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Title page

A neuronal microtubule interacting agent, NAP, reduces tau pathology and enhances cognitive function in a mouse model of Alzheimer's disease

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Abstract

Neurofibrillary tangles composed of aggregated, hyperphosphorylated tau in an abnormal conformation represent one of the major pathological hallmarks of Alzheimer's disease (AD) and other tauopathies. Recent data suggest however, that the pathogenic processes leading to cognitive impairment occur before the formation of classic tangles. In the earliest stages of tauopathy, tau detaches from microtubules and accumulates in the cytosol of the somatodendritic compartment of cells. Either as a cause or an effect, tau becomes hyperphosphorylated and aggregates into paired helical filaments that comprise the tangles. To assess whether an agent that modulates microtubule function can inhibit the pathogenic process and prevent cognitive deficits in a transgenic mouse model with AD-relevant tau pathology, we administered the neuronal tubulin-preferring agent, NAP. Three months of treatment with NAP at an early-to-moderate stage of tauopathy reduced the levels of hyperphosphorylated soluble and insoluble tau. A 6-month course of treatment improved cognitive function. While non-specific tubulin interacting agents commonly used for cancer therapy are associated with adverse effects due to their anti-mitotic activity, no adverse effects were found after 6 months of exposure to NAP. Our results suggest that neuronal microtubule interacting agents such as NAP may be useful therapeutic agents for the treatment or prevention of tauopathies.

Introduction

Neurofibrillary tangles containing hyperphosphorylated tau represent one of the principal pathological hallmarks of AD. Clinical progression of AD is tightly related to tau pathology (Braak and Braak, 1995), and a recent transgenic mouse study suggests that the pathogenic processes leading to cognitive impairment occur before the formation of classical tangles (Roberson, et al., 2007). Tau is a neuronal microtubule-associated protein and phosphorylation of tau regulates its ability to promote microtubule assembly (Lindwall and Cole, 1984). Abnormal hyperphosphorylation is thought to interfere with the normal biological function of tau by reducing its ability to bind to and stabilize microtubules (Bramblett, et al., 1993; Alonso, et al., 1994). Treatment with lithium chloride, a GSK3 inhibitor, reduced tau phosphorylation and prevented or reversed several aspects of tauopathy in mice (Noble, et al., 2005; Engel, et al., 2006; Plattner, et al., 2006) and flies (Mudher, et al., 2004). Nevertheless, it is not clear whether inhibitor-mediated reduction of phospho-tau improved tau binding to microtubules in these studies. Paclitaxel (taxol), a tubulin binding agent and FDA-approved potent anti-cancer drug, restored fast axonal transport and motor dysfunction in a tau transgenic mouse in vivo (Zhang, et al., 2005). However, paclitaxel is not neuron-specific, has the capacity to inhibit microtubule dynamics, has limited brain bioavailability and presents side effects including inhibition of axonal transport associated with peripheral neuropathy (Lee and Swain, 2006). Thus, more specific, brain penetrable microtubule-interacting drugs are required (Michaelis, 2006).

The octapeptide NAPVSIPQ (NAP) preferentially interacts with neuronal and glial tubulin, promotes microtubule assembly, influences microtubule dynamics in post-mitotic cells and reduces phosphorylated tau in vitro at femtomolar concentrations (Divinski, et al., 2004; Divinski, et al., 2006; Gozes and Divinski, 2004). Peripheral administration of NAP has shown significant efficacy in various in vivo models, and penetration into the brain at therapeutic levels (Gozes, et al., 2005). Phase I clinical trials indicate that intranasally-administered NAP is well tolerated and

produces blood levels of the peptide expected, on the basis of animal studies, to reflect therapeutic concentrations in brain (personal communication, Allon Therapeutics Inc.).

We have shown that chronic intranasal NAP treatment in a triple transgenic mouse model with AD-type Abeta and tau pathology (Oddo, et al., 2003) reduced Abeta accumulation and tau hyperphosphorylation when administered at an early pathological stage (9-12months of age) (Matsuoka, et al., 2007), Here, we extend these studies to examine the effect of increased NAP dosage on tau pathology and cognitive function in the same mouse model at a later stage of disease (12-18 months of age).

Materials and Methods

Animals, treatment protocol, and sampling

Triple transgenic mice (3xTg-AD) expressing three mutant transgenes, i.e., amyloid precursor protein (APP, double Swedish, K670M/N671L), presenilin-1 (M146V), and tau (P301L), were generated by co-microinjection of APP and tau transgenes under the control of Thy 1.2 promoter into mutant PS-1 knock-in mice (Oddo, et al., 2003). Mice were bred and maintained at the Georgetown University animal facility. NAP was synthesized as previously reported (Bassan, et al., 1999) and was dissolved in 7.5 mg NaCl, 1.7 mg citric acid monohydrate, 3.0 mg Na_2PO_4 $2H_2O$, 0.2 mg benzalkonium chloride solution (50%), per 1 ml USP sterile water (Alcalay, et al., 2004).

Mice were treated with NAP for 3 or 6 months starting from 12 months of age, and sacrificed at 15 or 18 months of age, respectively. We used 12 mice each (6 female and 6 male mice) at 12.1 ± 0.3 (vehicle) and 12.1 ± 0.2 (NAP) months of age for 3 months treatment; and 12 mice (5 male and 7 female) at 11.6 ± .0.4 (vehicle) and 13 mice (5 male and 8 female) at 11.6 ± 0.4 (NAP) months of age for 6 months treatment. Mice were intranasally treated with either NAP (2 µg NAP/mouse/day, 2.5 µl for each nostril, 5.0 µl/mouse/day) or vehicle (2.5 µl for

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each nostril, 5.0 µl/mouse/day) daily from Monday through Friday between 10 am and 2 pm. We monitored overall health status on a daily basis during the entire treatment period.

Mice were sacrificed by cervical dislocation, and the brain was quickly isolated after the olfactory bulb and cerebellum were discarded. Each hemi brain was snap-frozen in dry ice, or immersion fixed in 4% paraformaldehyde in 100 mM phosphate buffer for biochemical and histochemical studies, respectively.

Biochemical analysis

Levels of phospho-tau were analyzed by immunoblotting. The gel system used allowed the comparison of 26 samples simultaneously, and all samples from two groups were run on one gel and compared within the gel. The brain was homogenized in a 5-fold volume of 50 mM Tris-HCl buffer, pH 7.4, consisting 1% Nonidet P40, 150 mM NaCl, 1mM ethylenediaminetetraacetic acid, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethyl-sulfonyl fluoride, and protease inhibitor cocktail (crude brain homogenate). The homogenate was centrifuged at 20,000 g for 20 min at 4 °C, and the supernatant was used as a crude tau fraction. The crude tau fraction was heat-treated at 100 °C for 5 minutes, centrifuged at 20,000 g for 20 minutes at room temperature, and the supernatant was used as heat-stable soluble tau fraction. The crude tau fraction was treated with sarkosyl (1% final concentration) for 30 minutes at room temperature and ultracentrifuged at 100,000 g for 1 hour at 4 °C. The pellet contained the sarkosyl-insoluble tau fraction.

Samples were solubilized in sample buffer, Tris-HCl buffer, pH 7.0, consisting of 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and protease and phosphatase inhibitor cocktail, and run on a 4-15% gradient SDS-PAGE gel (Criterion, BioRad, Hercules, CA). Proteins were transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was probed with one of the primary antibodies. 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). For phosphorylated tau, we used clones AT8, AT180 and CP13, which detect phosphorylated tau at serine (Ser) 202 / threonine (Thr)205 (Mercken, et al., 1992), Thr231 (Greenberg and Davies, 1990) and Ser202, respectively (AT8 and AT180, 1 µg IgG/ml, were from Pierce, Rockford, IL; CP13, 1:200 dilution, was gift from Dr. Peter Davies, Elbert Einstein College of Medicine, New York, NY). The primary antibodies were detected by a horse radish peroxidase (HRP)-coupled anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA), and visualized with a chemiluminescence kit (Pierce). The protein bands were densitometrically analyzed (Quantity One, BioRad). Since the 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 (antibodies AT8, AT180 and CP13) were divided by densitometric measures of total tau bands (antibody tau46).

For Abeta quantification, the crude brain homogenate was centrifuged at 135,000g for 1 hour at 4°C, and the supernatant was used to determine the levels of soluble Abeta. To determine the levels of total Abeta, the crude brain homogenate was mixed with formic acid to yield 70% final concentration, sonicated, ultracentrifuged at 135,000 g for 45 min, and the supernatant was used. Levels of Abeta 1-40 and 1-42 were quantified using enzyme-linked immunosorbent assays (ELISAs) developed by our group (Horikoshi, et al., 2004). In brief, a 96-well plate was coated with either Abeta 40 or 42 specific antibody (clones 1A10 and 1C3, respectively, IBL, Takasaki, Gunma, Japan), then standards and samples were incubated overnight. The plate was then incubated with a HRP-coupled N terminus end-specific antibody (clone 82E1, IBL), and visualized using tetramethylbenzidine as a substrate (Pierce).

Histochemical analysis

After incubation with 15% sucrose containing phosphate buffer for cryoprotection, 30 µm-thickness saggital sections were prepared using a cryostat. Sections were treated with 0.3%

hydrogen peroxide in 10 mM phosphate buffered saline, pH 7.4, consisting 0.3% Triton X-100 (PBST), for one hour and then incubated with 1% normal goat serum for one hour to block non-specific binding. Sections were incubated with primary antibodies for phosphorylated tau (AT8 and AT180, 1 μg IgG/ml; CP13, 1:200 dilution) and Abeta (82E1, 1 μg IgG/ml) (Horikoshi, et al., 2004) overnight at 4 °C. Primary antibodies were detected by biotinylated anti-mouse IgG (1 μg IgG/ml in PBST, Vector Laboratories, Burlingame, CA) for 2 hours at room temperature, and then visualized using a kit (Vector stain ABC kit, Vector Laboratories). For semi-quantitative analysis, we used three hippocampal sections from each mouse; the first section at the Bregma -1.46 mm (Franklin and Paxinos, 1997) and the 10th and the 20th sections from the first section (section thickness: 30 μm; i.e., 10th and 20th sections are at 0.3 and 0.6 mm toward the back from the first section). The number of phosphorylated tau-bearing pyramidal neurons in the CA1 region and the number of Abeta plaques in the hippocampal formation were counted using a 20x objective lens. Slides were coded and the code was broken after all counts were completed.

Behavioral analysis

The Morris water maze (MWM) (Morris, et al., 1982) is a behavioral procedure designed to test spatial learning and memory by measurements of the time required to find a hidden platform in a pool (defined as latency). A pool (160 cm diameter and 50 cm high), was filled with white-colored water (1 liter of Crayola Premier™ Tempera white paint) and water temperature was maintained at 24°C. Visual cues were placed around the pool, and a hidden platform (15 cm diameter) was placed at the fixed location. Two daily training sessions, 120 sec each, were performed during 5 consecutive days. On the fifth day, immediately after the second daily training, the platform was removed from the pool and the time spent by the mice in the pool's quarter where the platform used to be was recorded (for a maximal period of 120 sec), termed probe test. Morris water maze was analyzed using a video-tracking software (Actimetrics, Wilmette, IL).

To control for animal's visual abilities, we tested the animals in the visible platform version of the MWM. In this variant of the task, the platform with very easily recognizable object was placed into the pool. Mice were released into the water and time taken to find the platform was recorded (maximum 60 seconds). Animals that did not climb on the platform during this test were excluded from the statistical analysis as animals with visual defects.

Olfactory habituation/dishabituation task

This test is designed to determine if a mouse can remember and distinguish between odors (Sanchez-Andrade, et al., 2005). The odors were presented on a suspended cotton swab to the test mouse placed into the clean cage with fresh shavings. Each mouse was tested during three consecutive 2 min. periods, for each odor with two min. interval between presentations. The time that the mouse smelled the swab was recorded (beginning whenever the animal oriented its nostrils toward the cotton swab - within 2 cm or less). The time period measured for water sniffing gave a baseline level comparable to the time period spent in sniffing other odors. After 3 trials (first block) with the odorless swab (water), the same 3 trials were repeated (second block) with the swab dipped in diluted vanilla (1:33, McCormick, Hunt Valley, MD). The increase in sniffing time represented the smell sensitivity of the mouse. Mice exhibiting impairment in smell sensitivity were excluded from the test. In general, mice habituate very quickly to odors and sniffing time declines when the mouse is exposed several times to the same odor. When a new odor is introduced, there is an increase in sniffing time, indicating that the animal can discriminate between odors. After the second block of trials, the swab was dipped in almond extract (1:100, McCormick) and presented to the animals (third block). Increase in sniffing time in the first trial of the third block represented the ability to distinguish between odors and/or the ability to remember the previous odor.

Statistical analysis

Statistical significance was determined by t-test (SPSS, Chicago, IL).

Results

The transgenic mice used in this study, 3xTg-AD (Oddo, et al., 2003), were housed at Georgetown University. Since different mouse colonies may show different rates of progression of tau and Abeta pathology, we examined the progression of pathology using mice at 3, 6, 9, 12, 16, 20 and 23 months of age (n=6 at each age, age variance ± 0.0-0.4 months of age depending on the time points) and found slower progression of pathology in our mouse colony (Hirata-Fukae et al., submitted) as compared to the original 3xTg-AD mouse colony (Oddo, et al., 2003). In brief, at the time we started treatment, 12 months of age, extracellular Abeta plaques were initially detected in the subiculum, and intraneuronal Abeta was seen in the cerebral cortex. Phosphorylated tau was detected in both heat-stable soluble and sarkosyl-insoluble tau fractions, and phosphorylated tau-bearing neurons were seen in the hippocampus and cerebral cortex. Levels of Abeta load, plaques, phosphorylated tau, and phosphorylated tau-bearing neurons increased with aging, and more advanced pathology was seen at the end points of the study; i.e., 15 and 18 months of age. In our colony, Thioflavin T-positive mature neurofibrillary tangles were initially detected at 20 months of age.

During the treatment period, we monitored the overall health status of the mice in each treatment group. We assigned 12 mice to vehicle-treatment and 12 mice to NAP-treatment groups for the 3-month-treatment period (12 → 15 months). In this experiment, all mice survived throughout the entire testing period, showing no abnormalities and maintaining their bodyweights. In the 6-month- treatment period (12 → 18 months), 12 and 13 mice were assigned to vehicle and the NAP treatment group, respectively. During the treatment, 2 mice in

the vehicle- and 1 mouse in the NAP-treated group died. These mice died without any change in the overall health status.

After 3 months of intranasal NAP treatment, the level of phosphorylated tau was significantly reduced in the brains of 15 month-old mice (**Fig. 1A-C**): 74% at Ser202/Thr205 (P<0.001), 74% at Thr231 (P<0.001) and 43% at Ser202 (P<0.001) in the crude tau fraction (**Fig. 1D-F**). Specifically, after NAP treatment the number of phosphorylated tau-bearing hippocampal pyramidal neurons was significantly reduced (**Fig. 1G-I** vs. **J-L**). Semi-quantitative analysis indicated that the number of phosphorylated tau-bearing neurons was significantly reduced after three months NAP treatment; AT8: 53 ± 13% (P<0.01), AT180: 63 ± 10% (P<0.01), CP13: 59 ± 18% (P<0.01). Both biochemical and histological analysis detected significant NAP-associated reduction at all tau phosphorylation sites that were examined in the current study. Note that our colony of triple transgenic mice shows a slower progression of pathology than the original colony from which it was derived (Oddo, et al., 2003), and no mature thioflavin T-positive neurofibrillary tangles were evident at 15 or 18 months of age (Hirata-Fukae et al., submitted).

We further examined the levels of phosphorylated tau in heat-stable soluble and sarkosyl-insoluble tau fractions after NAP treatment. The levels of all phosphorylated tau forms examined were significantly reduced in the heat-stable soluble tau fraction after 3 months of NAP treatment: 41% at Ser202/Thr205 (P<0.01), 33% at Thr231 (P<0.001), and 45% at Ser202 (P<0.001) (**Fig. 2D-F**). In the sarkosyl-insoluble tau fraction, NAP treatment significantly reduced tau phosphorylation at Ser202/Thr205 (50%, P<0.05), but the impact on tau phosphorylation at Thr231 was only marginally significant (42%, P=0.06). Tau phosphorylation at Ser202 was unchanged (P=0.89) (**Fig. 2J-L**). Hyperphosphorylation of tau is thought to precede conformational change; therefore we compared the levels of soluble and insoluble tau.

Sarkosyl-insoluble abnormal tau was significantly reduced after treatment with NAP (22%, *P*<0.01) (**Fig. 2P**), while heat-stable soluble tau showed a trend toward elevation (**Fig. 2O**).

We examined the levels of soluble and total Abeta using specific ELISAs. In contrast to our previous results with younger mice that showed statistically significant reduction in Abeta1-42 upon NAP treatment (Matsuoka, et al., 2007), in these experiments, the NAP-associated reduction in Abeta did not reach statistical significance in older mice. Results following three months of NAP treatment were as follows; soluble Abeta 1-40, 80 ± 17% (*P*=0.67); soluble Abeta 1-42, 75 ± 16% (*P*=0.72); total Abeta, 1-40, 96 ± 15% (*P*=0.89); total Abeta 1-42, 86 ± 13% (*P*=0.73) compared to the vehicle-treated mice. We detected a few extracellular Abeta plaques in the hippocampus and intraneuronal Abeta in the cerebral cortex in untreated and vehicle-treated control mice (data not shown), however, the paucity of plaques did not allow for precise determination of a possible effect of NAP treatment (data not shown).

Six-month daily NAP treatment resulted in significant cognitive enhancement in 18-month old mice as measured in the MWM and in the habituation/dishabituation test. In the MWM hidden platform probe test, NAP-treated mice remembered the hidden platform location (rescue point) after a five-day training period, spending significantly more time in the quadrant where the platform was originally placed (*P*<0.05, **Fig. 3A**) and crossing the platform location more frequently as compared to vehicle-treated mice (*P*<0.05, **Fig. 3B**).

In the habituation/dishabituation test that exposes the mice to water followed by two different odorants, each presented three consecutive times, both NAP- and vehicle-treated mice showed an apparent response to the first odorant (**Fig. 3D1**) and the arithmetic difference between first odorant and third water investigation time (i.e., **Fig. 3D1- 3C3**, *P* =0.435) was not statistically significant between the tested groups. In contrast, in response to the second odorant (**Fig. 3E1**), only the NAP-treated mice showed significantly more interest compared to their

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interest in the previous odorant (**Fig. 3E1- 3D3**, *P*<0.05), indicating significantly increased olfactory recognition memory as a consequence of NAP treatment.

Six months of NAP treatment increased levels of heat-stable soluble tau (**Fig. 4A**), while sarkosyl-insoluble, presumably abnormal, tau was unchanged (**Fig. 4B**). Quantitative analysis of the protein bands indicated that the NAP-related cognitive benefit after 6 months of treatment was associated with an increase of soluble tau; heat-stable soluble tau was significantly increased (by ~62%, *P*<0.05) (**Fig. 4C**), but sarkosyl-insoluble tau was unchanged (**Fig. 4D**), implicating a significant change in tau solubility as a consequence of NAP treatment.

An apparent decrease in tau phosphorylation at Ser202/Thr205 (~35%, *P*=0.067) was found in heat-stable soluble tau fraction (**Fig. 4E-F**) in the NAP-treated mice as compared to vehicle-treated mice. All other phosphorylation sites examined in this study were unchanged (*P*>0.10). Histological examination detected no changes in phosphorylated tau-bearing hippocampal neurons (data not shown). Histological examination is thought to represent the crude (total) tau fraction. Since the biochemical method did not detect significant changes in the phosphorylation levels of tau in the crude fraction, both biochemical and histological examinations were in agreement.

In contrast to results obtained with NAP treatment during the early pathological stage (9 → 12 months) which reduced Abeta content (Matsuoka, et al., 2007), and in agreement with results obtained after a shorter treatment at later stages (12 → 15 months, above), the levels of Abeta were not affected by 6 months of NAP treatment (12 → 18 months). Results for NAP treated mice compared to the vehicle-treated mice were as follows: soluble Abeta 1-40, 95 ± 13% (*P*=0.86); soluble Abeta 1-42, 93 ± 17% (*P*=0.76); total Abeta 1-40, 106 ± 13% (*P*=0.89); total Abeta 1-42, 87 ± 19% (*P*=0.81). As expected from the biochemical data, we did not find a difference in histologically-detected Abeta plaques between the vehicle- and NAP-treated mice after 6-months of treatment (data not shown).

Discussion

The current study shows for the first time, cognitive enhancement upon chronic intranasal NAP treatment in the aging 3xTg-AD mouse model in two different, independent paradigms. Furthermore, NAP treatment was significantly associated with changes in tau-related pathology. Specifically, we tested the potency of daily nasal administration of NAP (2 µg/mouse) for 3 or 6 months in the 3xTg-AD mouse model. Three months of treatment with NAP at an early-to-moderate stage of tauopathy (12 → 15 months of age) dramatically reduced the levels of phosphorylated tau (>70% for Ser202/Thr205 and Thr231) in protein fractions. Phosphorylated tau was also significantly reduced in the hippocampus when assessed by immunohistochemical methods. The impact on phosphorylated tau levels was most apparent in the heat-stable, soluble tau fraction, suggesting that NAP administration increased availability of tau for tubulin/microtubule interactions and reduced tau isoforms destined to form neurofibrillary tangle-like compromised conformational structures. NAP-related cognitive benefits on both spatial memory as well as olfactory discrimination were observed after 6 months of chronic application (12 → 18 months of age) and were associated with an increase of heat-stable soluble tau.

We previously tested a lower dose of NAP (0.5 µg/day/mouse) at an earlier pathological stage in the 3xTg-AD model (9 → 12 months of age), and found a significant ~40% reduction of phosphorylated tau and ~20% reduction of Abeta load after NAP treatment (Matsuoka, et al., 2007). Although the detailed mechanism is unknown, NAP is thought to modulate tau phosphorylation through interaction with neuronal and glial tubulin. As the pathology gradually increases with aging in the 3xTg AD model, we assumed that a higher dose of NAP might be required to alter tau pathology and behavioral outcomes in mice with more advanced pathology. For this reason, we used a higher NAP dose in the current studies, 2 µg/day/mouse.

A chronic three-month treatment (12 → 15 months of age) with the higher NAP dose significantly reduced the levels of phosphorylated tau and sarkosyl-insoluble abnormal tau. The most robust reduction was detected in Ser202/Thr205, a phosphorylation site on tau which was observed to respond to NAP treatment in previous studies (Gozes and Divinski, 2004; Matsuoka, et al., 2007; Vulih-Shultzman, et al., 2007). The reduction in tau phosphorylation at Ser202/Thr205 was maintained (although to a lower degree) also after the longer 6 months of treatment, under conditions of worsening pathology and increased sample variability (12 → 18 months of age). A recent study investigated the degree of tau phosphorylation in the cerebrospinal fluid (CSF) of AD patients. Levels of phosphorylated tau at Ser202/Thr205 (AT8 site) were significantly higher in AD patients compared with the non-demented control group; suggesting that tau phosphorylation at Ser202/Thr205 is associated with the presence of dementia in human AD cases (Maccioni, et al., 2006). Tau phosphorylation at Ser202/Thr205 antagonizes the tau-mediated nucleation of tubulin (Hashiguchi, et al., 2002), probably resulting in a paucity of microtubules leading to neuronal dysfunction and cognitive decline.

Although a direct head-to-head comparison is necessary to demonstrate dose-dependency, our experience across these two sets of studies is consistent with a NAP dose-related activity. This assumption is further corroborated by our preliminary study that indicates that 30 µg NAP/day/mouse may have a greater impact on the behavioral outcomes when compared head-to-head with a 2 µg/day/mouse regimen of treatment (data not shown).

The 2 µg/day/mouse NAP regimen used here, chronically applied for 6 months in the aged 3xTg-AD mice increased cognitive function as compared to vehicle treatment. These results extend and broaden previous studies using short-term treatment with NAP in other AD-related animal models. Thus, NAP enhanced spatial learning and memory in apolipoprotein E-deficient mice [apolipoprotein E is the most important risk factor for AD (Bassan, et al., 1999) and control apolipoprotein E-deficient mice show increased tau pathology (Gordon, et al.,

1996)]. Similar findings were also observed in rats exposed to the cholinotoxin, AF64A (Gozes, et al., 2000) that is associated with neuronal cell death and hence microtubule disruption.

Although there is a general consensus that reduction of hyperphosphorylated and conformationally abnormal tau will be beneficial, prior studies have not provided precise evidence linking specific forms of tau to cognitive dysfunction. More than 30 phosphorylation sites on tau have been identified (Ahlijanian, et al., 2000; Spittaels, et al., 2000), but the importance of each phosphorylation site is unclear. The current study may implicate Ser202/Thr205 as important for tau/microtubule involvement in cognitive functions and further suggests that the amount of soluble, presumably functional tau versus insoluble, presumably functionally impaired tau could be a useful index to evaluate the potency of tau therapeutic agents in tau models of cognitive deficits.

While the mechanisms are not fully understood, there is evidence that the Abeta and tau pathways influence each other and reduction of Abeta may be associated with reduced levels of phosphorylated tau in vivo (Oddo, et al., 2004). NAP treatment reduced phosphorylated tau levels and changed tau distribution throughout the course of the development of the pathology, without significantly altering Abeta load at later pathological states. As cognitive performance was also improved in NAP-treated mice and was associated with increased soluble tau, the current results suggest a correlation between tau pathology and cognitive function and support previous data that reduction of phosphorylated tau without altering Abeta load through genetic manipulation may lead to improvement of cognitive impairment (SantaCruz, et al., 2005). The results further imply that a significant pharmacological inhibition of tau pathology offers effective therapeutic benefits as exemplified by cognitive enhancement.

The NAP amino acid structure (NAPVSIPQ) has beta sheet breaker characteristics and NAP interferes with Abeta aggregation in vitro (Ashur-Fabian, et al., 2003). In our previous study using 3xTg-AD mice at an earlier pathological stage (9 → 12 months of age), we found that

three-month intranasal treatment significantly reduced the Abeta load (Matsuoka, et al., 2007), suggesting as indicated above, temporal impact of NAP on both the Abeta and the tau pathological hallmarks of AD at the early stages of the disease.

We administered NAP by intranasal drops. It should be noted that pharmacokinetic studies indicate that NAP enters the central nervous system through the blood, even with intranasal administration, i.e. NAP is found in the CSF concurrently with being found in the plasma regardless of the administration route (e.g., Gozes, et al., 2005; Morimoto et al., 2006). Measurements of NAP were conducted by a tandem liquid chromatography mass spectroscopy (LC-MS/MS method). In addition, preliminary data suggests beneficial effects of NAP on the habituation/dishabituation task when administered by IP injection (unpublished observation, Illana Gozes and Regina Pikman).

Initial memory formation and its short-term retention require the function of the olfactory bulb, the piriform and entorhinal cortices and the hippocampus. It is thus suggested that NAP treatment increased olfactory memory function rather than smell sensitivity (which may not be impaired in the tested 3xTg-AD mice). Normal control mice, young and aged (Frick, et al., 2000) behave in a similar manner to NAP-treated transgenic mice (data not shown). It is interesting to note that AD patients frequently have odor recognition pathology (Wilson, et al., 2007). Furthermore, deficits in olfactory detection thresholds and identification occur early in AD, before clinical symptoms are fully developed, and decline further over the course of the disease. High detection thresholds, together with impaired identification, may be useful as an early indicator of AD (Djordjevic, et al., 2007) in addition to being responsive to NAP administration. Interestingly, anosmia has been related to microtubule dysfunction (Kulaga, et al., 2004).

Previous studies have shown that NAP treatment transiently increases the amount of non-phosphosphorylated tau in a time course paralleling apparent increased microtubule stabilization (Gozes and Divinski, 2004) and that NAP treatment reduced tau

hyperphosphorylation and increased cognitive function in mice lacking its parent protein, activity-dependent neuroprotective protein (ADNP) (Vulih-Shultzman, et al., 2007). Taken together with the current data, these studies suggest that microtubule-targeting drugs may protect against tau pathology and cognitive decline. To date, two tubulin interacting agents, NAP and paclitaxel have been shown to reduce tau pathology in vivo. Paclitaxel was shown to diminish the NAP-tubulin interaction in vitro (Divinski, et al., 2006), suggesting that these two agents work, in part, through a related mechanism. While paclitaxel is a non-selective tubulin interacting agent and is associated with severe adverse effects in humans, NAP exhibits preferential interaction with neuronal tubulin (Divinski, et al., 2006) as well as brain bioavailability (Gozes, et al., 2000; Gozes, et al., 2005).

Finally, Phase I clinical testing has demonstrated the safety and tolerability of intranasally administered NAP (Gozes, et al., 2005). NAP may thus represent a prototype disease-modifying treatment for AD, targeting the major pathological hallmarks of the disease. The intranasal formulation of NAP (AL-108) is currently in phase II clinical trials in an AD program initially targeting elderly patients suffering from amnestic mild cognitive impairment (Allon Therapeutics Inc) (Melnikova, 2007).

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Ziga Elton (Elbaum) Laboratory for Molecular Neuroendocrinology, at Tel Aviv University. I.G. Serves as the Chief Scientific Officer of Allon Therapeutics Inc., Vancouver, Canada.

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Footnotes

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Legends for Figures

Figure 1. The neuronal tubulin interacting agent NAP reduced levels of phosphorylated tau in the crude tau fraction in AD transgenic mice.

After 3 months of intranasal treatment with NAP, levels of phosphorylated tau in the total tau fraction were examined. Typical protein bands were shown (**A-C**). Densitometry analysis indicates that phosphorylated tau levels were significantly reduced, ****P*<0.0001 (**D-F**). NAP treatment reduced phosphorylated tau-bearing neurons in the hippocampus compared to vehicle-treated mice (vehicle-treated, **G-I** vs NAP-treated, **J-L**). Bar=200 μm.

Figure 2. NAP-treatment reduced levels of phosphorylated tau in heat-stable soluble and sarkosyl-insoluble tau fractions.

After 3 months of intranasal treatment with NAP, levels of phosphorylated tau in the heat-stable soluble tau fraction were significantly reduced (**A-F**). Levels of phosphorylated tau at Ser202/Thr205 were also reduced in the sarkosyl-insoluble tau fraction (**G** and **J**). Reduction of phosphorylated tau at Thr231 was only marginally significant, *P*=0.06 (**H** and **K**), and phosphorylated tau at Ser202 was unchanged in the sarkosyl-insoluble tau fraction (**I** and **L**). Sarkosyl-insoluble total tau (non-phosphorylated and phosphorylated tau) was significantly reduced (**M** and **P**). Heat-stable soluble total tau showed a trend toward elevation not reaching significance (**M** and **O**). ****P*<0.001.

Figure 3. Six-month NAP-treatment improved cognitive impairment.

After 6 months treatment with NAP, effects on cognition were examined. NAP treated mice performed significantly better in the MWM (**A** and **B**) the habituation/dishabituation test (**C-E**) and as compared to vehicle-treated mice. **P*<0.05.

Figure 4. Heat-stable soluble tau was increased in association with cognitive benefit.

The levels of heat-stable soluble and sarkosyl-insoluble tau were examined after 6-months treatment with NAP. After 6 months treatment, the levels of heat-stable soluble tau were increased (**A**), while sarkosyl-insoluble tau was unchanged (**B**). Quantitative analysis indicated that the increase in heat-stable soluble tau was significant, $^{*}P<0.05$ (**C**), while the change in the sarkosyl-insoluble tau was not significant (**D**). Levels of phosphorylated tau at Ser202/Thr205 (AT8 site) was apparently reduced after 6-months NAP treatment compared to vehicle treatment ($^{\ddagger}P=0.067$, **E, F**).

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Figure 1

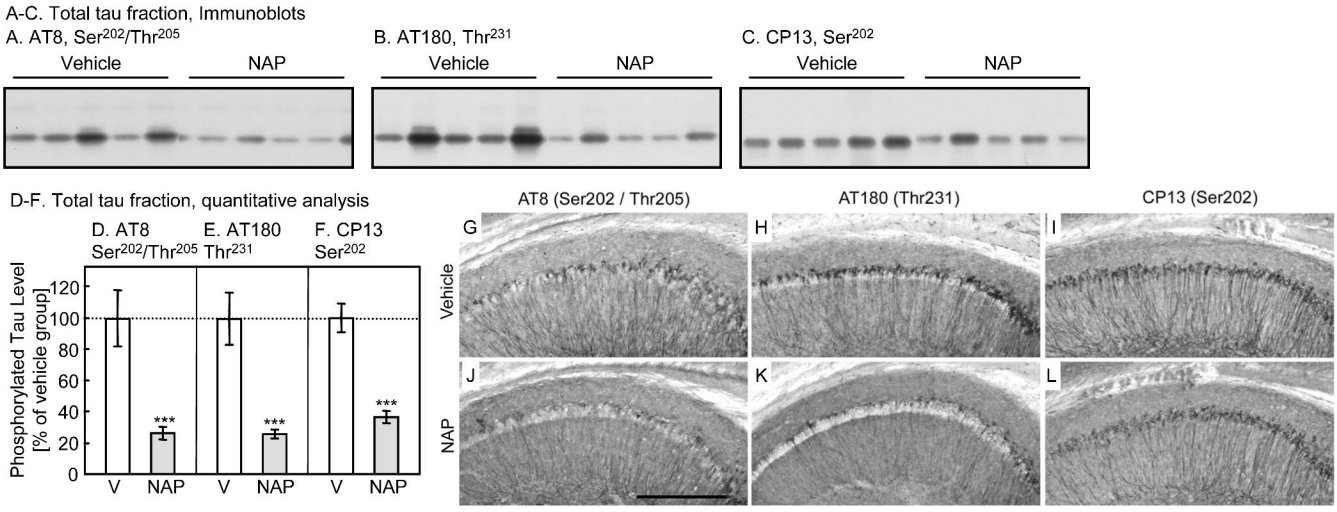

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Figure 2

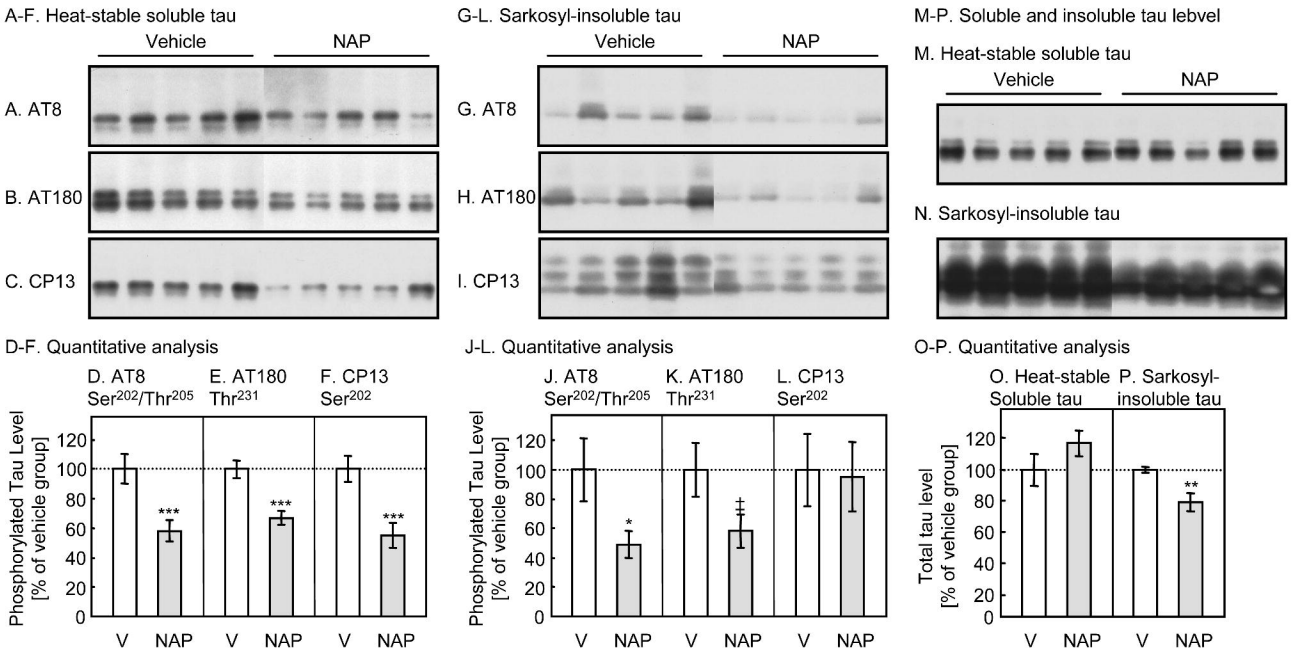

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Figure 3

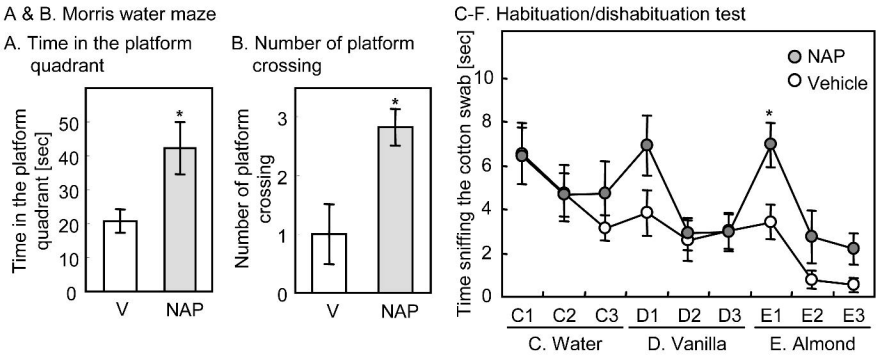

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Figure 4