and 4 females.

injected with Lenti-Nla 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-Nla spent more time in the target quadrant compared with that of Tg-APP/PS1 mice (Figure 1C). These results suggest that the expression of Nla through the Lentiviral 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 Nla 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 Nla improved the impaired memory retention to the level of the non-transgenic control (Figure 2). These results suggest that the expression of Nla in the brain of Tg-APP/PS1 mice significantly improved context-dependent memory as well.

In the marble burying test, Tg-APP/PS1 mice

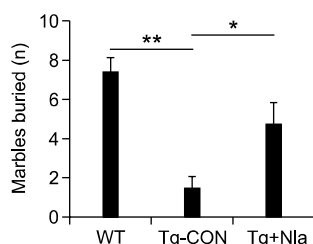


Figure 3. Behavioral changes in the marble burying test were restored by Nla expression. The numbers of glass marbles buried under wood chips by Tg-APP/PS1 mouse control (Tg-CON), Tg-APP/PS1 mice infused with Lenti-Nla (Tg+Nla), and their non-transgenic control (WT) for the 30-min period are presented. Nla 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+Nla, 4 males and 4 females.

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

Suppression of A β pathology in the brain with Nla expression

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

We recently demonstrated that the plant viral protease Nla effectively degraded A β *in vitro*, and that the expression of Nla in the brain of an AD model mice using a Lentiviral system reduced A β levels and plaque deposition (Han *et al.*, 2010). Because Nla is a potent exogenous protease, it was questioned whether or not overexpression of Nla produces any detrimental effects on the brain, other than the reduction in the levels of A β . After the infusion of Lenti-Nla 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 Nla 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 A β -degrading protease Nla, 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 A β in the brain. We believe that further studies on the effects of a more prolonged expression of Nla over a period of several months to a year, for example, are required in order to more fully elucidate the effects of A β regulation through Nla.

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 Nla (Figures 1D and 3), reducing the concern that overexpression of the foreign protease Nla in the brain could have detrimental effects on the emotional behavior.

Repression of A β pathology by Nla 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

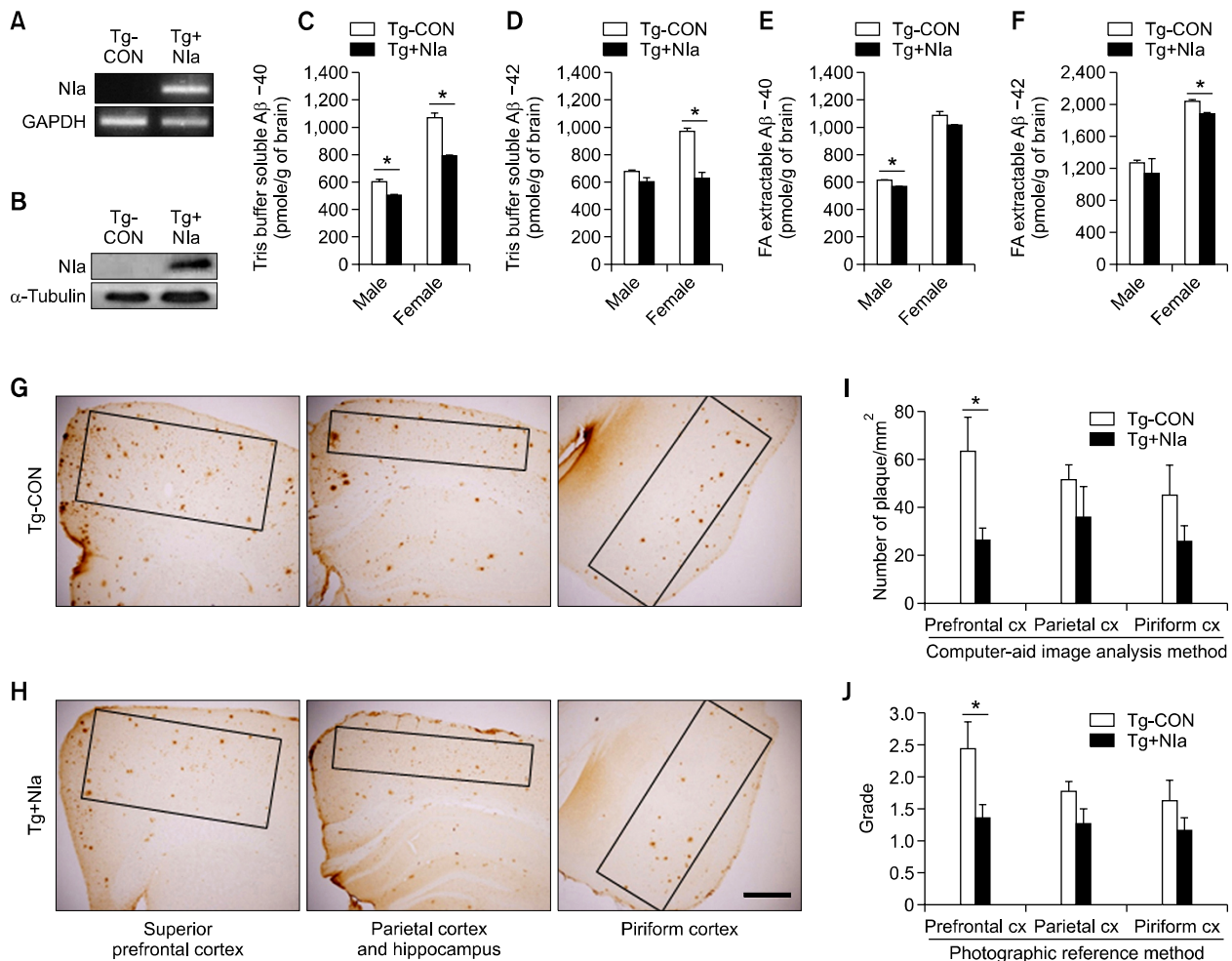


Figure 4. Suppression of A β pathology by Nla expression in the brain of Tg-APP/PS1 mice. (A) RT-PCR showing the expression of Nla transcript in the brain of Tg-APP/PS1 mice infused with Lenti-Nla (Tg+Nla). The expression of Nla was analyzed on the parietal cortex of Tg-APP/PS1 mice and their control mice (Tg-CON). GAPDH is a control. (B) Western blots showing the expression of Nla protein in the brain of Tg-APP/PS1 mice infused with Lenti-Nla (Tg+Nla). The expression of Nla (molecular weight: 28 kD) in the prefrontal cortex was examined using anti-Nla. Tg-APP/PS1 mouse control (Tg-CON). α -tubulin was used as a loading control. (C-F) The amounts of A β (40) (C, E) and A β (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-Nla (Tg+Nla). (G, H) Photomicrographs showing anti-A β (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-Nla (Tg+Nla; H). (I, J) Quantification of the levels of plaques (mm²) 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-Nla. 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 μ m. Data are presented as the means \pm 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 handling 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 A β 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 A β .

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 A β pathology, as evidenced that the phenotype also was reversed by N1a 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-N1a in Tg-APP/PS1 mice suppressed the A β 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-N1a in Tg-APP/PS1 mice suppressed A β 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 N1a in different brain regions is, in part, due to the uneven penetration of Lenti-N1a in different brain regions. It is also possibly attributed to the fact that the final viral titer used in the present study was 0.8×10^8 TU, which is slightly lower than the previous titer 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 A β levels was sufficient to provide a beneficial effect on behavior. In conjunction with this, it would be worth investigating whether local expression of N1a in specific brain regions produces specific or limited rescue of impaired brain function. Lenti-N1a expression in female Tg-APP/PS1 mice seemed to suppress A β 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 A β contents, but no evidence for the sexual difference was detected. There was also difference in Tris- and FA-extracted A β 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 N1a or A β availability to given N1a levels in the brains with different genders.

Considering that NEP and IDE do not effectively clear oligomeric A β (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 N1a cleaves both monomeric and oligomeric A β , and has a strict substrate specificity for A β , N1a is a good candidate to be used as a strategy for the improvement of A β pathology and the cognitive deficits of AD.

Methods

Transgenic AD model mice

Transgenic AD model mice, Tg-APP^{swe}/PS1^{dE9} (Tg-APP/PS1 for short), overexpressing human mutated APP and PS1 (APP^{swe}/PS1^{dE9}) were maintained in C57BL6 \times 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-N1a virus

The Lenti-N1a expression vector system was prepared as previously described (Han *et al.*, 2010). In brief, the cDNA for N1a was inserted in the pCD513B-1 lenti viral expression system (System Biosciences, Mountain View, CA). The resulting Lenti-N1a plasmid, together with the pPACK packing plasmid mix (System Biosciences), was transfected in HEK293T cells using a calcium (2.5 M CaCl₂)-phosphate method. After 3 days, the supernatant containing Lentiviral particles was collected after filtration with a 0.45- μ m 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-N1a and Lenti-GFP were estimated using a FACS analysis method, and the final titers obtained were 0.8×10^8 TU and 0.9×10^8 TU, respectively.

Intracerebroventricular injection of Lenti-N1a

Intracerebroventricular (i.c.v.) injection of Lenti-N1a was performed as previously described (Han *et al.*, 2010). Tg-APP/PS1 mice at 7 months of age were randomized into the Lenti-N1a ($n=8$; 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 μ l/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 intracerebroventricularly injected, on both sides with a total of 3 μ l of Lenti-N1a (0.8×10^8 TU in total) or Lenti-GFP with the same titer (0.8×10^8 TU) at the speed of 1 μ l/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 performed 15 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 μ m-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-A β (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 A β levels

ELISA assays for A β 42 and A β 40 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 A β . 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 A β . 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 A β 42 or A β 40 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-Nla were analyzed. Anti-Nla was produced in rabbits by repeated injections of Nla which was expressed in bacteria and purified. The detailed procedure for the production of anti-Nla will be described elsewhere. Anti-Nla 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: Nla: 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-Nla 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-day training 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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