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A pilot trial of the microtubule-interacting peptide (NAP) in mice overexpressing alpha-synuclein shows improvement in motor function and reduction of alpha-synuclein inclusions

Sheila M. Fleming,

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Caitlin K. Mulligan,

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Franziska Richter,

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Farzad Mortazavi,

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Vincent Lemesre,

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Carmen Frias,

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Chunni Zhu,

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Alistair Stewart,

Allon Therapeutics Inc. Vancouver BC V6B 2S2, Canada

Illana Gozes,

Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel; Allon Therapeutics Inc. Vancouver BC V6B 2S2, Canada

Bruce Morimoto, and

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Correspondence: Marie-Françoise Chesselet, M.D., Ph.D., Department of Neurology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, 310-267-1782, 310-267-1786 (fax), mchesselet@mednet.ucla.edu.

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AS (VP Commercial Research), BM (VP Drug Development and IG (Chief Scientific Officer) are employed/consult with Allon Therapeutics Inc. which is clinically developing NAP (generic name, davunetide).

Allon Therapeutics Inc. Vancouver BC V6B 2S2, Canada

Marie-Françoise Chesselet

Departments of Neurology and Neurobiology, The David Geffen School of Medicine at UCLA, 710 Westwood Plaza, Los Angeles, CA 90095-1769, USA

Abstract

Abnormal accumulation of α -synuclein is associated with several neurodegenerative disorders (synucleinopathies), including sporadic Parkinson's disease (PD). Genetic mutations and multiplication of α -synuclein cause familial forms of PD and polymorphisms in the α -synuclein gene are associated with PD risk. Overexpression of α -synuclein can impair essential functions within the cell such as microtubule-dependent transport, suggesting that compounds that act on the microtubule system may have therapeutic benefit for synucleinopathies. In this study, mice overexpressing human wildtype α -synuclein under the Thy1 promoter (Thy1-aSyn) and littermate wildtype control mice were administered daily the microtubule-interacting peptide NAPVSIPQ (NAP; also known as davunetide or AL-108) intranasally for two months starting at one month of age, in a regimen known to produce effective concentrations of the peptide in mouse brain. Motor performance, coordination, and activity were assessed at the end of treatment. Olfactory function, which is altered in PD, was measured one month later. Mice were sacrificed at 4.5 months of age, and their brains examined for proteinase K-resistant α -synuclein inclusions in the substantia nigra and olfactory bulb. NAP-treated Thy1-aSyn mice showed a 38% decrease in the number of errors per step in the challenging beam traversal test and a reduction in proteinase K-resistant α -synuclein inclusions in the substantia nigra compared to vehicle treated transgenics. The data indicate a significant behavioral benefit and a long lasting improvement of α -synuclein pathology following administration of a short term (2 month) NAP administration in a mouse model of synucleinopathy.

1. Introduction

The presynaptic protein α -synuclein accumulates intracellularly in Parkinson's disease (PD), dementia with Lewy bodies, and multiple system atrophy (MSA), diseases collectively known as synucleinopathies. In PD, α -synuclein-containing proteinaceous inclusions (Lewy bodies) are present in central and peripheral neurons (Spillantini et al., 1997; Braak et al. 2003). A direct role for α -synuclein in PD pathophysiology is strongly suggested by genetic evidence linking mutations, multiplications, and polymorphisms in the α -synuclein gene with familial and sporadic forms of PD (Cookson, 2009; Pankratz et al., 2009; Simon-Sanchez et al., 2009).

Multiple defects have been observed in cells overexpressing α -synuclein, including impaired endoplasmic reticulum-Golgi vesicular trafficking (Cooper et al., 2006). Microtubules are essential for vesicular movement and overexpression of α -synuclein in cells can lead to the disruption of microtubule-dependent trafficking (Lee et al., 2006). Conversely, impairments within the microtubule complex increase α -synuclein aggregation and toxicity (Kim et al., 2008). In a transgenic model of MSA, with α -synuclein inclusions in oligodendrocytes, α -synuclein binds to β -III tubulin leading to the accumulation of insoluble complexes and neuronal dysfunction, and this accumulation is suppressed by a microtubule depolymerizing compound (Nakayama et al., 2009). Thus, agents modulating the microtubule system may provide therapeutic benefits in synucleinopathies, including their most common form, PD.

NAPVSIPQ (NAP; also known as davunetide or AL-108) is a neuroprotective peptide derived from the activity-dependent neuroprotective protein (ADNP, Bassan et al., 1999) that interacts with both neuronal and glial tubulin to modulate microtubule assembly

(Divinski et al., 2004, 2006; Gozes and Divinski, 2004, 2007). Importantly, an interaction with β -III tubulin has been suggested (Divinski et al., 2006) and NAP protects neuronal cells in vitro against dopamine toxicity and severe oxidative stress, pathological mechanisms that likely contribute to PD (Offen et al., 2000).

Bioavailability and pharmacokinetic studies after intranasal administration with (3)H-labeled NAP show that it reaches the rodent brain, remains intact 30 min after administration, and dissipates 60 min after administration (Gozes et al., 2000). Similar results were obtained after intraperitoneal (Spong et al., 2001) or intravenous administration (Leker et al., 2002). A liquid-chromatography, mass spectrometry assay demonstrated that intact NAP reaches the brain after either intravenous or intranasal administration, in rat, dog and human. This was reviewed by Gozes et al. (2005) and recently updated to cite Phase II clinical results showing a positive impact on memory function in patients with amnesic mild cognitive impairment, a precursor to Alzheimer's disease, treated with intranasal NAP (davunetide) formulation (AL-108; Gozes et al., 2009). The bioavailability studies were extended to simultaneous measures in cerebrospinal fluid and plasma in rats as well as whole body autoradiography (Morimoto et al., 2009).

Intranasal NAP is also effective against brain pathology in mice. Indeed, NAP has been shown to reduce accumulation of amyloid peptide and, to a greater extent, tau pathology and improve cognitive function in a triple transgenic mouse model of Alzheimer's disease (Matsuoka et al., 2007, 2008). Similarly, in a mouse model of frontotemporal dementia with tau tangle-like formation, intranasal NAP treatment reduced the tau aggregate load (Shiryaev et al., 2009). NAP has also been shown to be beneficial in animal models of stroke (Leker et al., 2002) and fetal alcohol syndrome (Spong et al., 2001). These extensive studies indicate intranasal efficacy in the range of 0.5-30 micrograms of NAP per mouse (Gozes et al., 2005, 2009, Matsuoka et al., 2007, 2008 and unpublished data).

Here, we sought to determine whether NAP could improve behavioral and pathological anomalies in a mouse model of synucleinopathy. Mice overexpressing wildtype, human α -synuclein under the Thy1 promoter (Thy1-aSyn) show increased α -synuclein levels throughout the brain (Rockenstein et al., 2002) and develop proteinase K-resistant α -synuclein aggregates in several brain regions, including the substantia nigra and olfactory bulb (Fernagut et al., 2007; Fleming et al., 2008). Young Thy1-aSyn mice show progressive impairments in sensorimotor function and non-motor symptoms that are associated with the pre-manifest and early stages of PD such as deficits in olfaction, autonomic, digestive, and cognitive function (Fleming et al., 2004, 2008; Fleming and Chesselet, 2009; Wang et al., 2008). By 14 months, these mice show a 40% decrease in striatal dopamine and L-Dopa responsive behavioral deficits, indicating they reproduce canonical aspects of PD (Hean et al., 2010). While dopaminergic deficits occur at older ages, some of the early behavioral and pathological anomalies have high power to detect drug effects and provide powerful endpoint measures to assess potential treatments for α -synuclein-induced cellular dysfunction in younger mice. Here, NAP was tested for efficacy in Thy1-aSyn mice after intranasal administration at a dose known to produce effective drug levels in mouse brain.

2. Materials and Methods

2.1 Mice

Animal care was conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals, and procedures were approved by the Institutional Animal Care and Use Committee at the University of California Los Angeles (UCLA). Transgenic mice overexpressing human wildtype α -synuclein under the Thy-1 promoter (Thy1-aSyn) were developed previously and crossed into a hybrid C57BL/6-DBA/

2 background (Rockenstein et al., 2002). Animals were maintained on the hybrid C57BL/6-DBA/2 background by mating N7 female hemizygous for the transgene with male wildtype mice on the hybrid background obtained from Charles River Laboratories, Inc. (Wilmington, MA; Fleming et al., 2004, 2008; Fernagut et al., 2007). Male and female mice from the same litters were never bred together. As in our previous work, only male mice were used to avoid inconsistencies due to random inactivation of the x chromosomes (that harbors the transgene) in females. Male mice from 21 litters were included in the study and litter sizes ranged from 2-11 mice per litter. The genotype of all Thy1-aSyn and wildtype mice was verified by polymerase chain reaction (PCR) amplification analysis of tail DNA at the end of the experiment. Animals were maintained on a reverse light/dark cycle with lights off at 10 am and all testing was performed between 12- 4 pm during the dark cycle under low light. Food and water were available ad libitum except before and during buried pellet testing.

2.2 Intranasal Administration of NAP

The protocol for intranasal NAP administration followed previously published studies that show bioavailability and efficacy (e.g. Gozes et al., 2000, 2005 and 2009; Morimoto et al., 2009). The vehicle used for NAP administration was originally described by Alcalay et al., 2004 as detailed below. Vehicle rather than an irrelevant peptide was chosen as a control in this study based on extensive prior work showing lack of effect of scrambled or other control peptides compared to NAP in a variety of models in vivo and in vitro (Smith-Swintosky et al., 2005; Divinski et al., 2004; Gozes et al., 2000; Leker et al. 2002). The choice of the 2 microgram/mouse/day dose is based on extensive previous mouse model studies of intranasal efficacy which used doses in the range of 0.5-30 microgram per mouse/day (Gozes et al., 2005, 2009, Matsuoka et al., 2007, 2008 and unpublished data). For intranasal administration, NAP (0.4 mg/ml) was dissolved in sterile water containing 129 mM sodium chloride (7.5mg/mL), 8mM citric acid monohydrate (1.7mg/mL), 17 mM disodium phosphate dehydrate (3.0mg/mL) and 0.01% benzalkonium chloride (0.2mg/mL of a 50% solution). After addition of NAP, the pH of the dose formulation was adjusted to pH 5.0 ($\pm$ 0.5) using sodium hydroxide. Mice were administered 2 μ g per day in a volume of 5 μ l (2.5 μ l/nare/day) daily for 2 months. For intranasal administration, each mouse was cradled in a vertical position and the solution was applied using a pipette and tip (10 microliter tip). One droplet was released at the exterior naris and then absorbed by the mouse. This method has been extensively validated as producing reliable brain levels of the drug by investigators in the study.

Four groups were compared: wildtype + vehicle (n= 15); Thy1-aSyn + vehicle (n= 12); wildtype + NAP (n= 16), Thy1-aSyn + NAP (n= 13). The general condition of the mice was monitored daily during the two months of intranasal injections. Weights were measured daily and body temperature was measured weekly during drug administration.

2.3: Behavioral assessment

2.3.1. Challenging Beam Traversal—A primary end point measure for determining drug effects in this study was motor performance and coordination measured with the challenging beam traversal test adapted from Fleming et al. (2004). Although motor performance is often assessed by measuring movement along a narrow beam, this test is made more challenging by overlying a mesh grid on the beam, which allows the investigator to measure slips or “errors” per step taken by the mouse while traversing the beam. This measure is reliably altered in young Thy1-aSyn mice (Fleming et al. 2004, 2006); only 15 subjects are needed to detect a 50% effect on the first trial with 80% power. Briefly, the beam consists of four sections (25 cm each, 1 meter total length), each section having a different width. The beam starts at a width of 3.5 cm and gradually narrows to 0.5 cm by 1

cm increments. Animals were trained to traverse the length of the beam starting at the widest section and ending at the narrowest section. Animals received two days of training prior to testing; on the day of the test a mesh grid (one cm squares) of corresponding width was placed over the beam surface leaving approximately a one cm space between the grid and the beam surface. Animals were then videotaped while traversing the grid-surfaced beam and videotapes were rated on slow motion by an experimenter blind to genotype and drug condition. The number of errors per steps was measured for the first trial on the grid-surface beam for each mouse. In addition, the number of steps, and time to traverse the beam were measured although these are not reliably altered in young Thy1-aSyn mice, to insure that the drug did not have any unwanted effect on motor performance.

2.3.2 Pole Test—Additional motor behaviors are robustly altered in young Thy1-aSyn mice and were measured in this study. However, they were not considered primary measures because power analysis indicates that larger groups of animals would be required to detect significant drug effects (over 30 mice to detect a 50% effect on time to turn or descend in the pole test with 80% power). The pole test was used as previously described (Fleming et al., 2004). Briefly, animals were placed head upwards on top of a vertical wooden pole 50 cm long (1 cm in diameter). The base of the pole was placed in the home cage. When placed on the pole, animals orient themselves downward and descend the length of the pole back into their home cage. Thy1-aSyn and wildtype mice received two days of training that consisted of five trials for each session. On the test day, animals received five trials; time to orient downward (t-turn) and total time to descend (t-total) were measured for each trial. If a mouse fell off, slid down, or could not complete the task it was given a default score of 30 seconds for time to turn and 60 seconds to descend. The means of the 5 trials were used for analyses.

2.3.3 Spontaneous Activity—Similar to the pole test, spontaneous movements in a cylinder are reliably altered in Thy1-aSyn mice but the present study was not powered to detect a drug effect in this test, which requires 17 subjects to detect a 50% drug effect on hind limb impairment with 80% power. Although the study did not include large enough groups to detect drug-induced improvements in this test, it was also included to detect a potential worsening of the phenotype by the drug. Movements were measured in a small transparent cylinder (height, 15.5 cm, diameter, 12.7 cm; Fleming et al., 2004). The cylinder was placed on a piece of glass with a mirror positioned at an angle beneath the cylinder to allow a clear view of movements along the ground and walls of the cylinder. Videotapes were viewed and rated in slow motion by an experimenter blind to mouse genotype and drug condition. The number of rears, forelimb and hindlimb steps, and time spent grooming were measured.

2.3.4 Buried Pellet—The buried pellet test was performed as previously described (Fleming et al., 2008) except that only data from the first day of exposure to the food pellet were used to avoid compensatory strategies developed over repeated trials by the transgenic mice. Although the Thy1-aSyn mice showed deficits in several tests of olfactory function (Fleming et al. 2008), the buried pellet test has higher power to detect drug effects than other tests detecting deficiencies in the Thy1-aSyn mice. However, 20 subjects are needed to detect a 50% improvement with 80% power in trial 1. The olfactory testing was performed after completion of all motor tests, i.e. one month after cessation of drug administration. Briefly, mice were food restricted and maintained at ~90% body weight two days prior to and during testing. Food restricted mice were given 3-4 g of mouse chow per animal per day depending on weight and weights were monitored each day during food restriction. The surface pellet control test was performed one day after the buried pellet test. For the buried pellet test, a clean mouse cage (15 × 25 × 13 cm) was filled with 3 cm of clean bedding. One

piece of sweetened cereal (~250 mg; Cap'n Crunch®, Quaker, Chicago, IL) was buried along the perimeter of the cage approximately 0.5 cm below the bedding so that it was not visible. A mouse was then placed in the center of the cage and the latency to dig up and begin eating the cereal was measured using a stopwatch. After the trial, each animal was returned back to its homecage. If a mouse did not locate the food pellet within 5 minutes, the animal was removed, returned to its homecage, and given a score of 5 minutes. The bedding was changed between mice. The surface pellet test was set up in a similar way to the buried pellet test except that the piece of cereal was placed on top of the bedding.

2.4 α -Synuclein Immunohistochemistry

At 4.5 months of age (i.e. approximately 1.5 month after cessation of drug treatment) a subset of mice were deeply anesthetized with pentobarbital (100 mg/kg, ip) and intracardially perfused with 0.1M phosphate buffered saline (PBS) at room temperature followed by ice cold 4% paraformaldehyde. Brains were quickly removed, post-fixed for 2 hours in 4% paraformaldehyde, cryoprotected in 30% sucrose in 0.1M PBS, frozen on powdered dry ice and stored at -80°C . Free-floating coronal sections (40 μm thick) were collected for analysis. For assessment of insoluble α -synuclein aggregates, sections were washed in 0.1M PBS, incubated at room temperature for 10 minutes in 0.1M PBS containing 10 $\mu\text{g}/\text{mL}$ of Proteinase K (Invitrogen, Carlsbad, CA) and then washed with 0.1M PBS. An alternate set of sections did not receive Proteinase K treatment and were only washed in 0.1M PBS to assess overall staining for α -synuclein. All sections were incubated for 1 hour in a blocking solution containing 0.1M PBS and “mouse on mouse” blocking solution (Vector Laboratories, Burlingame, CA). Sections were then incubated overnight with a primary antibody that recognizes both mouse and human α -synuclein (4ml/mL mouse anti- α -synuclein, BD Biosciences, San Jose, CA), at 4°C in the presence of 2% normal goat serum. Sections were washed in 0.1M PBS followed by a 2-hour incubation with a biotinylated secondary antibody, goat anti-mouse IgG F(ab)₂ at room temperature in the presence of 2% normal goat serum. Sections were rinsed in 0.1M PBS and subsequently incubated in avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA) for 45 minutes and rinsed again in 0.1M PBS followed by an incubation in 0.05M TBS containing 3-3'-diamino benzidine (DAB; Sigma) and 0.3% H₂O₂ (Sigma) to reveal staining. Sections were rinsed with 0.1M PBS, mounted on gelatin coated slides, dehydrated, cleared with xylene and mounted with Eukit mounting medium (Calibrated Instruments, Hawthorne, NY).

2.5 Quantification of Proteinase K-Resistant α -Synuclein Inclusions

Two sections from both the substantia nigra and olfactory bulb that were stained for α -synuclein with proteinase K treatment were used for quantification of aggregates in each transgenic animal. Quantification was only performed on tissue from the transgenic mice because wildtype mice do not exhibit proteinase K-resistant aggregates of α -synuclein (Fernagut et al. 2007). Using the fractionator principle of stereology for unbiased population estimation, the contours of the substantia nigra and olfactory bulb were delineated at 5X objective using the Stereo Investigator software (MicroBrightField, Colchester, VT) coupled to a Leica DM-LB microscope with a Ludl XYZ motorized stage and z-axis microcator (MT12, Heidenheim, Traunreut, Germany). Following delineation of the regions, and to ensure no overlap of the sampling sites, as well as generation of an adequate number of sampling sites, the SRS layout of the substantia nigra and olfactory bulb was generated in Stereo Investigator (counting frame: 50 μm $\times$ 50 μm , and grid size set at 150 μm $\times$ 150 μm). Fifty-seventy images from each region in each animal were acquired using the “acquire SRS image series” function of the Stereo Investigator and using 100X oil objective with a delay of 5 seconds to adjust the focus manually prior to the acquisition of each image. Following the acquisition of images (in jpeg format from the Stereo Investigator) from both sides of the

substantia nigra, images were transformed to 8 bit files using ImageJ software (ImageJ software, version 1.38x, National Institutes of Health; <http://rsb.info.nih.gov/ij>). In order to perform the particle analysis in ImageJ and detect the necessary threshold, each picture was opened as a binary file and the contrast was enhanced by 0.5%. This step provides the visual cue for setting the threshold: the same image was also opened (in 8 bit format), and the threshold was manually adjusted in relation to the binary file (with enhanced contrast) to ensure the inclusion of all aggregates. Inclusions were defined by circularity (to avoid inclusion of dust or other artifact), and the number of aggregates as well as the percent area occupied by aggregates were generated by ImageJ.

2.6 Summary of Study Design

Behavioral Testing				
Daily NAP injections	Sensorimotor	Olfaction	Histology	
Age (months)				
1	2	3	4	4.5

2.7 Statistics

For motor performance and coordination, a 2×2 randomized design ANOVA was used to compare error per step scores, time to traverse, and number of steps on the challenging beam across genotype and drug treatment. Similarly, spontaneous activity in the cylinder was analyzed using a 2×2 randomized design ANOVA to compare mean number of rears, forelimb and hindlimb steps, and time spent grooming across genotype and drug treatment. Post hoc analysis for the challenging beam and spontaneous activity was done with Fisher's LSD. For the pole test, Thy1-aSyn and wildtype scores were compared using Mann-Whitney U. The latency to locate the pellet on the first trial and the latency to find the surface pellet were compared between wildtype and Thy1-aSyn mice using a Mann Whitney U test. For analysis of the quantification of proteinase K-resistant α -synuclein aggregates a Student's t-test was used to compare vehicle to drug treatment in Thy1-aSyn mice. All analyses were conducted with GB-STAT software (Dynamic Microsystems, Inc. Silver Spring, MD, 2000) for Macintosh. The level of significance was set at $p < 0.05$.

3. Results

In agreement with previous animal and human studies (Gozes et al., 2005; Gozes et al., 2009), the drug treatment was well tolerated throughout the experiment. As previously reported (Wang et al., 2008; Fleming et al., 2004), Thy1-aSyn mice did weigh less compared to wildtype mice but weights did not significantly differ between NAP and vehicle-treated groups. Body temperatures did not differ between genotypes or drug treatment (Table 1). No adverse reaction or difficulty were noted with the daily intranasal applications, which, based on previous in vivo and in vitro studies, leads to brain concentrations well within the effective dose (Matsuoka et al. 2008; Gozes et al., 2005; Gozes et al., 2009; Morimoto et al., 2009).

3.1 NAP improves motor deficits on the challenging beam

This study was designed to determine whether a short time intranasal treatment with NAP could improve the primary behavioral endpoint, i.e. the number of errors per steps on the challenging beam. Although a statistically significant improvement was detected with the average of five trials (Thy1-aSyn/Vehicle=0.62 $\pm$ 0.06, Thy1-aSyn/NAP= 0.49 $\pm$ 0.05), a larger effect was detected in the first trial, i.e. when Thy1-aSyn mice show the greatest

impairment. ANOVA indicated a significant effect of genotype $F(1,51)= 57.26$, $p<0.01$ for errors per step, with both NAP and vehicle-treated Thy1-aSyn mice making more errors per step compared to their respective wildtype controls ($p<0.01$). Importantly, post hoc analysis indicated that NAP-treated Thy1-aSyn mice made fewer errors per step compared to vehicle-treated Thy1-aSyn mice ($p<0.01$) (Figure 1). Thy1-aSyn mice treated with NAP showed a 38% improvement compared to vehicle-treated Thy1-aSyn mice.

3.2. Lack of adverse effects of NAP on motor behaviors in Thy1-aSyn mice

The number of steps and the time to traverse the beam were recorded to ensure that the drug did not have adverse effects on behavior, although they were not powered to detect improvements in this study. For steps, ANOVA revealed a significant main effect of genotype $F(1,52)= 33.85$, $p<0.01$ where both NAP and vehicle-treated Thy1-aSyn mice made fewer steps compared to wildtype controls ($p<0.01$; Fig. 2). There was no detectable effect of NAP treatment on the number of steps on the challenging beam. Time to traverse was not different between the groups ($p>0.05$) (Fig. 2).

Similarly, measures of performance on the pole and within the cylinder, that are profoundly altered in the Thy1-aSyn mice, were included to detect potential adverse effects, however the study was not powered to detect drug-induced improvements in these tests. As previously shown (Fleming et al. 2004, 2006), Mann-Whitney U indicated that Thy1-aSyn mice treated with vehicle showed a significant increase in the time to turn and time to descend the pole compared to their respective wildtype controls ($p<0.01$). The same effect was observed in Thy1-aSyn mice treated with NAP, and the treatment did not significantly affect either measure; ($p>0.05$) (Fig. 2). Behavior in the cylinder was measured to assess spontaneous activity in the mice, with spontaneous rearing, forelimb and hindlimb steps, and grooming being measured for three minutes. Rearing was markedly decreased in both NAP and vehicle-treated Thy1-aSyn mice compared to wildtype mice as revealed by ANOVA $F(1,52)= 6.07$, $p<0.05$. For forelimb stepping, ANOVA indicated a main effect of genotype $F(1, 52)= 10.51$, $p<0.01$ and post hoc analysis showed a significant reduction in the number of forelimb steps made by both NAP and vehicle-treated Thy1-aSyn mice compared to wildtype mice ($p<0.01$ and $p<0.05$, respectively). Similarly, a main effect of genotype $F(1,52)= 72.31$, $p<0.01$ was observed for hindlimb stepping, indicating a significant reduction in the number of hindlimb steps made by both NAP and vehicle-treated Thy1-aSyn mice compared to wildtype mice ($p<0.01$). Thy1-aSyn from both treatments spent less time grooming than wildtype controls with a main effect of genotype $F(1,52)= 10.33$, $p<0.01$. NAP treatment did not affect any of these measures (Fig. 3). Thus, NAP significantly improves motor deficits on the challenging beam and does not have detrimental effects on coordination or spontaneous activity.

3.3 Intranasal NAP treatment did not adversely affect olfaction

Thy1-aSyn mice show deficits in several tests of olfaction (Fleming et al. 2008). Because NAP was administered intranasally, a concern was that olfaction could be further impaired in transgenic mice, and/or altered in wildtype mice. Among the various tests showing olfactory deficits in young Thy1-aSyn mice (Fleming et al. 2008), the buried pellet test was used because it provides the highest power to detect drug effects, although the group sizes used in this study fell short of providing power to detect a 50% improvement in this test. Both NAP and vehicle-treated Thy1-aSyn mice took significantly longer to locate the buried pellet compared to WT mice (Mann Whitney U, $p<0.05$ and 0.01 , respectively) but this latency was not affected by the drug. Although not significant, NAP-treated wildtype mice had a tendency to find the pellet faster than vehicle-treated wildtype mice ($p=0.058$). Thy1-aSyn and wildtype mice did not differ in their ability to find the surface pellet indicating a similar interest, mobility, and attention for the pellet (Fig. 4).

3.4 NAP decreases proteinase K-resistant α -Synuclein aggregates in the Substantia Nigra

Analysis of sections of Thy1-aSyn mice stained for α -synuclein in the absence of proteinase K did not reveal any noticeable changes in intensity or distribution of the staining in NAP-treated compared to vehicle-treated mice (not shown). The presence of proteinase-K resistant α -synuclein aggregates in many brain regions was confirmed in the present study (Fernagut et al. 2007). These inclusions are difficult to detect in the cerebral cortex and the striatum where, if present at all, they are extremely small; however, abundant aggregates are present in the substantia nigra (Figure 5 A, C, E) and the olfactory bulb (Figures 5 B,D,F) but their size is much smaller in this region than in the substantia nigra. The number of proteinase K-resistant α -synuclein aggregates in the substantia nigra was slightly but significantly reduced in NAP-treated compared to vehicle-treated Thy1-aSyn mice ($p < 0.05$) 1.5 months after cessation of treatment (Figure 6A). The total surface area occupied by the inclusions in this region was also significantly reduced in NAP-treated Thy1-aSyn mice, indicating that the decreased number of aggregates was not due to a shift towards larger sizes (Figure 6B). In contrast, the number and surface area of the much smaller aggregates observed in the olfactory bulb did not differ at this age between NAP and vehicle-treated Thy1-aSyn mice (Figure 6 C,D). NAP did not alter cell morphology in either the substantia nigra or olfactory bulb (Fig. 5 E, F)

4. Discussion

In the present study, we show that intranasal administration of the microtubule-interacting peptide NAP for two months improves motor performance and coordination and reduces proteinase K-resistant α -synuclein inclusions in the substantia nigra of mice that overexpress wildtype human α -synuclein. NAP administration was well tolerated and had no detrimental effects on weight, body temperature, or behavior. This is the first study to demonstrate a beneficial effect of NAP in a mouse model of synucleinopathy and the data provide further support for a role of the microtubule system in synucleinopathies. Furthermore, these results indicate that early behavioral deficits and pathology can be improved by potential disease-modifying treatments in Thy1-aSyn mice.

Behavioral deficits similar to those previously reported in Thy1-aSyn mice were observed in this study including increased errors per step on the challenging beam, reduced spontaneous activity in the cylinder and increased time to turn and descend the pole (Fleming et al., 2004, 2006; Fernagut et al. 2007). These mice also show olfactory deficits as early as 3 months of age (Fleming et al. 2008), later digestive deficits (Wang et al. 2008), as well as other deficits in behavioral domains affected in PD (Fleming and Chesselet, 2009). Thy1-aSyn mice exhibit a loss of striatal dopamine and L-Dopa responsive deficits at 14 months of age (Hean et al. 2010). The early behavioral deficits chosen as end-point measures in this study occur when striatal dopamine levels are still normal (Hean et al. 2010) and they are distinct from “parkinsonism”, that requires loss of dopamine. However, they are extremely robust (approximately 7X increase in errors per step; 10 X increase in time to descend on the pole; 4X decrease in hindlimb stepping) and highly reproducible. These deficits provide evidence that overexpression of α -synuclein induces profound dysfunction in sensorimotor and olfactory behaviors from an early age in α -synuclein overexpressing mice. Although at the age examined Thy1-aSyn also present deficits in autonomic, circadian, affective, and cognitive function (Fleming and Chesselet, 2009 and unpublished observations), further supporting their use as a valid model of early stage “pre-manifest” PD, this study focused on deficits with the highest power to detect a drug effect and that could be used in a single cohort of mice.

Among the behavioral tests showing early deficits in Thy1-aSyn mice, errors per step on the challenging beam carries the highest power to detect a drug effect. We detected a 38%

improvement in errors per steps in transgenic mice treated with NAP compared to vehicle-treated mice. Based on our previous studies, this deficit is already apparent at 2 months of age in untreated mice, and progresses with age (Fleming et al. 2004). In the present study, mice were tested on the challenging beam immediately at the end of treatment, at three months of age. Therefore, it is possible that the main effect of the treatment was to prevent a worsening of the deficit rather than reverse it. It is also possible that NAP effects are due to a broad effect on motor performance, rather than a specific improvement of alpha-synuclein-mediated deficits. For example, NAP treatment has also been shown to enhance performance in the morris water maze in normal middle-aged rats (Gozes et al., 2002). This is unlikely to explain the beneficial effect observed in transgenic mice in this study, however, because NAP did not induce any significant effect in wildtype mice on any of the motor tests included here. Although the effect of NAP was modest, and limited to the behavioral measure with highest power to detect a drug effect, it is possible that a longer period of treatment could lead to greater improvement. In previous studies in the triple transgenic model of Alzheimer's disease, NAP was administered over varying lengths of time with behavioral improvement seen after 4.5 and 6 months of chronic treatment (Matsuoka et al., 2007, 2008; Shiryayev et al., 2009) in contrast to the short 2 month regimen used in this pilot study. In addition, larger cohorts will be necessary to detect drug-induced improvements in the broad range of behavioral deficits detected in these mice. Indeed, the number of mice included would have been too small to detect benefits in the pole or the cylinder of the magnitude observed on the challenging beam. The present data, however, clearly indicate that the drug produced a marked improvement in one high power measure of behavioral deficit and does not cause adverse effects on a variety of motor behaviors.

In addition to behavioral improvement, Thy1-aSyn mice administered NAP also showed a significant reduction in proteinase K-resistant α -synuclein inclusions in the substantia nigra. Importantly the area occupied by the inclusions showed a similar decrease, indicating that the decrease in number of inclusions is not offset by an increase in their size. In vitro NAP has also been shown to reduce β -amyloid aggregation (Ashur-Fabian et al., 2003). Although the role of α -synuclein inclusions in neuronal dysfunction is unclear, mutant and wildtype α -synuclein aggregates are associated with neuronal toxicity in vitro as well as in vivo (St. Martin et al., 2007; Kirik et al., 2003). However, the effect of NAP on aggregate size and behavior was modest and therefore it is not clear if the effects were mediated entirely by a decrease in α -synuclein aggregation or an effect on other parameters, for example α -synuclein clearance. Further, there is no data so far indicating whether reversal of α -synuclein aggregation would lead to behavioral improvements similar to those induced by NAP; this information may emerge from ongoing studies with antisense oligonucleotides directed to α -synuclein in the same model (Franich et al. 2010).

Interestingly, the effect of NAP in Thy1-aSyn mice appears to be long lasting since the decrease in inclusions was observed 1.5 months following cessation of NAP treatment. This is consistent with observations that NAP administration to ADNP heterozygous mice, a model of tauopathy, for two weeks resulted in reduced tau phosphorylation in the cortex 6 weeks after the cessation of NAP treatment (Vulih-Shultzman et al., 2007). A durable effect after treatment cessation is also seen in a Phase II study of NAP in the treatment of amnesic mild cognitive impairment (Gozes et al., 2009).

While NAP had a beneficial effect on sensorimotor function, it did not improve olfactory function, a behavior not powered to detect drug effect in the present study, or reduce α -synuclein inclusions in the olfactory bulb. However, despite the use of intranasal route, no worsening of olfactory function was seen either in wildtype or transgenic animals. Instead, a trend towards a NAP treatment effect was observed on olfaction in wildtype mice. Importantly, the concentration of NAP is unlikely to have been greater in the olfactory bulb

than the rest of the brain despite the use of intranasal administration. Whole body autoradiography with radio-labeled drug has shown general penetration of NAP into the brain but no accumulation in any one brain area. Furthermore, liquid chromatography mass spectrometry analysis showed no difference between intravenous and intranasal administration regarding potential accumulation in the olfactory system (Morimoto et al., 2009). Olfactory testing was performed when the mice were already ~6 weeks off the drug, while motor testing was performed at three months of age, just days after the last drug administration. Thus, potential olfactory benefits may have waned by the time this parameter was tested. Furthermore, olfactory deficits occur very early in PD, often years before the onset of motor symptoms, and do not progress with the course of disease (Ross et al., 2008; Doty, 2007). It is possible that the deficits were already established and irreversible by the time the treatment was started in the present study.

Based on previous extensive studies of NAP and control peptides, it is unlikely that NAP effect is nonspecific, because previous work utilizing scrambled or additional control peptides showed no effect both in vitro and in vivo (Smith-Swintosky et al., 2005; Divinski et al., 2004; Gozes et al., 2000; Leker et al., 2002), which led us to use vehicle alone as the control for NAP in the present study. We cannot exclude, however, that in this particular model, other peptides may have an effect. Mechanistically, NAP interacts with both neuronal and glial tubulin to promote microtubule assembly (Divinski et al., 2004, 2006; Gozes and Divinski, 2004). Although no direct measure of NAP's effect on microtubule function in Thy1-aSyn mice was performed in this study, previous work has shown NAP to be highly effective in reducing pathology related to the microtubule associated protein tau and amyloid in various genetic models (Matsuoka et al., 2007, 2008; Shiryayev et al., 2009; Vulih-Shultzman et al., 2007). The relationship between the microtubule system and α -synuclein is not as well defined as that of tau, however it does appear to be reciprocal. Specifically, tubulin can initiate and promote α -synuclein fibril formation whereas overexpression of α -synuclein has been shown to impair microtubule-dependent trafficking (Alim et al., 2002; Lee et al., 2006). In both cases, α -synuclein interaction with microtubules may start a cascade of events that lead to cellular dysfunction and ultimately cell loss. It is of particular interest that several recent genome-wide association studies have identified variations in MAPT, the gene encoding tau, as the second highest association with sporadic PD, just after α -synuclein (Simon-Sanchez et al., 2009). In this respect, we have shown that NAP reduced tau aggregate load in a model of tauopathy mimicking frontotemporal dementia (Shiryayev et al., 2009). In addition, amyloid beta has been shown to drive α -synuclein aggregation and NAP reduces amyloid beta load in vitro and in vivo (Masliah et al., 2001; Ashur-Fabian et al., 2003; Matsuoka et al., 2007). The present data provide a rationale for additional studies with longer treatments examining drug effects on the progression of behavioral deficits as well dopamine loss in the striatum in older mice (Hean et al., 2010).

5. Conclusion

The present study reveals a beneficial effect on sensorimotor function and reduced α -synuclein pathology following a short-term administration of the microtubule-interacting protein NAP. These findings point to a potentially important therapeutic target in PD and to the overall usefulness of the Thy1-aSyn mouse in preclinical drug studies. Given that the microtubule system is essential to overall cellular function it is not surprising that NAP has broad beneficial effects in a variety of disease models including cholinotoxicity, apolipoprotein E deficiency, stroke, and fetal alcohol syndrome and now a model of synucleinopathy (Gozes et al., 2000; Bassan et al., 1999; Leker et al., 2002; Spong et al., 2001). NAP is currently being tested in clinical trials for cognitive impairments showing significant efficacy in several tests of visual memory and minimal side effects indicating a

clear path toward advanced clinical testing (Gozes et al., 2009). Together these studies will help expedite the path from bench to bedside of this drug in PD and other synucleinopathies.

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Non-standard Abbreviations

Thy1-aSyn	mice overexpressing human wildtype alpha synuclein under the Thy1 promoter
NAP	microtubule assembly promoting peptide NAPVSIPQ

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Figure 1. Effect of NAP administration on motor performance and coordination in Thy1-aSyn and wildtype mice

Errors per step on the challenging beam in WT (Veh=15; NAP=16) and Thy1-aSyn (Veh=12; NAP=13) mice treated with intranasal Vehicle or NAP. Scores are $\pm$ SEM, ** represents $p < 0.01$ compared to respective WT and †† represents $p < 0.01$ compared to Thy1-aSyn Vehicle (2×2 ANOVA followed by Fisher's LSD).

A

B

C

D

Figure 2. No adverse effects of NAP administration on the challenging beam and pole tests in Thy1-aSyn and wildtype mice

Steps (A) and time to traverse (B) on the challenging beam and time to turn (C) and descend (D) the pole in WT (Veh=15; NAP=16) and Thy1-aSyn (Veh=12; NAP=13) mice treated with intranasal Vehicle or NAP. Scores are $\pm$ SEM, ** represents $p < 0.01$ compared to respective WT (2×2 ANOVA followed by Fisher's LSD (Beam) and Mann-Whitney U (Pole)).

B

D

Figure 3. No adverse effect of NAP administration on spontaneous activity in Thy1-aSyn and wildtype mice

Forelimb stepping (A), hindlimb stepping (B), rears (C), and grooming (D) in WT (Veh=15; NAP=16) and Thy1-aSyn (Veh=12; NAP=13) mice treated with intranasal Vehicle or NAP. Scores are $\pm$ SEM, *, ** represents $p < 0.05$, 0.001 compared to respective WT (2×2 ANOVA followed by Fisher's LSD).

Figure 4. No adverse effect of NAP administration on olfaction in Thy1-aSyn and wildtype mice Latency to find the pellet in the buried pellet (A) and surface pellet (B) test in WT (Veh=15; NAP=16) and Thy1-aSyn (Veh=12; NAP=13) mice treated with intranasal Vehicle or NAP. Scores are $\pm$ SEM, *, ** represents $p < 0.05$, 0.01 compared to respective WT (Mann-Whitney U).

Figure 5. Photomicrograph of Proteinase K-resistant α -synuclein inclusions in the substantia nigra and olfactory bulb in Thy1-aSyn mice

Proteinase K resistant α -synuclein staining in the substantia nigra (SN, A, C and E) and olfactory bulb (B, D and F) of Thy1-aSyn mice treated with vehicle (A, B C and D) or NAP (E and F). The contours of the SN (A) and glomerular layer of olfactory bulb (C) are marked by dotted lines. Higher magnification showing proteinase K resistant α -synuclein aggregates staining in the SN (C and E) and olfactory bulb (D and F) of Thy1-aSyn mice treated with vehicle (C and D) or NAP (E and F). Epl, external plexiform of olfactory bulb; Gl. Glomerular layer of olfactory bulb; ON, olfactory nerve layer. Scale bars in A, 100 μ m; B, 200 μ m; C-F, 30 μ m.

Figure 6. Effect of NAP on proteinase K-resistant α -synuclein aggregates in the substantia nigra and olfactory bulb

A, B: Average number (A) and percent surface area occupied by (B) proteinase K-resistant α -synuclein aggregates from two adjacent sections within the substantia nigra in Thy1-aSyn mice treated with vehicle (n=6) or NAP (n=7). C, D: Number (C) and percent surface area occupied (D) by proteinase K-resistant α -synuclein aggregates from one section within the glomerular layer of the olfactory bulb in Thy1-aSyn mice treated with vehicle (n=5) or NAP

(n=4). Scores are $\pm$ SEM, *, ** represents $p < 0.05$, 0.01 (respectively) compared to vehicle treated Thy1-aSyn mice (Student's t-test).

Table 1

Lack of effect of NAP on body weight and temperature

Age	Body Weight (g)					
	Vehicle			NAP		
	1 month	2 months	3 months	1 month	2 months	3 months
WT	16 ± 0.51	25 ± 0.55	29 ± 1.06	17 ± 0.40	26 ± 0.69	31 ± 1.03
Thy1	16 ± 0.74	24 ± 0.74	26 ± 0.86	15 ± 0.61	23 ± 0.68	26 ± 0.80

Age	Body Temp (°C)					
	Vehicle			NAP		
	1 month	2 months	3 months	1 month	2 months	3 months
WT	38 ± 0.19	38 ± 0.21	38 ± 0.12	38 ± 0.14	38 ± 0.16	38 ± 0.11
Thy1	38 ± 0.22	38 ± 0.22	38 ± 0.20	38 ± 0.22	38 ± 0.20	38 ± 0.16

Body weight and temperature in wildtype (WT) and Thy1-aSyn (Thy1) mice at the beginning of the experiment (1 month of age) and following two months of NAP or vehicle treatment (2 and 3 months of age).