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Research report

β -Amyloid(Phe(SO₃H)²⁴)25–35 in rat nucleus basalis induces behavioral dysfunctions, impairs learning and memory and disrupts cortical cholinergic innervation

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Abstract

Long-term behavioral effects, changes in learning and memory functions and aberrations of cholinergic fibers projecting to the parietal cortex were investigated after bilateral injections of β -amyloid(Phe(SO₃H)²⁴)25–35 peptide in rat nucleus basalis magnocellularis (*nbn*). The β -amyloid peptide used in these experiments contained the original β -amyloid 25–35 sequence which was coupled to a phenylalanine-sulphonate group at position 24. This additional residue serves as a protective cap on the molecule without influencing its neurotoxic properties and results in water-solubility, stability and low rates of peptide metabolism. In this paper, home cage, locomotor and open-field activities, passive shock-avoidance and 'Morris' water maze learning abilities were assessed throughout a 35-day survival period. Subsequently, acetylcholinesterase (AChE) histochemistry was used to visualize alterations of parietal cortical cholinergic innervation. In response to the neurotoxic action of β -amyloid(Phe(SO₃H)²⁴)25–35, a progressive hyperactivity developed in the rats in their home cages which were maintained throughout the 5-week post-injection period. This was accompanied by a significant hypoactivity in the novel environment of a locomotor arena. β -Amyloid(Phe(SO₃H)²⁴)25–35-treated animals showed greatly impaired cortical memory functions in the step-through passive shock-avoidance paradigm, while spatial learning processes remained unaffected. Moreover, β -amyloid(Phe(SO₃H)²⁴)25–35 injections in the nucleus basalis suppressed explorative behavior in rats and inhibited conditioned stress responses 28 days after surgery. Reductions of cortical cholinergic (AChE-positive) projections provided anatomical substrate for the behavioral changes. This indicated extensive, long-lasting neurodegenerative processes as a result of β -amyloid(Phe(SO₃H)²⁴)25–35 infusion. © 1998 Elsevier Science B.V.

Keywords: Acetylcholinesterase; β -Amyloid neurotoxicity; Behavior; Cholinergic system; Learning and memory; Parietal cortex

1. Introduction

One of the specific neuropathological hallmarks of Alzheimer's disease (AD) is the extensive extracellular aggregation and deposition of β -amyloid peptides (β AP), forming neuritic plaques and cerebrovascular

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deposits [41]. Senile plaque-forming β AP consist of 42 amino acid residues in mature form [13]. These are minor fragments of the large, transmembrane β -amyloid precursor protein (APP), located within the extracellular and membrane-spanning domains [17] of this receptor-like cell surface molecule [21]. In an aberrant proteolytic cleavage process [8] APP liberates various potentially amyloidogenic β AP fragments differing in distribution and in physiological and chemical properties [2,12,25,38]. While β AP are also produced during the normal cell metabolism [16], their physiological effects are very controversial. While β -amyloid fragments exert neurotrophic properties under certain conditions in vitro [47,48], ample evidence has recently accumulated on the direct neurotoxicity of β AP [1,6,7,18,19,24,25,43] occurring in a dose-dependent manner [48]. It has also been demonstrated that β AP enhance glutamate neurotoxicity and thus render neurons vulnerable to excitotoxic [31] or metabolic insults [22]. Moreover, selective cholinergic effects of the β -amyloid_(1–42) peptide (β AP_(1–42)) were demonstrated both in vitro [40] and in vivo [18]. Relative preservation of neurons containing Ca^{2+} -binding proteins after application of multiple β AP were observed. Changes of intracellular free Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) after β AP exposure [29–31] were also reported and indicates that a severe derangement of the intracellular Ca^{2+} homeostasis is pivotal in β -amyloid mediated neurotoxicity.

The search for amino acid sequences, critical for the neurotoxic mechanism of β AP, revealed that the 'active core' of the protein is localized within the 25–35 (β AP_(25–35)) [12,38,40,48] and possibly within the 31–35 sequence [38] of the peptide. Adjacent amino acid regions such as 1–24 and 36–42, respectively, may either lend conformational stability to the protein [12] or influence its solubility [2] and perhaps direct exoprotease cleavage. Giovannelli et al. [12] have recently reported that both histopathologic symptoms and behavioral dysfunctioning decreased 2 weeks after β AP_(25–35) injections, in the nucleus basalis magnocellularis (*nbm*) of rats, while β AP_(1–40) permanently altered higher brain functions and affected cholinergic neurotransmission.

The *nbm* plays a critical role in cognitive and memory functions [3], as shown in several behavioral tasks [35,45,46], by the persistent regulation of higher cortical processes [3]. Moreover, cholinergic neurons of the basal forebrain complex are particularly susceptible to excitotoxic injury and undergo rapid neurodegeneration both in rats [6,18,19,26] and humans [3]. Earlier papers have shown that the cortical cholinergic hypofunction, which is characteristic of AD, is primarily due to degeneration of the *nbm* [3] that maintains extensive cholinergic projections to cortical structures, such as the parietal cortex [6,26–28].

The aim of this paper was to determine behavioral and histochemical alterations after bilateral *nbm* lesions elicited by β AP in rats throughout a 5-week survival period (Fig. 1). The peptide containing the β AP_(25–35) sequence coupled with a phenylalaninesulphonate (Phe(SO₃H)) residue at position 24 (β AP(Phe(SO₃H)²⁴)_{25–35}) was used to determine whether β AP conformation, the rate of the peptide metabolism or water-solubility might play a principal role in β -amyloid mediated neurotoxicity. The additional Phe(SO₃H) group serves as a protective cap on the molecule, presumably reducing exoprotease cleavage but without influencing its neurotoxic potential (Penke et al., unpublished data). Similar modifications of other neuropeptides, such as pentagastrin or cholecystokinin octapeptide, were extensively used to study long-lasting effects of these proteins [23].

2. Materials and methods

2.1. Peptide synthesis

β AP(Phe(SO₃H)²⁴)_{25–35} was synthesized [18,19] with slight modifications. Peptide synthesis was ini-

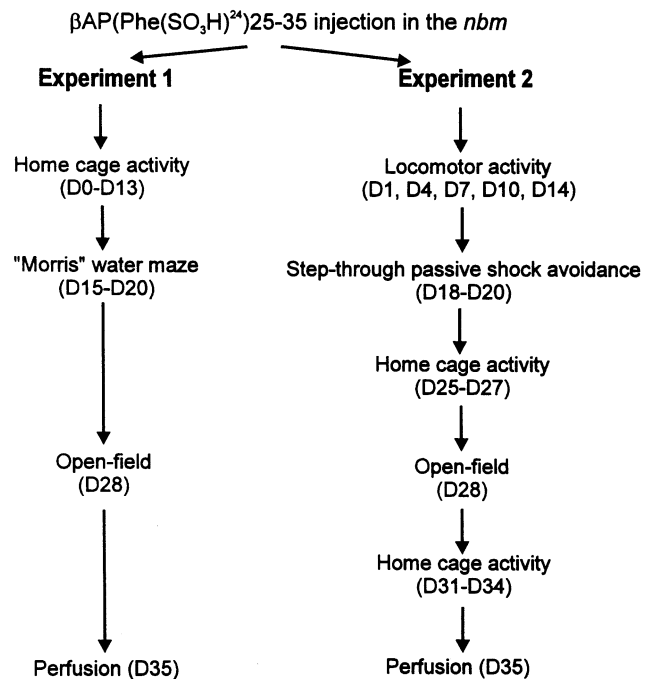


Fig. 1. Schematic outline of the time-course of the experimental protocol including particular steps in the behavioral analysis. β -Amyloid(Phe(SO₃H)²⁴)_{25–35} was bilaterally injected in the nucleus basalis magnocellularis (*nbm*). Behavioral changes induced by the peptide were determined by applying specific tests for the assessment of altered motor activities and/or arousal and retention/acquisition functions throughout a 5-week survival period. Subsequently, AChE histochemistry was used to visualize cholinergic fibre loss in parietal cortical sections.

tiated with an amide at the C-terminal by a solid-phase technique involving Boc chemistry. Peptide chains were elongated on MBHA resin (0.73 mmol/g) and the syntheses were carried out manually. Couplings were performed with dicyclohexylcarbodiimide with the exception of Phe(SO₃H), which was incorporated in HOBt-ester form. The Boc group was removed by treatment with 50% trifluoroacetic acid in CH₂Cl₂. After completion of the synthesis, the peptide was cleaved from the resin with liquid HF. Free peptides were dissolved in distilled water, filtered and lyophilized. The crude peptide was purified by reversephase high performance liquid chromatography (RP-HPLC) on a Vydac-300 C₁₈ column. The purity was checked by RP-HPLC on a W-Porex 5 C₁₈ column. Amino acid analysis demonstrated the expected amino acid composition (SO₃H-FGSNKGAIIGLM) and electrospray mass spectrometry (ES-MS) gave the expected molecular ion. Comparative in vitro studies on mixed glial/neuronal cultures prepared from basal forebrains of rat fetuses using both the native β AP(25–35) and the modified β AP(Phe(SO₃H)²⁴)25–35 peptides did not reveal significant differences in their neurotoxic potentials (data not shown).

2.2. Surgical procedure

Forty eight male Sprague–Dawley rats (280–310 g) were used in the experiments. The rats were caged individually at least 3 days prior to surgery and kept on a normal diet and tap water ad libitum, in an air-conditioned room (21 ± 2°C) with a 12 h daylight cycle (lights on at 08.00 h). They were anesthetized intraperitoneally with chloral hydrate (375 mg/kg body weight; Merck, Darmstadt, Germany), and their heads were fixed in a stereotaxic apparatus. Twenty animals received 0.4 nmol/2 μ l of β AP(Phe(SO₃H)²⁴)25–35, which was injected slowly (~0.1 μ l/min) into the *nbm* at both sides of the brain (AP-2.0 mm, lat. ± 3.5 mm, vent. 5.6 mm) [37] with a 10 μ l Hamilton microsyringe. The β AP(Phe(SO₃H)²⁴)25–35 was dissolved in physiological saline. Control rats were kept under normal conditions without any surgical treatment ($n = 8$), while sham-operated animals received bilateral injections of 2 μ l solvent only ($n = 20$). No significant difference in weight gain was observed between the β AP(Phe(SO₃H)²⁴)25–35-treated (378.50 ± 19.8 g) and sham-operated (380.89 ± 6.27 g) groups, although their weights were significantly less than those of the control animals ($P < 0.05$; 393.67 ± 10.7) at the end of the experiment. Behavioral testing started at 10.00 h

2.3. Home cage activity

The home cage activity apparatus was designed especially for studying animal mobility [44]. It consists of a

207 cm long, 170 cm high and 50 cm wide steel rack divided into 24 totally screened compartments. The home cages of the β AP(Phe(SO₃H)²⁴)25–35-injected and sham-operated animals were placed randomly into the compartments of the home cage activity apparatus. On top of each compartment a passive IR sensor was mounted powered by a 10 V DC power supply. The sensor was fitted with a fresnel lens splitting the IR beam into 16 zones which radiated across the floor of the cage. The 24 sensors were connected to separate switch inputs on an Interpak 2 Interface (Farnell, Leeds, UK) which was linked to an IBM computer equipped with a corresponding 'Home Cage Activity Monitor' software package (version 2.2, ©A. O'Halloran (1993), University College Galway, Ireland). When monitoring begun a sensor dosed if the animal moved from one IR zone into another and the total ambulation score for the compartment was incremented by one. This method using IR sensors avoids removal of the animals from their home cage during the testing period and permits continuous measurements of motor activity.

In addition, the total of ambulation scores during either the light or the dark periods (12/12 h) was used as the measure of the circadian rhythm of the animals.

2.4. Locomotor activity

Locomotor activity was recorded 1, 4, 7, 10 and 14 days post-surgery for the β AP(Phe(SO₃H)²⁴)25–35-treated, sham-operated and naive control animals according to Caldwell et al. [4]. Activity was monitored using an automatic system, which consisted of four circular perspex arenas, 525 mm in diameter and 350 mm high. Each had a central perspex cylinder of 125 mm in diameter and 350 mm high, providing an annular arena for exploration. Three IR photoswitches consisting of separate beam transmitters and receivers (Farnell, Leeds, UK) were mounted symmetrically (60° apart) outside the arena. An IR beam was transmitted to an IR receiver throughout the arena and the central cylinder, at a height of 1.0–1.5 cm above the floor of the arena. The three IR receivers in each box were connected to separate switch inputs on the individual Interpak 2 Interface (Farnell, Leeds, UK). The inter-paks were connected in pairs to a computer (OPUS PC V) via an IBM-intercard (Farnell, Leeds, UK). The software package 'Activity Monitor' (version 12.1, ©A. O'Halloran (1988)) [4] scanned each box for a beam interruption, which was registered as a change in the port status. If there had been a successive beam broken the total count for the box was incremented by one. The software allows for both clockwise and anticlockwise locomotion. Each animal was monitored for a 15 min period.

2.5. Step-through passive shock avoidance

For the passive shock-avoidance conditioned response test a two-compartment (one-way) step-through apparatus was used [45]. In the training trial the rats were placed in an illuminated chamber lit by a 40 W bulb and allowed to explore the boxes. The latency to step into the dark compartment was recorded (preshock latency). On the second day of the test the rats were placed again on the lit platform. Upon entry into the dark compartment a mild foot-shock (1 mA, for 3 s) was delivered through the grid floor and the rats were returned to their cages. Retesting was performed 24 h later and the latency to step into the dark chamber was recorded (post-shock latency) within a total of a 3 min retesting period.

2.6. 'Morris' water maze

The 'Morris' water maze is widely used to assess spatial navigation scores in rodents [34]. The rats were placed in a circular pool of water (100 cm in diameter, 80 cm high, water temperature $26 \pm 1^\circ\text{C}$) from which they could escape onto a hidden platform (11 cm in diameter and submerged 1.5 cm below the water surface). Both the pool and the platform were constructed of black polyvinyl plastic and therefore offered no clues to guide escape behavior. On the first day of the test rats were placed in the water and allowed to swim with no opportunity for escape for 60 s (pre-training acquisition). On the second and third day of the test, rats were placed in the apparatus facing the perimeter of the pool at one of three different locations used (45° apart) and allowed to escape on the fixed platform (fixed platform test). On days 4 and 5 of the test the platform was moved to the opposite of its original location. The rats were placed in a starting position also on the opposite side of the pool which resulted in a 180° rotation of the test conditions (altered platform test). Escape latencies were measured in s with a 60 s cut-off time over 5 daily test sessions with a 5 min inter-trial interval.

2.7. Open-field activity

The novelty-induced activities of all experimental groups were measured, initially described by Gray and Lalljee [15], in an open-field test box. This consisted of a circular arena 80 cm in diameter, which was divided into 20 sectors by faint brown lines and surrounded by a 60 cm high aluminum wall. Illumination was provided by a 30 W bulb positioned 60 cm above the floor of the apparatus. All measurements were carried out in a darkened and quiet room. The test was started by placing the rat in the center of the arena. Each test lasted for 3 min, during which (i) the degree of horizontal ambulation (the number of line-crossings), (ii) the

number of times and (iii) the total time the rat spent in an upright position (rearing) and (iv) the number of fecal boli deposited (defecations) were recorded. In fact, $\beta\text{AP}(\text{Phe}(\text{SO}_3\text{H})^{24})_{25-35}$ treatment did not influence the number of fecal boli deposited in the open-field (data not shown).

2.8. Tissue processing and histochemistry

Fixation of the brains was carried out under deep chloral hydrate anesthesia (500 mg/kg body weight) by transcatheterial perfusion with 300 ml fixative composed of 4% *para*-formaldehyde and 0.05% glutardialdehyde in 0.1 M phosphate buffer (PB, pH 7.4) at a perfusion speed of 15 ml/min. This was preceded by a short prerinse of heparinized saline. After removing the brains from the skull they were cryoprotected by overnight storage at 4°C in 30% sucrose in 0.1 M PB. Subsequently, the brains were coronally sectioned on a cryostat microtome at a thickness of 20 μm . Sections of $\beta\text{AP}(\text{Phe}(\text{SO}_3\text{H})^{24})_{25-35}$ -injected and sham-operated animals were processed simultaneously. Before sectioning, the brains of sham-operated rats were marked by punching a small needle hole in the corpus callosum. The free floating brain sections were post-fixed by immersion in a 2.5% glutardialdehyde solution in phosphate buffered saline overnight at 4°C . The cholinergic fibers were then visualized by staining for AChE (EC 3.1.1.7) according to Hedreen et al. [20] using a silver nitrate intensification procedure. Finally, the sections were mounted, dehydrated, coverslipped and examined by light microscopy.

To determine the effect of $\beta\text{AP}(\text{Phe}(\text{SO}_3\text{H})^{24})_{25-35}$ on the cholinergic projection neurons in the *nbm*, the AChE fiber density was measured in layer V of the posterior somatosensory (parietal) cortex in both the sham-operated and $\beta\text{AP}(\text{Phe}(\text{SO}_3\text{H})^{24})_{25-35}$ -lesioned animals according to a standard protocol [26] by using a Quantimet Q-600HR computerized image analysis system (Leica, Rijswijk, The Netherlands). Cortical fibers were counted in three parietal cortical sections, representing a cortical region receiving the densest cholinergic innervation from the damaged *nbm* division [26–28,45]. The relative value of fiber reduction as a result of neurotoxic cell damage was calculated from fiber crossings at both sides of the $\beta\text{AP}(\text{Phe}(\text{SO}_3\text{H})^{24})_{25-35}$ -lesioned brains as percentages of corresponding values of sham-operated animals. Earlier studies in our laboratory [6] revealed only minor effects of vehicle injections into the *nbm* on the cortical cholinergic innervation (data not shown). Representative photomicrographs depicting the changes in parietal cortical sections of $\beta\text{AP}(\text{Phe}(\text{SO}_3\text{H})^{24})_{25-35}$ and vehicle-injected animals were taken under identical light conditions and developed simultaneously under the same printing circumstances.

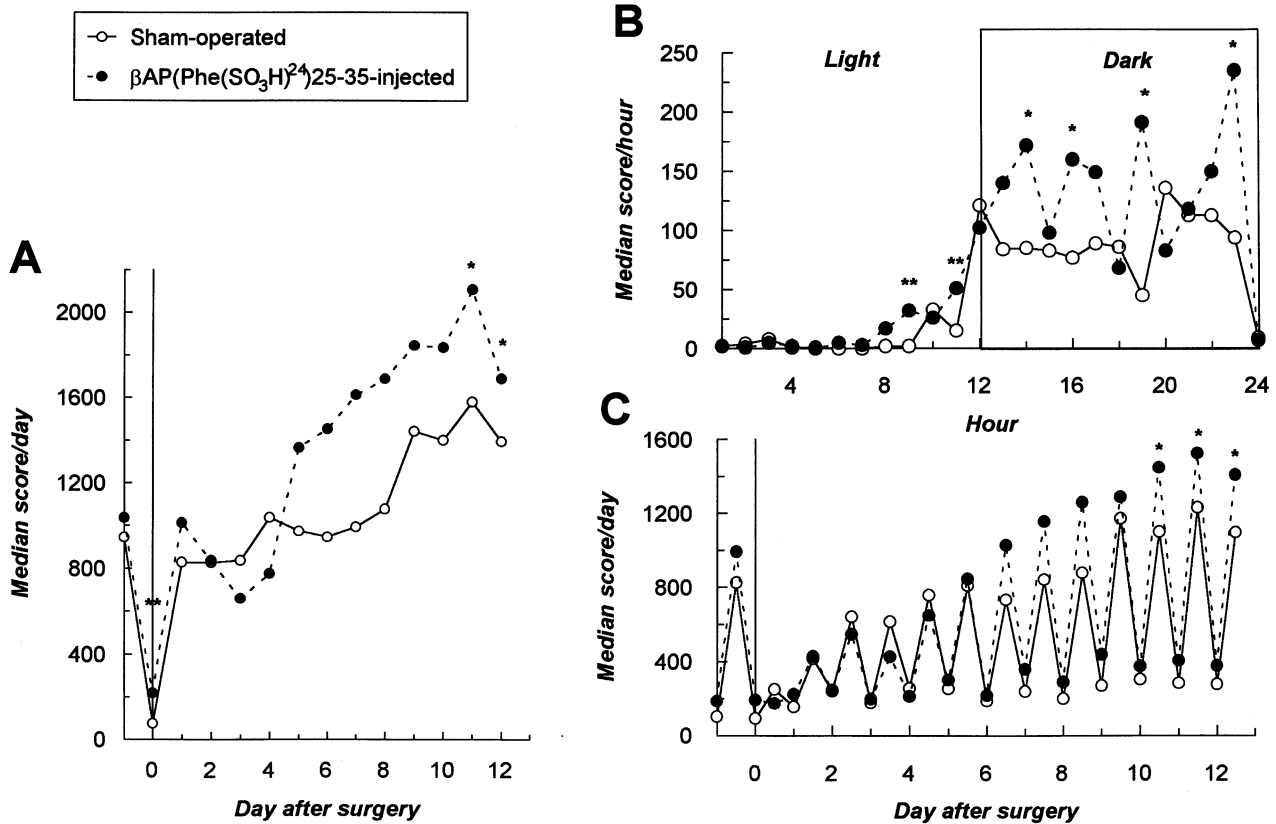


Fig. 2. Changes in home cage activity of β AP(Phe(SO₃H)²⁴)25–35-lesioned and sham-operated animals up to 13 days post-injection (a). Note the biphasic activity profile of β AP(Phe(SO₃H)²⁴)25–35-treated rats. Although hypoactivity occurred transiently in the initial phase of the experiment (day 2–4 post-surgery); β AP-injected animals developed a persistent hyperactivity, reaching the significant level on day 12 post-injection. Detailed analysis of motor activities assessed 12 days after β AP(Phe(SO₃H)²⁴)25–35 infusions (b) revealed a significant increase in ambulation of β AP-injected rats. β AP(Phe(SO₃H)²⁴)25–35 treatment did not alter behavioral reactivities in an illuminated environment and thus the peptide did not affect the circadian rhythm of the animals (c). Illuminated conditions were maintained from 08.00 to 20.00 h. ** $P < 0.01$, * $P < 0.05$ (Kruskal–Wallis test), relative to the sham-operated group. Data are presented as medians; $n = 10$ [β AP lesioned] and $n = 9$ [sham].

2.9. Statistical analysis

Results on altered animal behavior, expressed as medians, were statistically evaluated with the non-parametric Kruskal–Wallis or Mann–Whitney U -tests. Changes in body weight (means \pm S.D.) were analyzed using paired Student's t -test. The degree of cortical fiber reduction (means \pm S.E.M.) was calculated by the one-way analysis of variance (ANOVA) test. $P < 0.05$ was taken as indicative of statistical significance for the tests. This was calculated with a computer program for an IBM PC (MINITAB®, Release 9.2, 1993, Minitab, Staten College, PA).

3. Results

3.1. Behavioral changes

The measurement of motor behavior of rats in their home cage revealed a biphasic activity response as a result of β AP(Phe(SO₃H)²⁴)25–35 infusions (Fig. 2a,b).

Both β AP(Phe(SO₃H)²⁴)25–35 and sham-operated animals rapidly recovered from the operation trauma and had 24 h post-surgery ambulation scores similar to those recorded prior to the β AP injections. Although the rats were randomly injected either with β AP(Phe(SO₃H)²⁴)25–35 or with saline (sham-operated animals), the β AP(Phe(SO₃H)²⁴)25–35-lesioned group exhibited a significantly higher activity immediately after recovery from anesthesia (218[lesioned] vs. 77[sham] ambulation score; $P < 0.01$, Fig. 2a). This was followed by a transient period of decreased mobility of the β AP group between 2 and 5 days post-surgery. On post-lesion day 6 an overt, permanent hyperactivity was determined for the β AP-injected group (Fig. 5). The rapid development of this activity increase was noted. The median ambulation scores of β AP(Phe(SO₃H)²⁴)25–35-treated rats on days 4 and 5 post-operation differed significantly (776 vs. 1366, total daily activity values; $P < 0.01$, Fig. 2a). Furthermore, daily analysis of the home cage activity values for both the β AP-lesioned and sham-controls during the hyperactive period indicated significant alterations that mainly occurred dur-

ing the dark phase (from 20.00 to 08.00 h; $P < 0.01$, $P < 0.05$, Fig. 2b). β AP(Phe(SO₃H)²⁴)_{25–35} injections did not influence behavioral activities in the early light phase. Thus, this did not affect the circadian rhythm of the animals (Fig. 2c), although significant differences were also observed in the last 3 h of the light period ($P < 0.01$, Fig. 2b).

β AP-lesioned rats showed a significant reduction in novelty-induced locomotion 1, 4, 7, 10 and 14 days after β AP(Phe(SO₃H)²⁴)_{25–35} injection. Its maximum was reached 14 days after surgery, as compared to both control groups (69 [lesioned] vs. 150.5 [sham] or 204.5 [naive control] ambulation scores; $P < 0.05$ and $P < 0.01$, respectively, Fig. 3a). Although the rats were tested four times in the locomotor activity task no adaptation to the test conditions was observed. Baseline observations (3 days prior to surgery) of the animals tested under home cage circumstances did not reveal differences in the locomotor activity of the experimental groups (data not shown). Compared to the sham-operated and naive control groups ($P < 0.01$), the arousal of β AP-injected rats was greatly reduced in the initial phase of the 15 min test periods as shown in Fig. 3b, while sham-operated animals habituated normally to the test conditions.

We have previously demonstrated [6,18,19] that β AP_(1–42) exerts marked neurotoxic effects and induces cholinergic cell death after a 14-day survival period. Thus, in these experiments, alterations of retention mechanisms and acquisition were investigated 2 weeks post-surgery in the step-through passive avoidance and ‘Morris’ water maze paradigms (Fig. 4a,b). When tested for passive avoidance before re-entering the shock-compartment, β AP injected bilaterally in the *nbm* significantly decreased the post-shock latencies 24 h after the delivery of a mild foot-shock, compared to both sham-operated and control animals (59 [lesioned] vs. 180 [sham or naive controls] s; $P < 0.05$ or $P < 0.01$, respectively, Fig. 4a). This indicates the short-term impairment of ‘cortical’ retention mechanisms. The performance of naive and vehicle-injected rats did not differ from each other in the test. In accordance with the perturbed locomotor functions, preshock latencies of β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned rats were mildly increased (data not shown).

Behavioral effects of β AP(Phe(SO₃H)²⁴)_{25–35} lesions on spatial orientation were investigated in the ‘Morris’ water maze task. The performance in this test was expressed as the escape latency from the water maze to the submerged, invisible platform in both the fixed (trial 1–15) and altered (trial 16–25) positions. Fig. 4b shows that the acquisition, defined as the decreased escape latency over repeated trials, revealed no obvious differences between the β AP-injected and sham-operated control groups. It was noted, that the rats progressively learned the test across days, so long-

term retention appeared unaffected. Moreover, greatly increased escape latencies of β AP-lesioned rats were recorded in the sixth training session (first trial on day 2).

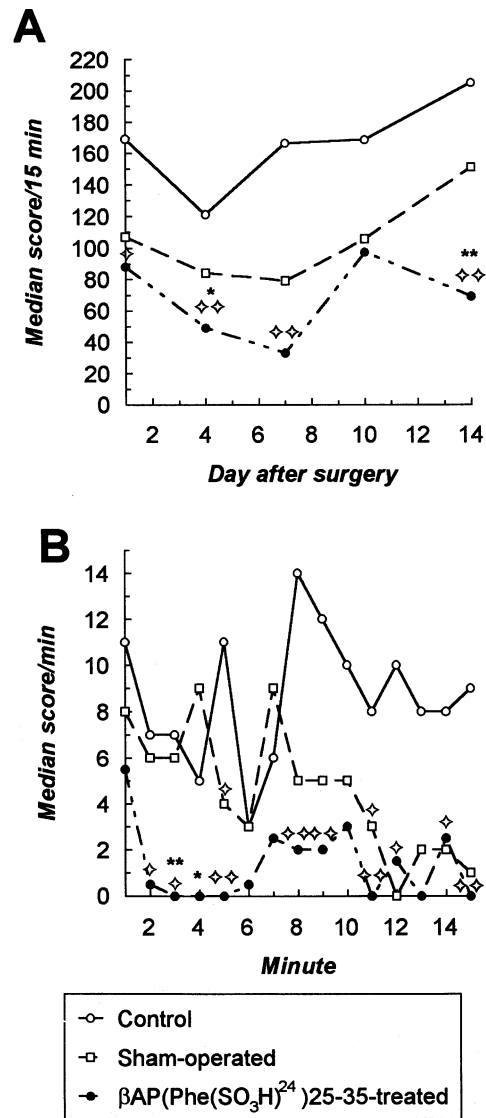


Fig. 3. Effects of β AP(Phe(SO₃H)²⁴)_{25–35} injected in the rat *nbm* on the locomotor activity 1, 4, 7, 10 and 14 days post-surgery (a). In the experiments ten β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned, nine sham-operated and eight naive control animals were used. β AP(Phe(SO₃H)²⁴)_{25–35}-treated rats showed a significant hypoactivity in the locomotor arena throughout the four test sessions, compared to both sham-operated and naive control groups. However, the explorative behavior of sham-operated animals was, albeit non-significantly, also decreased. Detailed analysis of locomotion 7 days post-injection (c) revealed a dramatic reduction in horizontal ambulation and rearing activities of β AP(Phe(SO₃H)²⁴)_{25–35} injected rats during the initial phase of the tests. There are obvious differences between the activity profiles of β AP(Phe(SO₃H)²⁴)_{25–35}-treated and sham-operated or naive control animals. * $P < 0.05$, ** $P < 0.01$ vs. the sham-operated group, while significant differences between β AP(Phe(SO₃H)²⁴)_{25–35} lesioned and control animals are indicated as follows: $\diamond P < 0.01$, $\diamond\diamond P < 0.05$ (Kruskal–Wallis test). Data are presented as medians.

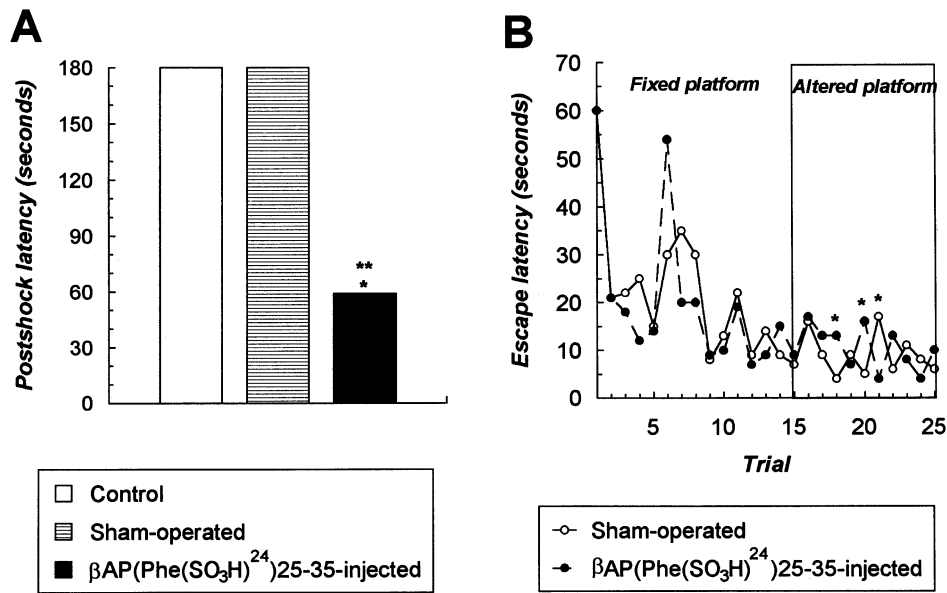


Fig. 4. Changes in post-shock latencies in the 24 h retention trial of the passive avoidance learning paradigm (a) and in spatial navigation learning in the 'Morris' water maze test, using a hidden platform task (b), after β AP(Phe(SO₃H)²⁴)25–35 injections in the *nbm*. Rats were tested for passive avoidance learning 16 days post-operation. β AP(Phe(SO₃H)²⁴)25–35 treatment resulted in a significant decrease in post-shock latencies indicating a marked impairment of short-term memory functions. In this experiment ten β AP(Phe(SO₃H)²⁴)25–35-lesioned, nine sham-operated and eight control animals were used in the experiment. In the water maze paradigm the time to find the platform was measured with a 60 s cut-off time over 5 daily test sessions with a 5 min inter-trial interval. Escape latencies are expressed in s. Spatial learning abilities of β AP(Phe(SO₃H)²⁴)25–35-lesioned rats did not differ from those of sham-operated animals. $n = 10$ and $n = 9$ for the β AP(Phe(SO₃H)²⁴)25–35 and sham-operated groups, respectively. In both experiments the results were expressed in s and are presented as medians. ** $P < 0.01$ vs. naive controls, * $P < 0.05$ vs. sham-operated animals (Kruskal–Wallis (a) or Mann–Whitney U (b) tests).

To determine the long-term effects of intracerebral β AP(Phe(SO₃H)²⁴)25–35 injections and a possible tendency towards recovery, home cage- and open-field activities were determined up to 5 weeks post-injection. Home cage activities of β AP-lesioned rats displayed a permanent and long-lasting increase as recorded up to 34 days post-surgery (Fig. 5). The increased activity response reached its maximum approximately 26 days after the β AP(Phe(SO₃H)²⁴)25–35 treatment (1916 [β AP] vs. 1414 [sham] or 1575[naive control] total daily activity scores; $P < 0.05$, Fig. 5). The activity values of β AP-lesioned rats between day 32–34 post-injection were still higher but no longer significantly different from those of sham-operated and naive control animals ($P = 0.174$, Fig. 5).

The behavioral responses of β AP(Phe(SO₃H)²⁴)25–35-injected and control animals to a novel environment were recorded in the open-field test 28 days after surgery. This task enables assessment of alterations of both motor activity and exploration. In fact, the behavioral scores in the discrimination and spatial learning paradigms predicted the outcome of the open-field test. The group of β AP(Phe(SO₃H)²⁴)25–35-injected rats, previously trained in the 'Morris' water maze paradigm, exhibited a hypoactive response in the open-field as shown by a significant decrease on explorative rearing activity (5 [lesioned] vs. 11 [sham] rearing score; $P <$

0.05, Fig. 6a). Conversely, another group of β AP-injected rats, initially trained in the step-through passive avoidance task, exhibited a marked hyperactivity in the novel environment of the open-field arena ($P = 0.083$, Fig. 6b). These β AP-lesioned animals were more active but showed no improvement in exploration.

3.2. AChE-positive profiles in the parietal cortex

Thirty five days after bilateral injections of β AP(Phe(SO₃H)²⁴)25–35 in the *nbm*, AChE histochemistry was used to visualize aberrations of cholinergic fibres in parietal cortical sections. In this paper, we confined our analysis to pathological changes of the cortical cholinergic innervation, since alterations in the intimate interactions of cholinergic *nbm* structures and neocortical regions have been previously demonstrated to direct behavioral and learning/memory functions [3,27,28,35,36,45,46]. As a result of β AP-(Phe(SO₃H)²⁴)25–35 injections, cholinergic projection neurons of the *nbm* exhibited characteristic signs of neurodegeneration (e.g. their fibres showed a beaded appearance; data not shown). This is similar to those described in our earlier studies after intracerebral β AP_(1–42) application [18]. Degenerating projection fibers scattered through the cortical area investigated resembled the loss of projection neurites following ei-

ther β AP_(25–35) or β AP_(1–42) infusion in the *nbm* [6,19]. β AP(Phe(SO₃H)²⁴)_{25–35} treatment elicited the loss of AChE-positive fibres in all layers of the parietal cortex. This was most apparent in superficial layers, for example in the areas of layer 4 and 5 of the cerebral cortical (Fig. 7a). Quantitative analysis of the density of AChE-positive projection fibers in the posterior somatosensory cortex revealed a $18.45 \pm 4.31\%$ loss of cholinergic neurons 35 days after β AP(Phe(SO₃H)²⁴)_{25–35} infusions, relative to the sham-operated animals ($P < 0.05$). It was noted that AChE-positive processes invading the parietal cortex showed decreased staining intensities as a consequence of β AP(Phe(SO₃H)²⁴)_{25–35} infusion into the *nbm* (Fig. 7a). This might also reflect malfunctioning of the cortical cholinergic system. No differences were found between the AChE-positive cortical staining patterns of sham-operated and naive control animals.

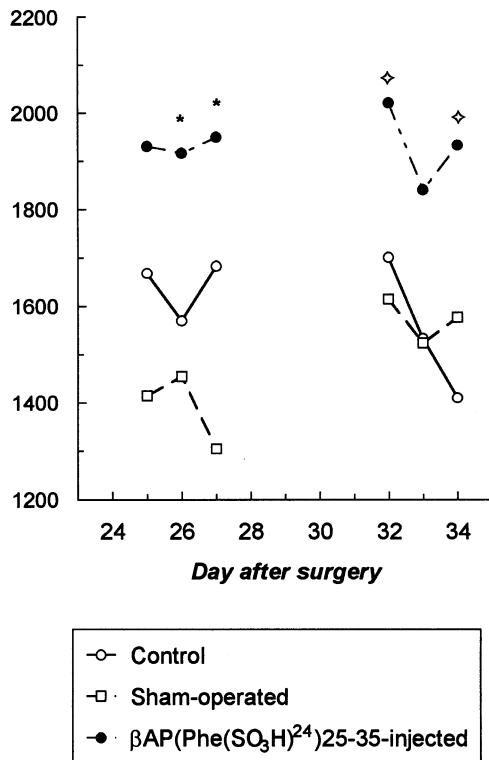


Fig. 5. Changes in home cage activities 4 and 5 weeks after β AP(Phe(SO₃H)²⁴)_{25–35} treatment. Note the permanent hyperactivity of β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned rats. The hyperactive response reached its maximum 26 days after β AP treatment, while still slightly higher activity values of β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned rats were observed between day 32–34 post-surgery. In the experiment ten β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned, nine sham-operated and eight control rats were used. * $P < 0.05$ vs. sham-operated, $\diamond P < 0.05$ vs. naive controls (Kruskal–Wallis test); data are presented as medians.

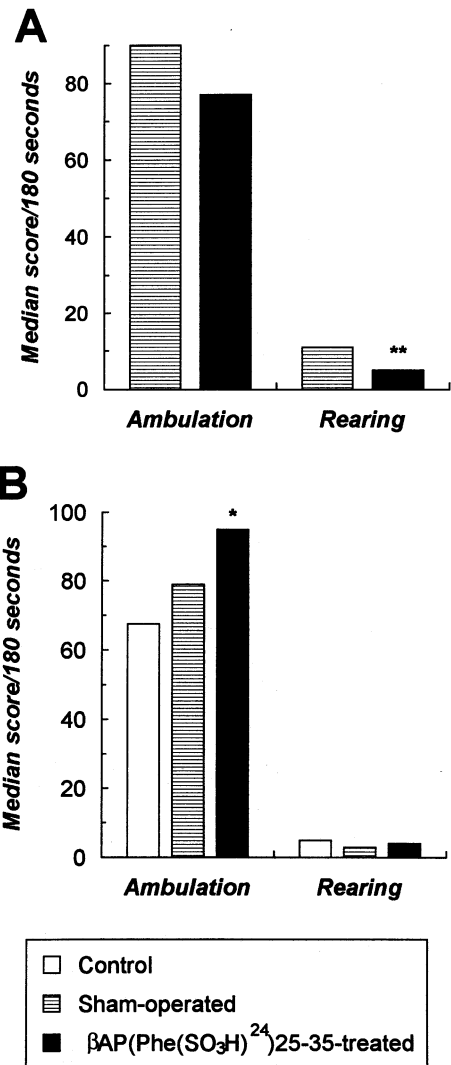


Fig. 6. The effects of β AP(Phe(SO₃H)²⁴)_{25–35} treatment on novelty-induced behaviors in the open-field 28 days post-surgery. The behavioral scores in the discrimination (Fig. 4) and spatial learning (Fig. 5) paradigms predicted the outcome of the open-field test. β AP-injected rats previously trained in the ‘Morris’ water maze paradigm, exhibited a hypoactive response in the open-field (a), while a marked hyperactivity of the rats earlier trained in the passive avoidance task was recorded (b). Open-field activities of the sham-operated groups were significantly different from each other. ** $P < 0.05$ vs. the sham-operated group, * $P = 0.083$ vs. the controls (Mann–Whitney U test). Data are expressed as medians, $n = 8–10$ per group.

4. Discussion

The family of β AP is a heterogeneous group of proteins, which might be generated in successive cleavage processes of different APP isoforms or from the mature β AP_(1–42) fragment itself [8,17]. The abundant appearance of several β AP fragments in AD tissues [2,41] indicates that the actual molecular milieu influences these cleavage mechanisms; e.g. an acidic compartment or unbalanced metabolic supply-induced acidosis might enhance these mechanisms [6]. A lot of

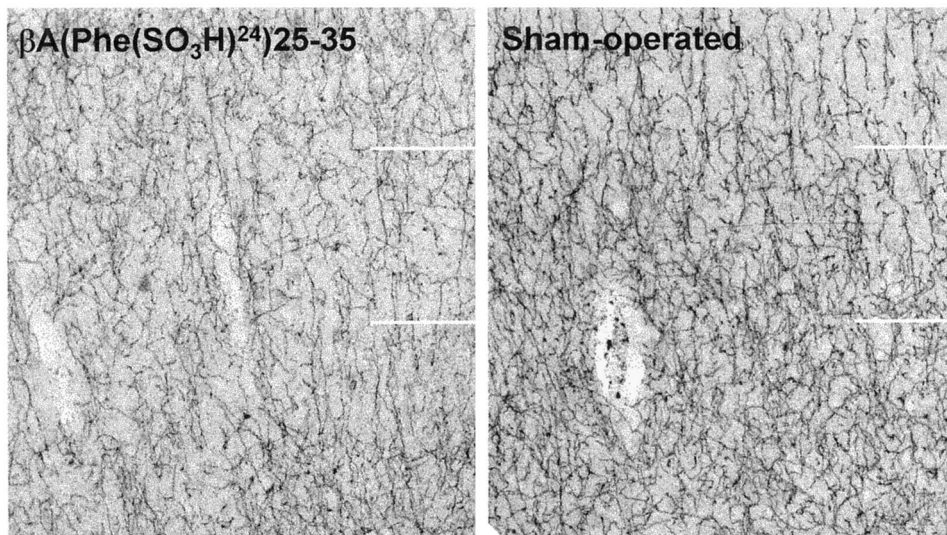


Fig. 7. Bright-field photomicrographs depicting aberrations of acetylcholinesterase (AChE)-positive fibres in parietal cortical sections 35 days after bilateral β AP(Phe(SO₃H)²⁴)_{25–35} infusions in the AChE histochemistry was performed according to the modified method of Hedreen et al. (1985). β AP(Phe(SO₃H)²⁴)_{25–35} injections resulted in a marked reduction of cholinergic innervation and that of AChE staining intensity in all layers of the cortical area studied. Note the obvious AChE-positive fiber loss in layer 5 of the parietal cortex (horizontal open lines). Scale bar = 80 μ m.

recent papers demonstrated that β AP fragments differ both in their chemical [2,38] and physiological properties [6,12,24,25,38] and elicit neurodegeneration [7,18,19], although the particular molecular events of this neurotoxic pathway are still to be ascertained. However, the state of peptide assembly has been proposed as an essential factor in the neurotoxic mechanisms [39]. Furthermore, Giovannelli et al. [12] reported different toxicity profiles for β AP_(25–35) and β AP_(1–40), which were recently corroborated by De Jong et al. [6]. In conclusion, the three dimensional peptide structure and the aggregation and water-solubility of β AP might determine the peptides' effects. To resolve the possible role of these chemical characteristics a modified peptide, namely the β AP(Phe(SO₃H)²⁴)_{25–35} fragment, initiated by coupling of the large but chemically inert Phe(SO₃H) group to the biologically active β AP_(25–35) sequence, was used in these studies. This modification resulted in a water-soluble peptide fragment which is protected against exoprotease cleavage and has a relatively low metabolic turnover, but bears the biological activity of β AP_(25–35) (Penke et al., unpublished observations). On the basis of the persistent behavioral and histochemical abnormalities observed we assume that aggregation and/or fibril formation is not a prerequisite for β AP neurotoxicity. β AP fragments released extracellularly may elicit neurotoxic cascades via acting on specific cell surface targets (receptors) or by their virtue to form free radicals [31], while peptide aggregation may exacerbate these mechanisms. Indeed, small β AP fragments without a discrete tertiary structure undergo rapid enzymatic degradation as suggested previously [12].

Loss of cholinergic projection neurons in the basal forebrain, particularly those of the *nbm*, may play a central role in the cognitive decline associated with aging and AD [3]. In this regard, lesions to the *nbm* induced by injections of excitotoxins [11,35,46] have been frequently used to induce cholinergic malfunctioning. Recent papers using either intracerebral β AP infusions [9,12,36] or transgenic animals [10,33] revealed marked deficits in learning skills and memory functions accompanied by extensive β AP deposition [6,36] and even AD-like neuropathology [10]. In these experiments alterations in spontaneous animal behavior and memory functions were investigated. Extensive changes in home cage behavior as a result of bilateral β AP(Phe(SO₃H)²⁴)_{25–35} injections became evident as early as 24 h after peptide infusions. They showed a biphasic profile and were maintained throughout the survival period. β AP treatment of hippocampal neurons was established to elicit a rapid and persistent elevation in $[Ca^{2+}]_i$. This was accompanied by a remarkable elevation of firing, potentiation of excitatory post-synaptic potentials and large increases in post-synaptic Ca^{2+} levels in vitro [29,31,38]. The early, brief hyperactive peak in β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned rats' home cage activities might be attributed, therefore, to an increased Ca^{2+} influx in the projection neurons of the *nbm*. The rise in $[Ca^{2+}]_i$ may result in hyperexcitability and an increased transmitter release [14] eliciting the hyperactivity that was observed. Furthermore, the transient hypoactivity, and the persistent overt mobility response occurring exclusively during the dark periods, may correlate with β AP-induced degeneration of *nbm* (cholinergic) projection neurons and the subse-

quent loss of the regulation of higher cortical functions. Similarly, transgenic mice expressing the 751-amino acid isoform of the human APP (APP₇₅₁) exhibited lower activity responses than wild type mice which became apparent exclusively during the dark periods [33].

Locomotor activity has been suggested as a possible behavioral screen for neuronal damage following brain injury [32]. In parallel with the increase in general activity, β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned rats exhibited a significant reduction in novelty-induced explorative behavior in the locomotor arena. In accordance with previously reported changes in locomotion after ischemia [4], activity changes might reflect the degree of neuronal damage. The neurotoxic action of β AP(Phe(SO₃H)²⁴)_{25–35} induced a significant hypoactivity which was of greatest magnitude during the first 5-min block of each test-period. Habituation of sham-operated and naive control rats to the test conditions masked these responses in later phases of the experiments. Studies using β AP_(1–42) fragments revealed similar reductions in locomotion in a small open-field task 14 days after surgery (De Jong and Harkany, unpublished data).

Magnocellular regions of the basal forebrain are recognized as critical sites of sleep-wake and arousal regulation [42]. Furthermore, recent research suggests that this cholinergic system plays an important role in attentional processes, as was determined in an associative learning framework after 192 IgG-saporin-induced immunotoxic lesions [5]. Cholinergic *nbm* neurons have recently been established to mediate neocortical and limbic arousal-associated and cognitive functions within the waking state [42]. While non-cholinergic, presumably GABAergic cells in the substantia innominata, maintaining intimate interactions with the cholinergic magnocellular nerve cells, are responsible for sleep-promoting *nbm* functions. Since GABAergic interneurons are relatively preserved after β AP infusions [18] they maintain the normal circadian rhythm of the animals. Conversely, cholinergic malfunctioning in the *nbm* may result in significantly higher mobility values and persistent hyperactivity during the dark periods. This is due to the increased activity of the cholinergic magnocellular projection neurons. The disturbances in sensory information processing (arousal regulation) may account for the loss of cholinergic nerve cells in the *nbm*, and that of the neocortical innervation because of β AP-induced excitotoxic damage, which results in decreased locomotor activities.

Several in vivo investigations demonstrated that β AP directly influence learning and memory functions. In fact, both acute injections into the basal ganglia or limbic structures [9,12] and prolonged peptide infusion [36] affect these mechanisms. In these experiments β AP(Phe(SO₃H)²⁴)_{25–35} injections in the *nbm* induced

marked impairment of cortical retention mechanisms, as was defined in the passive avoidance paradigm. It was noted that preshock latencies of β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned rats increased (data not shown) thus indicating a persistent decrease in explorative rearing. This was accompanied by a dramatic decrease in the latencies before re-entering the shock-compartment in the retention trial. In addition, escape latencies in the 'Morris' water maze showed unaltered long-term acquisition or retention in the test. Combining the data suggests a severe derangement of cortical memory functions which results in the decline of short-term memory and reflects specific, site-directed effects of the peptide. These results concur with the findings of Flood et al. [9] showing that β AP exert direct neurotoxic properties on *nbm* structures resulting in the impairment of 'cortical' learning and memory functions. However, recent studies using β AP_(25–35) or β AP_(1–40) have failed to show memory disturbances when tested for passive avoidance conditioned responses, although a shortening of the retest latency was found [12]. In fact, the tertiary structure and relatively low levels of peptide breakdown may play a pivotal role in the long-lasting neurotoxic actions.

Previous studies [18,19] demonstrate that β AP_(1–42) elicits cholinergic malfunctioning 14 days after intracerebral injections. Since only transient effects of β AP_(25–35) have been reported [12], an attempt was made in this investigation to determine long-lasting changes after β AP(Phe(SO₃H)²⁴)_{25–35} injections. In this respect, persistently increased home cage activities and altered open-field behavior indicated long-lasting disruption of neural functions. β AP(Phe(SO₃H)²⁴)_{25–35}-lesioned rats previously trained in the step-through passive avoidance test showed a marked hyperactivity in a novel environment, although without an improvement in exploration. The difference between β AP(Phe(SO₃H)²⁴)_{25–35}-treated and sham-operated or naive control groups may indicate a persistent amnesic effect of peptide infusions, since the illuminated arena did not elicit a conditioned fear response in rats injected with β AP(Phe(SO₃H)²⁴)_{25–35}. By contrast, ambulation scores of sham-operated and naive control animals were relatively suppressed, presumably as the long-lasting effect of the passive avoidance task. Open-field responses of rats initially trained in the light-independent 'Morris' water maze paradigm revealed persistent decreases in exploration, manifested as the loss in explorative rearing. These data concur with our findings in the locomotor activity task 1, 4, 7, 10 and 14 days post-surgery, reflecting the persistent, long-lasting depression of locomotion.

Extensive damages to the cortical AChE-positive fibre aberrations provided an anatomical substrate for the behavioral changes elicited by β AP(Phe(SO₃H)²⁴)_{25–35} injections. In accordance with

our previous observations [18] cholinergic projection neurons of the *nbm* appeared to be severely damaged by β AP(Phe(SO₃H)²⁴)_{25–35} infusions and underwent neurodegeneration (data not shown) 35 days post-injection. Pronounced morphological aberrations [6,7,18,19] and biochemical disturbances [1,19] after intracortical or intrahippocampal β AP injections, provide evidence for the direct cholinotoxic effects of the peptide fragments. Emre et al. [7] reported the most significant loss of cortical cholinergic innervation approximately 14 days post-injection. These observations have recently been corroborated by De Jong et al. [6] who found the most severe AChE-positive fibre loss 3 days after surgery which was maintained up to 21 days post-operation. In this investigation the analysis of AChE fibre distribution in all layers of the parietal cortex revealed extensive loss of cortical cholinergic innervation following β AP(Phe(SO₃H)²⁴)_{25–35} injections and the behavioral changes correlated with the extent of cholinergic cell death (data not shown). We assume, therefore, that the modified β AP(Phe(SO₃H)²⁴)_{25–35} peptide preserves the neurotoxic potential of β AP_(25–35) and exerts direct cholinotoxic properties.

In summary, microinjections of the non-aggregating, water-soluble β AP(Phe(SO₃H)²⁴)_{25–35} peptide elicit profound changes in spontaneous animal behavior and disrupt learning and memory functions. This stable tertiary structure with the Phe(SO₃H) group protecting against exoprotease cleavage may contribute to the persistent changes in animal behavior and the loss of AChE-positive structures in the rat somatosensory cortex. The different vulnerability of sleep-wake and attentional processes regulated by GABAergic or cholinergic mechanisms might display, even if indirectly, predominantly cholinotoxic properties of β AP. These data might indicate that potentially amyloidogenic β AP fragments, when released extracellularly, induce neurodegeneration prior to fibril or plaque formation. Furthermore, disturbances in yet unidentified protease activities in the brain directing the intra- and/or extracellular enzymatic degradation of β AP may trigger the accumulation and deposition of β AP initiating exacerbation of the neurodegenerative cascade. This new family of modified β AP allows the modelling of specific effects of truncated β AP fragments in long-term experiments and in the present model the effectiveness of neuroprotective drug therapies can be studied directly.

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