Neuroscience 315 (2016) 18–30

THE RECOMBINANT C-TERMINAL FRAGMENT OF TETANUS TOXIN PROTECTS AGAINST CHOLINOTOXICITY BY INTRASEPTAL INJECTION OF β-AMYLOID PEPTIDE (25–35) IN RATS

A. PATRICIO-MARTÍNEZ, [a] **L. MENDIETA,** [a,b] **I. MARTÍNEZ,** [a] **J. AGUILERA** [c,d] **AND I. D. LIMÓN** [a]*

[a] *Laboratorio de Neurofarmacología, FCQ-Benemérita Universidad Autónoma de Puebla, Puebla, Mexico*

[b] *Cajal Institute, CSIC, Madrid, Spain*

[c] *Departament de Bioquímica i de Biologia Molecular, Facultad de Medicina, Institut de Neurociències, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Spain*

[d] *Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Barcelona, Spain*

Abstract—The recombinant C-terminal domain of tetanus toxin (Hc-TeTx) is a new non-toxic peptide of the tetanus toxin that exerts a protective action against glutamate excitotoxicity in motoneurons. Moreover, its efficacy as a neuroprotective agent has been demonstrated in several animal models of neurodegeneration. The eleven amino acids in the β amyloid peptide ($A\beta_{25–35}$) mimic the toxic effects of the full β amyloid peptide ($A\beta_{1–42}$), causing the impairment of the cholinergic system in the medial septum (MS) which, in turn, alters the septo-hippocampal pathway and leads to learning and memory impairments. The aim of this study was to examine the neuroprotective effects of the Hc-TeTx fragment against cholinotoxicity. The Hc-TeTx fragment (100 ng) was injected into the rats intercranially, with the $A\beta_{(25–35)}$ (2 μg) then injected into their MS. The animals were tested for spatial learning and memory in the eight-arm radial maze. The brains were removed to assess cholinergic markers, such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), and to explore neurodegeneration in the MS and hippocampus, using amino-cupric silver and H&E staining. Finally, capase-3, a marker of apoptosis, was examined in the MS. Our results clearly demonstrate that the application of Hc-TeTx prevents the loss of cholinergic markers (ChAT and AChE), the activation of capase-3, and neurodegeneration in the MS and the CA1 and CA3 subfields of the hippocampus. All these improvements were reflected in spatial learning and memory performance, and were significantly higher compared with animals treated with $A\beta_{(25–35)}$. Interestingly, the single administration of Hc-TeTx into the MS modified the ChAT and AChE expression that affect cognitive processes, without inducing neurodegeneration or an increase in capase-3 expression in the MS and hippocampus. In summary, our findings suggest that the recombinant Hc-TeTx fragment offers effective protection for the septo-hippocampal pathway, given that it reduces the neurodegeneration caused by $A\beta_{(25–35)}$ and improves learning and memory processes.

Key words: medial septum, Hc-TeTx, amyloid-$\beta_{(25–35)}$, choline acetyltransferase, acetylcholinesterase, spatial memory.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of degenerative dementia to affect the human central nervous system (CNS) and is characterized by a severe decline in memory function. The two major morphopathological hallmarks of AD are the deposition of extracellular neuritic β-amyloid peptide-containing plaques (senile plaques), which is accompanied by the presence of intracellular neurofibrillary tangles (Selkoe, 1991; Maccioni et al., 2001). The toxic properties of the native full-length $A\beta_{(1–42)}$ peptide are retained in the 25–35 fraction of amyloid beta ($A\beta_{25–35}$) (Pike et al., 1995), for which reason this small peptide has been widely used to aid in understanding the physiopathology of AD. Previous studies by our research group have shown that the injection of $A\beta_{(25–35)}$ peptide into the hippocampus and temporal cortex of rats produces the serious decline in spatial memory associated with oxidative and nitrosative stress, apoptosis, and neuroinflammation resulting in neuronal death (Limón et al., 2009; Díaz et al., 2012; Ortega et al., 2014). The cholinergic neurons in the basal forebrain are particularly vulnerable to Aβ toxicity, something which has been corroborated in animal models (Abe et al., 1994; Giovannelli et al., 1995; Maurice et al., 1996; Harkany et al., 1995; Yamaguchi and Kawashima, 2001). The degeneration of the cholinergic neurons in the basal forebrain and the loss of cholinergic transmission in the cerebral cortex and hippocampus is the principal cause of cognitive dysfunction in patients with AD (Auld et al., 2002; Kar and Quirion,

*Corresponding author. Address: Laboratorio de Neurofarmacología, FCQ-Benemérita Universidad Autónoma de Puebla, 14 Sur y Av. San Claudio C.U. Edificio 105C, A.P. 72570 Puebla, Mexico. Tel/fax: +52-222-229-55-00x7528.

E-mail addresses: ilhlimon@yahoo.com.mx, dlimonperezdeleon@yahoo.com.mx (I. D. Limón).

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; A-Cu-Ag, amino-cupric-silver; AD, Alzheimer's disease; Aβ, β amyloid peptide; ChAT, choline acetyltransferase; CNS, central nervous system; Hc-TeTx, C-terminal domain of tetanus toxin; H&E, Hematoxylin and eosin; MS, medial septum; NGF, nerve growth factor; PBS, phosphate-buffered saline; RM, reference errors; SSI, isotonic saline solution; Trks, tropomyosin receptor kinases; VAChT, vesicular acetylcholine transporter; WM, working memory errors.

http://dx.doi.org/10.1016/j.neuroscience.2015.11.066
0306-4522/

2004; Kar et al., 2004). In addition, the progressive loss of cholinergic neurons in the basal forebrain leads to a reduction in a number of cholinergic markers, such as choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), and acetylcholinesterase (AChE), as well as levels of acetylcholine (ACh) (Whitehouse et al., 1981; DeKosky et al., 1992; Mesulam, 2004).

While AChE inhibitors have been used for treating solely the symptomatology of AD in order to maintain cholinergic tone and enhance cognitive functions, cognitive improvement is only transient. In recent years, considerable attention has been paid to neurotrophins and, in particular, the nerve growth factor (NGF), which has potential for the maintenance of cholinergic neuron function. NGF has been shown to up-regulate several cholinergic markers (i.e. ChAT activity) and the synthesis and release of ACh, thus enhancing cell survival and improving memory processes (Oosawa et al., 1999; Szutowicz et al., 2004; Conner et al., 2009). NGF is synthetized and released by cortical and hippocampal neurons, for which reason it serves as a target-derived neurotrophic factor for basal forebrain cholinergic neurons. Despite the fact that NGF may have a potential neuroprotective role in AD treatment, this neurotrophin is unable to cross the blood–brain barrier (BBB), meaning that its neuroprotective effects are restricted to the short-term and it requires continuous infusion.

It has been shown that the non-toxic C-terminal fragment of tetanus toxin (Hc-TeTx) acts as a potent neuroprotector, preventing the neuronal death caused by apoptosis (Chaib-Oukadour et al., 2004, 2009). The Hc-TeTx fragment corresponds to half of the heavy chain of tetanus toxin (TeTx), and is responsible for binding TeTx to the cell membrane and transporting the complete toxin retroaxonally to the CNS. This fragment is able to activate the neurotrophin receptors involved in the neural survival of cortical neurons, tropomyosin receptor kinases (Trks) (Gil et al., 2000, 2003). Different studies have shown that the Hc-TeTx fragment can be used as a biological carrier of neurotrophic factors in order to cause neuronal improvements *in vivo* (Larsen et al., 2006; Ciriza et al., 2008; Calvo et al., 2011); however, the Hc-TeTx fragment by itself has been shown to exert neuroprotective or restorative effects in animal models of neurodegeneration (Moreno-Igoa et al., 2012; Mendieta et al., 2009, 2012; Sánchez-González et al., 2014).

The aim of this study was to evaluate the neuroprotective effect of Hc-TeTx fragment against the cholinotoxicity induced by the injection of $A\beta_{(25–35)}$ into the medial septum (MS) through the examination of cholinergic markers (ChAT and AChE) and the neurodegenerative process, both of which impact on spatial learning and memory processes in rats.

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats (250–300 g) ($n = 48$) were obtained from the Claude Bernard animal facilities at the *Benemérita Universidad Autónoma de Puebla* (BUAP). They were individually housed in groups of 4–6 per cage in constant temperature conditions of 22 ± 2 °C, in a 12-h light/dark cycle (lights on at 8 AM), with free access to food and water. All experimental procedures conformed with the Guide for Care and Use of Laboratory Animals (*Norma Official Mexicana*, Official Mexican Standard, NOM-062-ZOO-1999) and were approved by the BUAP bioethics committee.

Hc-TeTx and $A\beta_{(25–35)}$ preparation

The Hc-TeTx fragment (50 kDa) was synthesized in accordance with Herrando-Grabulosa et al., 2013. The $A\beta_{(35–25)}$ and $A\beta_{(25–35)}$ fractions (Sigma–Aldrich Ltd., St. Louis, MO, USA) were dissolved in isotonic saline solution (SSI) in order to obtain a concentration of [1 μg/μL] for each. The aggregation of $A\beta_{(35–25)}$ and $A\beta_{(25–35)}$ was carried out by means of a 36-h incubation at 37 °C.

Stereotaxical surgery

Each animal was anesthetized with ketamine–xylazine (75:10 mg/kg, ip). Rats were randomly assigned to be injected with vehicle (SSI), Hc-TeTx, $A\beta_{(35–25)}$, Hc-TeTx + $A\beta_{(35–25)}$, $A\beta_{(25–35)}$, and Hc-TeTx + $A\beta_{(25–35)}$ into the MS ($n = 8$ per group). Each animal was placed in a stereotaxical apparatus (Stoelting Co., Wood Dale, IL, USA) for the administration of $A\beta_{(25–35)}$, Hc-TeTx fragment or vehicle. Each solution was injected using a 10 μL Hamilton syringe with each 1 μL infused for 300 s. 100 ng of Hc-TeTx was dissolved in 2 μL of vehicle, followed by (2 μg/2 μL) of $A\beta_{(35–25)}$, or $A\beta_{(25–35)}$ peptide was injected into the MS, with a 10-min interval between the injection of each agent and the following stereotaxic coordinates: AP: +0.6 mm from Bregma, L: 0.0 mm from midline, DV: −5.2 below dura (according to Paxinos and Watson's Stereotaxic Atlas, 1998). The vehicle group was administered only 2 μL of SSI into the MS. Proper post-operative attention was provided until the animal made a full recovery.

Histological examination

After the behavioral experiments, all animals ($n = 8$) were anesthetized and intra-cardially perfused with 200–250 mL of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Brains were removed and embedded in paraffin. Coronal brain sections of 5-μm were obtained for histological analysis using a microtome (Leica RM2125RT) at the level of the MS, approximately 0.7–0.1 from the bregma, and approximately −2.3 to −4.1 from the hippocampus.

Immunohistochemistry

The paraffin was removed by section, which was rehydrated using conventional histological techniques and rinsed with PBS pH 7.4. Nonspecific binding sites were blocked by means of incubation in IgG-free 2% bovine serum albumin (BSA, Sigma) for 40 min. Specimens were then incubated for 10 min with 0.2% Triton X-100 in PBS at room temperature, with the slices then rinsed with PBS. The sections were incubated overnight at 4 °C with a polyclonal rabbit