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The effect of tachykinin neuropeptides on amyloid β aggregation

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ABSTRACT

A hallmark of Alzheimer's disease is production of amyloid β peptides resulting from aberrant cleavage of the amyloid precursor protein. Amyloid β assembles into fibrils under physiological conditions, through formation of neurotoxic intermediate oligomers. Tachykinin peptides are known to affect amyloid β neurotoxicity in cells. To understand the mechanism of this effect, we studied how tachykinins affect Aβ(1–40) aggregation *in vitro*. Fibrils grown in the presence of tachykinins exhibited reduced thioflavin T (ThT) fluorescence, while their morphology, observed in transmission electron microscopy (TEM), did not alter. Cross linking studies revealed that the distribution of low molecular weight species was not affected by tachykinins. Our results suggest that there may be a specific interaction between tachykinins and Aβ(1–40) that allows them to co-assemble. This effect may explain the reduction of Aβ(1–40) neurotoxicity in cells treated with tachykinins.

1. Introduction

Peptides and proteins convert under some conditions from their native soluble state into highly ordered fibrillar aggregates. Such transitions are associated with numerous illnesses such as Parkinson's, Huntington's, and Alzheimer's diseases (AD) [1]. AD is a common neurodegenerative disorder, which affects mainly the elderly. Deposition of insoluble fibrous masses in the brain, termed amyloid plaques, is one of the main characteristics of AD. The major constituents of amyloid plaques are fibrils formed from amyloid beta (Aβ), which is a 38–43 residue peptide derived from the amyloid precursor protein [2]. Aβ is in dynamic equilibrium between soluble monomers and insoluble fibrils [3,4]. The aggregation pathway involves formation of intermediate oligomers that elongate to form fibrils. These oligomers are believed to be the major neurotoxic species [5]. Existing anti-AD drugs are able only to give temporal relief to the symptoms of the disease, and none of these drugs are able to alter the aggregation process of Aβ. Currently efforts are aimed at designing new drugs that will reduce neurotoxicity caused by Aβ oligomers by inhibiting Aβ aggregation, inhibiting Aβ formation, or increasing Aβ degradation [6].

Tachykinins are a family of neuropeptides that possess the common C-terminal sequence: Phe–X–Gly–Leu–Met–NH_2, where X is an aromatic or aliphatic residue [7]. Tachykinins reverse the neurotoxic effect of Aβ(1–40) in cultured neurons, and play a role in neurodegenerative diseases [8–10]. Nevertheless, the mechanism by which they affect Aβ neurotoxicity is still not understood. Here we studied the mechanism by which the tachykinins substance P, neurokinin A, neurokinin B and physalaemin affect Aβ (1–40) aggregation, and whether they physically interact with Aβ(1–40) *in vitro*. Our data suggests that tachykinins co-assemble with Aβ to form fibrils with a morphology that is similar to the pure Aβ fibrils.

2. Materials and methods

2.1. Materials

Aβ(1–40) was purchased from Sigma–Aldrich (Rehovot, Israel). Tachykinin peptides were synthesized on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA), or on a Liberty Microwave-Assisted peptide synthesizer (MAPS, CEM), using standard Fmoc chemistry. Peptides that do not include Trp or Tyr in their original sequence were labeled with Trp at their C-termini for UV spectroscopy (see Table 1 for sequences). The peptides were purified on a Gilson HPLC (Middleton, WI) with a reverse phase C8 semipreparative column (ACE) with a gradient of acetonitrile in water (both containing 0.01% (v/v) trifluoroacetic acid). The specific gradient was optimized for each peptide separately. Peptide concentrations were determined by using a UV spectrophotometer (Shimadzu, Kyoto, Japan).

Abbreviations: Aβ, Amyloid β; AD, Alzheimer's disease; PICUP, photo-induced cross linking of unmodified proteins; CD, circular dichroism; ThT, thioflavinT; TEM, transmission electron microscopy; HFIP, hexafluoroisopropanol; PBS, phosphate buffer saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LMW, low molecular weight.

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Table 1
Sequences of the tachykinin peptides used in this study.

Neurokinin A + Trp (NKAW)	HKTDSFVGKMW
Neurokinin B + Trp (NKBW)	DMHDFFVGLMW
Substance P + Trp (SPW)	RPKPQQFFGLMW
Physalaemin	pEADPNKFYGLM*

* pE denotes pyroglutamic acid.

2.2. Aβ(1–40) pretreatment and sample preparation

Prior to experiments Aβ(1–40) was dissolved in hexafluoroisopropanol (HFIP) to a concentration of 1 mg/ml and incubated at room temperature for at least 4 h, to remove any pre-existing aggregates. HFIP was then removed by evaporation under a gentle stream of nitrogen gas. The remaining peptide film was dissolved in 50 mM phosphate buffer (PBS), at pH 7.5, containing 0.02 M NaCl and 0.01% NaN_3, and bath sonicated for 5 min, to remove small aggregates that may have formed during dissolution. The peptide concentration was determined using UV spectroscopy, according to the absorbance of the single tyrosine residue at position 10 in the sequence of Aβ(1–40). Lack of ThT fluorescence at $t = 0$ shows that there were indeed no preformed fibrils in the solution prior to our experiments.

2.3. Thioflavin T (ThT) fluorescence spectroscopy

A solution of 80 μM Aβ(1–40) was prepared in PBS. 5 mM ThT stock solutions were prepared by dissolving ThT in 50 mM glycine–NaOH buffer at pH 8.5. On the day of the experiment, ThT stock was diluted to 4 μM with glycine–NaOH buffer. Tachykinin stock solutions were freshly prepared for each experiment, by dissolving the lyophilized peptide in 50 mM PBS at pH 7.5 containing 0.01% NaN_3, and adjusting the peptide concentration. The ThT and neuropeptides were added ca. 20 min after dissolution of Aβ(1–40) in PBS buffer. Experiments were performed on a spectrofluorimeter (Tecan, Maennedorf, Switzerland). All experiments were conducted in a 96-well plate (Nunc). Reagents were added to each well in the following order: 25 μl ThT, 50 μl of 50 mM PBS buffer or tachykinin solution and then 25 μl of Aβ(1–40). Final concentrations of ThT and Aβ were always 1 μM and 20 μM, respectively. Tachykinin concentration varied. Measurements were performed at 37 °C, with a 10 min interval between successive measurements. The plate was shaken for 5 min between measurements. Emission at 485 nm following excitation at 450 nm was monitored for up to 40 h.

2.4. Circular dichroism (CD) spectroscopy

A solution of 100 μM Aβ(1–40) was prepared. At selected time points, CD spectra were recorded at ambient room temperature by using a CD J-810 spectropolarimeter (Jasco, Easton, MD) with a 100-QS 1 mm path-length quartz cuvette (Hellma, Müllheim, Germany). Recordings were taken at 0.5 nm intervals in the spectral range 200–260 nm. The sample was incubated at 37 °C between measurements, without shaking.

2.5. Transmission Electron microscopy (TEM)

Preparation of samples for electron microscopy was as previously described [11]. Briefly, five microliter of the sample to be imaged were spotted on a glow-discharged, carbon-coated grid (Electron Microscopy Sciences, Hatfield, PA) for 2 min, stained with 0.5% (w/v) aqueous uranyl acetate for 20–30 s, and then washed with three drops of distilled water. Uranyl acetate solutions were filtered through 0.2-μm sterile syringe filters before use. TEM analysis was performed using a Tecnai G^2 Spirit Twin T-12, 120 kV (FEI, Netherlands), equipped with a 4 K FEI Eagle CCD camera.

2.6. Photo-induced cross linking of unmodified proteins (PICUP)

Solutions of 20 μM Aβ(1–40) in the presence or absence of tachykinins at a 1:1 molar ratio (at which significant ThT effects were observed) were prepared and incubated at 37 °C, with or without shaking. At selected time points an aliquot of 25 μl was taken from each sample and centrifuged at 16,800 g for 30 min. Then, 18 μl of the supernatant were added to 1 μl of 1 mM Tris(2,2′-bipyridyl)dichlororuthenium(II) and 1 μl of 20 mM ammonium persulfate in 10 mM sodium phosphate at pH 7.4. The mixture was irradiated for 60 s with visible light by exposure to a 150-watt halogen lamp at 23 cm distance and the reaction was terminated with 5 μl of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer with β-mercaptoethanol as a quencher (50% (v/v) glycerol, 1% (w/v) SDS, 25% 0.5 M TrisHCl pH 6.8 + 0.4% SDS, 25% (v/v) β-mercaptoethanol and traces of bromo phenol blue). The cross-linked oligomer mixtures were fractionated by SDS–PAGE on 16% tricine gels. The gels were visualized by silver staining (SilverQuest, Invitrogen, Carlsbad, CA, USA).

3. Results

3.1. Aβ(1–40) exhibits a random coil to β-sheet transition

To establish conditions for monitoring the fibrillation of Aβ(1–40), the CD spectrum of Aβ(1–40) was measured as a function of time as an indication for its secondary structure. Aβ(1–40) was initially in a random coil conformation when dissolved in buffer. As fibrils formed, a continuous conformational shift towards extended β-sheet was observed. This shift is indicated by the decrease in elipticity at ca. 220 nm, and the increase in elipticity at 200 nm (Fig. 1a). The β-sheet minimum is usually at 215 nm, but it has been previously shown that β-sheets formed by Aβ may exhibit a shift of the minimum toward higher wavelengths [12]. These results are in agreement with earlier studies [12] and indicate that Aβ(1–40) fibrils are formed under the conditions used.

3.2. Aβ(1–40) fibril morphology was not affected by the presence of tachykinins

To study the effect of the tachykinin peptides on the Aβ(1–40) fibrils, TEM was used. Samples of 20 μM Aβ(1–40) alone or in the presence of the tachykinins at a 1:1 M ratio were prepared and incubated at 37 °C. After 15 days grids were prepared for TEM measurements as described. The presence of the tachykinins had no significant effect on the structure of the fibrils formed, within the resolution of our images (Fig. 1b–f). The average diameter of the observed fibrils was 12 ± 2 nm, regardless of tachykinin presence. The variations in length are typical for Aβ(1–40) fibrils. Images of the tachykinins alone were also taken, showing that no apparent structure was developed (data not shown).

3.3. The presence of tachykinin peptides caused a reduction in ThT fluorescence at equilibrium

To test whether the tachykinins have an effect on the kinetics of Aβ(1–40) aggregation, ThT fluorescence was monitored. Sigmoidal curves representing the constitutive fibrillation kinetics of Aβ(1–40) were observed (Fig. 2). Neurokinin B, neurokinin A and substance P did not affect the lag time but lowered the emission amplitude, at equilibrium, in a concentration dependent manner.

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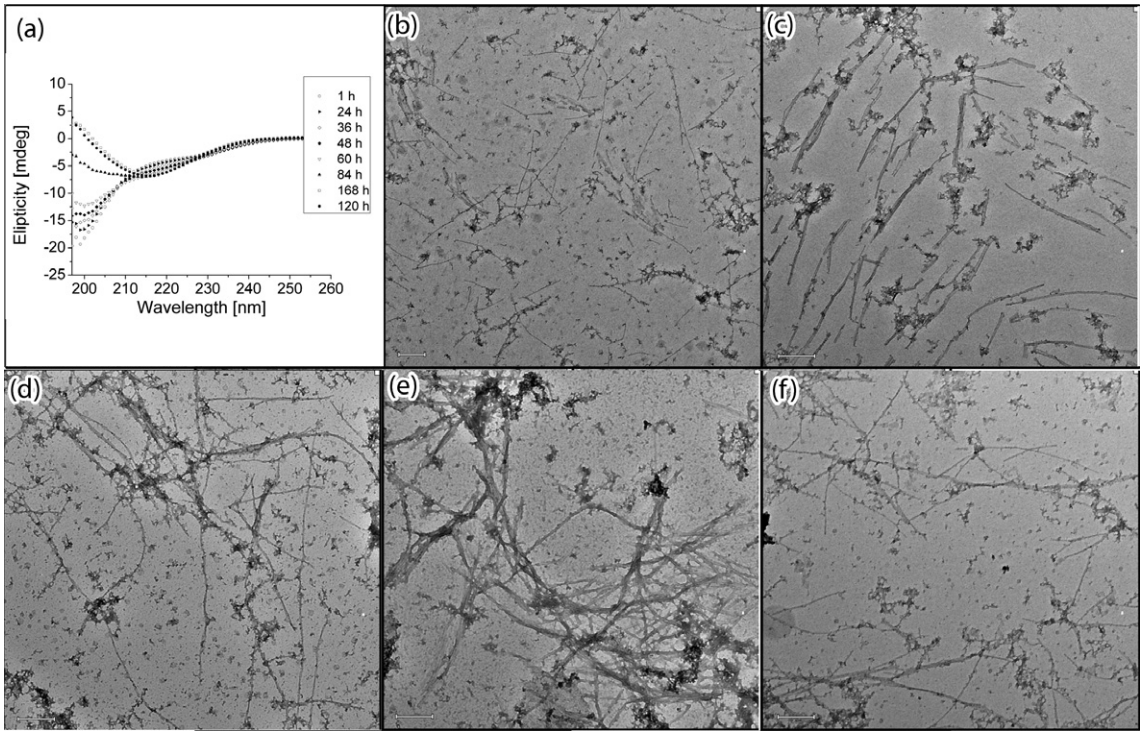

Fig. 1. (a) CD spectra of Aβ(1–40) at different time points during the aggregation process; 100 μM Aβ(1–40) were incubated at 37 ° and CD spectra were recorded at the times indicated in the figure; TEM images 15 days after 20 μM Aβ(1–40) were assembled alone (b); with 20 μM NKBW (c); with 20 μM NKAW (d); with 20 μM SPW (e); or with 20 μM physalaemin (f); Scale bars equal 200 nm.

This result is consistent with a specific interaction between these neuropeptides and Aβ(1–40). The lower emission amplitude resulted from fewer ThT molecules that could bind the mature Aβ(1–40) fibrils in the presence of these peptides. The effect was particularly significant when 1:1 molar ratio was exceeded. Due to difficulties in obtaining reproducible results, we could not obtain reliable data for the kinetics in the presence of physalaemin. Since the kinetics of fibril formation was monitored indirectly, the observations can be interpreted in a number of ways: (i) Formation of fewer fibrils (ii) Reduced affinity of ThT to the fibrils, or (iii) Competition of the tachykinins with the ThT on amyloid binding.

3.4. Tachykinins exhibited no significant effect on low molecular weight species of Aβ(1–40)

Reduced ThT fluorescence in the presence of tachykinin peptides could in principle result from a reduction in the amount of fibrils. In such a case, we could expect to find a higher concentration of monomers or oligomers at equilibrium, due to a shift of the equilibrium. To test this hypothesis, we used PICUP (Fig. 3) [13], and monitored the distribution of the low molecular weight (LMW) species of Aβ(1–40) during the aggregation reaction. Bands corresponding to monomers, dimers, and trimers of Aβ(1–40) were visualized. Precipitation of the LMW species was observed as equilibrium was approached. The migration distances of the monomers, dimers and trimers were not affected by the presence of the neuropeptides, indicating that they did not participate in the cross linking reaction. The neuropeptides themselves were not visualized in any of the samples, probably due to their small size (1–1.5 KDa) that enabled them to diffuse out through the pores of the gel. NKAW and physalaemin did not change the amount of LMW species of Aβ(1–40) during the lag phase. Samples with Aβ(1–40) + NKBW exhibited relatively poor silver staining development, but seemed to show the same trend. Due to the early precipitation of samples containing Aβ(1–40) + SPW, the effect of SPW

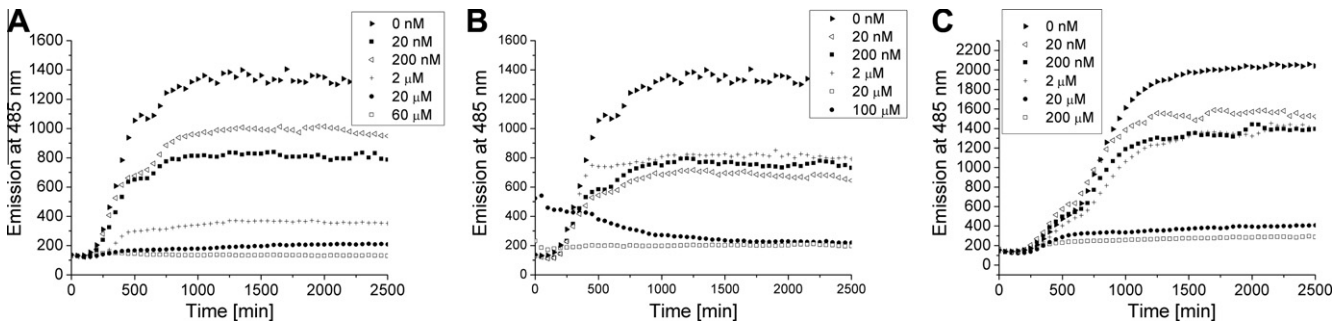

Fig. 2. ThT fluorescence as a function of time of Aβ(1–40) alone or in the presence of different concentrations of SPW (A); NKBW (B); or NKAW (C); $t = 0$ indicates the time of Aβ(1–40) dissolution in the working buffer (see text). The neuropeptide concentrations are indicated next to each curve.

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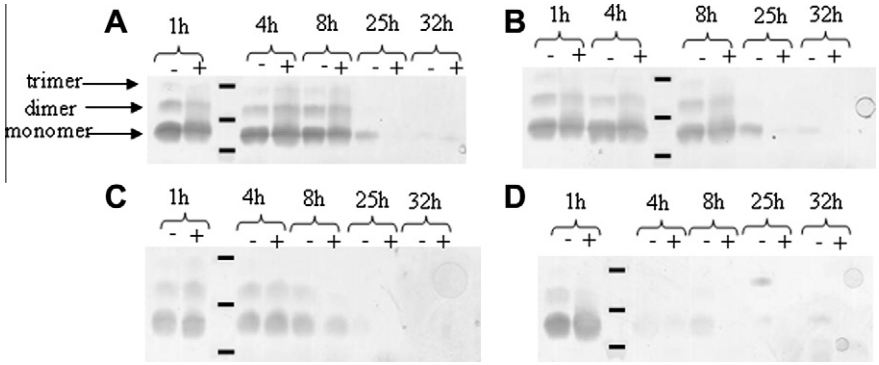

Fig. 3. Silver stained gels of 20 μM Aβ(1–40) with 20 μM NKAW (A); 20 μM physalaemin (B); 20 μM NKBW (C); or 20 μM SPW (D); (−) corresponds to samples containing Aβ(1–40) alone; (+) corresponds to samples of Aβ(1–40) in the presence of the tachykinin; Protein markers (in black) correspond to 3, 6 and 14 kDa. Each sample contained 25% glycine–NaOH buffer at pH 8.5 and samples were shaken for 5 min every 10 min in order to match the conditions used for ThT fluorescence experiments.

on the LMW species could not be interpreted. The Aβ(1–40) monomer concentration at equilibrium dropped to a level that was barely detectable by silver staining, both in the presence and in the absence of the tachykinin peptides.

4. Discussion

Our results demonstrate that NKBW, NKAW and SPW have a significant effect on the ThT fluorescence of Aβ(1–40) fibrils at equilibrium, suggesting that a specific interaction between the peptides and Aβ(1–40) may exist.

TEM images revealed fibrils in all the samples that contained Aβ(1–40) and neuropeptides, although at the same molar ratios little or no ThT fluorescence was observed. This observation demonstrates that lower ThT fluorescence does not necessarily indicate attenuation of Aβ(1–40) aggregation. Small structural variations, at the molecular level (that cannot be detected by TEM), however, remained to be discovered. The lower ThT fluorescence is probably not due to a direct interaction between the tachykinins and ThT. Such interaction, which could alter the binding of ThT to Aβ(1–40), is unlikely. In solution, the benzothiazole and benzaminic rings of the ThT molecule rotate freely around a shared C–C bond. Upon binding to amyloid fibrils, this free rotation is inhibited, resulting in a blue shift of the excitation and emission peak wavelengths and increased quantum yield. ThT molecules were shown to bind to the peripheral site of the acetylcholine esterase enzyme (AChE), and a similar spectral shift was observed upon binding [14]. It is reasonable to assume that if ThT interacts with the neuropeptides its rotational freedom should be at least partially inhibited and shift its spectral properties. We tested this hypothesis and found no change in the absorbance spectrum of ThT in the presence of increasing concentrations of NKAW and physalaemin (Fig. 4), suggesting that there is no interaction between the tachykinins and ThT.

A possible interpretation to the reduction in ThT fluorescence is that less Aβ(1–40) monomers were incorporated into fibrils. If the tachykinins had a significant effect on the stability of the fibrils, we would expect the concentration of monomers, at equilibrium, to be significantly higher in their presence, due to a shift in the oligomerization equilibrium of Aβ(1–40). PICUP results demonstrated that when equilibrium was reached, bands corresponding to Aβ(1–40) monomers were barely visible, regardless of the presence or absence of tachykinins. These results are consistent with the TEM results that showed formation of fibrils even in samples that showed no ThT fluorescence. We note, however, that TEM is imited since it is not a bulk technique, and additional studies may improve our statistics and further support our conclusion that the amount of fibrils is unchanged following tachykinin treatment. Nonetheless, our results demonstrate that there is little or no shift in the equilibrium of the LMW Aβ(1–40) species due to the presence of tachykinin peptides. We therefore infer that the effect of the tachykinins on the ThT fluorescenc at equilibrium cannot be explained by a reduction in fibril formation.

Based on our ThT fluorescence, TEM and PICUP data we propose that the tachykinins may co-assemble with Aβ(1–40) monomers to form heterogeneous fibrils. The tachykinins used in this study contain amino acids that have been shown to contribute to amyloidogenicity [15]. Aromatic amino acids such as tryptophan, tyrosine and phenylalanine have the highest intrinsic propensity for aggregation. The sequences of the tachykinins each contain at least two aromatic residues (Table 1). These residues may contribute to strong aromatic interactions with Aβ(1–40). The diphenylalanine that exists in the central hydrophobic core of Aβ(1–40) has been previously shown to be crucial to fibrillogenesis [16]. This effect was attributed to an important role of π-stacking interactions in the aggregation process [17], or to other properties of the aromatic moieties, such as their hydrophobicity [18]. The sequence similarity between Aβ(25–35) fragment and tachykinin peptides also strengthens our hypothesis (Table 2). A heterogeneous fibril may possess reduced affinity for ThT binding because ThT exhibits different binding affinities to fibrils that differ morphologically [19]. Another possibility arises from the suggestion that ThT interaction with β-sheets is mediated by docking onto surfaces formed by a single tyrosine ladder, consisting of at least 4–6 contiguous tyrosine residues [20]. Aβ(1–40) contains a tyrosine in position 10 and the tyrosine residues are in close proximity in the assembled fibril. If the tachykinins indeed co-assemble with Aβ(1–40), the tyrosine ladders should be formed from less than four tyrosine residues at a time. This effect is expected to become more significant as the concentration of the tachykinin grows, as observed. ThT binding is most significantly reduced when the molar ratio exceeds 1:1, meaning that there is more of the tachykinin peptide than Aβ(1–40). In this case, the probability for the formation of a ladder containing over four tyrosine residues is obviously very small. Our proposed model also provides an explanation to the TEM and PICUP results: Mixtures of tachykinins and Aβ(1–40) monomers at a 1:1 M ratio formed fibrils, even though at this molar ratio ThT fluorescence was markedly reduced, and the tachykinins showed no detectable effect on the distribution of the Aβ(1–40) LMW species.

It has been reported that the neurotoxicity caused by Aβ(1–40) was reduced in cells treated with tachykinins [9]. Our data provides evidence that a specific interaction is formed between tachykinins and Aβ(1–40). Deeper understanding of this interaction requires further studies. Nevertheless, this interaction

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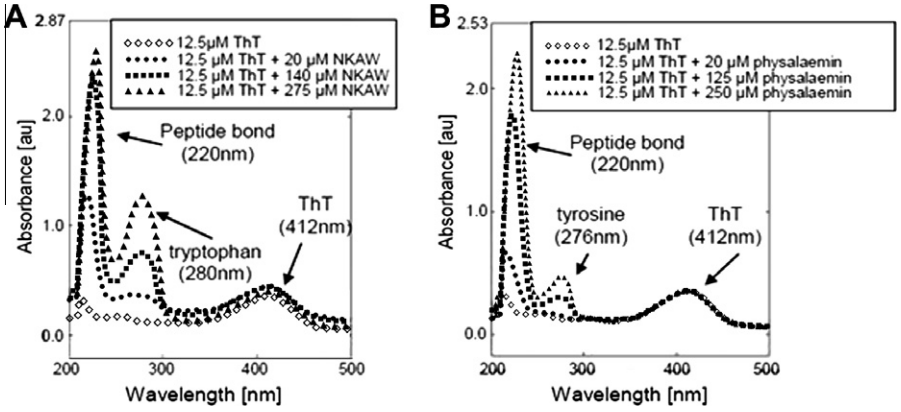

Fig. 4. Absorption spectra of 12.5 μM ThT in the presence of increasing concentrations of NKAW (A) and physalaemin (B). The absorbance of ThT is unchanged, suggesting no interaction with the tachykinins.

Table 2
Sequences of Aβ(1–25) and tachykinin peptides. Identical residues and conserved changes are in bold [9].

Peptide	Sequence
Aβ(25–35)	G**S**NK**G**A**II**G**LM**
Neurokinin B	DM**HD**F**FV**G**LM**
Neurokinin A	H**K**TDS**FV**G**LM**
Substance P	R**PK**PQQ**FF**G**LM**
Eledoisin	E**PSK**D**A**F**IG**L**M**
Kassinin	DV**PK**SDQ**FV**G**LM**
Physalaemin	pEADPNK**FY**G**LM***

* pE denotes pyroglutamic acid.

may explain the reduced Aβ(1–40) neurotoxicity in cells treated with tachykinins. The design of peptides that are able to specifically interact with Aβ and disrupt its neurotoxicity may serve as a strategy for developing anti-Alzheimer's lead compounds.

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