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Research report

# Neuroprotective efficiency of tetanus toxin C fragment in model of global cerebral ischemia in Mongolian gerbils

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ABSTRACT

The tetanus toxin C (TTC) fragment capacity of being transported in a retrograde way through motoneurons and its nontoxic nature opens the door to a new promising therapeutic strategy for neurodegenerative diseases. In this study, the TTC effect was tested for the first time in animal model of global cerebral ischemia induced by 10-min occlusion of both common carotid arteries. The aim was to evaluate the effect of TTC gene therapy treatment on the development and expression of global cerebral ischemia/reperfusion-induced oxidative stress and motor hyperactivity in Mongolian gerbils. Several oxidative stress and motor behavioral parameters were investigated between 2 h and 14 days after reperfusion. Neuroprotective efficiency of TTC was observed in the forebrain cortex, striatum, hippocampus, and cerebellum at the level of each examined oxidative stress parameter (nitric oxide level, superoxide production, superoxide dismutase activity, and index of lipid peroxidation). Additionally, TTC significantly decreased ischemia-induced motor hyperactivity based on tested parameters (locomotion, stereotypy, and rotations). As judged by biochemical as well as behavioral data, treatment with TTC for the first time showed neuroprotective efficiency by reduction of ischemia-induced oxidative stress and motor hyperactivity and can be a promising strategy for ischemia-induced neuronal damage treatment.


## 1. Introduction

Stroke and cerebral ischemia are the leading cause of death and permanent disability, with still no effective treatment. Transient global cerebral ischemia occurs during cardiac arrest, cardiopulmonary bypass surgery and other situations that deprive the brain of oxygen and glucose for short periods. The events that follow during ischemia are glutamate mediated excitotoxicity, $Ca^{2+}$ overload, oxidative stress, neurovascular pathophysiology and inflammation, cell death mode, and gene expression (Mehta et al., 2007). In both humans and animals, ischemia damages neurons in vulnerable structures of the brain, including the hippocampus, striatum, cerebral cortex, and cerebellum. Considering the very important role of these brain structures in control of different types of motor behavior it is expected that, apart from the well known morphological changes, global cerebral ischemia also leads to functional changes that can be assessed by behavioral studies (Block, 1999). It has already been established that behavioral studies are a valuable completion of morphological studies, especially concerning a simpler way of the evaluation of neuroprotective efficacy of drugs. Actually, the search for an efficient therapeutic treatment in nervous system pathologies is not an easy task due to the blood–brain barrier. Dealing with possible neuroprotectants in cerebral ischemia, until now a wide range of choices has been described (Kluska et al., 2005; Sjakste et al., 2005; Reiter et al., 2007; Nagel et al., 2008; Janac et al., 2008; Hyun et al., 2011; Pendharkar et al., 2010; Selakovic et al., 2010).

The non-toxic C-terminal fragment of the tetanus toxin heavy chain (TTC), which is obtained by protease digestion, has been shown to be transported through neurons in a similar manner to the native toxin transport, without causing clinical symptoms (Evinger and Erichsen, 1986). Some authors have implicated TTC in neurotrophic signaling pathways and anti-apoptotic processes in neuronal cultures (Gil et al., 2003; Chaib-Oukadour et al., 2004; Ciriza et al., 2008b). Furthermore, TTC is sufficient for neuron binding, internalization, and retrograde and trans-synaptic transport (Sinha et al., 2000). In particular, the TTC capacity of being transported in a retrograde way through motoneurons has been

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exploited in previous studies, in which TTC has been used as a retrograde carrier of active molecules, such as neurotrophic factors, to central nervous system (Coen et al., 1997; Bordet et al., 2001; Larsen et al., 2006). Interestingly, this potential use of TTC as a neurotrophic agent has been demonstrated for neurodegeneration *in vivo* in a mouse model of amyotrophic lateral sclerosis (Moreno-Igoa et al., 2010). So far, TTC was proven as a valuable protein carrier in the central nervous system, but the effect of TTC itself was neither tested before nor in the model of experimental ischemia.

To address this fact, the present study was carried out to analyze the potential neuroprotective effect of TTC gene therapy treatment on the development and expression of global cerebral ischemia/reperfusion-induced oxidative stress and motor hyperactivity in Mongolian gerbils. The possibility of using TTC as non-viral therapeutic target may shed light on the novel molecular pathways involved in the pathogenesis of cerebral ischemia and provide a steady and safe tool for the treatment of this disease in humans.

## 2. Materials and methods

### 2.1. Animals

Adult male Mongolian gerbils (*Meriones unguiculatus*, 60–75 g) were housed in an air-conditioned room, at a temperature of 23 ± 2 °C, with 55 ± 10% humidity, and with lights on 12 h/day (07:00–19:00). Food and tap were given water *ad libitum*. Animals used for procedures were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985), European Communities Council Directive (86/609/EEC), as well as with approval of the local Ethical Committee and with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2. Induction of global cerebral ischemia

Experimental global ischemia occurs when both common carotid arteries were clamped for 10 min, and reperfusion was confirmed visually (for details, see Selakovic et al., 2010). Post ischemic temperature was carefully monitored due to the fact that the gerbil model typically shows an intrinsic hyperthermic response during the initial hours of recirculation (Kuroiwa et al., 1990). Since the changes in body temperatures are known to have an impact on the consequences of global ischemia, it was maintained at 37 ± 0.3 °C throughout the surgical procedure.

### 2.3. Construction of recombinant plasmid carrying TTC DNA and its application

A TTC-encoding gene was cloned into the pcDNA3.1 (Invitrogen S.A.) eukaryotic expression plasmid under control of the cytomegalovirus (CMV) immediate-early promoter. The TTC gene was removed from pGex-TTC plasmid (Ciriza et al., 2008a) with *BamHI* and *NotI* restriction enzymes and inserted into pCMV to create the pCMV-TTC plasmid. After sequencing, vectors were expanded in chemically competent *Escherichia coli* (*DH5α*) and purified using Genelute maxiprep-kit (Sigma–Aldrich Química, S.A., Madrid, Spain). Using an insulin syringe, each animal received in tongue double injection of 0.71 mol/L pCMV-TTC, a naked DNA of TTC gene (nDNA-TTC, Fig. 1B) dissolved in 50 μL ultrapure water 4 days before the occlusion, because it has been described as a higher plasmid expression time (Miana-Mena et al., 2005). Appropriate control animals were injected with the same amount of empty plasmid, naked DNA (nDNA, Fig. 1A). Sham-injected animals received 50 μL ultrapure water.

### 2.4. Experimental procedure

The gerbils were randomly divided into seven experimental groups. Control groups were intact, sham-operated, and naked DNA (nDNA, 200 μg) injected, while the treatment groups were naked DNA encoding for TTC (nDNA-TTC, 200 μg) injected, submitted to 10-min global cerebral ischemia or treated with nDNA 4 days before the occlusion (Fig. 2). Intact gerbils were not submitted to any type of surgical and injection procedures. Sham-operated gerbils were exposed to the same surgical intervention as ischemic gerbils, but without occlusion of both common carotid arteries. nDNA injected gerbils were treated as nDNA-TTC injected gerbils, but with the same amount of empty plasmid.

After 10-min global cerebral ischemia, neurological status of gerbils without and with TTC treatment was monitored. Also, the effect of TTC on survival of ischemic gerbils was estimated 48 h after occlusion on a separate group of animals.

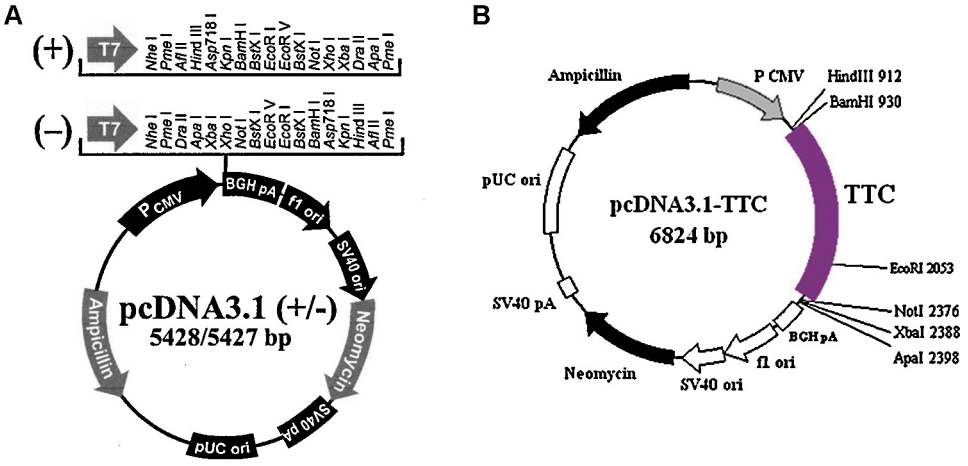


**Fig. 1.** Restriction maps of empty plasmid and recombinant plasmid pcDNA3.1-TTC. A TTC-encoding gene was cloned into the pcDNA3.1 (Invitrogen S.A., Prat de Llobregat, Spain) eukaryotic expression plasmid (A) under control of the cytomegalovirus (CMV) immediate-early promoter. Forward (GGATCCCCAGTCATGGTTTTT) and reverse (GCGGCCGCTCGAGTCGACCCG) primers were used to amplify the sequence of TTC (B).