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ORIGINAL ARTICLE

Intranasal NAP Administration Reduces Accumulation of Amyloid Peptide and Tau Hyperphosphorylation in a Transgenic Mouse Model of Alzheimer's Disease at Early Pathological Stage

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Abstract

Accumulation of β-amyloid (Aβ) peptide and hyperphosphorylation of tau in the brain are pathological hallmarks of Alzheimer's disease (AD). Agents altering these pathological events might modify clinical disease progression. NAP (Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln) is an octapeptide that has shown neuroprotective effects in various in vitro and in vivo neurodegenerative models. Previous studies showed that NAP protected against Aβ-induced neurotoxicity, inhibited Aβ aggregation, and, by binding to tubulin, prevented disruption of microtubules. In this study, we investigated the effect of NAP on Aβ and tau pathology using a transgenic mouse model that recapitulates both aspects of AD. We administered NAP intranasally (0.5 μg/mouse per day, daily from Monday through Friday) for 3 mo, starting from 9 mo of age, which is a prepathological stage in these mice. NAP treatment significantly lowered levels of Aβ 1–40 and 1–42 in brain. In addition, NAP significantly reduced levels of hyperphosphorylated tau. Of particular interest, hyperphosphorylation at the threonine 231 site was reduced; phosphorylation at this site influences microtubule binding. Our results indicate that NAP treatment of transgenic mice initiated at an early stage reduced both Aβ and tau pathology, suggesting that NAP might be a potential therapeutic agent for AD.

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Index Entries: Alzheimer's disease; tau; phosphorylation; β-amyloid peptide; NAP; neuroprotection; intranasal administration; transgenic mouse; therapy.

Introduction

Activity-dependent neuroprotective protein (ADNP) and activity-dependent neurotrophic factor (ADNF) were identified as major proteins secreted by glial cells that mediate the neuroprotective effects of vasoactive intestinal peptide (VIP) (Brenneman and Gozes, 1996; Said, 1996; Bassan et al., 1999; Gozes,

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2001; Gozes et al., 2003; Furman et al., 2004). In addition to its neuroprotective effects, ADNP was shown to be critical for brain formation, i.e., mice lacking the ADNP gene do not form a brain during embryonic development and consequently die *in utero* (Pinhasov et al., 2003). Studies of peptide fragments of ADNP identified an active domain, which consists of eight amino acids, Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln. This octapeptide, referred to as NAP (from the single-letter codes for the first three amino acids in the sequence), showed neuroprotective activity at the femtomolar (10^{-15} *M*) concentration range in various in vitro models (Gozes et al., 2005). Despite the difficulties commonly encountered with peptide-based drugs, NAP has shown promise as a neurotherapeutic agent. Peripheral administration of NAP showed significant efficacy in various in vivo models, indicating penetration into the brain at therapeutic levels (Gozes et al., 2005).

Alzheimer's disease (AD) is a neurodegenerative affliction associated with cognitive dysfunction. Neurofibrillary tangles and senile plaques are hallmarks of AD neuropathology. Neurofibrillary tangles are composed of hyperphosphorylated tau; tau is involved in microtubule assembly under physiological conditions (Avila et al., 2004). Senile plaques are composed primarily of β-amyloid (Aβ) peptide, which is generated by sequential proteolytic cleavage of the amyloid precursor protein (APP) (Hardy and Selkoe, 2002). Agents reducing these neuropathological lesions are pursued as potential disease-modifying effects for AD.

NAP has shown beneficial effects in cell-culture models and cell-free assays of relevlance to the pathogenesis of AD: (1) NAP prevented Aβ-induced neuronal toxicity (Bassan et al., 1999; Zemlyak et al., 2000); (2) NAP inhibited Aβ aggregation and dissociated preformed Aβ aggregates (Ashur-Fabian et al., 2003); and (3) NAP promoted microtubule assembly, stabilized microtubules, and increased the ratio of nonphosphorylated to phosphorylated tau (Divinski et al., 2004, 2006; Gozes and Divinski, 2004). Although NAP has shown protection against cholinotoxicity in rats (Gozes et al., 2000), its effects on other AD pathologies in vivo have not been assessed previously.

Generating mice with both plaques and tangles is crucial for studies of the molecular relationship between Aβ peptide and tau and to test the effectiveness that anti-AD interventions have on both pathologies. LaFerla et al. were successful in generating a model that mimics both AD neuropathologies (Oddo et al., 2003b). The group comicroinjected two transgenes (encoding APP_{Swe} and tau_{P301L} under the control of the Thy 1.2 promoter) into single-cell embryos harvested from presenilin-1 $(PS\text{-}1)_{M146V}$ knock-in mice (Guo et al., 1999). The resulting mice are triple transgenic mice (and referred to as 3xTg-AD mice). These mice develop tau pathology that is driven, at least in part, by amyloid accumulation (Oddo et al., 2003a, 2006).

In this study we investigated the effect of peripheral administration of NAP on Aβ peptide and tau pathology at an early pathological stage using the 3xTg-AD mouse model of AD.

Materials and Methods

Animals and Treatment Protocol

Transgenic mice expressing three mutant transgenes, i.e., APP (double Swedish, K670M/N671L), PS-1 (M146V), and tau (P301L), were generated by comicroinjection of APP and tau transgenes under the control of Thy 1.2 promoter into mutant PS-1 knock-in mice (Oddo et al., 2003b). Mice were bred and maintained at the Georgetown University animal facility. NAP was prepared as published previously (Bassan et al., 1999) and was dissolved in 7.5 mg NaCl, 1.7 mg citric acid monohydrate, and 3.0 mg $Na_2PO_42H_2O$, 0.2 mg benzalkonium chloride solution (50%) per 1 mL USP sterile water (Alcalay et al., 2004). For this study, mice were treated with NAP for 3 mo, starting from 9 mo of age (40 ± 1 wk of age), and sacrificed at 12 mo of age. Mice ($n = 12$) were treated intranasally with either NAP (0.5 μg NAP/mouse per day) or vehicle (2.5 μL for each nostril, 5.0 μL/mouse per day) daily from Monday through Friday between 10 AM and 2 PM. All chemicals were purchased from Sigma (St. Louis, MO) unless specified otherwise.

Sample Preparation

Because 2 mice in the NAP treatment group died during the treatment (for details, *see* Results), 10 and 12 mice in the NAP and vehicle treatment groups, respectively, were used for analysis. Mice were sacrificed by cervical dislocation. The brain was quickly isolated, the olfactory bulb and cerebellum were discarded, and the hemibrain was snap-frozen in dry ice. The brain was homogenized in a fivefold volume of 50 m*M* Tris-HCl buffer (pH 7.4), consisting of 1% Nonidet P-40, 150 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid, 0.25% sodium deoxycholate, 1 m*M* NaF, 1 m*M* Na_3VO_4, 1 m*M* phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. This

master homogenate was used to prepare samples for tau phosphorylation and Aβ peptide analysis.

Analysis of Tau Phosphorylation Status

The master brain homogenate was centrifuged at 20,000*g* for 20 min at 4°C, and the supernatant was used as a crude tau fraction. Samples were run on a 4%–15% gradient SDS-polyacrylamide gel (Bio-Rad, Hercules, CA), and the proteins were transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was probed with one of the primary antibodies. For phosphorylated tau, we used clones AT8, AT180, and CP13, which detect phosphorylated tau at serine (Ser)^{202}/threonine (Thr)^{206} (Mercken et al., 1992), Thr^{231} (Greenberg and Davies, 1990), and Ser^{202}, respectively (AT8 and AT180 [1 μg IgG/mL] were from Pierce [Rockford, IL]; CP13 [1:200 dilution] was from Dr. Peter Davies [Albert Einstein College of Medicine]). For total tau, we used clone tau46, which binds to an epitope within the phosphorylation-independent region of tau (1.2 μg IgG/mL, Sigma) (Kosik et al., 1988). The primary antibodies were detected by HRP-coupled anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) and visualized with a chemiluminescence kit (Pierce). The membranes were densitometrically analyzed (Quantity One, Bio-Rad). Because tau expression level in transgenic mice is not completely consistent among animals, we standardized levels of phosphorylated tau by total tau level, i.e., densitometric measures of phosphorylated tau bands (clones AT8, AT180, and CP13) were divided by densitometric measures of total tau bands (clone tau46). Statistical significance was determined by *t*-test (SPSS, Chicago, IL).

Analysis of Ab Peptide Load

The master brain homogenate was mixed with formic acid to yield 70% final concentration, sonicated, and ultracentrifuged at 135,000*g* for 45 min. The supernatant was collected, neutralized with Tris buffer, and subjected for Aβ ELISA as described previously (Matsuoka et al., 2003). Levels of full-length Aβ 1–40 and 1–42 were quantified using ELISAs developed by our group (Horikoshi et al., 2004). A 96-well Maxisorp plate (Nunc, Rochester, NY) was coated overnight with either clone 1A10 or 1C3, which are specific for Aβ 40 and 42, respectively. After blocking with Block Ace (Serotec, Raleigh, NC), samples were incubated overnight at 4°C. The plate was then incubated with HRP-coupled N-terminal end-specific antibody, clone 82E1, for 4 h, and visualized using TMB as a substrate (Pierce). Statistical significance was tested by *t*-test (SPSS).

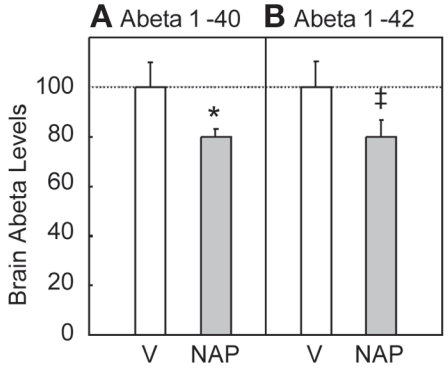

Fig. 1. NAP treatment reduces levels of Aβ 1–40 and 1–42 in a mouse model of AD. Triple transgenic mice were treated with NAP (0.5 μg NAP/mouse per day) for 3 mo, starting from 9 mo of age, and levels of brain Aβ 1–40 and 1–42 were determined by ELISA. NAP treatment ($n = 10$) reduced both Aβ 1–40 and 1–42 levels to a similar degree, 20% as compared with controls ($n = 12$). Reduction of Aβ 1–40 was significant ([*] $p < 0.05$), whereas reduction of Aβ 1–42 was only marginally significant ([‡] $p = 0.06$). Statistical difference was determined by *t*-test.

Results

During the 3-mo period of treatment, two mice in the NAP treatment group died. The cause of death was unknown, but we do not suspect that death was related to NAP treatment, because no mouse died in another 3-mo-long study using a fourfold higher NAP dose in older animals (unpublished observation).

After 3 mo of intranasal treatment with NAP (0.5 μg NAP/day per mouse), the brains were isolated and samples were prepared. Levels of full-length Aβ 1–40 and 1–42 in the brain homogenates were determined by ELISA. Unlike other Aβ ELISAs, the ELISAs used here are composed of both N- and C-terminal end-specific antibodies, and provide highly accurate measures of Aβ peptide levels (Horikoshi et al., 2004). NAP treatment reduced both Aβ 1–40 and 1–42 to a similar degree (20%), compared with vehicle-treated control mice (Fig. 1). Although the reduction of Aβ 1–40 achieved significance ($p < 0.05$) (Fig. 1A), reduction of Aβ 1–42 was only marginally significant ($p = 0.06$) (Fig. 1B) due to relatively large variance.

We also examined the effect of NAP on tau phosphorylation using the crude tau fraction. NAP treat-

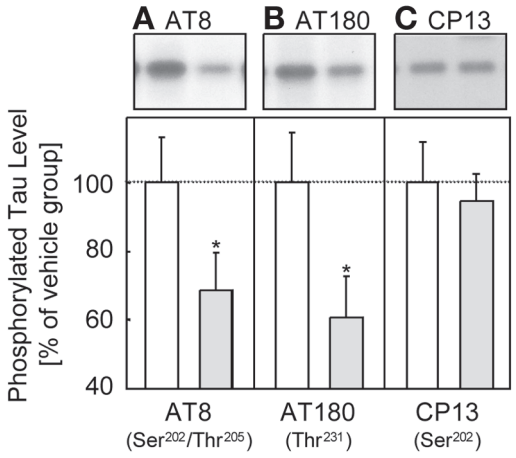

Fig. 2. NAP treatment reduces tau phosphorylation level in a mouse model of AD at pre- to early pathological stage. Triple transgenic mice were treated with NAP (0.5 μg NAP/mouse per day) for 3 mo, starting from 9 mo of age, and levels of phosphorylated tau were determined ($n = 10$, NAP treated [gray bars]; $n = 12$, controls [white bars]). Because tau level is not completely consistent among transgenic mice, levels of phosphorylated tau were standardized by total tau level; i.e., phosphorylated tau levels (AT8, AT180, and CP13) were divided by total tau level, which was detected using phosphorylation-independent tau antibody (clone tau46). NAP treatment reduced phosphorylated tau significantly at Ser^{202}/Thr^{205}, and Thr^{231} (AT8 **[A]** and AT180 **[B]** sites, respectively) but did not alter the level of phosphorylated tau at Ser^{202} (CP13 site) **(C)**. Typical protein bands are shown in the box.

ment significantly reduced phosphorylated tau at Ser^{202}/Thr^{205} (31% reduction, $p < 0.05$, Fig. 2A) and Thr^{231} (39% reduction, $^*p < 0.05$, Fig. 2B). Levels of phosphorylated tau at Ser^{202} were not altered (Fig. 2C).

Discussion

Currently, two classes of drugs are approved by the Food and Drug Administration (FDA) for the treatment of AD (Aisen, 2005). Acetylcholinesterase inhibitors are the first FDA-approved class of anti-AD drugs, and three of these drugs, approved between 1997 and 2001, are commonly prescribed: donepezil (Aricept®), rivastigmine (Exelon®), and galantamine (Razadyne®). Memantine (Namenda®), representing a new class of AD therapeutics, was approved in 2003 for the treatment of moderate-to-severe AD. Memantine is a noncompetitive low-to-moderate affinity *N*-methyl-D-asparate (NMDA) receptor antagonist. Although acetylcholinesterase inhibitors are symptomatic medications, NMDA receptor antagonists were thought to be neuroprotective, i.e., disease modifying. However, clinical evidence suggests that the benefits of memantine, like the cholinesterase inhibitors, are symptomatic (Reisberg et al., 2003). Thus, all currently available medications are marginally effective but only show symptomatic efficacy, and some patients do not tolerate the medications. Effective disease-modifying agents are needed to slow the clinical progression of AD.

Loss of neuronal function is the cellular basis of dementia. Thus, NAP, which showed neuroprotective effects in various in vitro and in vivo models, including Aβ-induced cell toxicity (Bassan et al., 1999; Zemlyak et al., 2000), might be a useful therapeutic agent. In this study we tested the potency of NAP against AD pathology in vivo. Triple transgenic mice housed at Georgetown University showed slower progression of Aβ and tau pathology compared with the original report (Oddo et al., 2003b; unpublished data), and we suspect this is due to the effect of differing housing environments as seen in other AD models (Jankowsky et al., 2003). We treated 3xTgAD mice for 3 mo, from 9 mo of age, and sacrificed at 12 mo of age. Although no Aβ plaque is observed in any brain region at 9 mo of age in this colony, some extracellular Aβ plaques are detectable in hippocampal formation by the end of the treatment period, 12 mo of age. Some hippocampal neurons are positive for early tau phosphorylation markers, such as Ser^{202} (AT8 and CP13 sites) at 9 mo of age, and the number of phosphorylated tau-bearing neurons increases steadily with aging. Therefore, the results of this mouse treatment protocol are most applicable to pre- to early-stage AD.

Three-month treatment with NAP lowered levels of Aβ 1–40 and 1–42 significantly in the brain. In in vitro studies, NAP inhibited Aβ aggregation and dissociated preformed Aβ aggregates (Ashur-Fabian et al., 2003). Inhibiting Aβ aggregation with other agents has been associated with reduced Aβ levels in transgenic mouse brain (Gervais et al., 2006), presumably by increasing Aβ cleavage and/or augmenting transport from the brain.

NAP significantly reduced levels of phosphorylated tau in vitro (Gozes and Divinski, 2004), and here we show for the first time a significant effect in vivo, one that might be linked to microtubule stabilization. This effect might be linked to the fact that both NAP and tau are known to influence microtubule dynamics (Divinski et al., 2004, 2006; Gozes and Divinski, 2004). Tau promotes tubulin assembly (Weingarten et al., 1975), and phosphorylation of tau regulates the binding of tau to microtubules

(Wagner et al., 1996). Although many kinases are known to phosphorylate tau, glycogen synthesis kinase 3 (GSK3) plays an important role in regulating tau phosphorylation under both physiological and pathophysiological conditions, and is considered to be a potential therapeutic target in AD (Bhat et al., 2004). Two types of GSK3-induced tau phosphorylation have been proposed: primed (following prior phosphorylation by another kinase), and unprimed phosphorylation (Frame et al., 2001). Primed phosphorylation occurs at Thr^{231} and affects microtubule binding, whereas unprimed phosphorylation can take place at Ser^{396} or Ser^{404} and does not affect microtubule binding (Cho and Johnson, 2003). Previous studies have shown that NAP binds to tubulin and reduces microtubule disruption (Divinski et al., 2004). Our in vivo findings, particularly with regard to phosphorylation at Thr^{231}, further support the potential of NAP in reducing tau pathology in AD.

Our study shows the potential efficacy of NAP in the treatment of AD using an animal model that recapitulates both Aβ and tau pathology. Unlike other peptide-based drugs, intranasally administered NAP enters the brain at therapeutic levels and treatment reduces both Aβ and tau pathology. Overall, our study suggests that NAP is a promising disease-modifying treatment candidate for early-stage AD.

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