Neuropharmacology 59 (2010) 334–342

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Calpain inhibition prevents amyloid-β-induced neurodegeneration and associated behavioral dysfunction in rats

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ARTICLE INFO

Article history:
Received 24 November 2009
Received in revised form
13 April 2010
Accepted 13 July 2010

Keywords:
Calpain
Neurodegeneration
Nucleus basalis
Amyloid-β
Excitotoxicity

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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0028-3908/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2010.07.013

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

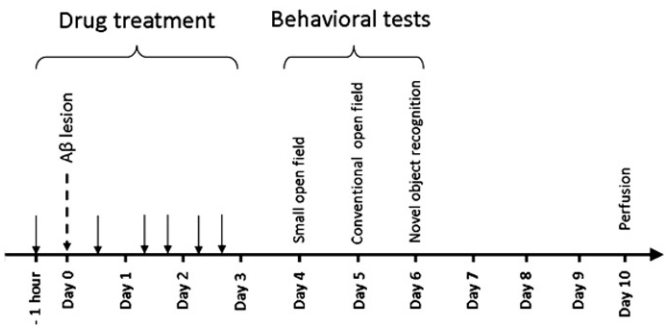

Fig. 1. Schematic outline of the experimental setup for the in vivo experiments. Rats received in total 6 intraperitoneal injections of the calpain inhibitor A-705253 or saline. The first injection was given 1 h prior to unilateral Aβ lesions into the nucleus basalis magnocellularis, the other 5 injections 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.