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ABSTRACT

Amyloid-β (Aβ) is toxic to neurons and such toxicity is – at least in part – mediated via the NMDA receptor. Calpain, a calcium dependent cystein protease, is part of the NMDA receptor-induced neurodegeneration pathway, and we previously reported that inhibition of calpain prevents excitotoxic lesions of the cholinergic nucleus basalis magnocellularis of Meynert. The present study reveals that inhibition of calpain is also neuroprotective in an *in vivo* model of Aβ oligomer-induced neurodegeneration in rats. Aβ-induced lesions of the nucleus basalis induced a significant decrease in the number of cholinergic neurons and their projecting fibers, as determined by analysis of choline-acetyltransferase in the nucleus basalis magnocellularis and cortical mantle of the lesioned animals. Treatment with the calpain inhibitor A-705253 significantly attenuated cholinergic neurodegeneration in a dose-dependent manner. Calpain inhibition also significantly diminished the accompanying neuroinflammatory response, as determined by immunohistochemical analysis of microglia activation. Administration of β-amyloid markedly impaired performance in the novel object recognition test. Treatment with the calpain inhibitor, A-705253, dose-dependently prevented this behavioral deficit.

In order to determine whether pre-treatment with the calpain inhibitor is necessary to exhibit its full protective effect on neurons we induced Aβ toxicity in primary neuronal cultures and administered A-705253 at various time points before and after Aβ oligomer application. Although the protective effect was higher when A-705253 was applied before induction of Aβ toxicity, calpain inhibition was still beneficial when applied up to 1 h post-treatment.

We conclude that inhibition of calpains may represent a valuable strategy for the prevention of Aβ oligomer-induced neuronal decline and associated cognitive deterioration.

1. Introduction

According to the amyloid-β-hypothesis of Alzheimer's disease (AD) accumulation of Aβ in brain parenchyma – possibly in its soluble form – causes a degeneration of neurons and their processes in brain areas involved in memory formation (Selkoe, 2008). Among the first regions to be affected are the hippocampus and the nucleus basalis of Meynert. The latter provides the majority of cholinergic input to neocortical structures and plays an essential role in attention and information storage (Blokland, 1995; Van der Zee and Luiten, 1999). Damage and the selective degeneration of the nucleus basalis of Meynert provide the morphological correlate of the cortical cholinergic deficiency in AD. The loss of this discrete cholinergic neuronal population leads to an impairment of higher cortical functions, which is directly related to the progressive deterioration of memory and attention, and cognitive processes in affected patients.

A number of studies suggest that Aβ-induced toxicity in AD is caused by excessive glutamate stimulation, over activation of the NMDA receptor, and subsequent calcium accumulation in the postsynaptic neuron (Harkany et al., 2000; Molnár et al., 2004; Mattson et al., 2000). Recently, the pathology of Aβ has been

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correlated to oligomeric forms of the peptide (for review see Walsh and Selkoe, 2007), and studies indicate an involvement of the NMDA receptor also in oligomer toxicity (Shankar et al., 2007). Although the exact mechanism of this process is not fully understood, there is evidence that calpains, Ca^{2+}-dependent cystein proteases, are components of the downstream cascade. Inhibition of calpains prevents excitotoxic neuronal cell death *in vitro* (Caba et al., 2002; Ray et al., 2006) and *in vivo* (Chiu et al., 2005; Takano et al., 2005), and there is evidence that calpain cleaves several downstream targets that are critical for the progression of excitotoxic neurodegeneration (Hou et al., 2006; Wu et al., 2004). Calpains have therefore been discussed as a target for interference in the neurodegenerative diseases that are associated with neuronal loss (for review see Huang and Wang, 2001; Goll et al., 2003; Zatz and Starling, 2005).

Using a specific low molecular weight inhibitor, A-705253 (Lubisch et al. 2003), we have recently shown that inhibition of calpain completely prevents NMDA-induced excitotoxic lesions of the nucleus basalis magnocellularis (NBM), the rat analog of the nucleus basalis of Meynert in humans. A-705253 also fully protected from behavioral deficits that accompany such lesions (Nimmrich et al., 2008). Although excitotoxicity is likely to contribute to the pathology of AD, this study did not reveal whether neuronal decline could also be prevented, if the insult was induced by Aβ. To provide this missing link we assessed whether calpain inhibition would protect from Aβ-induced degeneration of the NBM, and whether such treatment would protect from associated cognitive decline of the rats.

As oligomeric Aβ is now thought to underlie the pathology of the disease, we generated Aβ-oligomers *in vitro* and used such oligomer preparation – rather than the monomeric peptide – to induce NBM degeneration in rats. Lesioning of the NBM causes a decline of cholinergic projections, mimicking the characteristic loss of forebrain cholinergic innervation in AD (Bartus et al., 1982; Gaykema et al., 1992).

Here we present data showing that calpain inhibition prevents Aβ oligomer-induced neurodegeneration of NBM and associated decrease of cortical cholinergic innervation. Furthermore, calpain inhibition attenuates cognitive deficits that occur as a result of such neurodegeneration.

NMDA receptor activation is an early step in the excitotoxicity cascade, and compounds targeting the NMDA receptor have to be administered in close time proximity of the toxic stimulus. Calpain activation lies further downstream in this cascade, thus offering an opportunity to interfere with cell death signaling at later time points. We therefore added to this study an *in vitro* analysis of the time course of the calpain application relative to the point of insult. Calpain inhibition is also neuroprotective when initiation of the toxic insult has already been initiated.

2. Materials and methods

2.1. Calpain inhibitor

Calpain inhibitor *A-705253* was solubilized in DMSO (Sigma–Aldrich, St. Louis, USA) and stored as 1 M stock at -20 °C. Working stock solutions with different concentrations of A-705253 were prepared in ultrapure water containing 0.9% sodium chloride with a pH between 5 and 5.5. The solution was prepared freshly before use.

2.2. Preparation of Aβ-oligomers

Oligomeric $A\beta_{42}$ was prepared as was described by Dahlgren et al. (2002). In short, solid $A\beta_{42}$ peptide (EZBiolabs, Carmel, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma–Aldrich, St. Louis, USA) to a concentration of 1 mM. The peptide solution was aliquoted and the HFIP removed by evaporation in a SpeedVac (Savant Instruments, Hyderabad, India). The dry peptide films were stored at −20 °C until further processing. Before use $A\beta_{42}$ films were dissolved in anhydrous DMSO to 5 mM and subsequently diluted in neurobasal medium to a final concentration of 100 μM (stock solution). The stock solution was incubated at 4 °C for 24 h to enable $A\beta_{42}$ oligomerization.

2.3. Animals

All animals were purchased from Harlan (Horst, The Netherlands). For the *in vivo* experiments, we used male Wistar rats of 3.5 months of age. For the *in vitro* experiments we used female C57BL/6J mice (12 weeks old). 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 at 07.00 h) with food and tap water ad libitum. All care and treatments were carried out in accordance with the European Communities Council Directive on the use of experimental animals.

2.4. Nucleus basalis lesion

Surgery was performed as described in Luiten et al. (1995). The animals were anaesthetized with Nembutal (sodiumpentobarbital, 60 mg/kg i.p.). The coordinates for the injection in the nucleus basalis magnocellularis (NBM) were 1.5 mm posterior to bregma, 3.2 mm lateral to midline as defined by the atlas of Paxinos and Watson (1986). A 5 μL Hamilton syringe was lowered into the brain, followed over 10 min by two injections of 0.5 μL of the freshly prepared solution of $A\beta_{1-42}$ oligomers (250 pmol each) diluted in 0.01 mM phosphate buffer pH 7.4 unilaterally at two dorsoventral positions, 6.0 mm and 6.7 mm ventral to the dura. The final injected amount of the peptide therefore was amounted to 500 pmol per animal. For sham-operated animals two times 0.5 μl phosphate buffered physiological saline solution were infused (0.01 mM pH 7.4 PBS) containing equivalent amount of DMSO, which served for sham-injection. After each injection the needle was left *in situ* for another 10 min to allow for diffusion and to limit spread of the solution during withdrawal of the needle. Brain injections were performed only in the right hemisphere and the left hemisphere was left undisturbed and served for the self-control side for the histological examinations.

The animals received the calpain inhibitor A-705253 intraperitoneally in doses of 1, 3, and 10 mg/kg of body weight, 1 h before, 12 h after and twice a day for two consecutive days after surgery (for experimental design see Fig. 1).

2.5. Small open-field behavior

A moderate novelty-induced behavioral activation and habituation to a dimly lit home-cage like novel environment was tested in this paradigm (Nimmrich et al., 2008). The test also reflected the general behavioral condition after experimental manipulations, since it was performed 3 days after the surgery. Every 10 s the following behaviors were scored by behavioral sampling technique: a) rearing, b) sniffing with head turning, c) walking, d) grooming, and e) immobility (resting). Exploration was expressed by a combined score of rearing, sniffing and walking (exploration = 3 × rearing + 1 × sniffing + 2 × walking scores). The representative scores of each behavioral component were summed up in 5 min blocks and analyzed statistically.

2.6. Novel object recognition

Testing the ability of rats to recognize a novel object in an otherwise familiar environment represents a sensitive and discriminating test to assess memory performance. Novel object recognition was measured in a conventional cylindrical

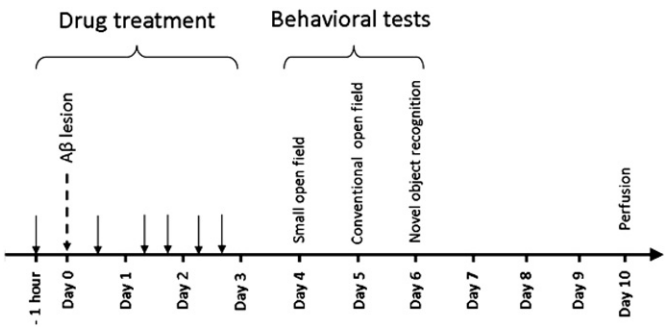

Fig. 1. Schematic outline of the experimental setup for the in vivo experiments. Rats received in total 6 intraperitoneal injections of the calpain inhibitor A-705253 or saline. The first injection was given 1 h prior to unilateral Aβ lesions into the nucleus basalis magnocellularis, the other 5 injections followed within two consecutive days after the lesion. From the fourth day on (after the lesion) the rats were subjected to different behavioral tests. Ten days after the lesion the animals were transcardially perfused and the brains removed for immunohistochemical analysis.

open-field box of 80 cm diameter, in which the floor was divided into 20 sectors and surrounded by a 60 cm high reflective aluminum wall (Nyakas et al., 2009).

During the first day the rats were allowed to habituate to the apparatus and their open-field behavior was recorded for 3 min (conventional open field). The test was carried out the day following to the 3 min open-field test and consisted of 2 sessions. During the 1st session the rats were allowed to explore in the open-field apparatus for 5 min while two identical objects (A + A) were placed into the arena. During the 2nd session, carried out 2.5 h after the 1st session, one of the two objects was replaced by a novel one (B + A). The ratio of visiting the novel versus known objects indicated the object recognition ability, i.e. attention behavior. The number and the period in sec spent with exploration of the objects during the 1st and 2nd sessions were recorded. The recognition ability of the novel object at the 2nd session was calculated in the following way: duration of exploration of the novel (B) object was divided by the duration of exploration of both novel (B) and familiar (A) objects and expressed in percentage. The criterion to pass the test was that both objects had to be visited at least 5 times during the first session representing sufficient interest for object exploration. From the 57 animals only one failed to perform the test, thus statistical analysis was conducted on 56 animals.

2.7. Brain tissue processing

At postoperative day 10 the animals were transcardially perfused under deep pentobarbital anesthesia with a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 after a short pre-rinse with heparinized saline. The brains were removed, post fixed for two days in the same fixative and stored in phosphate buffer at 0 °C, cryoprotected by 30% sucrose for 4 days and sectioned on a cryostat microtome at a thickness of 20 μm. Vials processed for immunostaining contained every 10th section at the level of NBM, i.e. the serial sections positioned 200 μm apart. Free floating brain sections were processed for immunocytochemical staining.

2.8. ChAT staining of cholinergic fibers

Immunostaining procedure was applied to visualize choline-acetyltransferase (ChAT) positive cholinergic neurons in NBM and their axon ramifications in the target brain areas selecting parietal neocortex. Goat anti ChAT primary antibody (AB144P, Lot: LV1359401; Chemicon International, Temecula, CA, USA) was used in dilution 1:500. Biotinylated rabbit anti goat antibody and Vectastain ACB 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.

2.9. Microglial activation

Mouse anti rat integrin αM [CD11b] monoclonal antibody (CLB1512, Lot: 0604026553; Chemicon International, Temecula, CA, USA) was used as first antibody in a dilution rate of 1:1500 to recognize activated microglia. The biotinylated second antibody and the ABC kit were obtained from Vector Labs (CA, USA, see above). DAB reaction was enhanced by nickel ammonium sulfate.

2.10. Quantification of cortical cholinergic innervation

2.10.1. Reduction in cholinergic fiber density and cholinergic neurons

The quantification procedure for cholinergic fiber density in the parietal neocortex was established in our laboratory and described in detail in a series of previous publications (Horváth et al., 2000; Harkany et al., 2001; Dolga et al., 2009). Briefly, parietal neocortex which is topographically the target of afferent cholinergic pathway from the NBM sites where the $A\beta_{42}$ oligomers were injected, was analyzed for ChAT positive fiber density with a Quantimet 600HR (Leica, Germany) image analysis program. The exact measurement took place in the superficial sublayer of the layer V cortical area representing the densest zone of cortical cholinergic innervation. In addition we determined the number of ChAT positive neurons in the NBM. Three brain sections were analyzed for each experimental animal and the results were averaged. Percent surface area of positively stained fibers against zero background and number of positively stained neurons were computed in both sides of the brain section. The ChAT positive fiber density and NBM cell count ipsilaterally to the lesion was compared to the intact contralateral side and the percent decrement was calculated as an indicator of cholinergic degeneration.

2.10.2. Magnitude of microglial reaction

The magnitude of microglial activation was quantified with by image analysis measuring the extent of CD11b-immunoreactive microglia in a specific volume at the level of the lesion site (total infiltration volume). Therefore, a series of positively stained sections were used to reconstruct the extent of activation area. At each section the surface area of core structure around the injection channel and the size of infiltration area were measured with the Quantimet 600 system (Leica, Germany) by means of manual delineation of the affected area. Based on these measurements the total volume of the infiltrated brain area was computed: $(x_1 + x_2 + \ldots x_n) \cdot 200\ \mu m$; where '$x$' was the cross-sectioned stained area in μm^2 and 200 μm was the distance between two consecutive sections. The volume of affected brain area was expressed in μm^3.

2.10.3. Primary cortical neuron culture

Primary cortical neurons were prepared from embryonic brains (E14) of C57BL/6J mice. The cortices were carefully dissected, meninges were removed and the neurons separated by trituration. Cells were plated on poly-*D*-lysine pre-coated plates at a density of 1.2×10^5 cells/well (96 well plates). Neurobasal medium supplemented with 2% (v/v) B27-supplement, 0.5 mM glutamine, 1% (v/v) penicillin/streptomycin was used as a culture medium. After 48 h neurons were treated with 10 μM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged with fresh medium and after 6 days of *in vitro* culture, the neurons were used for experiments.

2.10.4. Treatment of cells

The neuroprotective effect of A-705253 was assessed by incubating neurons (cultured in 96 well plates) for 24 h with 25 μM or 50 μM oligomeric Aβ in the presence or absence of different concentrations of A-705253. After the treatment the cell viability was determined by an MTT-assay. All treatments were performed in triplicates and the experiments were repeated at least two times.

2.10.5. Determination of cell viability by MTT-assay

Neuronal viability was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide] assay as described previously (Mosmann, 1983). 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2 h of incubation, cells were lysed in DMSO. The absorbance of each well was measured with an automated ELISA plate reader (Bio-Rad, Munich, Germany) at 595 nm with a reference filter at 630 nm.

2.10.6. Statistics

The Statistica 8 package was applied for one-way ANOVA evaluation of the results of independent groups which was followed by Dunnett *post hoc t*-test to reveal differences between two selected groups. The level of $p < 0.05$ was accepted as significant. Data are presented as means ± S.E.M.

3. Results

3.1. A-705253 protects neurons against Aβ-induced toxicity in vitro

To investigate the neuroprotective properties of A-705253 against Aβ-induced toxicity, we treated primary cortical neurons with different concentrations (0.04–5 μM) of the calpain inhibitor and challenged with 25 μM or 50 μM of oligomeric $A\beta_{42}$ for 24 h. Our results show that the calpain inhibitor A-705253 in the present condition protects primary cortical neurons against oligomeric $A\beta_{42}$. Moreover, we found that concentrations in the range of 1 μM of A-705253 in the current experimental setup are most effective (Fig. 2A).

Furthermore, we were interested if the temporal dynamics of the treatment is important for the neuroprotective effect. Therefore we applied 1 μM A-705253 2 h and 1 h before, 1 h and 2 h after, or together with the $A\beta_{42}$ challenge. We found that pre-treatment, or simultaneous treatment of A-705253 with $A\beta_{42}$ was fully protective against Aβ induced toxicity (Fig. 2B). Interestingly, when neurons were treated 1 h after the insult, calpain inhibition still exerted a significant neuroprotective effect. However, application of the compound could not prevent neuronal damage when applied 2 h after the insult.

3.2. Calpain inhibition prevents Aβ-induced degeneration of cholinergic fibers and neurons after lesion of the nucleus basalis magnocellularis

Recently, we demonstrated that the inhibition of calpain can protect cholinergic neurons against an NMDA-initiated excitotoxic insult (Nimmrich et al., 2008). Although excitotoxicity is likely to contribute to the pathology of AD, this study did not reveal whether neuronal decline could also be prevented, if the insult was induced by Aβ.

To assess the sensitivity of cholinergic neurons to Aβ-induced toxicity we injected oligomeric $A\beta_{42}$ into the NBM and quantified the loss of cholinergic neurons and their cortical innervations that originate from the NBM (Fig. 3) (Gaykema et al., 1992). Cholinergic neurons and fibers were visualized with the cholinergic marker

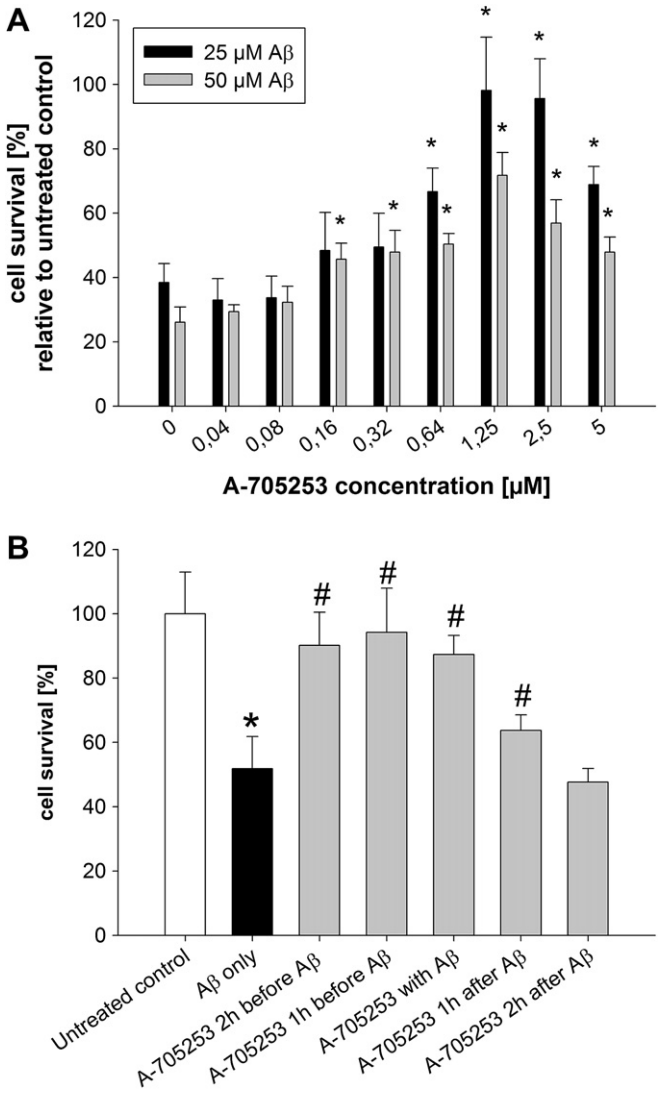

Fig. 2. A) Neuroprotection was determined by co-incubating increasing concentrations of calpain inhibitor A-705253 with 25 µM or 50 µM $A\beta_{42}$ for 24 h. B) The effect of the time point of treatment relative to the insult was assessed by incubating primary cortical neurons were with 1 µM A-705253 for 2 h and 1 h before, after or together with 20 µM oligomeric Aβ for 24 h. Cell viability determined by MTT-assay. Bars indicate the mean cell viability in % relative to untreated controls ± S.E.M. (*$p < 0.05$ vs untreated control and #$p < 0.05$ vs Aβ only).

choline-acetyl transferase (ChAT, EC3.2.1.6) (Harkany et al., 2000). The fiber density was measured in the superficial sublayer of the layer V as described earlier (Horváth et al., 2000). The cholinergic fiber density (Fig. 3A and B) and the number of neurons (Fig. 3C and D) ipsilaterally to the lesion was compared with the intact contralateral side respectively. The percentage of fiber and neuronal loss in the various experimental groups is summarized in Fig. 4. Injection of oligomerized $A\beta_{42}$ into the NBM led to a loss of 40.6 ± 2.3% in cholinergic neurons, which is a 26.78% higher depletion compared to sham-injection (Figs. 3C, D and 4A) and a concomitant decrease of 26.4 ± 3.4% in cholinergic fiber density (18.03% compared to sham injected control) in the cortex (Fig. 4B, C). Pre-treatment with 3 and 10 mg/kg of the calpain inhibitor A-705253 significantly prevented the Aβ-induced cell death and cholinergic fiber loss, respectively. The smallest dose of 1 mg/kg A-705253 was ineffective. The density of cholinergic neurons and fiber innervation was not affected in sham-treated animals, which received 10 mg/kg A-705253.

3.3. Reduced microglia activation in A-705253 treated rats

In neurodegenerative diseases, microglial activation is an early sign that often precedes neuronal death and sustained local inflammatory responses. For that reason we determined the effect of calpain inhibition on microglia activation. We calculated the amount of CD11b-immunoreactive microglia, as a measure of brain inflammation, in a specific volume at the level of the lesion site (infiltration volume) (Fig. 5B). Injection of oligomerized $A\beta_{42}$ resulted in a strong activation of microglia at the infusion site (Fig. 5C), which was significantly reduced in animals treated with 3 mg/kg A-705253 as measured by CD11b expression (Fig. 5A).

3.4. Calpain inhibition as well as NBM lesion does not affect explorative behavior in rats

We further examined whether drug treatment or the Aβ-induced NBM lesion had an effect on the explorative behavior of the animals. Therefore, the rats were tested in a small open field and a conventional open-field paradigm to asses explorative behavior. Neither the drug treated nor the Aβ-induced NBM lesioned animals showed any significant differences in behavior in the tested paradigms. Analysis of the different ambulatory behaviors in the open-field tests, i.e. rearing walking, sniffing was not different from sham-treated rats. Exploration time, immobility as well as time spent grooming was also unaffected and reflects the behavior of healthy animals in such a paradigm (Fig. 6 A–C).

3.5. A-705253 prevents Aβ-induced memory deficits in a novel object recognition paradigm

Cholinergic neuronal projections from the NBM are well known to directly modulate neocortical attentional and memory functions. The novel object recognition task is a learning task which depends, in part, on proper neocortical information processing. In a previous study we could show that a lesion of the NBM in rats leads to attention and memory impairments in a novel object recognition paradigm (Nimmrich et al., 2008). Therefore, we used this behavioral test to investigate whether the preservation of cortical cholinergic function by calpain inhibitor A-705253 can prevent the cognitive deficits induced by Aβ-mediated lesion of the NBM.

The novel object recognition test sensitively assesses attention, or discrimination ability between a novel object placed nearby to a familiar one in a habituated environment. The $A\beta_{42}$-lesioned group showed less attention towards the novel object as compared to the controls treated with vehicle. The amyloid-injected and drug treated groups performed all better than the lesioned control, and even the dose of 1 mg/kg A-705253 was effective in preventing $A\beta_{42}$-induced memory deficits (see Fig. 6D).

4. Discussion

Soluble, oligomeric forms of $A\beta_{42}$ are increasingly associated with the neuropathological mechanisms of AD. Whereas the toxicity of Aβ oligomers has been demonstrated in multiple studies, mainly *in vitro*, the mode of action of those peptide aggregates remains largely unknown. However, a greater understanding of the underlying process of amyloid toxicity is pressing in order to develop medication that specifically interferes with the Aβ-induced disease cascade. A number of authors reporting *in vitro* studies have suggested that the pathological effect of Aβ oligomers is mediated by the NMDA receptor (Shankar et al., 2007; Kelly and Ferreira, 2006; De Felice et al., 2007), and that over activation of the NMDA receptor should be considered as a common principle for some major neurological diseases, including AD (Lipton and Rosenberg, 1994; Harkany et al., 2000;

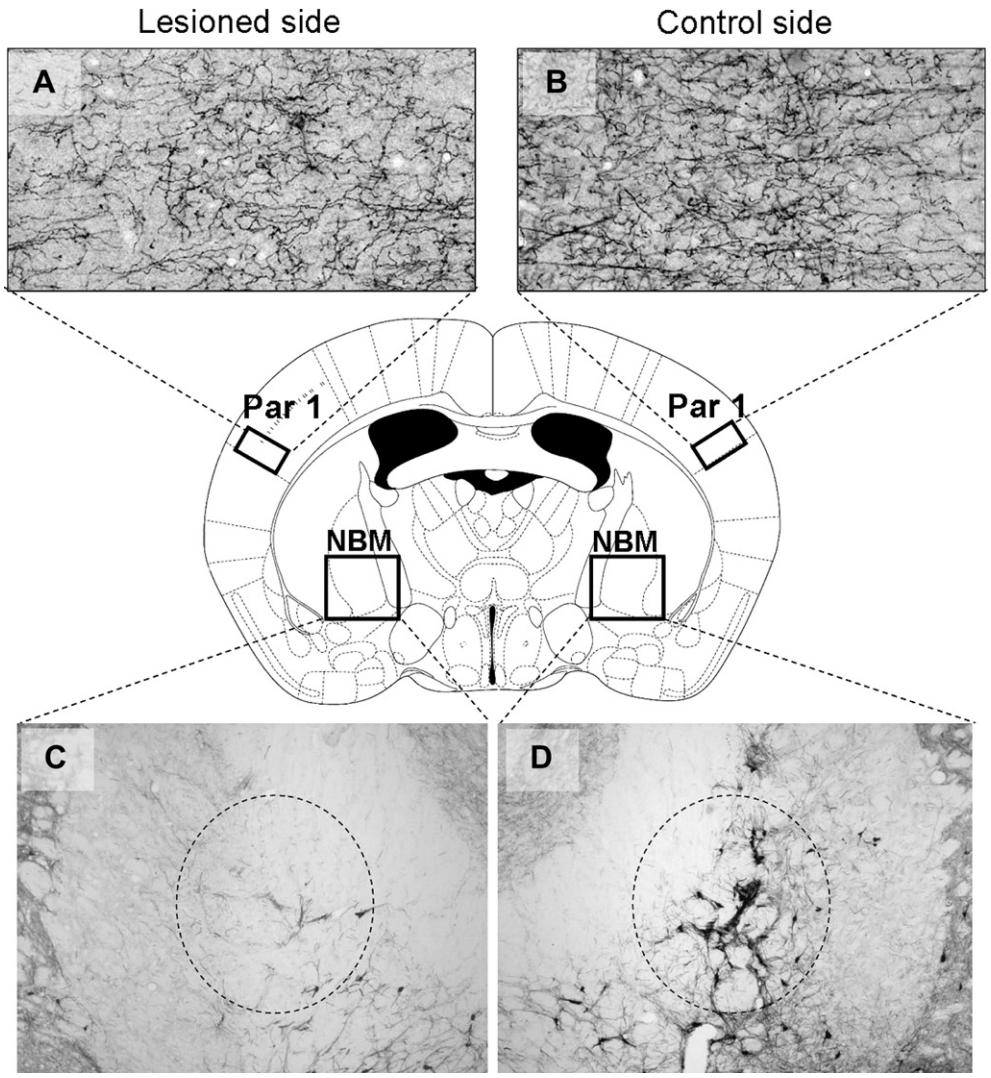

Fig. 3. Anatomical description of studying the cholinergic system after NBM lesion with oligomerized $A\beta_{1-42}$. The upper panel shows ChAT positive fibers in the parietal neocortex of the lesioned rat. A) $A\beta_{1-42}$-lesioned side B) ipsilateral control side. The $A\beta_{1-42}$-injection site in the NBM (dashed circle) is depicted in figure C) the intact ipsilateral NBM (dashed circle) is shown in D).

Mattson, 2004). Stimulation of the NMDA receptor leads to excessive entry of calcium into the cell, which activates proteases that are involved in cell death signaling. Calpain is a calcium-activated cysteine protease that has been implicated to contribute to NMDA-mediated excitotoxic cell death in various studies. For example, inhibition of calpain is neuroprotective after NMDA-exposure in hippocampal slice cultures (Caba et al., 2002). *In vivo*, calpain inhibition reduces neurodegeneration in the rat retina after NMDA-injection (Chiu et al., 2005). Calpain has therefore been discussed as target for neurodegenerative disorders (Huang and Wang, 2001; Zatz and Starling, 2005; Saez et al., 2006). Using the specific calpain inhibitor A-705253 we recently reported that inhibition of calpain prevents NMDA-induced lesioning of the NBM in rats, whereas physiological NMDA-cascades remained unaffected (Nimmrich et al., 2008). However, although these studies suggested that calpain inhibition can prevent excitotoxic neurodegeneration in various *in vitro* and animal models, evidence that calpain inhibition could be protective against $A\beta_{42}$ oligomer-induced neuronal deterioration remained elusive.

The present study provides now evidence that calpain inhibition is neuroprotective against Aβ oligomer-induced cholinergic cell lesion. Injection of oligomeric Aβ to the nucleus basalis caused a strong deterioration of cortical cholinergic projections leading to deficits in learning behavior. Behavioral decline was prevented by application of the calpain inhibitor A-705253. The neuroprotective effect of calpain inhibition was also shown by morphological analysis. Cholinergic denervation in the parietal cortex and the extent of microglia activation around the injection in the NBM were greatly attenuated by A-705253. The present findings expand previous findings in animal models of excitotoxicity, and allow the conclusion that inhibition of calpain is effective *in vivo* against neurodegeneration triggered by oligomeric Aβ. Interestingly, our *in vitro* studies indicate that calpain inhibition is not only effective when the compound is applied prior to the insult. Efficacy of A-705253 against Aβ-induced cell damage was demonstrated – albeit less pronounced – up to 1 h after administration of Aβ. This indicates that the Aβ-induced cell death cascade – once initiated – may not instantly kill the neuron, but requires some time until degeneration is irreversible. If this holds true also for other acute forms of neurodegeneration, this could offer a promising avenue for the treatment of acute neurodegenerative disorders. It is likely that drugs targeting the NMDA receptor may only be effective up to the

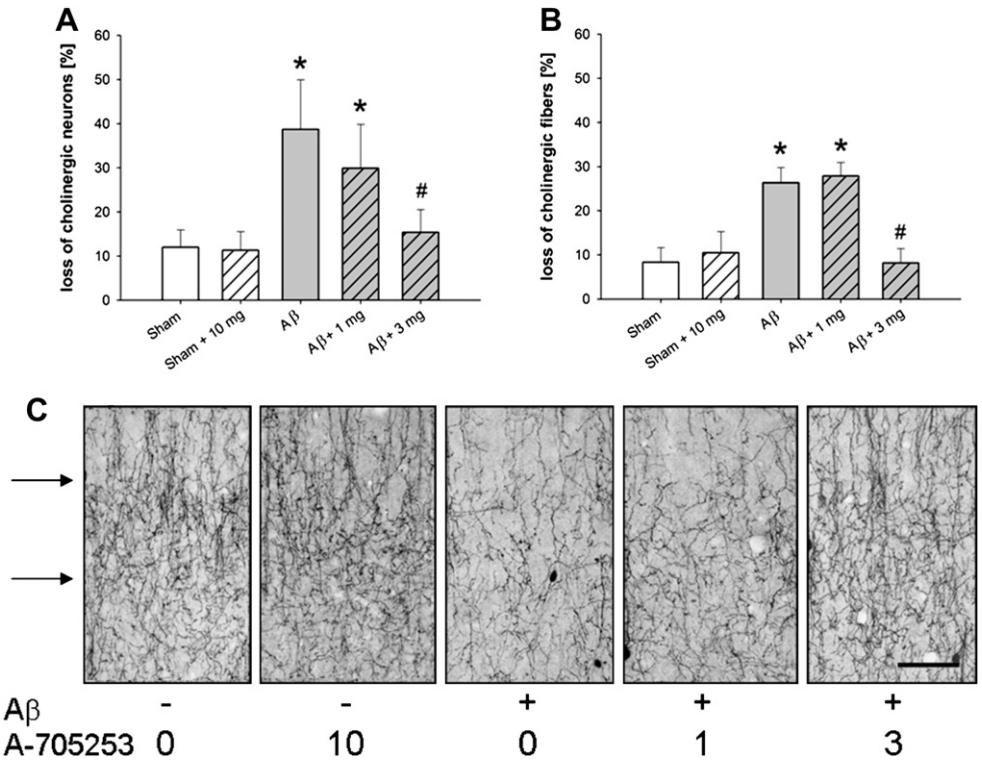

Fig. 4. Cholinergic neurons in the NBM and their innervation of the parietal neocortex was measured in 6 groups of animals: vehicle-treated and sham-lesioned (Sham); sham-lesioned injected with 10 mg/kg A-705253 (Sham + 10 mg); vehicle-treated and $A\beta_{1-42}$-lesioned (Aβ); $A\beta_{1-42}$-lesioned and injected with A-705253 in doses of 1 and 3 mg/kg (labeled Aβ + 1 or 3 respectively). A) $A\beta_{1-42}$-injection into the NBM resulted in decrease in cholinergic neurons. B) Decrease in cholinergic fiber density in the parietal neocortex after sham-injection and in the cholinergic NBM region. $A\beta_{1-42}$-injection (Aβ) increased the decline of cholinergic neurons and cortical innervation, which was not modulated by the smallest dose of 1 mg/kg, but greatly attenuated at 3 mg/kg. $^*p < 0.01$ vs. Sham; $\#p < 0.01$ vs. Ab. C) Representative photomicrographs of cholinergic innervation of the parietal neocortex. Quantitative measurement of fiber density was performed between the two arrows in the superficial part of layer V. The scale bar represents 100 μm.

time of the insult. At least from studies on long-term potentiation (LTP), a neurophysiological paradigm considered to mimic neuronal memory processing, it is known that NMDA receptor-related cascades cannot be reversed by NMDAR blockers once the cascade is initiated. This could be the reason why patients suffering from acute neurodegenerative processes do not benefit from current pharmacological treatment: the drug is administered once the NMDAR-dependent cell death cascades have already started. Future studies need to reveal whether inhibition of calpain is also effective *in vivo* after NBM-lesioning, which is currently hampered by the fact that the time of Aβ diffusion to target cells cannot exactly be determined. The time point of treatment, however, may be less relevant for more chronic neurodegenerative diseases, like those accompanying amyloidoses. In any case, calpain inhibition might be superior to drugs targeting the NMDA receptor for another reason: calpain inhibition does not affect physiological processes like LTP (Nimmrich et al., 2008). Thus, it is likely that calpain inhibition does not interfere with memory processes, but indeed contributes solely to the prevention of cognitive deficits. This is also indicated by the fact that the highest dose of A-705253 did not impair rat memory in several tasks (e.g. novel object recognition test) (Nimmrich et al., 2008).

The compound used here was introduced by Lubisch and co-workers (Lubisch et al., 2003) and does not affect caspases or the proteasome (Ki calpain = 27 nM; Ki proteasome > 10,000 nM; Ki caspases > 10,000 nM). Therefore, it is likely that the protective effects against Aβ-oligomer toxicity are mediated by inhibition of calpain, and not by reducing proteasome or caspase activity. The proteasome complex (Rubinsztein, 2006; Pan et al., 2008) as well as caspases (Cribbs et al., 2004) potentially contribute to neurodegeneration, and a discrimination from these targets mandatory. It is likely that A-705253 has fully reached the target in our experiments as it rapidly penetrates tissues, affecting its cellular target within minutes after perfusion (Neuhof et al., 2003, 2004). It also effectively prevented Aβ-induced toxicity at doses previously shown to inhibit calpain (Nimmrich et al., 2008). It should be noted that A-705253 also inhibits other molecules like cathepsin B to some extend. We therefore cannot fully exclude that at least some of the observed protective effects may be attributed to mechanisms other than calpain inhibition. In reverse, it is also possible that the reduction of efficacy at higher concentration is caused by inhibition of other proteins. Future synthesis of more selective compounds is highly desirable for dissecting the molecular components of neuroprotection.

The model presented here reflects some essential hallmarks of the AD pathology. The NBM is one of the early regions to be affected during AD and the overall neuropathology in this model, including cholinergic denervation and microglial activation, features much of the pathology found in AD patients.

Beyond this, Aβ oligomers – rather than amyloid plaques or monomeric amyloid-β – are thought to underlie the neurotoxic initiator of the disease. They occur in patients prior to plaque formation and correlate much better with cognitive deficits than amyloid plaques. Aβ oligomers have been extracted from brains of AD patients and correlate well with the severity of the disease. Aβ oligomers are therefore – to the best of our knowledge – currently the most appropriate conformation of β-amyloid to induce pathology. The oligomer preparation used here has been introduced by Stine and colleagues (Stine et al., 2003) and was shown to

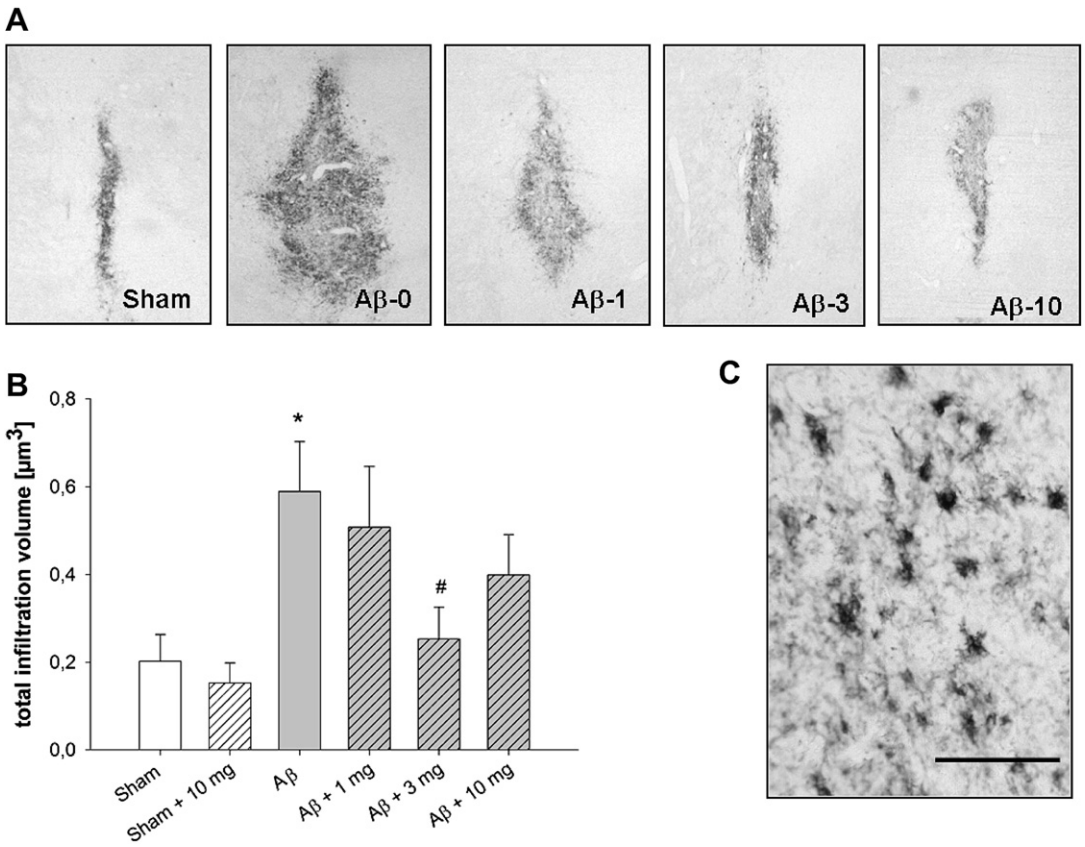

Fig. 5. Extent of microglia activation after $A\beta_{1-42}$-injection decreased under treatment with A-705253. A) Representative photomicrographs show the extent of microglia activation at the level of the brain injection site measured in 6 groups of animals: vehicle-treated and sham-lesioned (Sham); sham-lesioned injected with 10 mg/kg A-705253 (Sham + 10 mg); vehicle-treated and $A\beta_{1-42}$-lesioned (Aβ); $A\beta_{1-42}$-lesioned and injected with A-705253 in three different doses of 1, 3 and 10 mg/kg (labeled Aβ + 1, 3 or 10 respectively). The volume of total microglia infiltration volume around the injections (PBS or oligomers) is shown in B) ($^*p < 0.05$ vs. Sham and $\#p < 0.05$ vs. Aβ). C) High-magnification image shows robust microglia reaction around lesion site (scale bar 100 μm).

be toxic to neurons (Lambert et al., 1998). They also impair LTP (Lambert et al., 1998; Trommer et al., 2005) and have been demonstrated to exhibit their pathogenic action via the NMDA receptor (Lacor et al., 2007). The preparation is similar in their biophysical properties to brain derived Aβ oligomers, underlining their physiological relevance (Gong et al., 2003). In spite of all similarities to AD it must be noted, however, that the experimental model presented here involves a rather rapid decline of neurons and thus differs from the slowly progressive neurodegeneration observed in AD. It would be challenging to examine whether calpain inhibition also prevents more chronic neurodegeneration processes, which is currently only feasible in transgenic mouse models. Most of these transgenic models, however, suffer from a lack of neurodegeneration and decline of cholinergic fibers. Hence, it may be an adequate approach to combine the effect of calpain inhibition on neurodegeneration in an acute *in vivo* model, and the prevention of other deficits – like synaptic decline – in chronic mouse models. The latter effects were explored by Battaglia and colleagues using another, less specific calpain inhibitor, E64 (Battaglia et al., 2003), who showed that long-term (5 mo) intraperitoneal application of E64 in APP(K670N/M671L)/PS1(M146L) mice prevented synaptic impairment as measured by LTP. Chronic calpain inhibition also attenuated the development of deficits in spatial behavior of these mice. Thus, it is likely that calpain inhibition serves to protect from acute and chronic neuronal decline, and from a variety of pathological features observed in AD.

Interestingly, protection from behavioral deficits in our study is more sensitive to calpain inhibition than the prevention of cholinergic denervation and microglia activation. A significant improvement in cognitive function measured by novel object recognition was already accomplished with 1 mg/kg of A-705253 whereas the best protection from Aβ-induced cholinergic denervation was achieved with a dose of 3 mg/kg. This did not further improve with the highest dose of 10 mg/kg (data not shown). This suggests that the efficacy of calpain inhibitor A-705253 reaches a plateau at 3 mg/kg in our *in vivo* model.

In addition, the highest dose (10 mg/kg) A-705253 did not significantly prevent microglia activation. This could indicate that neither cholinergic denervation nor microglia activation do fully contribute to the behavioral outcome measured by the novel object recognition test. It is very likely that the microglia reaction might follow a different dose–response course, which might facilitate other molecular pathways, specific for microglia independent from functional changes behind the behavioral effects.

Whether the effect on behavior at 1 mg/kg may be due to an improvement of physiological function (and hence remain undetected by neuroanatomical analysis) should be assessed in further studies. Recent data showing the involvement of calpain in Aβ-mediated dysfunction of the synaptic vesicle machinery (Kelly et al., 2005; Kelly and Ferreira, 2006) could support such view.

In summary the presented study add evidence that calpain inhibition significantly decreases acute Aβ oligomer-induced degeneration of NBM and its associated cholinergic fiber projections, and attenuates behavioral deficits associated with such lesions. Furthermore, *in vitro* data suggest that inhibition of calpain is still effective when performed shortly after the insult. Our findings indicate that

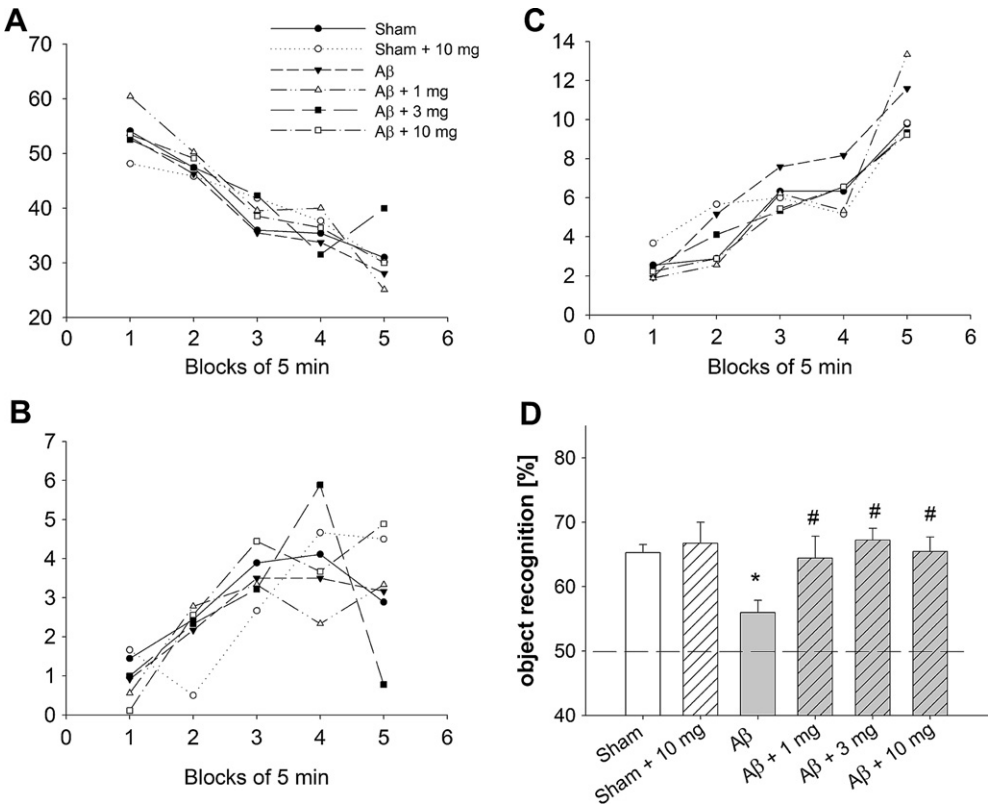

Fig. 6. Behavioral activity was compared in a small open-field paradigm. Exploration is expressed as a combined score of rearing, walking and sniffing in A), grooming is depicted in B), and immobility is shown in C). Scores were collected by behavioral sampling every 10 s for 25 min. No statistical difference could be observed between the groups. Attention was estimated by measuring novel object recognition ability in a habituated open field. The results shown in D). Fifty percent performance means chance level since both the novel and familiar objects are explored by equal amount of time. $A\beta_{1-42}$-lesioned and vehicle-treated rats performed significantly worse than controls (Sham) ($^{*}p < 0.05$; Dunnett post hoc test). Treatment with different doses of A-705253 prevented the behavioral deficit caused by $A\beta_{42}$-injection ($\#p < 0.05$ vs. Aβ).

inhibiting calpain could be a promising strategy for the therapeutic intervention of amyloid-β related neuropathology in AD.

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