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The Neuroprotective Peptide NAP Does Not Directly Affect Polymerization or Dynamics of Reconstituted Neural Microtubules

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Abstract

NAP (Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln) is a neuroprotective peptide that shows cognitive protection in patients with amnesic mild cognitive impairment, a precursor to Alzheimer's disease. NAP exhibits potent neuroprotective properties in several *in vivo* and cellular models of neural injury. While it has been found in many studies to affect microtubule assembly and/or stability in neuronal and glial cells at fM concentrations, it has remained unclear whether it acts directly or indirectly on tubulin or microtubules. We analyzed the effects of NAP (1 fM-1 μ M) on the assembly of reconstituted bovine brain microtubules *in vitro* and found that it did not significantly ($p < 0.05$) alter polymerization of either purified tubulin or of a mixture of tubulin and unfractionated microtubule-associated proteins. NAP also had no significant effect ($p < 0.05$) on the growing and shortening dynamics of steady-state microtubules at their plus ends, nor did it alter the polymerization or dynamics of microtubules assembled in the presence of 3-repeat or 4-repeat tau. Thus, the neuroprotective activity of NAP does not appear to involve a direct action on the polymerization or dynamics of purified tubulin or microtubules.

Keywords

microtubule dynamics; NAP; tau; tubulin polymerization

INTRODUCTION

NAP (Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln) is an octapeptide derived from activity-dependent neuroprotective protein (ADNP) that is being developed as a synthetic neuroprotective drug [1]. In a Phase IIa clinical study, NAP significantly improved measures of memory in patients diagnosed with amnesic cognitive impairment, a precursor to Alzheimer's disease, and is currently in a Phase II clinical trial for schizophrenia [2]. The octapeptide exhibits potent protection in several *in vivo* models of neural injury including those of frontotemporal dementia [3], Alzheimer's disease [4], fetal alcohol syndrome [5], and head trauma [6]. In these models, NAP improves memory, reduces learning and motor disabilities, and also prevents alcohol-induced fetal death and apoptosis. It also protects neuronal cells in culture from damage caused by oxidative stress [7], oxygen-glucose deprivation [8], amyloid- β (A β) [9,10], tetrodotoxin [11,12], HIV glycoprotein 120 [11], and cyanide toxicity [8].

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NAP appears to affect microtubule function in neuronal cells at very low concentrations. For example, at femtomolar concentrations it protected astrocytes exposed to cytotoxic zinc chloride in association with microtubule rearrangements [13]. Also, in neuron-enriched cell cultures treated with the microtubule-depolymerizing drug nocodazole, NAP protected the nocodazole-treated neurons by causing rapid repolymerization of the microtubules [14]. These effects have been suggested to be due to an interaction between NAP and microtubules/tubulin or microtubule associated proteins (MAPs), as NAP co localizes with neuronal microtubules [15]. Also, tubulin has been suggested as a NAP associating protein in neuronal cells since tubulin in cell extracts specifically binds to NAP-affinity columns [16]. The microtubule stabilizing activity of NAP in cells has also been reported in a cell-free system using microtubule associated protein (MAP)-rich tubulin [13].

Microtubules display two types of tightly controlled dynamic behaviors, treadmilling and dynamic instability, that are critical to their cellular functions. The most prominent is dynamic instability, which is the stochastic switching between growing and shortening phases at microtubule plus ends [17], while treadmilling is the unidirectional flux of tubulin with a net addition at one end and loss at the other end [18]. Microtubules in neurons are highly regulated to be either relatively stable or very dynamic based on their neuronal locations, their functional requirements and the stage of neuronal development. Relatively stable microtubules are normally associated with axons [19] and, when present in a neurite, can favor axon formation and neuronal polarization [20]. On the other hand, dynamic microtubules are required for growth cone steering in response to guidance cues, axon branching [19,21], axonal elongation [22], and polarized axonal transport of cellular cargo [23].

Since microtubule polymerization and dynamics play a critical role in neuronal function, it is reasonable to hypothesize that agents such as NAP might exert their neuroprotective effects by modulating microtubule polymerization dynamics. For example, paclitaxel, which suppresses microtubule dynamics [24], protected neurons against A β toxicity, perhaps by balancing the loss of microtubule stabilizing activity by defective tau action [25,26]. Tau is a stabilizing MAP that dissociates from microtubules when hyperphosphorylated and forms neurofibrillary tangles, a classical hallmark of Alzheimer's disease [27]. Alternative mRNA splicing of tau produces "3-repeat" (3R) or "4-repeat" (4R) tau isoforms with 3 or 4 microtubule binding repeats respectively. Both isoforms bind and stabilize microtubules though they differ in their binding affinities and the extent to which they regulate microtubule dynamics [28]. It is conceivable that the effects of NAP are mediated through an interaction with tau. Recently NAP has been reported to reduce tau hyperphosphorylation in animal models of Alzheimer's disease [4], frontotemporal dementia [3], and ADNP (its parent protein)-deficiency [29].

NAP crosses the blood brain barrier and exhibits strong neuroprotective effects and no toxicity making it potentially an ideal therapeutic intervention [1,15]. While its neuroprotective activity is considered to be due to an action on neuronal tubulin or microtubules, it remains unclear whether its actions occur through a direct effect on microtubule polymerization or dynamics or are exerted by an indirect effect mediated by cell signaling mechanisms or MAPs. Its effects on polymerization of tubulin in the presence and absence of MAPs or on microtubule dynamics have not been analyzed systematically.

To elucidate the potential interaction between NAP and purified neural tubulin, we analyzed the effects of NAP at concentrations between 10^{-15} and 10^{-6} M on the polymerization and dynamics of bovine brain tubulin devoid of MAPs *in vitro*. We also analyzed the ability of NAP to modulate polymerization or dynamics of microtubules assembled in the presence of unfractionated brain MAPs and purified short forms (with the shortest projection domains) of 3R tau and 4R tau *in vitro*. We found that NAP did not affect assembly into microtubules of either purified brain tubulin or MAP-rich tubulin and also did not affect *in vitro* dynamic

instability at the plus ends of microtubules assembled from purified tubulin. We further determined that NAP did not have any significant effect on 4R and 3R- tau induced microtubule assembly or dynamics *in vitro*. These results indicate that the action of NAP on microtubule assembly and stability in neurons does not involve a direct effect on microtubules assembled with purified tubulin or on microtubules assembled with bovine brain MAPs that co-polymerize with tubulin, or on microtubules in the presence the short forms of 3R or 4R tau. We suggest that NAP may exert its effects on microtubules in neurons and its neuroprotective effects by an indirect mechanism, perhaps involving signaling to the microtubule cytoskeleton.

MATERIALS AND METHODS

Materials

NAP was a kind gift from Allon Therapeutics Inc, Vancouver, Canada. NAP was dissolved in nanopure water and stored at -70°C. PIPES, MES, GTP, EGTA, cytochrome C, and all other chemicals were purchased from Sigma (St. Louis, MO) except for P11 cellulose phosphate (Whatman Inc., New Jersey), glutaraldehyde (Ernest F. Fullam, Inc., New York), uranyl acetate (Ted Pella, Inc., Redding, CA), and formvar carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA).

Purification of tubulin

MAP-rich tubulin consisting of ~70% tubulin and ~30% MAPs was isolated from fresh bovine brain by three cycles of polymerization and depolymerization without glycerol [30]. MAP-free tubulin (> 99% pure) was purified from MAP-rich tubulin by elution through phosphocellulose columns equilibrated in 50 mM PIPES, 1 mM EGTA, 1 mM MgSO₄, 0.1 mM GTP (pH 6.8); peak flow through fractions were collected; and the tubulin was drop-frozen in liquid nitrogen and stored at -70°C. Protein concentration throughout this work was determined by the Bradford assay [31] using bovine serum albumin as the standard.

Purification of tau isoforms

Expression vectors containing the human cDNA sequences for the shortest 3-repeat tau and 4-repeat tau isoforms were kind gifts from Dr. Kenneth Kosik (University of California, Santa Barbara). Tau was expressed in bacteria and HPLC- purified as described previously [32]. Purified tau was lyophilized and resuspended in BRB-80 buffer (80 mM PIPES, 1 mM EGTA, 1 mM MgSO₄ pH 6.8) with 0.1% β-mercaptoethanol [32]. The concentration of each tau sample was determined by SDS-PAGE comparison with a tau mass standard, the concentration of which was established by amino-acid analysis [28].

Microtubule polymerization and determination of polymer mass

To determine if NAP could stimulate assembly of purified MAP-free tubulin, the tubulin (4 μM) was mixed with different concentrations of NAP (1 fM–10 μM) in assembly buffer (100 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8 and 1 mM GTP) in the absence of any microtubule seeds that would initiate assembly. Paclitaxel (10 μM) was used as a positive control for microtubule assembly. For tau-assembled microtubules, purified tubulin (20 μM) was mixed with different concentrations of NAP (0.1 μM–10 μM) in assembly buffer containing 1 μM 4R tau or 3R tau that do not contain the alternatively-spliced N-terminal exons 2 and 3 (1:20 tau/tubulin), in the absence of “microtubule seeds”. In all cases, polymerization was monitored for 60 min at 350 nm as absorbance with a Beckman DU 640 Spectrophotometer at 30°C. Results are mean and SD of between one and seven experiments.

Three approaches were used to determine the effect of NAP on the microtubule mass. Microtubules were assembled from MAP-free tubulin in the presence of 3R or 4R tau as

described above or with “microtubule nucleating seeds” or using MAP-rich tubulin in the presence of different concentrations of NAP in assembly buffer for 60 min at 30°C. Polymerization of purified tubulin (27 μM) was initiated by “microtubule seeds” prepared from purified tubulin, 20% DMSO and 10% glycerol (1:6 dilution seeds/tubulin, final glycerol and DMSO concentration was 3.3% and 1.7 % respectively) while polymerization of MAP-rich tubulin (18 μM) was carried out in the absence of “microtubule seeds”. The microtubules were collected by centrifugation at $35,000 \times g$ for 60 min at 30°C, resuspended in assembly buffer and incubated overnight at 4°C to induce depolymerization and the protein content determined [31]. Results are mean and SD of between one and seven experiments.

Electron microscopy

MAP-rich tubulin (18 μM) was polymerized in the presence or absence of 1 μM NAP for 60 min as described above. Microtubules were fixed and negatively stained as described previously [33]. Microtubules were observed and digitally photographed with a JEOL 1230 transmission electron microscope at $3000 \times$ magnification (80 kV).

Determination of microtubule dynamic instability parameters in vitro

The dynamic instability behavior at plus ends of microtubules at steady state was analyzed by video-enhanced differential interference contrast (DIC) microscopy using an Olympus IX71 inverted microscope as described previously [34]. Tubulin (14.8 μM) was mixed with sea urchin axonemal seeds and polymerized to steady state (30 min, 30°C) in 87 mM PIPES, 36 mM MES, 1.4 mM MgCl_2 , 1 mM EDTA, 2 mM GTP, pH 6.8 in the absence or presence of 2 pM or 2 μM NAP. For studies with tau isoforms, tubulin (10.5 μM) was polymerized as above, in the presence of 1 μM NAP and 4R/3R tau (0.21 μM ; 1:50 tau/tubulin). Appropriate tau-containing controls without NAP were used in addition to a vehicle control. Microtubules were recorded for a maximum of 45 min after reaching steady state. The plus ends were distinguished from minus ends on the basis of their faster growth rates and longer time spent growing [33]. The positions of microtubule plus-ends were tracked and analyzed. The transition when microtubules switched from a phase of growth or attenuation to shortening was considered as “catastrophe” and the switch from shortening to growth or attenuation was considered as a “rescue” as described previously. Dynamicity was the rate of total measurable tubulin exchange at the microtubule ends (attributable to growing and shortening) [33]. Between 19 and 34 microtubules were analyzed for each experimental condition.

RESULTS

NAP does not affect the polymerization or lengths of microtubules assembled from tubulin

To examine if NAP could stimulate microtubule assembly, we used low concentrations of MAP-free tubulin (4 μM) at which microtubule assembly will not occur in the absence of “microtubule seeds” or stabilizing agents. The tubulin was mixed with a broad range of NAP concentrations (1 fM-10 μM) in the absence of “microtubule seeds” and polymerization was monitored turbidimetrically at 350 nm. NAP did not stimulate polymerization of tubulin at any concentration tested. For example, 1 fM NAP increased the light scattering signal at steady state (at 40 min) by an insignificant 3.4% (Fig. 1A) and 10 μM NAP decreased the light scattering by 11.9%. On the other hand, paclitaxel (10 μM) which was used as a positive control strongly stimulated microtubule assembly, increasing the absorbance by 11 fold from 0.02 in controls to 0.22 (at 40 min) (Fig. 1A). NAP also did not alter the polymerization kinetics of purified tubulin (27 μM) assembled with “microtubule seeds” or MAP-rich tubulin (18 μM) (data not shown). To examine the effect of NAP on the mass of assembled microtubule polymer, we pelleted the microtubules at $35,000 \times g$ and measured the protein content in the microtubule pellets. There was a decrease in the microtubule mass assembled from purified tubulin (27 μM) at different concentrations of NAP (0.1 %-10.9 %), but these decreases were not

significant at $p < 0.05$ when compared to controls (Fig. 1B). Similarly, NAP did not have any significant effect on the mass of microtubules assembled from MAP-rich tubulin (Fig. 1C). We also analyzed the possible effects of NAP on microtubule nucleation. If NAP could stimulate or suppress microtubule nucleation, the microtubule lengths would be expected to decrease or increase respectively. The lengths of the microtubules were not affected by NAP. Specifically, the mean microtubule length was $7.6 \pm 2.6 \mu\text{m}$ in untreated controls (Fig. 2A) and $7.4 \pm 3.1 \mu\text{m}$ in the presence of $1 \mu\text{M}$ NAP (Fig. 2B) ($n=200$). NAP also did not induce formation of any tubulin aggregates.

NAP does not modulate the dynamic instability behavior of microtubules made of purified tubulin

To determine whether NAP alters the dynamic instability of microtubules made from purified tubulin, purified MAP-free tubulin was assembled to steady state from axoneme seeds in the presence or absence of NAP, and length changes over time at plus ends were analyzed using DIC microscopy. Two different tubulin preparations (both at $14.8 \mu\text{M}$) were used due to lack of sufficient quantity of a single tubulin preparation for analyzing the effects of NAP at two different concentrations (2 pM and $2 \mu\text{M}$). Since the dynamic properties of microtubules can vary somewhat from preparation to preparation, we needed to run controls for each NAP data set. NAP at either concentration tested did not significantly ($p < 0.05$) alter any of the dynamic instability parameters (Table 1). Specifically, it did not significantly alter the mean growth rate, which was between 4.1 and $4.4 \mu\text{m}/\text{min}$ in all experimental conditions. At the highest concentration tested ($2 \mu\text{M}$), NAP increased the mean shortening rate by only 4.2% ; an amount that was not statistically significant. NAP also did not affect the catastrophe or rescue frequencies or the dynamicity. It decreased the time spent by microtubules growing by 3% and 2.6% at $2 \mu\text{M}$ and 2 pM respectively. The time spent shortening was increased by 1% by $2 \mu\text{M}$ NAP while 2 pM NAP decreased (0.3%) the same parameter. Dynamicity in the presence of 2 pM NAP was $1.7 \pm 0.1 \mu\text{m}/\text{min}$ when compared to $1.9 \pm 0.1 \mu\text{m}/\text{min}$ in controls. A 10^6 -fold increase in NAP concentration to $2 \mu\text{M}$ also did not alter the dynamicity.

NAP does not affect tau-induced microtubule assembly

Since NAP did not have any effect on polymerization of MAP-free tubulin or of a mixture of unfractionated brain MAPs, we next sought to test if its microtubule activity observed in neurons might be due to an effect on the assembly promoting activity of tau. We used both the shortest forms of 3R and 4R tau isoforms, which do not contain the alternatively-spliced N-terminal exons 2 and 3. In the absence of tau, NAP did not induce microtubule initiation or polymerization, as evaluated by both light scattering and sedimentation assays (data not shown). One μM NAP increased 3R tau-induced microtubule polymerization (60 min) by only 3.2% as determined by light scattering (Fig. 3A). There was no concentration dependent effect of NAP on 3R tau assembly with a 4 - 6% decrease in polymerization observed at $10 \mu\text{M}$ and $0.1 \mu\text{M}$ NAP. There was also no significant effect ($p < 0.05$) of NAP on the mass of microtubules assembled with 3R tau (Fig. 3B). Similarly NAP did not affect microtubule assembly induced by 4R tau. NAP ($1 \mu\text{M}$) increased the light scattering of microtubules assembled by 4R tau by only 0.6% and increased the protein concentration in the microtubule pellet from $7.05 \mu\text{M}$ in 4R tau control to $7.7 \mu\text{M}$ (data not shown). These results indicate that NAP does not alter polymerization of microtubules assembled in the presence of tau.

NAP does not alter the microtubule stabilizing properties of tau

Dynamic instability is strongly modulated by low concentrations of tau in the absence of significant effects on the mass of assembled microtubules [28,35]. Because the neuroprotective effects of NAP associated with microtubule assembly or organization in neurons might be mediated by an action of NAP on tau, we further evaluated the role of NAP ($1 \mu\text{M}$) on

microtubule dynamics at plus ends in the presence of 4R and 3R tau isoforms (0.21 μM). Both 3R tau and 4R tau suppressed the shortening rate of individual microtubules at steady state. For example, 3R tau decreased the shortening rate by 25.3% and 4R tau decreased it by 34.7% ($p < 0.05$ and $p < 0.001$ respectively when compared to controls). Previously, a similar decrease in the shortening rate by tau isoforms has been reported at near steady state conditions [28]. When NAP (1 μM) was added to tau-stabilized microtubules, it had no significant effect ($p < 0.05$) on any dynamic instability parameter (Table 2). NAP slightly decreased the mean growing and shortening rates in the presence of 3R tau from $2.5 \pm 0.3 \mu\text{m/min}$ and $7.1 \pm 0.6 \mu\text{m/min}$ to $2.3 \pm 0.3 \mu\text{m/min}$ and $6.4 \pm 0.4 \mu\text{m/min}$ respectively, but these decreases were not significant when we compared NAP plus 3R tau with the respective 3R tau group. NAP increased the time microtubules spent growing in the presence of 3R tau by 9.1%. But the overall dynamicity was unaffected when NAP was added along with 3R tau. The dynamicity was $0.9 \pm 0.1 \mu\text{m/min}$ in both conditions with 3R tau alone or 3R tau and NAP. Similarly NAP slightly affected 4R tau-modulated dynamic instability parameters, but these changes were also not statistically significant (Table 2). Specifically, there was a small increase in the mean growing rate in the presence of 4R tau, and a small decrease in the mean shortening rate. NAP also did not have any effect on the dynamicity. We conclude that NAP does not modulate the effect of the short forms of 3R and 4R tau on dynamics of microtubules made from of pure bovine tubulin.

DISCUSSION

Previous studies have indicated that the potent neuroprotective peptide, NAP, modulates microtubule organization and stability in neurons. It has been reported to interact with tubulin or microtubules, to co-localize with microtubules in PC12 cells [15], and to promote microtubule assembly at femtomolar concentrations in neurons, astrocytes and in cell free conditions with reconstituted tubulin [13,15]. The mechanism by which NAP affects microtubules in neuronal cells has remained unclear. Here we have analyzed the effects of NAP on the polymerization and dynamics of microtubules made from purified (> 99% purity) bovine brain tubulin, in the presence of unfractionated brain MAPs obtained by co-assembly beginning with crude brain extracts, and in the presence of short tau 3R and 4R isoforms.

We find that NAP did not affect the polymerization of MAP-free tubulin. NAP also had no effect on dynamic instability at plus ends of individual steady state microtubules assembled from purified brain tubulin, indicating that it does not directly affect the rates or extents of growth or shortening, the switching frequencies between growth and shortening, or overall dynamicity of the microtubules. We also did not detect any effects of NAP on polymerization of tubulin assembled with unfractionated brain MAPs, or the assembly or dynamic instability behavior of microtubules in the presence of short 3R or 4R tau isoforms.

The effects of NAP on microtubule organization in neuronal cells does not appear to occur by a direct action on tubulin

The effects of NAP on microtubule organization in neural cells occur at extremely low concentrations, in the femtomolar range. Because the concentration of tubulin in neural cells is in the micromolar range, it is difficult to imagine how NAP might act directly on tubulin itself because the NAP concentration would be so far below the tubulin concentration. The results described here indicate that NAP does not directly affect tubulin polymerization or the dynamics of individual microtubules made from purified tubulin over a broad range of NAP concentrations. It has been suggested [15] that NAP may exert its action in neurons by acting on the neuron specific class III β -tubulin isotype [35]. However, it seems unlikely that NAP exerts its action solely on the III β -tubulin isotype because the bovine brain tubulin preparation used in the present study contains ~25% β -III tubulin [36]. NAP is effective at much lower

concentrations (femtomolar) in astrocytes, which do not express β -III tubulin, than in neurons where picomolar concentrations are required to produce similar effects [37]. These findings suggest that NAP action could involve mediators/targets which are more abundant in glial cells than in neurons rather than a direct tubulin or β -III tubulin interaction.

The dynamic properties of microtubules play an important role in developing and mature neurons [19]. For example, the amount of stable polymer is higher in axons [19] and in contrast, microtubules near the growth cones are highly dynamic [19,21]. Reasoning that regulation of microtubule dynamics could contribute to neuroprotection, we examined the effect of NAP on dynamics of microtubules assembled from purified tubulin. We found that NAP did not significantly affect dynamic instability behavior at the plus ends of steady state microtubules made from tubulin devoid of detectable MAPs. The percentages of time the microtubules spent growing and shortening in the absence or presence of NAP were very similar. A very small, but not significant decrease in the mean growth rate was noted at 2 μ M NAP. NAP has been compared with paclitaxel for its microtubule stabilizing properties, but paclitaxel has very strong effects on both polymerization and dynamicity at plus ends at substoichiometric concentrations. Unlike NAP, paclitaxel suppresses dynamics and potentially suppresses microtubule shortening events at nanomolar concentrations [24].

Possible action of NAP through neural MAPs

Unfractionated brain MAPs—Previous experiments showed that NAP stimulates microtubule assembly at low concentrations and since all of these experiments included MAPs [13,14], another possibility is that NAP might mediate its effects through a neuronal MAP. However, we did not find any evidence in support of this possibility. Specifically, we examined the effects of NAP on polymerization of tubulin consisting of ~70% tubulin and 30% unfractionated brain MAPs obtained by co-assembly with microtubules through 3 successive cycles of assembly and disassembly. MAPs known to be present in the unfractionated mixture include tau, MAP1 and MAP2, and a large number of uncharacterized MAPs. These results indicate that NAP does not act through any of the MAPs that copolymerize with tubulin or these specific tau isoforms. While the possibility remains that NAP might affect microtubule assembly or organization in neurons by acting on a minor MAP, a different tau isotype than the ones analyzed here, or a post translationally modified MAP not present in the preparations, our results are most consistent with the conclusion that NAP's activity on microtubules in neuronal cells occurs through an indirect mechanism.

3R and 4R tau—As with purified tubulin, NAP did not alter the polymerization or dynamics of microtubules in the presence of the short forms of 3R and 4R tau at low ratios of tau to tubulin in the microtubules. Though NAP did not directly alter tau-mediated dynamics, it could also have an indirect positive effect on microtubule activity in cells by other effects on tau. For example, NAP's ability to increase non-phosphorylated tau as observed in astrocyte cultures [37] might enhance microtubule stabilizing activity in cells. NAP is also reported to reduce hyperphosphorylation of tau in *in vivo* models of tauopathy and Alzheimer's disease [3,4,29]. A decrease in hyperphosphorylation can increase tau binding to microtubules [27] leading to microtubule stabilizing function. However, NAP also exhibits neuroprotective effects in normal conditions not involving tau hyperphosphorylation, but rather involving developmental processes and plasticity. For example, it promotes neurite outgrowth and synaptophysin expression in cultured cortical and hippocampal cells [38]. Given these observations it is not clear if NAP's primary protective mechanism is modulation of cellular microtubule assembly or dynamics by reducing tau pathology.

NAP as a neuroprotective drug

From the results obtained in the present study, we can conclude that NAP does not alter microtubule polymerization or dynamics either directly or by interacting with tau in a cell-free system. However in cells, NAP might be indirectly involved in the assembly or dynamics of microtubules, as it acts on several targets such as amyloid- β [9] and MAP2 [8] in addition to tau [4]. Other mechanisms for the action of NAP have also been proposed such as activation of signaling pathways and possible nuclear regulation. For example, NAP activates MAP kinase/extracellular signal-regulated protein kinase and cAMP responsive element binding protein [39] and reduces the mRNA levels of proteins such as Mac-1 in closed head injury [40]. It also activates poly(ADP-ribose)polymerase-1 (PARP-1) in a similar manner to neurotrophins [41]. It is interesting to note that NAP is highly neuroprotective at very low concentrations. Peptides such as vasoactive intestinal peptide or pituitary adenylate cyclase-activating polypeptide also provide neuroprotection at pico- to femtomolar concentrations, but these peptides act through receptors and secondary mediators such as cytokines [42] while NAP penetrates and protects cells [15,43]. Further studies will be required to identify the primary neuroprotective mode of action of NAP since it has the potential to be an important neuroprotective drug.

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Figure 1.

NAP does not alter polymerization of purified tubulin. **A**) Highly polymerization-competent purified tubulin (>99 % pure, 4 μ M) was polymerized in the absence ($\square$) or presence of 1 fM (x), 1 pM (o), 10 μ M ($\blacktriangle$) NAP, or 10 μ M paclitaxel ($\blacklozenge$) at 30°C. Polymerization was monitored turbidimetrically by the change in absorbance at 350 nm. The arrow indicates addition of 10 μ M paclitaxel. **B**) Purified tubulin (27 μ M) was assembled with “microtubule seeds” in the presence or absence of NAP. Microtubules were sedimented at $35,000 \times g$ for 1 h at 30°C, depolymerized overnight at 4°C and the protein content determined by Bradford assay. Results are mean and SD of 3 experiments. **C**) The microtubules assembled from MAP-rich tubulin (18 μ M) in the absence or presence of NAP were pelleted and depolymerized at

4°C and the protein content determined (Materials and Methods). Error bars are very small for 1 fM NAP concentration. Results are mean and SD of 3 experiments.

Figure 2.

Effect of NAP on microtubule length distributions. Microtubule protein (MAPs plus tubulin, 18 μM tubulin) assembled in the presence or absence of NAP at steady state were fixed in 0.2% glutaraldehyde, and stained with 0.5 % uranyl acetate and analyzed by transmission electron microscopy (Materials and Methods). **A)** Distribution of lengths of control microtubules; mean microtubule length is 7.6 ± 2.6 μm; **B)** Distribution of lengths of microtubules assembled in the presence of 1 μM NAP; mean microtubule length is 7.4 ± 3.1 μm. The number of microtubules measured in each condition was 200.

Figure 3.

Effect of NAP on 3R tau induced microtubule assembly. **A)** MAP-free tubulin (20 μ M) was polymerized by 3R tau (1 μ M) in the absence or presence of different concentrations of NAP (0.1-10 μ M) and the reaction was monitored turbidimetrically at 350 nm at 30°C. Results are mean and SD of between 3-7 experiments. **B)** Microtubules assembled by 3R tau in the absence or presence of NAP were collected by sedimentation at 35000 $\times$ g for 1 h at 30°C. The microtubules were depolymerized overnight at 4°C and the protein content was determined [31]. Control = No tau. Results are mean and SD of between 3 and 7 experiments.

Table 1Effect of NAP on *in vitro* steady state dynamics at plus ends of microtubules

Parameter (Units)	Control for 2 pM NAP	NAP 2 pM	Control for 2 μ M NAP	NAP 2 μ M
Growth Rate (μ m/min)	4.4 $\pm$ 0.3	4.4 $\pm$ 0.3	4.4 $\pm$ 0.2	4.1 $\pm$ 0.2
Shortening Rate (μ m/min)	10.7 $\pm$ 0.6	10.7 $\pm$ 0.6	9.6 $\pm$ 0.8	10.0 $\pm$ 0.7
Percentage of time growing	41.6	39.0	21.5	18.5
Percentage of time shortening	6.1	5.8	11.1	12.1
Percentage of time attenuated	52.3	55.2	67.4	69.3
Catastrophe Frequency (per min)	0.3 $\pm$ 0.0	0.3 $\pm$ 0.0	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1
Rescue Frequency (per min)	5.4 $\pm$ 0.1	5.4 $\pm$ 0.6	8.0 $\pm$ 0.6	6.5 $\pm$ 0.7
Dynamicity (μ m/min)	1.9 $\pm$ 0.1	1.7 $\pm$ 0.1	1.4 $\pm$ 0.1	1.3 $\pm$ 0.1

Values are expressed as mean $\pm$ SEM. There were no statistically significant differences between microtubules assembled with NAP and their corresponding control microtubules assembled in the absence of NAP ($p < 0.05$ by Students t-test). Tests of significance were not done on transition frequencies and dynamicity which are overall variables. Results are from 28-30 individual microtubules tracked for 10 min for each condition.

Steady state *in vitro* dynamics at plus ends of microtubules in the presence and absence of 3R or 4R Tau and NAP

Table 2

Parameter (Units)	Control	3R Tau	3R Tau + NAP	4R Tau	4R Tau + NAP
Growth Rate ($\mu\text{m}/\text{min}$)	3.4 ± 0.6	2.5 ± 0.3	2.3 ± 0.3	3.1 ± 0.4	4.0 ± 0.5
Shortening Rate ($\mu\text{m}/\text{min}$)	9.5 ± 0.8	7.1 ± 0.6	6.4 ± 0.4	6.2 ± 0.4	5.7 ± 0.4
Percentage of time growing	42.4	32.7	41.8	36.8	28.8
Percentage of time shortening	9.5	8.4	8.0	9.3	9.1
Percentage of time attenuated	48.1	59.0	50.1	53.9	62.2
Catastrophe Frequency (per min)	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Rescue Frequency (per min)	2.0 ± 0.3	2.1 ± 0.3	2.1 ± 0.3	2.3 ± 0.3	2.4 ± 0.4
Dynamicsity ($\mu\text{m}/\text{min}$)	1.2 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.1 ± 0.1

Values are expressed as mean $\pm$ SEM. The tau: tubulin molar ratio was 1:50. The NAP concentration was $1 \mu\text{M}$. Comparisons were made between Tau (4R/3R) and the respective Tau (4R/3R) + NAP group. Statistical significance was analyzed at $p < 0.05$ by Students t-test and no significant difference was observed. Tests of significance were not done on transition frequencies and dynamicsity which are overall variables. Results are from 19-34 individual microtubules tracked for 10 min for each condition.