The basal forebrain cholinergic system in aging and dementia

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Research report

The basal forebrain cholinergic system in aging and dementia. Rescuing cholinergic neurons from neurotoxic amyloid-β42 with memantine

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ABSTRACT

The dysfunction and loss of basal forebrain cholinergic neurons and their cortical projections are among the earliest pathological events in the pathogenesis of Alzheimer's disease (AD). The evidence pointing to cholinergic impairments come from studies that report a decline in the activity of choline acetyltransferase (ChAT) and acetylcholine esterase (AChE), acetylcholine (ACh) release and the levels of nicotinic and muscarinic receptors, and loss of cholinergic basal forebrain neurons in the AD brain. Alzheimer's disease pathology is characterized by an extensive loss of synapses and neuritic branchings which are the dominant scenario as compared to the loss of the neuronal cell bodies themselves. The appearance of cholinergic neuritic dystrophy, i.e. aberrant fibers and fiber swelling are more and more pronounced during brain aging and widely common in AD. When taking amyloid-β (Aβ) deposition as the ultimate causal factor of Alzheimer's disease the role of Aβ in cholinergic dysfunction should be considered. In that respect it has been stated that ACh release and synthesis are depressed, axonal transport is inhibited, and that ACh degradation is affected in the presence of Aβ peptides. β-Amyloid peptide 1–42, the principal constituent of the neuritic plaques seen in AD patients, is known to trigger excess amount of glutamate in the synaptic cleft by inhibiting the astroglial glutamate transporter and to increase the intracellular Ca^{2+} level. Based on the glutamatergic overexcitation theory of AD progression, the function of NMDA receptors and treatment with NMDA antagonists underlie some recent therapeutic applications. Memantine, a moderate affinity uncompetitive NMDA receptor antagonist interacts with its target only during states of pathological activation but does not interfere with the physiological receptor functions. In this study the neuroprotective effect of memantine on the forebrain cholinergic neurons against Aβ42 oligomers-induced toxicity was studied in an *in vivo* rat dementia model. We found that memantine rescued the neocortical cholinergic fibers originating from the basal forebrain cholinergic neurons, attenuated microglial activation around the intracerebral lesion sides, and improved attention and memory of Aβ42-injected rats exhibiting impaired learning and loss of cholinergic innervation of neocortex.

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1. Introduction

1.1. Organization of the basal forebrain cholinergic system (BFChS)

The cholinergic innervation of the cortical mantle, olfactory bulb, hippocampus and amygdala in the mammalian brain originates from cholinergic cell groups in the basal forebrain and medial septal region. Its anatomical organization of its connectivity has well been studied with various methods like staining of cells and projection fibers with the cholinergic markers acetylcholinesterase and choline acetyltransferase [30,75], next to visualizing projection patterns with intra-axonal tract tracing methods [39]. Basal forebrain cholinergic cellgroups, also designated as the nucleus basalis

magnocellularis (MBN) in the rodent brain or the human homologue nucleus basalis of Meynert sends well developed projections to basically all layers of all cortical regions. The MBN and its subdivisions send their efferents to the entire cortical mantle although a clear antero-posterior organization can be distinguished. Anterior MBN innervates prefrontal and olfactory regions, and noteworthy also the amygdaloid complex. The more intermediate and posterior MBN subdivisions send their efferents to the more neocortical areas [39]. The general innervation pattern originating from the MBN complex is characterized by high terminal fiber densities in all cortical layers but appears to be particularly dense in layer V. The medial septum, vertical limb (VDB) and medial part of the horizontal limb of the diagonal band of Broca (HDB) provides the cholinergic innervations to the hippocampal regions including entorhinal cortex divisions [19] whereas the lateral part of the HDB sends their output to the olfactory bulb. The medial HDB is also a major cholinergic source of the prefrontal cortex [19].

Important from a functional point of view were the more detailed observations on how presynaptic terminals associate with their target structures. When anterogradely filled with tracer compounds like Phaseolus vulgaris leucoagglutinin positively labeled terminal boutons were seen making contact with dendritic or cell soma structures of the receptive nerve cells in the innervated regions. These synaptic contacts could not only be observed with light microscopic resolution but also be confirmed with electronmicroscopy [18]. Double labeling electronmicroscopy furthermore revealed presynaptic MBN terminals making contact with postsynaptic spines positively labeled for muscarinic receptor protein [18,69,70]. Another major detail was the direct contact of presynaptic boutons making contact with mainly microvascular and capillary structures in all innervated forebrain areas [21]. However more closer study of cholinergic innervations of cortical microvessel reveal that cholinergic terminals do not synapse directly on vascular endothelium or the surrounding basement membrane but to the adjacent perivascular astrocytic endfeet that proved to be endowed with muscarinic receptors [25]. We think that such detailed anatomical information is critical for understanding the consequences of cholinergic breakdown as occurs in Alzheimer's disease. These observations are highlighted here with respect to the prominent loss of cholinergic forebrain innervations in Alzheimer's disease and in aging to be further discussed elsewhere in this journal see also [11,20,39].

1.2. Effects of aging on the basal forebrain cholinergic system (BFChS)

Aging has a profound effect on the integrity of the forebrain fiber patterns that find their origin in general modifying transmitter systems such as the serotonergic, noradrenergic, dopaminergic and cholinergic cellgroups in midbrain and basal forebrain. We will not go into great detail in the present report. In view of the scope of the theme of this special issue we will confine our interest to the fate of the cholinergic basal forebrain system (BFChS) and its innervation networks in the cortical mantle and associated regions. Next to the fate of these fiber pathways during aging in general we will shortly address the profound loss of cholinergic presynaptic wiring in Alzheimer's disease.

During normal aging, that is aging not accompanied by overt behavioral or cognitive dysfunctions associated with the cholinergic forebrain system, clear changes can be discerned with respect to the anatomical integrity of fiber pathways penetrating the various cortical layers, a phenomenon that is not limited to the cortical regions but affects structures like thalamus and brainstem as well [20]. Characteristic for such aging related changes are conspicuous beadlike swellings within the cholinergic fibers. Electronmicroscopic observations of such enlarged thickenings reveal swollen axonal varicosities that often occurred in grape-like clusters. Occasionally swollen presynaptic endings were encountered but most of such thickenings were axonal swellings. The fact that we could induce such thickened fiber swellings by applying damage to the parent cell bodies point to an intermediary state of slow cell and fiber degeneration of affected neurons and their projecting axons [21,20].

1.3. The BFChS in Alzheimer's disease

The dysfunction and loss of basal forebrain cholinergic neurons and their cortical projections are among the earliest pathological events in pathogenesis of Alzheimer's disease [62]. In addition to the extensive neuronal loss in these brain regions, the evidence pointing to cholinergic impairments come from studies that report a decline in the activity of choline acetyltransferase (ChAT) and acetylcholine esterase (AChE), acetylcholine (ACh) release and the levels of nicotinic and muscarinic receptors in AD brain [30,50,3]. When taking amyloid-β deposition as the ultimate causal factor of Alzheimer's disease we should consider the role of Aβ in cholinergic dysfunction. In that respect it has been stated that ACh release and synthesis are depressed and ACh degradation is affected in the presence of Aβ peptides [7]. Although AChE levels are reduced in AD brain, its activity is increased around plaques and in neurofibrillary tangles (NFT) bearing neurons [67]. The increase in activity of this enzyme is likely to be due to an indirect effect of Aβ (notably of Aβ42), mediated via oxidative stress [45], via voltage dependent calcium channels or via nicotinic acetylcholine receptors (nAChRs) of the Ca^{2+} permeable α7 subtype [16]. This latter phenomenon links this Aβ effect to overexcitation of the nerve cell when exposed to amyloid peptides, which may boost the intracellular calcium accumulation induced by overstimulation by glutamate in the amyloid deposition domain [73,37]. Muscarinic AChR signaling pathways are also impaired by Aβ. In APP/PS1 double-transgenic mice, the density of mAChRs was lowered, which undergoes an age related decline that is not attributable to mAChR depletion alone, but rather to a malfunction in mAChR-G-protein coupling [40]. Such a mAChR decline may play a substantial role in the cognitive dysfunctions of the BFChS in AD. Interestingly, it was reported that AChE promotes Aβ aggregation, possibly through Aβ-AChE interaction by a hydrophobic environment close to the peripheral anionic binding site of the enzyme, thus promoting fibril formation [29]. When AChE becomes associated with amyloid fibrils, some of its characteristics, like sensitivity to low pH, change. Therefore, AChE might play an important role in neurotoxicity induced by Aβ. This notion is supported by the observation that Aβ-AChE complexes are more toxic than amyloid fibrils alone [2].

However, the relationship between Aβ and the cholinergic system is not unidirectional. There is a considerable amount of evidence that cholinergic dysfunction influences APP metabolism and consequent Aβ production. For example, it has been shown that stimulation of the M1 and M3 muscarinic receptor subtypes increased the release of APP through activation of PLC/protein kinase C (PKC) cascade [52]. BACE (beta-site APP-cleaving enzyme) expression was also increased by activation of these receptor subtypes [77].

1.4. Aberrant sprouting of cholinergic fibers, inhibition of axonal transport and neuritic pathology in AD

Alzheimer's disease (AD) pathology is characterized by an extensive loss of synapses and neuritic branchings which are the dominant scenario as compared to the loss of the neuronal cell bodies themselves [4,5]. However, the size of cholinergic neuronal cell bodies is getting smaller with age [46], thus neuronal cell
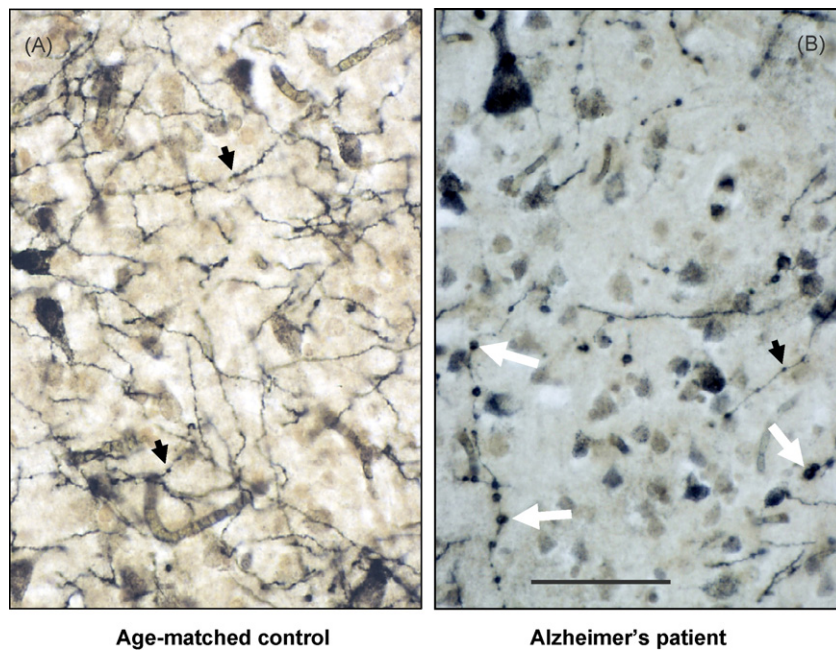
Fig. 1. Cholinergic fibers and en passant boutons along the fibers in parietal neocortex of Alzheimer's (panel B) and aged-matched control (panel A) cases stained with acetylcholine esterase (AChE) histochemical technique. Note that in the Alzheimer's case much less fibers are present and even these fibers contain lot of enlarged boutons (marked by white arrows), which might be considered as aberrant en passant synapses. The normal sized en passant boutons pointed by black arrows can be found in both the control and Alzheimer's cases, although much less are present in latter case. Scale bar is 50 μm.

body shrinkage is another sign of pathology. The breakdown of the cholinergic neuronal system is probably most extensively studied neuronal system among the different neurotransmitter systems in AD pathology. Obviously, the normal turnover and renewal of neuronal structures, first of all synapses, escapes physiological control processes, especially in response to the increased formation of beta amyloid (Aβ) peptides and tau phosphorylation. The result is that there are not only less and less cholinergic fibers in the target cortical areas as shown in Fig. 1, but also that the remaining synapses are deteriorated. These observations may lead to the conclusion that structural and functional aberrations of synapses are one of the primary pathological events in AD (Fig. 2).

Aβ42 triggers cholinergic dysfunction by promoting aberrant neuritic sprouting [42]. While, in general, GAP-43 immunoreactivity as a marker of sprouting is decreased in the cortical areas of AD brain, aberrant sprouting is accompanied by intense GAP-43 production in response to Aβ peptides [44]. Aberrant sprouting might lead to the formation of other pathologies, like fiber swelling and swollen profiles forming grape-like structures [20]. The abnormal appearance of sprouting cholinergic fibers and their underlying processes may be interpreted as an adaptive restorative response as a preliminary stage of subsequent neuritic degeneration.

The appearance of aberrant fibers and fiber swelling are more and more pronounced during brain aging and widely common in AD. It was found that Aβ inhibits the fast axonal transport of vesicular ACh transporter (VAChT). This finding supports the idea that major aspects of AD neuropathology results of failures in axonal transport [31], which is also endorsed by the impaired microtubule-based neuronal transport pathways during the progression of AD. Reversely, the reduction of microtubule-dependent axonal transport may stimulate pathological cleavage of APP resulting in Aβ deposition and plaque formation [66].

It was shown in neuronal cell culture experiment that fiber swelling was coupled with axonal transport block in response to exposure to Aβ fibrils [63]. Such observations suggest that an important cause of the formation of fiber swelling of cholinergic neurons in aging and AD will be the axonal transport block or pathological slowdown of the molecular transport processes. Moreover, the chronic deterioration of intra-axonal transport capacity of cholinergic neurons and their cortical projections can be considered as hazard that could promote the degeneration of the BFChS in AD in a retrograde fashion. It has been demonstrated that axonal fast transport, which is critical for normal neuronal functioning is inhibited by Aβ peptide oligomers, which cause bidirectional axonal transport inhibition as a consequence of endogenous casein kinase 2 activation [58]. Furthermore these authors showed that neither nonaggregated nor fibrillar amyloid beta Aβ affected axonal fast transport which makes understandable why the oligomeric form of Aβ proves to be the most neurotoxic.

Many neurodegenerative diseases exhibit axonal pathology, transport defects, and aberrant phosphorylation and aggregation of the microtubule binding protein tau. Regarding axonopathy processes directly interfering with axonal transport are sufficient to activate stress kinase pathways initiating a biochemical cascade that drives normal tau protein into the pathological hyperphosphorylated state [15] which is a prominent feature of AD neuropathology.

It may be concluded that axonal pathologies including abnormal accumulations of proteins and organelles within the swollen structures point to the importance of axonal transport in a neurodegenerative disease like AD [14]. In the direct vicinity of amyloid depositions and amyloid plaques the pathologic malformations of neuritic fibers are most prominent, often designated as dystrophic neurites [28,55,57,65]. With respect to cholinergic cortical fiber pathology such aberrations are not exclusively confined to the neuritic plaque domain and present all over the cortical mantle [20] and most likely a stage representing ongoing destruction of the BFChS.

1.5. The glutamatergic overexcitation theory of AD progression

β-Amyloid peptide 1–42, the principal constituent of the neuritic plaques seen in Alzheimer's disease (AD) patients, is known to trigger excess amount of glutamate in the synaptic cleft by inhibiting the astroglial glutamate transporter and to increase the
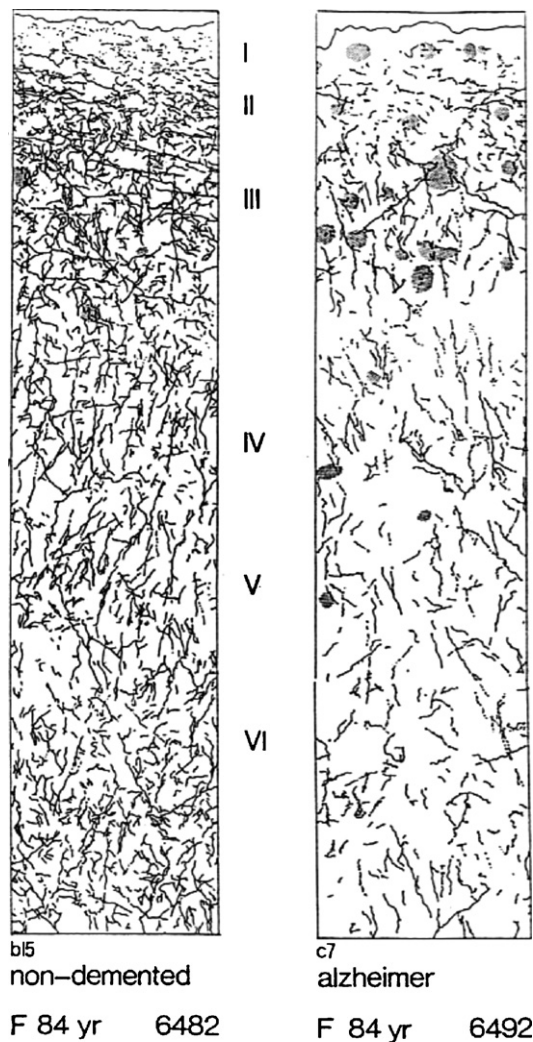
Fig. 2. Camera lucida drawings of the cholinergic innervation pattern in a strip of tissue in the prefrontal cortex from an Alzheimer patient and from an age-matched control case. AChE staining. Numbers refer to the various cortical layers. Grey dots in the right panel indicate the presence of amyloid plaques.

intracellular Ca^{2+} level [24] through enhancement of NMDA receptor activity [49]. Other mechanisms leading to excitotoxicity may include the induction of oxidative stress and the direct impact of Aβ on the glutamatergic NMDA receptor [9,34,43]. Whatever the precise underlying pathogenic processes, overstimulation of the nerve cell by glutamate and intracellular calcium accumulation will eventually cause neuronal apoptosis, disrupt synaptic plasticity (e.g., long-term potentiation, LTP) [34], and as a result of such dysregulation will profoundly impair learning and memory functions.

If synaptic concentration of glutamate is overbalanced, overexcitation of postsynaptic neurons may lead to cellular stress which is especially demanding if the energy maintenance of that neurons is compromised. During aging, and especially during pathological aging a number of endotoxic metabolic factors are bombarding the neurons (hypoxia, ischemia, insulin resistance, oxygen radicals, etc.) in addition to the genetic predisposition to pathological intracellular molecular events. Under these progressing condition during aging overexcitation of neurons by glutamate may be considered as one of the options to find pharmacological targets [36,72]. The pathogenesis of degenerative neural disorders including stroke, head and spinal-cord trauma, and chronic neurodegenerative diseases like Alzheimer's disease have all been linked to excitotoxic processes due to inappropriate overstimulation of the N-methyl-D-aspartate (NMDA) receptor [9,36].

1.6. NMDA receptor antagonists in AD treatment

Based on the glutamatergic overexcitation theory of AD progression for a long period of time a number of NMDA receptor antagonists served as candidates for clinical treatment. Previous attempts to use high affinity NMDA receptor antagonists as neuroprotectants have been hindered by serious side-effects in patients. The leading principle for any optimal drug is to develop well tolerated and effective compounds which should interact with their target only during states of pathological activation but should not interfere with the target if it functions physiologically [37]. Memantine, a moderate affinity uncompetitive NMDA receptor antagonist meets this requirement and does not interfere with the normal NMDA receptor function [10,73]. Memantine is well tolerated and shows clinically relevant efficacy in patients with Alzheimer's disease and preferentially blocks excessive NMDA receptor activity without disrupting normal activity. It slows cognitive, behavioral and functional (activities of daily living) decline and attenuates agitation/aggression and delusion in AD patients and is approved for the treatment of moderate to severe Alzheimer's disease [61,68,17]. The mechanisms by which memantine exerts its beneficial effects in AD are under continuous further investigation. Besides Alzheimer's disease and even Parkinson's disease, memantine is currently in trials for additional neurological disorders, including other forms of dementia, depression, glaucoma, and severe neuropathic pain [35]. In experimental studies beyond the cognitive enhancing effect memantine exerts an anxiolytic type of action [48], which opens further indications for future therapy.

In experimental studies memantine has been shown to provide neuroprotection and improves performance using several pharmacological models of impaired learning and memory [36]. It has been shown to reduce the secretion of Aβ peptides in primary fetal rat cortical neurons [1], in human neuroblastoma cells [60] and in triple-transgenic AD model mice [41]. Memantine improves hippocampus-based learning in APP/PS1 double-transgenic mice with higher than normal levels of brain Aβ42 and formation of Aβ plaques [47,41].

In this study, we report the neuroprotective effects of memantine in rats with multiple Aβ42 oligomer injections into the cholinergic nucleus basalis magnocellularis (MBN) and neocortex.

1.7. Aβ induced BFChS degeneration as an in vivo animal test model

In the present paper we have shortly reviewed the particular breakdown of the basal forebrain cholinergic system in aging and specially in Alzheimer's disease. The prominent breakdown of this system in AD prompted us to employ the damage and loss of cholinergic neurons and their forebrain projection induced by Aβ exposure as an in vivo test model to evaluate the neuroprotective potential of drugs or other treatments for therapeutic purposes in AD. There are a number of reasons to adapt to such a model. First of all the breakdown of cholinergic forebrain innervations is particularly relevant to AD neuropathology. Second, the loss of BFChS components in AD can be specifically be associated with cognitive decline characterized by derangement of memory performance, loss of learning capacity, and impaired behavioral attention [57,53,54]. Thirdly, our detailed knowledge of this transmitter system in the rodent brain allows highly reproducible quantification of Aβ induced neuronal and neuritic injury and concomitant cognitive deficits, which allows assessment of neuroprotective potential of novel drug treatment as measured on neuronal system rescue and their cognitive functions [53,26,38].

Recently, the pathology of Aβ has been correlated to oligomeric forms of the peptide which is the most neurotoxic form in the line of aggregated metabolic products (for review see [71]). Aβ oligomers destroy dendritic spines and are involved in the NMDA receptor-mediated intracellular toxicity [64]. In the present paper we will report our findings of the neuroprotective effects of the NMDA channel antagonist memantine in this *in vivo* rodent model applying Aβ oligomers as neurotoxin.

2. Protection of cholinergic neurons against Aβ-induced excitotoxicity by NMDA antagonist memantine

In the above chapters of Section 1 the vulnerability of cholinergic neurons in AD, the Aβ induced overexcitation of neurons including cholinergic types and the neuroprotective action of memantine against Aβ related pathologies were highlighted. Up till now, however, no study has been performed directly investigating the putative neuroprotective effect of memantine on the forebrain cholinergic neurons against Aβ induced toxicity. In this *in vivo* study we investigated the neuroprotective and behavioral effects of memantine in an advanced dementia model of Aβ42 oligomers-induced cholinergic toxicity. We report that memantine rescues the neocortical cholinergic fibers and improves attention and memory of Aβ42-injected rats exhibiting impaired learning and loss of cholinergic innervation of neocortex.

2.1. Animals and memantine drug treatment

Male Harlan Wistar rats weighing 250–280 g outbred in our laboratory were used in this study. During the experiment animals were kept under normal laboratory conditions in an air-conditioned room (21 ± 2 °C) with a 12/12 h light–dark cycle (lights on from 07.00 a.m. to 07.00 p.m.) with food and tap water ad libitum. The animal experiments were carried out in accordance with the European Community Council Directive directive 86/609 for the care and use of laboratory animals and were approved by the local institutional Scientific Ethical Committee at the University.

Memantine (Forest Research Institute, Jersey City, NJ, USA) was administered orally through drinking water at a dose of 35 mg/kg/day starting 3 days before surgery and continued throughout 10 days postsurgery. The solution was replaced every day and the daily memantine intake was controlled by adjusting the concentration in the drinking water according to individual water consumption. A separate pilot study preceding the main experiment was undertaken to determine the therapeutic dose of memantine producing a steady-state plasma drug level of around 1 μM in rat. This concentration corresponds to the human therapeutic dose. The *per os* dose of 35 mg/kg/day was selected and administered in the drinking water throughout the entire experiment. In the pilot study the plasma levels of memantine were analyzed using a gas-chromatographic system coupled with a mass spectrometer [33].

2.2. Surgery and Aβ$_{1-42}$ injection into the brain

Surgery was performed as described in [38]. The animals were anesthetized with Nembutal (sodium pentobarbital, 60 mg/kg i.p.) and placed in a stereotaxic frame (Narishige, Japan). The neurotoxicity of Aβ has been attributed to oligomeric conformations of the peptide [71,58], and additional studies indicate an involvement of the NMDA receptor also in Aβ-oligomer toxicity [64]. As oligomeric Aβ is now thought to underlie the pathology of the disease, we generated Aβ oligomers *in vitro* and used this oligomer preparation – rather than the monomeric or polymeric forms of the peptide – to induce acetylcholinergic degeneration in the MBN in rats. Blotting

analysis showed mainly dimer and trimer conformations as major constituents of the injected amyloid solution [23].

Injections of Aβ42 oligomers in a concentration of 1 μg/μl vehicle were performed into the right MBN and in the right frontoparietal neocortex at the rate of 0.1 μl/min. Into both anatomical locations 2 times 0.5 μl injections were delivered. The rationale for the double injections was to mimic the multiple anatomical Aβ depositions characteristic in the brain of Alzheimer's disease. The atlas of [56] was used for the stereotectic coordinates of the two injections: AP: Bregma −1.0 and −1.6; *L* 2.5 and 3.4; and for deepness of injections the two anatomical sites were differently approached: MBN 6.9, cortex 1.3 from the dura at both injection sites. The oligomeric Aβ42 was prepared according to [12] and as described in detail elsewhere [23].

2.3. Behavioral testing

All behavioral tests were performed blindly and the individual animals from the different groups and cages were selected randomly for the testing. The test arenas were observed through an optical camera positioned 1.5 m above the arenas, which also allowed to carry out video recording. By this way the experimental animals could not see the experimenter. The behavioral tests started 3 days after operation.

2.3.1. Novel object recognition

It served to test attention ability in a novel environment. On postsurgery day 3 the animals were habituated to handling and transport to the experimental room and to an open-field apparatus where the novel object recognition test was carried out next day as described earlier by us [53]. The test included two sessions. During the 1st session the rats were allowed to explore the same open-field arena for 5 min while two identical objects were placed in an asymmetric position with respect to the center of the arena. These objects became familiar objects. After a 4-h inter-session interval spent in the home-cage, the rats were replaced into the open-field arena for the 2nd session that lasted another 5 min. During the 2nd session one of the familiar objects was replaced by a markedly different object. The time (in s) spent on visiting the objects were recorded. The total time of visits towards both objects served as measure of general exploratory activity. The measure of novel object recognition was the percentage of time exploring the novel object divided by total time spent exploring both the novel and familiar objects. Fifty percent represented the chance level with no discrimination or no recognition of novelty.

2.3.2. One-way step-through passive avoidance learning

For the passive shock-avoidance conditioned response test a two-compartment (one-way) step-through apparatus was used [54]. The behavioral procedure started at 6th day after surgery and lasted for 3 days. In the initial trial at the first day the rats were placed in an illuminated chamber lit by a 40 W bulb and allowed to explore the apparatus. The latency to enter the dark compartment was recorded (1st trial latency). On the second day of experimental schedule the animals were placed again on the lit platform and allowed to entry freely into the dark compartment (2nd trial). During the 3rd trial after entering the dark compartment a mild foot-shock (0.8 mA, 3 s) was delivered through the grid floor and the rats were returned into their home-cage afterwards. The retention test was performed 24 h later and the latency to step into the dark chamber was recorded (postshock latency) within a total of a 5-min retesting period.

2.4. Brain tissue analysis

Brain fixation was carried out at postoperative days 10 with transcardial perfusion of heparinized phosphate

buffered saline (PBS) pH7.4 followed by 4% paraformaldehyde (Sigma–Aldrich, Hungary) solution containing 0.05% glutaraldehyde (Sigma–Aldrich, Hungary) in 0.1 M phosphate buffer (PB pH7.4) after adequate deep pentobarbital anaesthesia. After postfixation of the brains for 48 h in the same fixative the brains were stored in 0.1 M PB containing 0.1% Na-azide until histological examinations. For histological processing the brains were cryoprotected by storage in 30% sucrose and sectioned in a Leica cryostat microtome at a thickness of 20 μm to obtain coronal sections at the AP level of lesioned areas.

2.4.1. Cholinergic fiber innervation: staining and quantification

Immunostaining procedure on free floating coronal sections was applied to visualize choline acetyltransferase (ChAT) positive cholinergic neurons in MBN and their axon ramifications in the target brain areas, i.e. in the parietal neocortex. The primary antibody was a goat anti-ChAT (AB144P, Chemicon) which was used in a dilution rate of 1:500 to visualize cholinergic fibers [27]. Biotinylated rabbit anti-goat IgG and Vectastain ABC kit was obtained from Vector Laboratories (CA, USA). The staining was completed with nickel-enhanced diaminobenzidine (DAB) reaction in the presence of H_2O_2.

The quantification procedure for cholinergic fiber density is established in our laboratory and was described in greater detail previously [26,24]. Briefly, parietal neocortex, topographically corresponding to the anterior–posterior site of the Aβ42 lesion in the MBN and with the highest level of cholinergic fiber loss were analyzed for fiber density with the Quantimet 600HR (Leica, Germany) image analysis program. The exact measurement took place in the superficial sublayer of the layer V cortical area which receives high density of cholinergic innervation. Three brains sections were analyzed per animal and the results averaged. Fiber density expressed in percent surface area of positively stained fibers were computed in both sides of the brain sections. The ChAT positive fiber density ipsilateral to the lesion was compared to the intact contralateral side and the percentage loss was calculated as an indicator of cholinergic neuron and fiber loss.

2.4.2. Activated microglia: staining and magnitude of microglia reaction

The antibody against the integrin CD11b was selected to label activated microglia. In the control brain baseline level activity of microglia is minimal and hardly visible after immunocytochemical staining. Microglia activation becomes markedly enhanced in and around the lesion or degeneration of the nervous tissue. Mouse anti-rat CD11b (MAB1405Z, Chemicon), as primary antibody in a dilution rate of 1:1000 was used in the microglia immunoassay. The biotinylated secondary horse anti-mouse antibody and the ABC kit were obtained from Vector Labs (USA, see above) and used in 1:500 dilution rate. DAB (3,3′-diamino benzidine, Sigma) reaction was enhanced by nickel ammonium sulfate as described above.

Microglial activation was localized remarkably in and around the lesions and could be quantified with a computerized technique measuring the size of the brain region infiltrated by the activated microglia (Quantimet, Leica). Area of microglial activation was manually delineated and measured in the MBN and neocortex at the anterior–posterior level of the overstained injection channel. Here again three sections were selected for measurements. Selection of sections and the measurements were unbiased and carried out blindly.

Comparing different treatment groups one-way ANOVA was applied, followed by the Dunnett *post hoc t*-test comparing two groups. All statistical analyses were performed with Statistica 8.0 software. The statistical significance was set at $p < 0.05$. Means and standard errors of means (SEM) are presented to demonstrate the results.

2.5. Effects of memantine on Aβ-induced cholinergic degeneration

2.5.1. Behavioral effects: novel object recognition

The results are shown in Fig. 1. With one-way ANOVA no difference could be obtained among groups in exploration of objects in general: $F_{4,64} = 1.06$, $p = 0.38$, which shows that the exploratory behavior per se was not influenced by the treatments or the lesion (see upper panel). By analyzing the discrimination capability between novel *versus* familiar objects one-way ANOVA revealed a significant difference among groups ($F_{4,64} = 4.21$, $p < 0.005$). Aβ42-injected animals performed close to the chance, i.e. fifty percent level. *Post hoc* analysis of attention abilities between paired groups showed that against Aβ42 injection group (Abeta) both sham + memantine ($p < 0.01$) and Aβ42 + memantine ($p < 0.05$) groups performed better. Memantine treatment, therefore, was preventive against the Aβ42 oligomers-induced lesion effect. Sham-lesioned rats treated with memantine were not different from the sham control animals.

2.5.2. Behavioral effects: passive avoidance learning

Latency of entering the dark box 24 h after the learning trial was different among the groups ($F_{4,64} = 4.49$, $p < 0.005$) caused mainly by
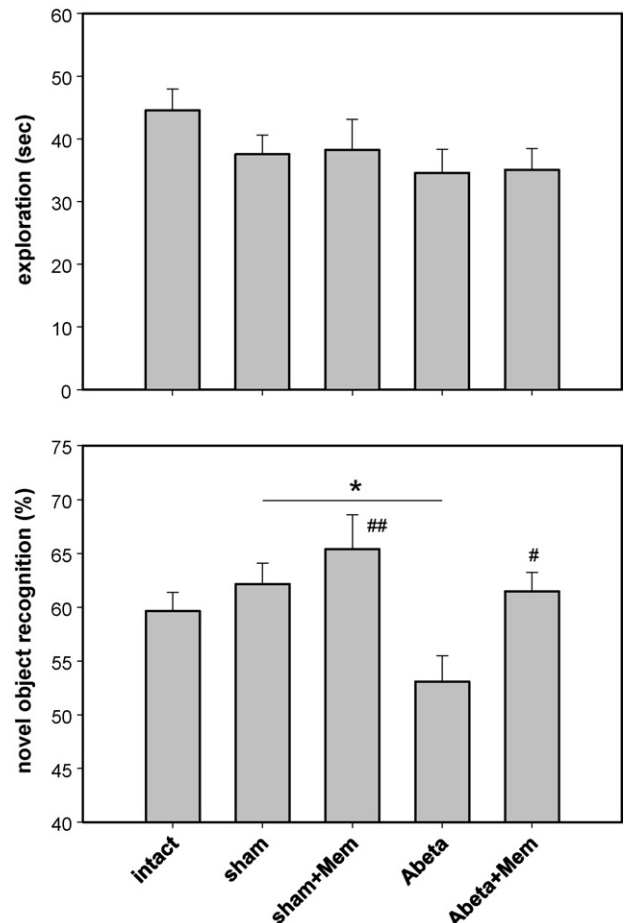
Fig. 3. Results on exploration of objects in a habituated open-field arena (upper panel) and discrimination between novel *versus* familiar objects (novel object recognition, lower panel) are shown. Groups are: intact, sham-operated, sham-operated and memantine-treated (sham + Mem), Aβ42-injected (Abeta), and Aβ42-injected and memantine-treated (Abeta + Mem). The number of animals in the groups varied from 10 to 18. ANOVA revealed no difference in exploration but significant group differences in novel object recognition: $F_{4,64} = 4.21$, $p < 0.005$. *Post hoc* Dunnett test showed a difference between groups of Abeta and sham control (*$p < 0.05$). Differences against Abeta group are indicated by #$p < 0.05$ and ##$p < 0.01$.

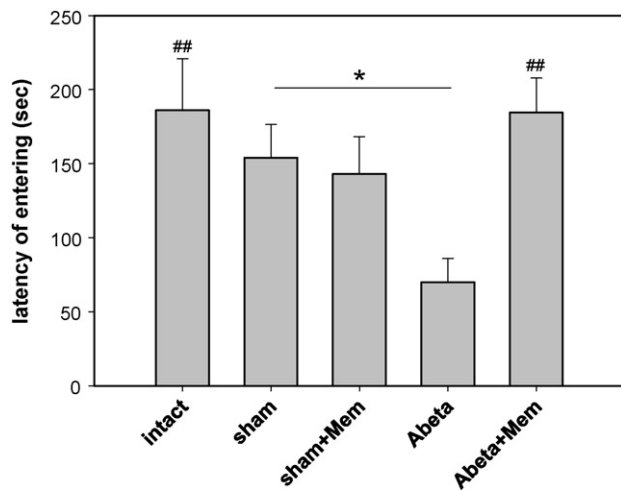**Fig. 4.** Memory expressed as latency of entering the dark shock-compartment tested in the passive avoidance learning paradigm is shown. Groups are: intact, sham-operated, sham-operated and memantine-treated (sham + Mem), Aβ42-injected (Abeta), and Aβ42-injected and memantine-treated (Abeta + Mem). The number of animals in the groups varied from 10 to 18. Entering latency 24 h after the learning trial (electric foot-shock) was significantly shorter in the Aβ42-injected (Abeta) rats against the sham control group (*$p < 0.05$). The performance of Abeta-injected and memantine-treated group was significantly better as that of Abeta group. ##$p < 0.01$ significant difference from Abeta.

the Aβ42 injection (Fig. 2). Retention of passive avoidance learning response was diminished after Aβ42 injection *versus* sham controls ($p < 0.05$). The Aβ42-lesioned animals differed significantly against other two groups including Aβ42-injected + memantine-treated animals ($p < 0.01$). The Aβ42-injected and memantine-treated animals performed the task indistinguishable from sham controls. In this behavioral test like in the novel object recognition test memantine prevented the behavioral deficit caused by amyloid peptide injection. Memantine treatment alone, in sham-lesioned rats, did not change the learning performance.

2.5.3. Memantine effects on the cholinergic lesion

The Aβ42-induced cholinergic fiber loss in the ipsilateral parietal neocortex was attenuated by memantine (Fig. 3A). One-way ANOVA showed a difference among groups ($F_{3,55} = 10.09$, $p < 0.001$). Paired comparisons are also shown in the figure, i.e. Aβ42 injection caused a significant 19.6% loss of cholinergic fibers ($p < 0.001$), memantine pre- and postlesion treatments attenuated this effect by 72% ($p < 0.005$). The fiber loss in the Aβ42-injected and memantine-treated group was not different from that of sham control. Sham animals treated with memantine were not different from sham controls. The photomicrographs of representative animals in Fig. 3 show the density of ChAT positive magnocellular neurons in the MBN and their corresponding axon arborizations in the parietal neocortex. Aβ42 treatment resulted in a loss of ChAT positive neurons in MBN and a decreased cholinergic fiber density
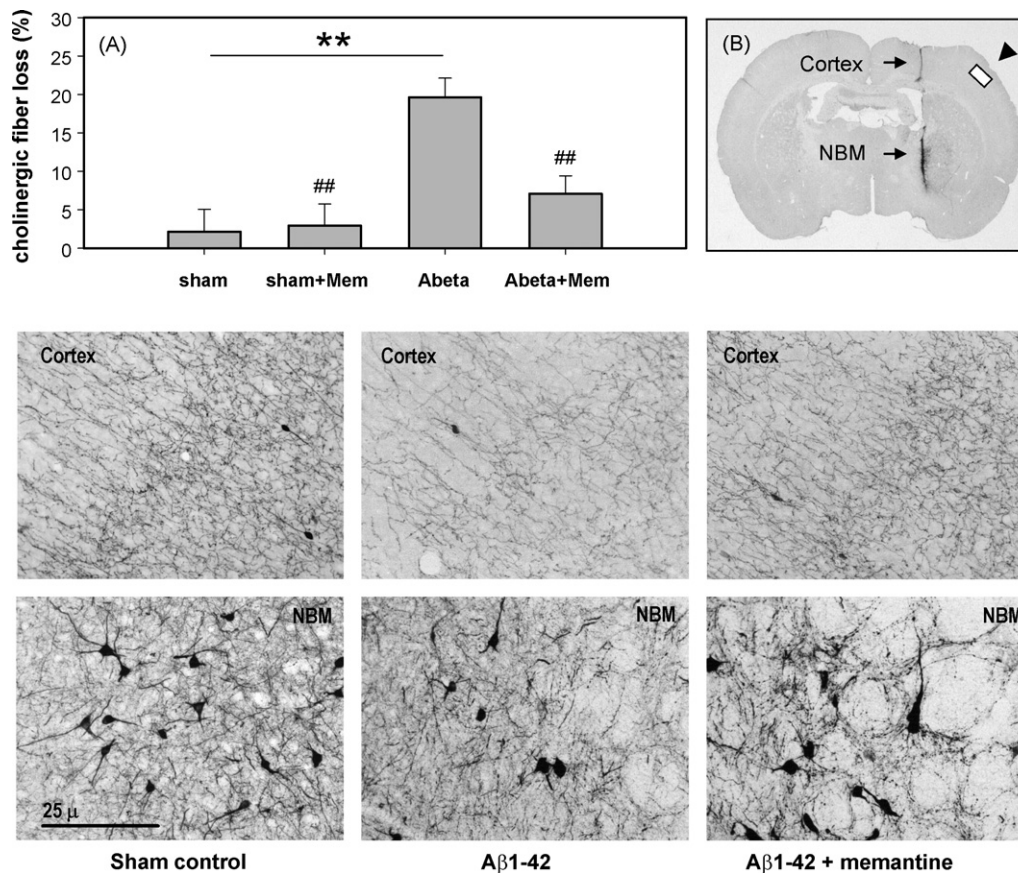

Fig. 5. Attenuation of cholinergic lesion caused by Aβ42-injections into the regions of nucleus basalis magnocellularis (NBM) and neocortex by NMDA antagonist memantine. In microphotograph B (upper right panel) the ipsilateral injection tracks are shown in cortex and nucleus basalis next to the area (white box) in the parietal neocortex where the ChAT positive fiber quantifications were performed (black arrow). The graph shows the degree of cholinergic fiber loss in the cortex as a result of the nucleus basalis lesions. Abeta (Aβ42 oligomers) injection increased fiber loss against sham control (**$p < 0.01$), while additional memantine treatment largely attenuated the amyloid induced neural damage (##$p < 0.01$ *versus* Abeta group). Two rows of photomicrographs below show the degree of ChAT positive neuronal loss in response to Aβ in the NBM and a fiber loss in the cortex compared to sham controls at the left. Aβ42-injected and memantine-treated rats represent an attenuated lesion effect shown both in the NMB where the cholinergic neurons are located and in the cortex where the forebrain cholinergic neurons project unilaterally.
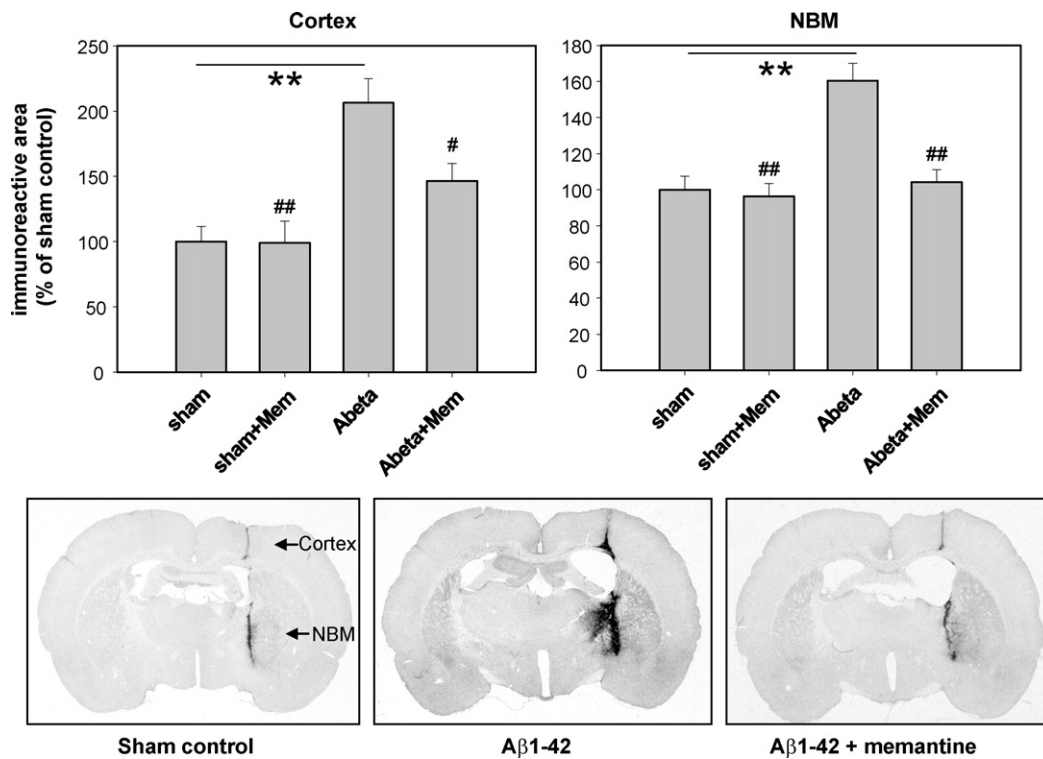
Fig. 6. Area of microglial activation evoked by amyloid peptide-induced lesion in the cortex and nucleus basalis magnocellularis (NBM). The upper part of the figure shows in columns that Aβ42 increased the activated area in both regions (**$p < 0.01$), and memantine interfered with this effect significantly: differences against the Abeta group: #$p < 0.05$ (in cortex) and ##$p < 0.01$ (NMB). The Abeta group was also different against sham + Mem group (##$p < 0.01$). Photomicrographs below show three groups: sham control, Abeta (Aβ42)-injected, and peptide-injected and memantine treated. Aβ42 increased the activated area represented by the middle photo in both regions and memantine interfered with this effect (photo at the right) and reduced the size of microglia activation.

in the neocortex. Memantine treatment attenuated both the loss of neurons and the loss of fibers.

2.5.4. Memantine effects on microglia activation

The Aβ42-induced lesion and also the sham-lesion resulted in microglia activation detected by immunostaining of the activity marker CD11b (Fig. 4). The upper graphs show the immunoreactive areas of microglia activation in percentage of sham controls in both the cortex and in MBN. The lower three photomicrographs are representative pictures demonstrating the extend of activation areas in sham, Aβ42-injected (Abeta) and Aβ42-injected + memantine-treated groups. The Abeta lesion increased the size of microglia activation around the injection channels in both the cortex and MBN. The quantitative data on the graphs show that the amyloid peptide treatment increased the activation area in both anatomical regions: cortex: $F_{3,55} = 11.62$, $p < 0.001$; MBN: $F_{3,55} = 16.23$, $p < 0.001$. Comparing Abeta and sham groups with *post hoc* test resulted in significant differences ($p < 0.01$). Memantine treatment markedly attenuated this effect in the cortex ($p < 0.05$) and practically prevented it in the MBN ($p < 0.01$). Memantine treatment alone in sham animals did not change the degree of microglia activation compared to sham controls (Figs. 5 and 6).

3. Discussion

In the presently applied Aβ42 lesion model, cholinergic neurons originating from the magnocellular basal nucleus (MBN) showed significant axonal degeneration in the parietal neocortex. Aβ42 oligomers also enhanced microglial reaction around the lesion site both in the MBN and in the neocortex. Treatment with a therapeutically relevant dose of memantine significantly attenuated Aβ42-induced loss of cholinergic fibers and microglia activation

in the neocortex and MBN. Memantine also reversed the attention and learning deficits in the Aβ42-treated rats.

These data indicate the ability of memantine to rescue brain cells from the neurotoxic *in vivo* effect of Aβ42 oligomers. Oral treatment of memantine was continuous for 3 days before Aβ injections and during the entire postsurgery period of 10 days. Thus, molecular actions of memantine on NMDA receptors and on a number of neuronal protective mechanisms should be taken into account to explain the present findings. Regarding the diffusion of intracerebrally injected peptide oligomers we have found that the diameter of Aβ peptide infiltration approached 2.0 mm in the nervous tissue 1 h after injection which gradually declined by the 3rd postinjection day allowing approximately 48 h exposure of the neurotoxin (unpublished data). Based on our previous findings [24] and findings of others as discussed in detail in the above section of this paper "The glutamatergic overexcitation theory of AD progression", we propose that memantine attenuated the postsynaptic glutamatergic overexcitation of the NMDA receptor leading to an attenuation of the increase of the intracellular Ca^{2+} concentration.

Aβ is known to exert a number of cellular and molecular pathologies leading to cognitive deficits, LTP disruption (disruption of synaptic plasticity), oxidative stress and apoptosis as summarised above. Memantine can interfere with all these pathological processes. It improves learning and memory in entorhinal cortex lesioned [74] and aged [8] rats and improves spatial learning in APP/PS1 transgenic [47] and triple-transgenic AD mice [41] overexpressing Aβ. Memantine increases the maintenance of long-term potentiation in the hippocampus of old rats [8] and of transgenic mice overexpressing Aβ [41]. In addition it prevents Aβ-evoked decrease in hippocampal somatostatin and substance P level, two neuropeptides that otherwise support LTP in the hippocampus [6]. In this later study the Aβ-induced activation of peptidases in

microglia and astrocytes and the activation of inducible nitric-oxide synthase were also significantly attenuated by memantine treatment. A number of authors have suggested that the NMDA receptor mediates the pathological effect of Aβ oligomers *in vitro* [64,32,13]. In addition, memantine is able to inhibit truncation of glycogen synthase kinase-3 triggered by activated calpain, which is believed to play a key role in the pathogenesis of Alzheimer's disease and notably the process of tau phosphorylation [22]. Calpain, a calcium dependent cysteine protease, is a downstream link of the NMDA receptor-induced neurodegeneration pathway [51]. These results all suggest that the neuroprotective effect of memantine found in this study had to be largely mediated through the stabilization of NMDA receptor function. However, it cannot be excluded that there might be other, NMDA independent neuroprotective pathways operated by memantine which could potentiate its neuroprotective type of action in the present study. Along this line it may be mentioned that there are reports showing that memantine reduces serotonergic 5-HT3 receptor-mediated inward currents in a same noncompetitive manner as for blocking of NMDA receptors [59], which action may add to the cognition enhancing effect of memantine. In addition, memantine can evoke neurotrophic types of action as well as was shown in cell culture experiments [76]. In this study the neurotrophic effect of memantine could be observed as increased production of glial cell line-derived neurotrophic factor (GDNF) in astroglia. In addition, memantine also displays neuroprotective effects against LPS-induced dopaminergic neuronal damage through inhibition of microglia activation as showed by both integrin alphaM (OX-42) immunostaining, and reduction of pro-inflammatory factor production. The latter include extracellular superoxide anions, intracellular reactive oxygen species, nitric oxide, prostaglandin E$_2$, and tumor necrosis factor-α [76]. In that respect we observed in the present experiments a reduced activation of microglia in response to memantine treatment which corroborates this finding.

In summary, in a novel *in vivo* dementia model applying toxic oligomers of beta amyloid peptide 1–42 into different brain areas including the basal cholinergic forebrain region a neuroprotective action of memantine has been demonstrated. Memantine rescued cholinergic neurons and their efferentation to the neocortex, and attenuated the lesion-induced attention and learning deficit. In addition the drug treatment reduced the activation of microglia around the lesion sides in the NBM and neocortex, which may be either the result of the decreased neuronal degeneration or a direct effect of memantine on microglia. Our data for the first time show that memantine may improve cognition by protecting cholinergic neurons from Aβ toxicity.

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