Pharmacology, Biochemistry and Behavior 153 (2017) 147–159

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

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

Research article

Calcineurin inhibitors improve memory loss and neuropathological changes in mouse model of dementia

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ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form 8 December 2016
Accepted 31 December 2016
Available online 4 January 2017

Keywords:
Dementia
STZ
Age
Cyclosporine
Tacrolimus
Calcineurin

ABSTRACT

Aim: The present study was designed to investigate the potential of Cyclosporine (CsA) and Tacrolimus, the inhibitors of calcineurin (CaN) in cognitive deficits of mice.
Methods: Streptozotocin [STZ, 3 mg/kg, injected intracerebroventricular (*i.c.v.*)] was used to induce memory deficits in NIH mice, while aged mice separately taken served as a natural model of dementia. Morris water maze (MWM) test was employed to evaluate learning and memory of the animals. A battery of biochemical and histopathological studies was also performed. Extent of oxidative stress was measured by estimating the levels of brain glutathione (GSH) and thiobarbituric acid reactive species (TBARS). Brain acetylcholinestrase (AChE) activity was estimated to assess cholinergic activity. The brain level of myeloperoxidase (MPO) was measured as a marker of inflammation.
Results: STZ *i.c.v.* and aging results in marked decline in MWM performance of the animals, reflecting impairment of learning and memory. STZ *i.c.v.* treated mice and aged mice exhibited a marked accentuation of AChE activity, TBARS and MPO levels along with a fall in GSH level. Further the stained micrographs of STZ treated mice and aged mice indicate pathological changes, severe neutrophilic infiltration and amyloid deposition. Cyclosporine and Tacrolimus treatment significantly attenuated STZ induced and age related memory deficits, biochemical and histopathological alterations.
Conclusion: The findings demonstrate the potential of CaN inhibitors Cyclosporine and Tacrolimus in memory dysfunctions which may probably be attributed to anti-cholinesterase, anti-amyloid, anti-oxidative and anti-inflammatory effects. It is concluded that CaN can be explored as a potential therapeutic target in dementia.

1. Introduction

Dementia is a syndrome, usually of chronic or progressive nature, caused by a variety of brain illnesses that affect memory, thinking, behavior and ability to perform everyday activities. Alzheimer's disease (AD) is the most common form of dementia and possibly contributes to 60–70% of cases (World Alzheimer's Report, 2009). It is one of the major causes of disability and dependency among older people worldwide. The number of people living with dementia worldwide is currently estimated at 35.6 million. This number is expected to double by 2030 and more than triple by 2050 (W.H.O., 2012). The high global prevalence, economic impact of dementia on families, caregivers and communities, and the associated stigma present a significant public health challenge. The global health community has recognized the need for action and need to place dementia on the public health agenda. Characteristic feature of AD is progressive loss of memory (Dao et al., 2013). Currently only two classes of drugs i.e. acetyl cholinesterase inhibitors like rivastigmine, donepezil etc. and NMDA antagonist mementine are available for clinical use (Fang et al., 2013). These drugs have only limited benefits so there is utmost need for a sure-shot remedy which will not only provide symptomatic relief but also halt the progression of disease.

Calcineurin (CaN), also known as protein phosphatase 2B (PP2B), is a calcium (Ca^{2+})-sensitive serine/threonine phosphatase. CaN is highly expressed in the central nervous system (CNS) and originally isolated from mammalian brain (Klee and Krinks, 1978). CaN is directly activated by Calmodulin (CaM), making it uniquely and exquisitely responsive to Ca^{2+} fluctuation (Rusnak and Mertz, 2000). CaN has been reported to affect the release of neurotransmitters from the pre-synaptic site and is intimately involved in a number of pathways that modulate neuronal excitability and synaptic activity (Jovanovic et al., 2001; Reese and Taglialatela, 2011; Utreja et al., 2013). Dysregulation of this complex system would likely have far-reaching consequences on synaptic connectivity (Reese and Taglialatela, 2011). Increase in CaN level has been shown to play an important role in altered synaptic plasticity, neuronal apoptosis, neuroinflammation, elevation in glutamate, excitotoxic cell death, hyperphosphorylated tau, neurodegeneration and behavioral impairments observed in mouse models of AD (Abdul et al., 2009;

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http://dx.doi.org/10.1016/j.pbb.2016.12.018
0091-3057/

Agostinho et al., 2008; Loo et al., 1993; Reese et al., 2008; Wu et al., 2010). Further immunohistochemical analyses of aged murine hippocampi show intense CaN staining in activated astrocytes (Norris et al., 2005), providing a possible role for CaN hyperactivity in triggering astrogliosis and inflammatory pathways; leading to age related memory loss in mouse. CaN is also found to play an important role in amyloid β-levels, tau proteins (prominent markers of AD) as well as neuronal and cell death in AD (Reese and Taglialatela, 2011; Wu et al., 2010; Yu et al., 2008). Recent clinical report shows significant reduction in incidence of dementia in organ transplant patient treated with calcineurin inhibitors (Taglialatela et al., 2015), thereby suggesting a central role of CaN in AD onset and/or clinical progression (Abdul et al., 2009; Braithwaite et al., 2012; Reese and Taglialatela, 2011; Reese and Taglialatela, 2011). Therefore, it is prudent to consider the possibility of CaN inhibition as a pharmacological target in the development of novel AD therapies.

So, far there is no conclusive evidence regarding the potential of CaN inhibitor in dementia of AD type, and there is a need to explore the potential of CaN inhibitors in dementia.

Therefore the present study has been undertaken to investigate potential of Cyclosporine and Tacrolimus, selective CaN inhibitors in mouse model of *i.c.v.* STZ and aging induced dementia.

2. Materials and methods

2.1. Experimental animals

NIH mice of either sex weighing 20–35 g were employed in the present study. Young mice (around 4 month old) and aged mice (15 month and above) were procured from Central Research Institute, Kasauli, Himachal Pardesh, India. They were housed in the departmental animal house, and were exposed to 12 h light and dark cycle. Animals were freely provided with standard laboratory diet and water ad libitum. The animals were acclimatized to the laboratory conditions 4 days before experiments. The experimental protocol was duly approved by the institutional animal ethical committee and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No. 107/1999/CPCSEA).

2.2. Drugs and chemicals

All drugs were freshly prepared before use. Streptozotocin (Sigma Aldrich Co. Ltd., St. Louis, U.S.A.) was dissolved in freshly prepared artificial cerebrospinal fluid (ACSF). Donepezil (Ranbaxy Laboratories Limited, Railmajra, Nawanshahar, India) was dissolved in saline. Cyclosporine (Novartis Pharmaceuticals UK Ltd) was diluted in ACSF, Tacrolimus (Panacea Biotec India Ltd.) was dissolved in 0.5% DMSO solution. All the reagents used in the present study were of analytical grade. Tacrolimus and Donepezil were administered *i.p.*, and Cyclosporine and Streptozotocin were administered *i.c.v. i.c.v.* route of Cyclosporine administration was selected based on the previous studies (Dougherty and Dafny, 1988; Francischi et al., 1997). The main reason behind this is that blood brain permeability of cyclosporine is poor, therefore we opted for *i.c.v.* route. Donepezil is FDA approved a well-established drug for the management of dementia of AD and being clinically used for memory deficits of AD patient. Studies have shown beneficial effects of donepezil in dementia of AD and other etiologies (Singh et al., 2013). Hence, donepezil has been taken as a positive control in this study.

2.3. Induction of experimental dementia by streptozotocin (STZ)

Experimental dementia was induced in young mice (around 4 months old) by intracerebrovascular (*i.c.v.*) injection of STZ (3 mg/kg) in two divided doses, on the first day and the third day (Sharma et al., 2008; Singh et al., 2013). The control group mice were given *i.c.v.* injection (10 μl) of artificial cerebrospinal fluid.

2.4. Aged mouse model of dementia

Mice of age 15 months and above served as natural model of dementia (Neha et al., 2014; Parle and Singh, 2007).

2.5. Morris water maze test (MWM)

MWM test was employed to assess learning and memory of the animals (Morris, 1984; Parle and Singh, 2004). MWM procedure was based on the principle where animal was placed in a large pool of water divided into four equal quadrants, as animal dislikes swimming, its tendency to escape was accomplished by finding a hidden escape platform. Each animal was subjected to four consecutive training trials (with an inter trial gap of 5 min) each day for four consecutive days in search for a hidden platform as shown below. The mouse was gently placed in the water between quadrants, facing the wall of the pool with drop location changing for each trial as shown below and allowed 120 s to locate the submerged platform. Then, it was allowed to stay on the platform for 20 s. If it failed to find the platform within 120 s, it was guided gently onto the platform and allowed to remain there for 20 s. The day 4 escape latency time (ELT) to locate the hidden platform in water maze was taken as the index of acquisition or learning. On the fifth day, the hidden platform was removed. Each animal was allowed to explore the pool for 120 s. Mean time spent in all the quadrants in search of the hidden platform was noted. The mean time spent by the animal in the target quadrant (TSTQ) was taken as an index of retrieval or memory.

The entire MWM test was video-graphed and path length/distance travelled and swim speed of each animal was measured by using ANY-maze video tracking system (v - 4.99).

Day 1	Q1	Q2	Q3	Q4
Day 2	Q2	Q3	Q4	Q1
Day 3	Q3	Q4	Q1	Q2
Day 4	Q4	Q1	Q2	Q3

2.6. Biochemical estimations

Animals were sacrificed by cervical dislocation at the end of the experiment, brain were collected and homogenized in phosphate buffer (pH 7.4, 10% w/v) using homogenizer and centrifuged at 3000 rpm for 15 min to obtain clear supernatant. The clear supernatant and pellet were then used for different biochemical estimations. Intact brains from each group were preserved in Bouin's solution for histopathological examinations.

2.6.1. Estimation of brain acetyl cholinesterase (AChE) activity

The whole brain AChE activity was measured by the method of Ellman et al. (1961). This was measured on the basis of the formation of yellow color due to the reaction of thiocholine with dithiobisnitrobenzoate ions. 0.5 ml of supernatant of the brain homogenate was pipetted out into 25 ml volumetric flask and dilution was made with a freshly prepared DTNB {5,5′-dithiobis (2-nitro benzoic acid)} solution. From this two 4 ml portions were pipetted out into two test tubes. Into one of the test tube, 2 drops of eserine solution was added. 1 ml of substrate solution (75 mg of acetylthiocholine iodide per 50 ml of distilled water) was pipetted out into both of the test tubes and incubated for 10 min at 30 °C. The test-tube containing eserine solution was taken as blank and change in absorbance per min of the sample was read spectrophotometrically (DU 640B spectrophotometer, Beckman Coulter Inc., CA, USA) at 420 nm. AChE activity was calculated using the following formula: