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Research report

L-type calcium channel blockade alleviates molecular and reversal spatial learning and memory alterations induced by entorhinal amyloid pathology in rats

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HIGHLIGHTS

- Entorhinal cortex amyloid pathogenesis leads to reversal spatial learning and memory deficit.
- Injection of beta amyloid into entorhinal cortex activates calcium-calpain-caspases mediated apoptotic pathway.
- L-type calcium channel blockers, isradipine and nimodipine reduce deteriorative effect of beta amyloid.

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ABSTRACT

The entorhinal cortex (EC) is one of the most vulnerable brain regions that is affected by beta amyloid (Aβ) in the early phases of Alzheimer's disease (AD). Calcium dyshomeostasis is one reason of Aβ pathology and the role of calcium channel blockers (CCBs) in this phenomenon has not fully understood. In this study, we investigated the possible neuroprotective effect of CCBs, nimodipine and isradipine against amyloid pathogenesis in EC. The Aβ 1-42 was injected bilaterally into the EC of male rats and spatial performance was assessed between 7 and 12 days after Aβ injection by Morris water maze test. Animals were daily treated by injection of various doses of nimodipine or isradipine (both at 3, 10, or 30 μg/2 μl) or their vehicles into the lateral ventricle until the start of behavioral test. Lesion in EC was assessed by measuring some proteinases involved in calcium dependent apoptotic pathway (calpain 2, caspase 12 and 3). Despite normal performance in probe test, Aβ treated rats showed delayed acquisition in a spatial reference memory task. Aβ treated rats revealed delayed acquisition in reversal memory and had deficit in probe test. The observed impairments were attenuated by isradipine (10 and 30 μg but not 3 μg) and nimodipine (30 μg). Calpain 2, caspase 12 and 3 were increased in the Aβ treated animals which was partially antagonized by isradipine and nimodipine. It is concluded that CCBs might have beneficial therapeutic effects in AD especially in the early phases of this disease.

1. Introduction

Alzheimer's disease (AD), an age-related neurodegenerative disease, is a main cause of dementia in the elderly [1,2]. AD is characterized by the over-production of amyloid β-peptide (Aβ) and related degeneration of neurons in brain regions that are involved in learning and memory including entorhinal cortex (EC) and hippocampal formation [3]. Aβ injection into the brain leads to degeneration, neurite dystrophy, synaptic integrity loss, microglia and astrocyte activation and neuronal death [4–6].

Various forms of memory are encoded in the entorhinal-hippocampal circuit [7,8]. Thus, aberrant function of this network in AD has deteriorative effect on learning and memory. In fact, the EC is particularly vulnerable and the early target in AD. It has been shown that in the beginning stages of AD a significant loss of neurons occurs in layer II of EC [9]. Furthermore, patients with mild cognitive impairment or early-stage AD suffer from early, selective atrophy and declined metabolism in their EC [10,11]. It has been postulated that AD originates in the EC and spreads from this region to the hippocampus and other cortical areas as the disease progresses [12].

The precise mechanism that Aβ induces neuronal cell death in AD is still unclear. One mechanism could be destabilization of Ca^{2+} homeostasis in neurons affected by Aβ [13]. It has been reported

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that Aβ induces intracellular Ca^{2+} dyshomeostasis by increasing its influx through voltage-dependent calcium channels (VDCCs). For example, Rovira et al. reported that L-type calcium channels are affected by Aβ25-35 [14]. Moreover, Macmanus et al. showed that calcium influx via its L-, N- and P-type channels is increased by Aβ 1-40 [15]. On the other hand, Whitson and Appel stated VDCCs are not affected by Aβ-induced neurotoxicity in hippocampal cell culture [16]. Intracellular Ca^{2+} dyshomeostasis has been shown in patient and in animal and cell culture models of AD that may play a pivotal role in inducing synaptic dysfunction and neuronal death [17–20]. Aβ induces elevated intracellular Ca^{2+} concentration and increased vulnerability of the neurons to excitotoxicity [17,21,22]. Since drugs used for treatment of AD have shown only limited effect [23] thus, finding a new approach to prevent or slow the rate of AD progression is of major interest. Using calcium channel blockers as a neuroprotective agent in neurodegenerative diseases such as stroke [24] and Parkinson [25] might give a possible promising therapeutic approach for AD treatment.

The endoplasmic reticulum (ER) is an essential intracellular organelle involved in regulating intracellular calcium homeostasis, in protein folding and in cell death activation [26]. Under pathological conditions including AD, intense and prolong increase of intracellular Ca^{2+} leads to a phenomenon known as ER-stress in which the unfolded proteins are accumulated and then the ER-stress-induced apoptosis pathway is activated [27,28]. Calpains and several caspases including caspase 12 [29] and 3 [30] have been shown to be involved in the ER stress. It has been proposed that caspase-3 cleaves amyloid precursor protein (APP) and therefore increases the amount of Aβ1-42 which in turn leads to further caspase activation that accelerates cell death in AD [31].

In spite of evidences suggesting that calcium channel blockers could prevent [32] or slow AD progression rate [33,34], large clinical trials have not shown any therapeutic effect of nimodipine for AD [35]. Because of high first-pass metabolism for calcium channel blockers, the failure of nimodipine in clinical trials could be associated to its low brain bioavailability [36]. On the other hand Anekonda et al. reported neuroprotective effect of VDCCs including isradipine and nimodipine in Aβ treated MC65 neuroblastoma cells [36]. Since injection of Aβ into the EC induces a neuroinflamation status and cognitive deficits resembling those observed in the early stages of AD [37], in this study we investigated the possible protective effects of CCBs, isradipine and nimodipine, on spatial learning and memory against amyloid pathology in the EC using Morris water maze (MWM) task. Meanwhile in order to verify the EC amyloid pathogenesis, histological staining and measurement of apoptotic proteins such as calpain 2, caspase 12 and caspase 3 were performed.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (180–230 g) were obtained from our own breeding colony. They were caged in groups of four with free access to food and water and were maintained on a 12 h-light/dark cycle (light on at 07:00 h), at a temperature of 23 ± 1 °C. All efforts were made to minimize the number of animals and their suffering during the experiments. All experiments were executed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996).

2.2. Drug administration

Human β-Amyloid 1-42 (Tocris, UK) was prepared as stock solutions in 0.1 M phosphate-buffered saline (PBS; pH 7.4) and aliquoted (10 μl per vial), and stored at −70 C until use. The peptide was still perfectly soluble after defrosting the aliquots, and 2 μl of freshly prepared (non-aggregated) β-amyloid (0.5 μg/μl) was used for each injection. The animals were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). They were injected bilaterally under the stereotaxic surgery by Aβ or vehicle into the entorhinal cortex (AP: −5.05, L: ±6.6 and DV: −8.2) according to the Atlas of Paxinos and Watson [38]. Injections were made over 2 min using a 5-μl Hamilton syringe fitted with a 30-gauge blunt-tipped needle, and the needle remained in place for an additional 1 min before it was slowly retracted. After entorhinal injection, animals were implanted with cannula (8 mm, 23 gauge) located 1 mm above the right ventricle (AP: −0.96 L: 1.8 DV: −3.4). The cannuula was fixed to the skull with a screw and dental cement. Every day, starting from the first day of the operation until the 6th day rats were given i.c.v. nimodipine (3, 10, 30 μg/2 μl, Tocris, UK), isradipine (3, 10, 30 μg/2 μl, Tocris, UK) or vehicle (DMSO, Sigma–Aldrich, USA) using a 30 gauge needle attached to a 5-μl Hamilton syringe by a polyethylene tube.

2.3. Morris water maze

2.3.1. Apparatus

The water maze was consisted of a pool (155 cm diameter) filled with water (21 ± 1 °C) until 10 cm from the edge of the tank. A transparent plexiglas platform (10 cm diameter) was located 1.5 cm below the water surface in the tank's eastern quadrant (target quadrant, Q_1). The platform was the only escapable thing from the water. The walls surrounding the pool were decorated with distinct extramaze (spatial) cues. These cues were kept in the fixed positions with respect to the swimming pool during the whole experiment to allow the animals to find the hidden platform. Animal movements was recorded by a CCD camera (Panasonic Inc., Japan) hanging from the ceiling above the MWM apparatus and locomotion tracking was measured by the Ethovision software (version XT7, Netherland), a video tracking system for automated analyzing of animal's behavior.

2.3.2. Habituation

Twenty-four hours before starting the hidden platform training, rats were given a 60 s swim in the tank without platform for adaptation to the environment.

2.3.3. Procedure

2.3.3.1. Reference spatial learning and memory. This test was done according to previous procedure conducted in our laboratory with little modification [39,40]. For training, the platform was submerged in the eastern quadrant (target quadrant, Q_1). The platform place remained the same throughout training, but animals released into the water (while facing to tank wall) from different location chosen from the north, south, west, northwest and southwest randomly between the trials. Rats received one training session for 3 consecutive days. Each session consisted of four trials with 10 min inter-trial intervals. Animals were allowed to swim 60 s to localize platform position in the tank. Rats that did not find the platform within 60 s were directed to it and allowed to rest on it for 10 s. Twenty-four hours after the 3rd session the spatial probe test was given. In spatial probe test, the platform was removed, and rats were allowed to swim for 60 s before they were removed. Animals were released in the water in a location that was exactly opposite from where the platform was placed. Behavior was recorded with a video tracking system. Escape latencies, distance traveled and percentage time spent in each quadrant and swim speeds were recorded for subsequent analysis.

2.3.3.2. Reversal spatial learning and memory. For the reversal learning and memory test, twenty four hour after spatial probe test, platform was placed in the opposite quadrant (western quadrant, Q_3) and rats were trained to find the new platform location during two blocks. Each block consisted of four trials with 10 min inter-trial intervals and separated by 30 min. Platform location remained the same throughout training and the drop location varied between trials. Twenty-four hours later, the reversal probe test was conducted during which platform was removed and rats were allowed to swim in the tank for 60 s.

2.3.4. Visual test

After the reversal probe trial, the platform was elevated above the water surface and covered by bright color aluminum foil and placed in a different zone (north quadrant) and rats were allowed to swim and find the visible platform during 60 s in order to test their visual ability. All experiments were conducted between 10:00 and 14:00.

2.4. Western blot analysis

One week after Aβ injection animals were decapitated and their brains were removed. Horizontal brain slices (500 μm) were prepared by vibroslicer (Campden Instruments, UK). Then, ECs were separated and homogenized in the lysis buffer containing a protease inhibitor cocktail. Total proteins were electrophoresed in 12% SDS-PAGE gels, then transferred to polyvinyl idene fluoride membranes and probed with calpain 2, caspase 3 (1/1000 dilution, Cell Signaling Technology, Beverly, MA, USA) and caspase 3 (1/1000 dilution, Abcam, Cambridge, UK). Immunoreactive polypeptides were detected by chemiluminescence using Electro ChemiLuminescence reagents (Amersham Bioscience, USA) and subsequent autoradiography. Quantification of results was performed by densitometric scan of the films. Data analysis was done using ImageJ, after background subtraction. Protein concentration was determined by Bradford method [41], using bovine serum albumin as a reference standard.