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ORIGINAL ARTICLE

The C-terminal domain of tetanus toxin protects motoneurons against acute excitotoxic damage on spinal cord organotypic cultures

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Abstract

The C-terminal domain of tetanus toxin (Hc-TeTx) has been suggested to act as a neuroprotective agent by activating signaling pathways related to neurotrophins and also to exert anti-apoptotic effects. Here, we show the beneficial properties of the recombinant protein Hc-TeTx to protect spinal motoneurons against excitotoxic damage. *In vitro* spinal cord organotypic cultures were used to assess acute glutamate excitotoxic damage. Our results indicate that Hc-TeTx treatment improves motoneuron survival within a short therapeutical window (the first 2 h post-injury). Within this interval, we found that p44/p42 MAP kinase (ERK1/2) and glycogen synthase kinase-3 (GSK3β) signaling pathways play a crucial role in the neuroprotective effect. Moreover, we demonstrated that Hc–TeTx treatment initiate autophagy which is ERK1/2- and GSK3β-dependent. These findings suggest a possible therapeutical tool to improve motoneuron survival immediately after excitotoxic insults or during the secondary injury phase that occurs after spinal cord trauma.

Keywords: autophagy, C-terminal domain of tetanus toxin, ERK1/2, glutamate excitotoxicity, GSK3β, motoneurons spinal cord organotypic cultures.

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Secondary damage induced after acute spinal cord injury (SCI) involves several physiopathologic molecular mechanisms. Excitotoxicity is one of the main processes implicated in neuronal death (Park *et al.* 2004; Kuzhandaivel *et al.* 2011). Some studies have suggested that excitotoxicity could be responsible of the induction of other processes, such as ischemia, energy collapse, and vascular tone autoregulation (McAdoo *et al.* 1999). Spinal motoneurons (MNs) are especially vulnerable to excitotoxic damage (Rothstein *et al.* 1993; Nakamura *et al.* 1994; Van Den Bosch *et al.* 2006; Ferraiuolo *et al.* 2011).

The C-terminal domain of tetanus toxin (Hc-TeTx) is a non-toxic fragment of the tetanus toxin responsible of the protein binding to the cell membrane (Bizzini *et al.* 1977; Manning *et al.* 1990; Pellizzari *et al.* 1999; Schiavo *et al.* 2000). Several studies have demonstrated that Hc–TeTx has the capacity to bind specifically to MNs (Price *et al.* 1975; Lalli and Schiavo 2002; Lalli *et al.* 2003; Roux *et al.* 2005), preferently by *lipid rafts* microdomains (Herreros *et al.* 2001), through gangliosides (Halpern and Loftus 1993; Benson *et al.* 2011) and glycoprotein acceptors (Yavin and Nathan 1986; Herreros *et al.* 2000). It is still unclear which protein acceptors are implicated in the binding, but is well known that Hc-TeTx share trafficking vesicles with some neurotrophins and their receptors to be transported retrogradally to the central nervous system (Deinhardt *et al.* 2006).

However, other actions of Hc-TeTx have been described. Hc-TeTx protects cerebellar granule neurons (CGNs) against

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Abbreviations used: BCA, bicinchoninic acid assay; DAPI, 4′-6-Diamidino-2-phenylindole; DIV, days *in vitro*; FPLC, Fast Protein Liquid Chromatography; GBSS, Gey's Balanced Salt Solution; GFAP, glial fibrillary acidic protein; HBSS, Hank's Balanced Salt Solution; HRP, horseradish peroxidase; MEM, minimal essential medium; PBS, phosphate buffered saline; SCOC, spinal cord organotypic culture.

apoptotic cell death caused by potassium deprivation (Chaib-Oukadour *et al.* 2004) and also reduces neuronal 1-methyl-4-phenylpyridinium (MPP^+) toxicity *in vitro* (Chaib-Oukadour *et al.* 2009) and *in vivo* (Mendieta *et al.* 2009). Furthermore, several studies performed by using the naked DNA of this domain demonstrated its beneficial effect in a mouse model of Amyotrophic Lateral Sclerosis (ALS) (Moreno-Igoa *et al.* 2010, 2012; Calvo *et al.* 2011). Previous results from our laboratory have demonstrated that Hc-TeTx induces the activation of the p21ras/MAPK (mitogen-activated protein kinase), PI3-K (phosphatidylinositol 3-Kinase)/Akt and PLCγ/PKC signaling pathways in rat synaptosomes (Gil *et al.* 2001), rat cortical neurons (Gil *et al.* 2003) and CGNs in culture (Chaib-Oukadour *et al.* 2004), and that these cascades activation correlated with tyrosine kinase receptors (Trks; tropomyosin-related kinases) induction and also with an anti-apoptotic effect of Hc-TeTx in neurons.

To determine whether Hc-TeTx might reduce some of the secondary effects of SCI, we have used an acute excitotoxic damage model on spinal cord organotypic cultures (SCOCs) (Guzman-Lenis *et al.* 2009). We have assessed the specific binding of Hc-TeTx on MNs in the tissue culture. Then, we have focused the present work to determine the neuroprotective properties of Hc–TeTx on the MNs and its biochemical mechanism of action.

Materials and methods

Hc-TeTx purification

Escherichia coli BL21 cells were transformed with pQE3 (Qiagen, Chatsworth, CA, USA) vector encoding for (6xHis)-tagged Hc-TeTx and were grown in Luria Bertani medium containing 100 μg/mL ampicillin as reported previously (Gil *et al.* 2001). Protein expression was induced by the addition of 0.4 mM isopropyl β–D–thiogalactoside (IPTG). After 3 h, cells were pelleted by centrifugation at 4000 *g* for 20 min at 4°C, re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 1% Triton-X-100; pH 8) and sonicated on ice for six 30 s periods. The suspension was centrifuged at 30 000 *g* for 30 min at 4°C. The clear supernatant, which contains the His-tagged protein, was purified by cobalt affinity chromatography. Mixed proteins were injected in a Fast Protein Liquid Chromatography (FPLC), which contains a cobalt-agarose resin (TALON Metal Affinity resin; Clontech Laboratories, Palo Alto, CA, USA), previously equilibrated (50 mM $NaH_2PO_4 \cdot H_2O$ and 300 mM NaCl; pH 7). The proteins, without His-Tags, were eluted by washing the resin with elution buffer (50 mM $NaH_2PO_4 \cdot H_2O$ and 300 mM NaCl; pH 7). Hc-TeTx contains six histidines, which is retained in the resin forming a Co-complex. Hc-TeTx was eluted with the elution buffer (50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl and 150 mM Imidazole; pH 7). Fractions collected were 0.5 mL volume. The elution process can be followed with FPLC system, that measures the absorbance at 280 nm constantly. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 12%. Gel was stained with GelCode Blue Stain Reagent (Pierce Chemical Co., Rockford, IL, USA) and those fractions containing purified Hc-TeTx protein were dialyzed (40 mM Na_2HPO_4, 10 mM NaH_2PO_4 and 150 mM NaCl; pH 7.4), overnight at 4°C, and for 2 h with new buffer. Protein concentrations were determined using the bicinchoninic acid assay (BCA; Pierce Chemical Co.) and lyophilized. The Hc-TeTx was stored in aliquots at −20°C. Alexa Fluor®555 was attached to Hc-TeTx following the manufacturer's protocol from Alexa Fluor®555 Labeling Kit (Invitrogen, Carlsbad, CA, USA) and stored at −20°C.

Spinal cord organotypic culture

Organotypic spinal cord cultures were prepared from lumbar spinal cords of 8-day-old Sprague-Dawley rat pups (P8), as previously described (Rothstein *et al.* 1993; Guzman-Lenis *et al.* 2008) with some modifications. After decapitation, the spinal cord was collected under sterile conditions and placed in ice-cold high glucose-containing (6.4 mg/mL) Gey's Balanced Salt Solution (GBSS) (Sigma-Aldrich Corp., St Louis, MO, USA). Meninges and roots were removed and the spinal cord was transversely sectioned in 350 μm slices with a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co., Surrey, UK). Four or five sections were carefully transferred on Millicell-CM porous membranes (0.4 μm) (Millipore, Billerica, MA, USA) into six-well plate containing 1 mL of incubation medium [50% minimal essential medium (MEM), 25 mM Hepes, 25% heat-inactivated horse serum, 2 mM glutamine, and 25% Hank's Balanced Salt Solution (HBSS) supplemented with 25.6 mg/mL glucose; pH 7.2]. Cultures were incubated at 37°C in a 5% CO_2/95% air humidified environment. Cultures were let to stabilize for 1 week, and after this point the medium was changed twice per week until 15 days *in vitro* (DIV). Under these conditions, the MN population reaches a steady number from 1 to 4 weeks as previously described (Guzman-Lenis *et al.* 2009). All procedures involving animals were approved by the Ethics Committee of Universitat Autònoma de Barcelona and followed the European Communities Council Directive 86/609/EEC.

Pharmacological treatments

Excitotoxic damage was induced at 15 DIV transferring the insert plates on modified Locke's buffer (137 mM NaCl, 2.5 mM $CaCl_2$, 5 mM KCl, 5.6 mM D-glucose, 0.3 mM KH_2PO_4, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 0.01 mM Glycine, and 10 mM Hepes; pH 7.4) with glutamate 1 mM and incubating at 37°C in 5% CO_2 for 30 min (Guzman-Lenis *et al.* 2008). Briefly, insert plates were replaced to fresh incubation medium. Modified Locke's buffer was added also in control treatments without glutamate. Hc-TeTx was added to the warm-fresh culture medium at 10 nM (Gil *et al.* 2003; Chaib-Oukadour *et al.* 2004, 2009) before, during or after excitotoxic damage. Motoneuronal loss was analyzed 5 days after excitotoxic damage.

Immunoblotting

To perform western blot analysis, spinal cord slices were collected in lysis buffer (50 mM Tris, 2 mM EDTA and 0.5% Triton-X-100; pH 6.8) at different time-points. Lysates were homogenated with Pellet pestle (Sigma-Aldrich Corp.), sonicated and quantified by BCA assay (Pierce Chemical Co.). Equal amounts of protein (20 μg/well) were resolved in SDS-PAGE and transferred to nitrocellulose membrane (Whatman, Dassel, Germany). Membranes were blocked with 6% non-fat dry milk in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot H_2O$ and 1.5 mM

KH_2PO_4) for 1 h at 20–25°C and incubated overnight with the corresponding primary antibody diluted in blocking buffer: rabbit anti-diphospho (Thr^{202}/Tyr^{204}) p44/p42 MAP kinase (P-ERK1/2) and anti-ERK1/2 (1 : 1000), rabbit anti-phospho-glycogen synthase kinase-3 (Ser^9) (5B3)(P-GSK3β), anti-GSK3 β (1 : 1000) (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-LC3I/II (1 : 1000) (Abcam, Cambridge, UK), and mouse anti-β-tubulin (Becton-Dickinson, Franklin Lakes, NJ, USA). After several washes, membranes were incubated for 1 h with an appropriate secondary antibody conjugated with horseradish peroxidase (1 : 3000) (anti-mouse-HRP; Dako Denmark, Glostrup, Denmark) and anti-rabbit-HRP (Invitrogen Corp.). Blots were developed using a chemoluminiscent mix 1 : 1 (0.5 M luminol, 79.2 mM p-coumaric acid, 1 M Tris-HCl; pH 8.5) and (8.8 M hydrogen peroxide, 1 M Tris-HCl; pH 8.5), and exposed to enzymatic chemoluminiscence (ECL) films (Amersham Pharmacia Biotech, Buckinghamshire, UK). The apparent molecular weight of proteins was determined by calibrating the blots with pre-stained molecular weight markers (All Blue; Pierce Chemical Co.). Densitometry was carried out using ImageJ software (National Institute of Health, Bethesda, MD, USA). Total content of each specific protein was assayed after membrane stripping (0.1 mM Glycine; pH 2.3) for 1 h at 20–25°C, blocked again and incubated with corresponding primary antibody.

Immunohistochemistry

Spinal cord slices were fixed with 4% paraformaldehyde at 20–25°C for 1 h. Slices were then washed twice with PBS for 15 min, blocked with 5% normal horse serum and 0.2% Triton-X-100 in PBS, and incubated overnight at 4°C with antibody against mouse anti-neurofilament heavy-chain (NF-H) (SMI-32, 1 : 1000) (Sternberger Monoclonals Inc., Baltimore, MD, USA), rabbit anti-P-GSK3β (Ser^9) (5B3)(P-GSK3β) (1 : 200) (Cell Signaling Technology), and rabbit anti-LC3I/II (1 : 50) (Abcam). Cultures were then thoroughly washed in PBS with 0.2% Tween-20 (PBS-T) and incubated with appropriate secondary antibody Alexa Fluor®555 goat anti-rabbit IgG (1 : 500), Alexa Fluor®488 goat anti-mouse IgG (1 : 1000) or Alexa Fluor®546 goat anti-rabbit IgG (1 : 500) (Invitrogen Corp.), diluted in blocking buffer for 1 h at 20–25°C. Then, slices were washed two times with PBS-T, incubated for 20 min with 4′-6-Diamidino-2-phenylindole (DAPI) diluted in PBS, and washed several times. Finally, slices were mounted in Superfrost®Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) with Fluoromount-G mounting medium (SouthernBiotech, Birmingham, AL, USA) and fluorescence was visualized under epifluorescence microscope (Nikon Eclipse 90i; Nikon Instruments Inc., Melville, NY, USA) or confocal microscope (Leica TSC SP5; Leica Microsystems, Deerfield, IL, USA or Olympus FluoViewTM FV1000; Hamburg, Germany). Motoneurons in the organotypic spinal cord slices were identified by SMI-32 immunostaining on the basis of their morphology and size (> 20 μm) and their localization in the ventral horn. A minimum of 15 sections were used for MN counting for each experimental condition.

Statistical analysis

Statistical significance was determined by one-way ANOVA followed by Bonferroni's *post-hoc* test. Differences were considered to be significant if $p < 0.05$.

Results

Motoneuron specific binding

Hc-TeTx was purified by optimized liquid chromatography. To visualize the purified protein, it was attached to the small molecule Alexa Fluor®555 (Alx^{555}) through its reaction between succinimidyl esters and the primary amines of the protein.

To analyze the activity of the purified complex Hc-TeTx-Alx^{555}, we first analyzed the capacity of Hc-TeTx-Alx^{555} to specifically enter into the MNs in the spinal cord explants during culture. By immunohistochemical analysis, the presence of Hc-TeTx-Alx^{555} attached to the periphery of the MNs was observed exclusively by 30 min after its addition at 10 nM into the culture medium. Larger amount of the immunofluorescence was observed inside the MN somas and along their processes at 2 h (Fig. 1). In contrast, Hc-TeTx-Alx^{555} did not co-localize with the astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. 1). When the same amount of the uncoupled Alexa Fluor®555 fluorophore was added, no labeling was observed.

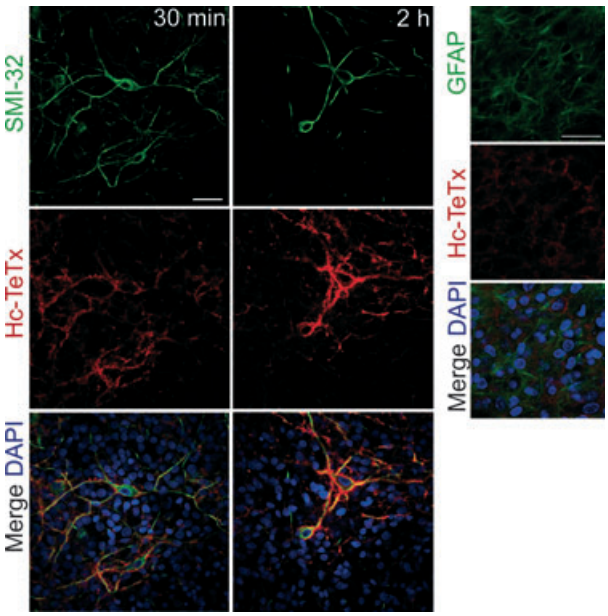

Fig. 1 C-terminal domain of tetanus toxin (Hc-TeTx) recombinant protein binds specifically to motoneurons in organotypic spinal cord cultures. Sections were incubated with Hc-TeTx coupled to Alexa Fluor®555 (red) for 30 min or 2 h. MNs are immunolabeled using SMI-32 antibody (green) and astrocytes with glial fibrillary acidic protein (GFAP) (green, right-side column). Note in merged figures that Hc-TeTx-Alx^{555} is internalized exclusively within the MNs. Merge panel also shows no binding of Hc-TeTx to glial cells after 3 h. Images were captured using confocal microscopy. Images were from at least three slices per condition. Scale bar represents 30 μm.

Motoneuron survival after excitotoxicity

Glutamate excitotoxicity can be induced to the SCOCs by the transient addition of high amounts of glutamate. The highest concentration of glutamate (1 mM) used yielded 60 ± 6% of spinal MN loss in these culture conditions as assessed by counting SMI-32 positive cells 5 days after the insult (Fig. 2). Lower glutamate concentrations were needed to induce similar MN loss in similar culture media serum compositions (Guzman-Lenis *et al.* 2009). With the aim to investigate whether Hc-TeTx recombinant protein can act as a neuroprotective agent for spinal cord MNs, we added 10 nM Hc-TeTx to the SCOCs exposed to acute excitotoxic damage. Hc-TeTx added 2 h before or 2 h after excitotoxic damage promoted survival of the MNs but not significantly (Fig. 3d and f). However, adding Hc-TeTx protein immediately following the excitotoxic insult resulted in a significant MN survival of 89 ± 21% (Fig. 3e). The addition of Hc-TeTx protein alone had no effect on SMI-32 positive cell number in the control SCOCs (Fig. 3b).

These results indicate that Hc-TeTx protein induces protection to MNs after suffering an excitotoxic insult, and that the pro-survival signaling triggered is critical within the first 2 h after the treatment.

Hc-TeTx induces ERK1/2 and GSK3β phosphorylation

To determine the signaling cascades involved in the neuroprotective effect of Hc-TeTx on MNs, we first investigated elements related to the previously described pathways activated by Hc-TeTx, PI3-K/Akt, and p21ras/MAPK (Gil *et al.* 2003; Chaib-Oukadour *et al.* 2004). We performed a time course immunoblot analysis after acute excitotoxic damage in the slices immediately treated with Hc–TeTx protein. ERK1/2 and GSK3β phosphorylation showed a transitory increase during the first 2 h (Fig. 4a and b). Under control conditions, no ERK1/2 phosphorylation was observed (Fig. 4c). We also confirmed that GSK3β phosphorylation occurred within the MN soma after 2 h of incubation by immunohistochemistry and vehicle treated slices exposed to excitotoxic damage showed no phosphorylation of GSK3β (Fig. 4d).

To further address the relevance of these pathways in the SCOC excitotoxic model, we used specific inhibitors of MEK1 and PI3–K kinases. Simultaneous treatment at 15 DIV with either PD98059 (inhibitor of MEK1) or LY294002 (inhibitor of PI3–K) abolished any neuroprotective effect induced by Hc-TeTx protein (8.20 ± 1.32 and 9.75 ± 1.30 survival MNs, respectively) (Fig. 5a–c). MN loss was analyzed five days post-treatments. Treatment of SCOCs with both inhibitors at normal and excitotoxic conditions alone had no effect on MN survival.

Hc-TeTx protein induces autophagosome formation

To determine the contribution of macroautophagic mechanisms to the neuroprotection elicited by Hc–TeTx protein following the excitotoxic insult on the SCOC model, we examined the expression and cellular localization of LC3-I and LC3-II, Beclin1 and p62. Western blot analyses revealed that Hc-TeTx up-regulates LC3-II during the first 2 h after excitotoxic damage (Fig. 6a). By immunohistochemistry punctuate LC3-II related with autophagosome formation was also observed after addition of Hc–TeTx (Fig. 7). No LC3-II labeling was detected at excitotoxic control condition.

Consistently, Beclin1, involved in autophagy initiation, was also enhanced after Hc-TeTx treatment following glutamate excitotoxicity. Finally, p62 protein accumulation declined at 24 h (Fig. 6a). Furthermore, we observed that the macroautophagy triggering was disturbed by addition of both kinase pathways inhibitors, PD98059 and LY294002, as revealed by a significant reduction in the expression of LC3-II (Fig. 6b and c).

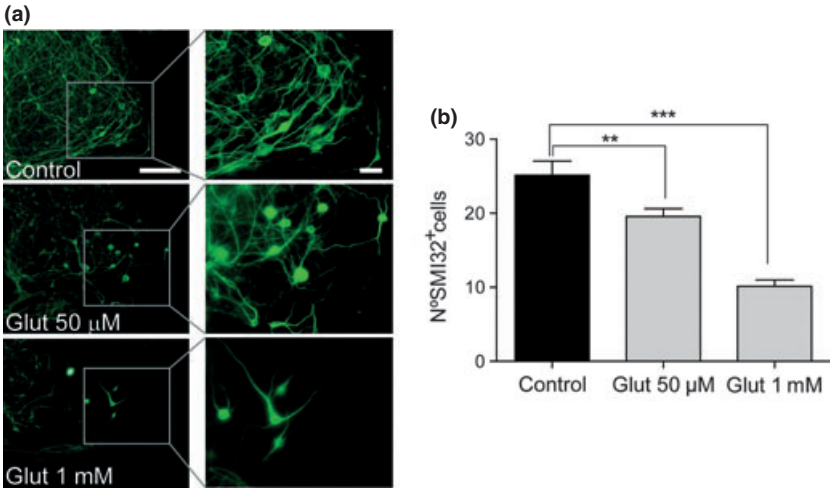

Fig. 2 Acute excitotoxic damage to spinal cord motoneurons. (a) Fluorescent microphotography representatives of MN distribution in the ventral horn hemisection of the spinal cord following glutamate toxicity (Glut 50 μM or 1 mM) or in control situation. Right column shows magnified pictures of the ventral horn hemisection. Scale bars represents 100 μm (left column) and 50 μm (right-column). (b) Bar graph showing the number of SMI-32 positive cells at the ventral horns with a diameter > 25 μm. Values are the mean ± SEM of at least 15 sections per treatment. ***$p < 0.001$ and **$p < 0.01$ by Bonferroni's *post-hoc* test.

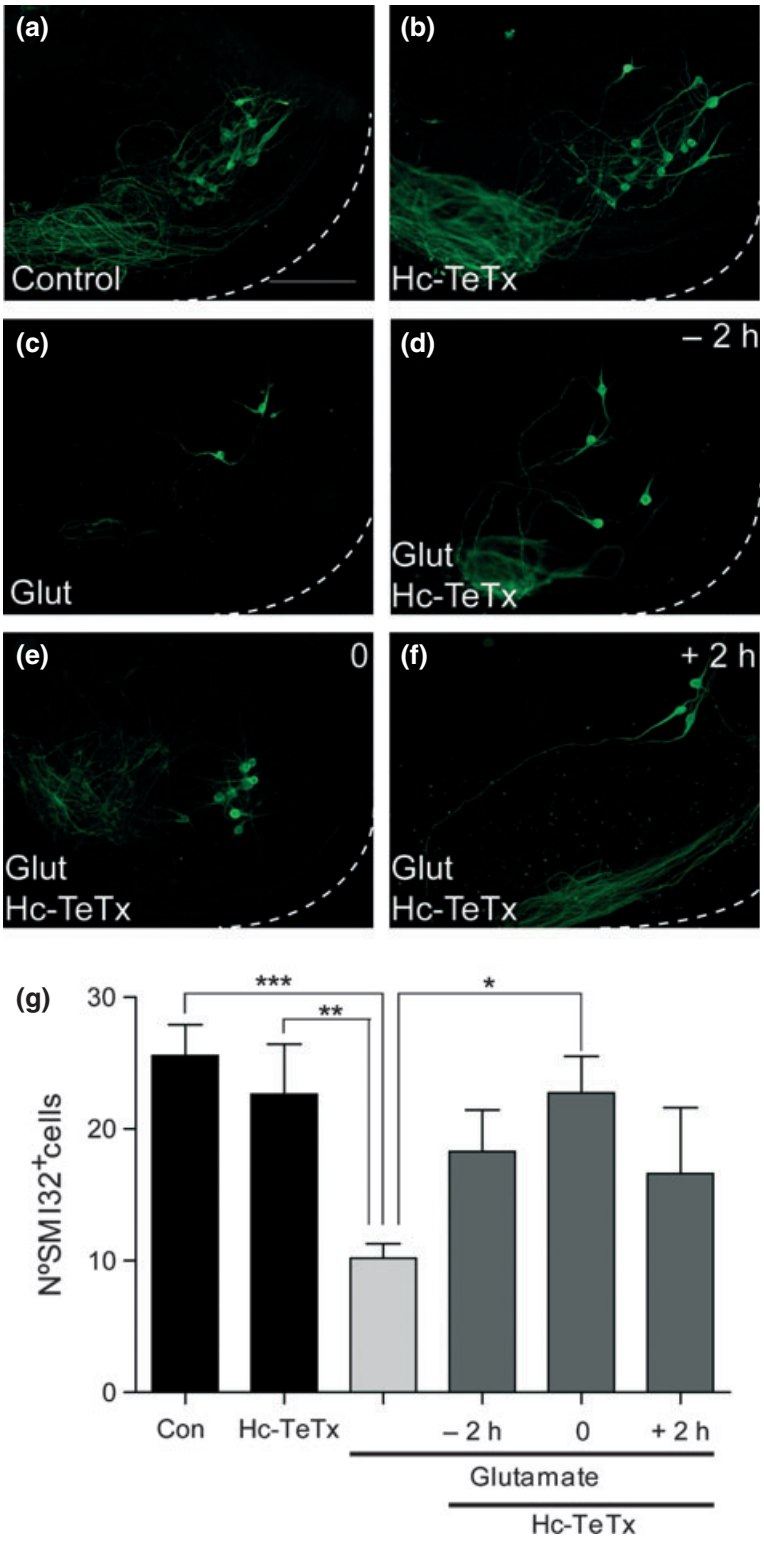

Fig. 3 Hc-TeTx protects spinal cord motoneurons against glutamate-induced acute excitotoxicity. Spinal cord cultures were treated at 15 DIV with 1 mM glutamate. Either vehicle or Hc-TeTx 10 nM was added to the culture medium 2 h before (−2 h), just at the time of excitotoxic damage (0 h) or 2 h later (+2 h). Motoneurons were identified by SMI-32 immunostaining and on the basis of their morphology, size, and localization. (a) Control, (b) Hc-TeTx 10 nM, (c) Glutamate 1 mM, (d–f) Glutamate 1 mM plus Hc-TeTx 10 nM. Scale bars represents 100 μm. (g) Representative micrographs of SMI-32 positive cells in the ventral horn of the spinal cord. Bar graph showing the number of SMI-32 positive cells present in the ventral horns under each condition. Values are the mean ± SEM of at least 15 sections per treatment. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ versus Glutamate 1 mM by Bonferroni's *post-hoc* test.

Discussion

Spinal cord trauma elicits a secondary phase injury starting minutes after the primary damage, with extracellular glutamate levels markedly increasing during the first 3 h after the injury (Park *et al.* 2004). Several studies have pointed out that the time of application of effective neuroprotective agents after SCI is short, probably restricted

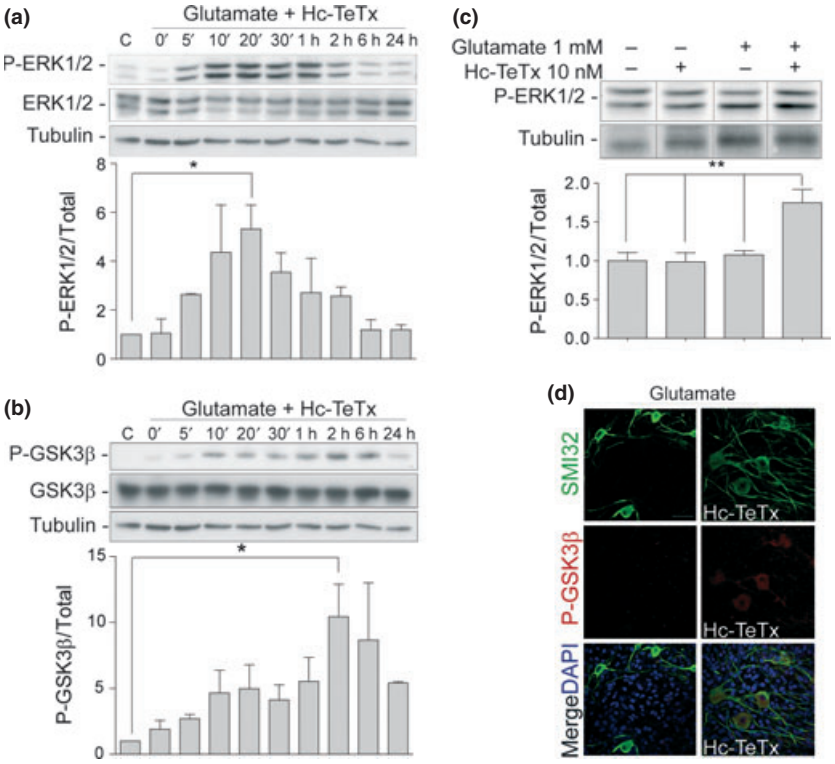

Fig. 4 Activation of ERK1/2 and GSK3β proteins after Hc-TeTx treatment under excitotoxic conditions. (a) Western blot analysis reveals an ERK1/2 transitory protein phosphorylation after Hc–TeTx treatment, which was added to the culture medium immediately after acute excitotoxic damage. (b) GSK3β protein also presents a transitory phosphorylation by western blot after Hc-TeTx treatment. (c) ERK1/2 phosphorylation was not detected at control conditions. (d) Immunohistochemistry against P-GSK3β under excitotoxic damage was only detected 2 h after Hc-TeTx treatment into the cytoplasm and the neurites of the motoneurons labeled by SMI-32. All results were from at least three independent experiments. $*p < 0.05$ and $**p < 0.01$ by Bonferroni's *post-hoc* test.

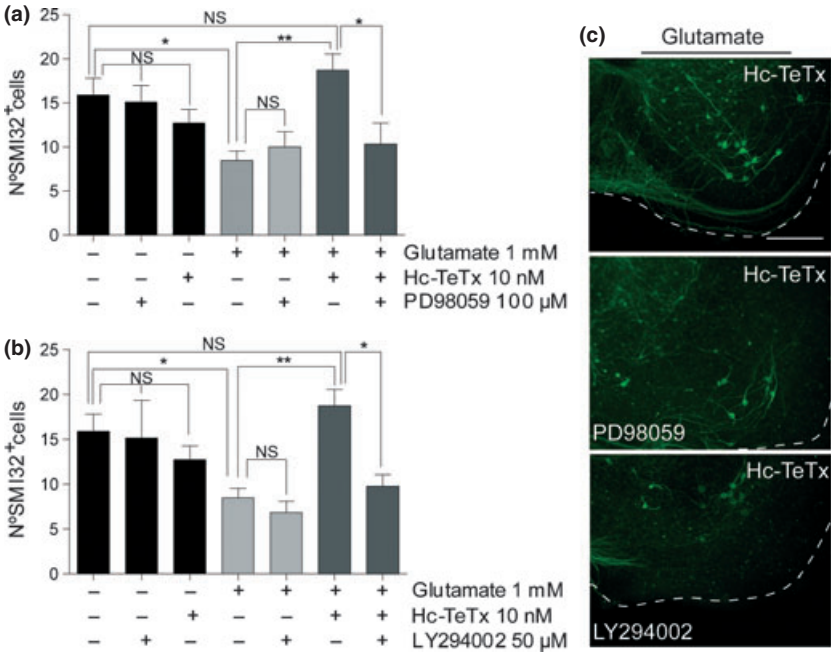

Fig. 5 Neuroprotective effects of Hc-TeTx were blocked after ERK1/2 and GSK3β pathways inhibition. (a) Addition to the culture medium together with Hc-TeTx (10 nM) after acute excitotoxic damage of the p21ras/MAPK pathway inhibitor, PD98059 (100 μM) and (b) the PI3K/Akt pathway inhibitor, LY294002 (50 μM) blocks the neuroprotective effects of the Hc-TeTx on the motoneurons. No effects on the survival were observed with both inhibitors under control or excitotoxic conditions. Values are the mean ± SEM of at least 15 sections per treatment. $**p < 0.01$ and $*p < 0.05$ versus Glutamate 1 mM sections by Bonferroni's *post-hoc* test. (c) Motoneurons at the ventral horn identified by SMI-32 labelling of the Hc-TeTx treatment cotreated with PD98059 and LY294002 inhibitors under glutamate-excitotoxic conditions.

to the first hours after the trauma (Fehlings *et al.* 2001; Kuzhandaivel *et al.* 2011). The results of this study show that Hc-TeTx treatment is able to significantly enhance MN survival in front of a excitotoxic insult if applied within a short therapeutic window of less than 2 h. After SCI, cellular damage expands far from the initial injury site. Although the capacity of Hc–TeTx to be retrogradally transported from peripheral to central nervous system could be interesting for

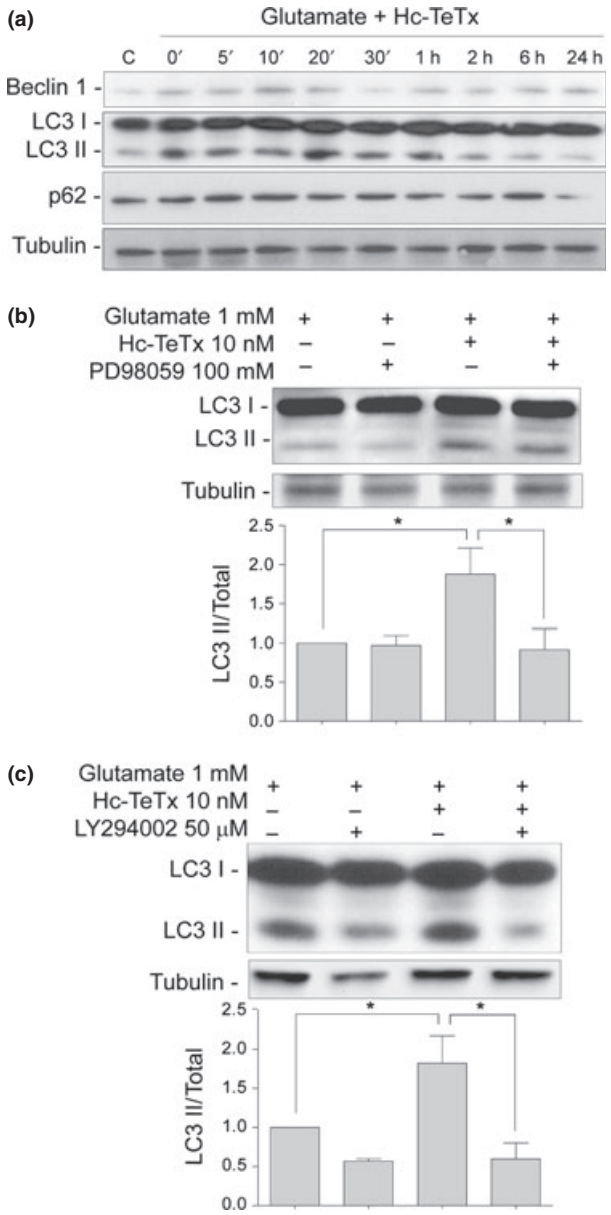

Fig. 6 Western blot analysis of the autophagic system induction after Hc-TeTx treatment. (a) Hc-TeTx treatment under excitotoxic conditions induces enhanced and moderate levels of Beclin1, LC3II formation, and p62 disappear which indicate an activation of the autophagic system. (b, c). Autophagosome or the LC3II formation was blocked by addition of the p21ras/MAPK (PD98059) and PI3K/Akt (LY294002) inhibitors. Autophagy was not activated under excitotoxic conditions. All results were from at least three independent experiments. *$p < 0.05$ by Bonferroni's *post-hoc* test.

developing a non-invasive therapeutical approach, the expansion of the damage will probably limit the effective targeting of the damaged MNs. The SCOC model allowed us to demonstrate that Hc-TeTx might also be applied by intrathecal injection because of its specific capability for binding to the plasma membrane of the MNs.

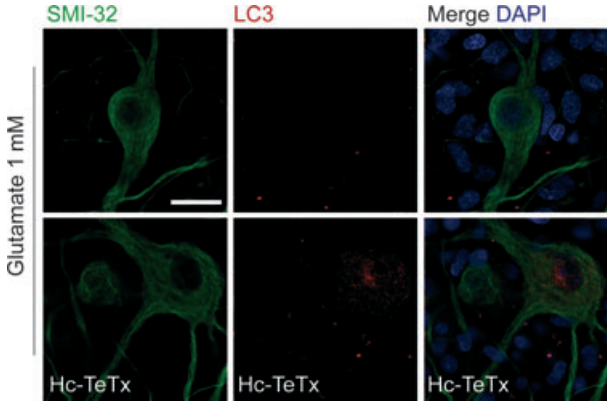

Fig. 7 LC3 immunohistochemistry shows autophagosome formation in the motoneurons after Hc-TeTx treatment. Under excitotoxic conditions, Hc-TeTx promotes moderate autophagosome formation into the cytoplasm of the motoneurons revealed by an increased number of LC3-positive dots. Scale bar represents 20 μm.

Our previous results showed that Hc-TeTx causes tyrosine phosphorylation of Trk receptors in rat synaptosomes (Gil *et al.* 2001), cultured cortical neurons (Gil *et al.* 2003), and cerebellar granule neurons (Chaib-Oukadour *et al.* 2004).

Although it is still unclear which is the protein receptor/s of TeTx in plasma membrane, TeTx and its carboxyl-terminal fragment Hc-TeTx might mimic the natural internalization pathway of neurotrophins (Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), NT3, and NT4/5) sharing the neurotrophin receptors (TrkA, TrkB, TrkC, and $p75^{NTR}$) to be internalized by endocytic vesicles (Deinhardt *et al.* 2006).

Neurotrophin Trk receptors activation promotes the activation of several signaling pathways, such as PI3–K/Akt and p21ras/MAPK. The present work confirms the action of Hc-TeTx upon activation of ERK1/2 and phosphorylation of GSK3β in the spinal MNs, as previously shown in other cell types (Gil *et al.* 2001, 2003; Chaib-Oukadour *et al.* 2004, 2009). Hc-TeTx promoted transient phosphorylation of both proteins in the SCOC after acute excitotoxicity damage, in addition of early activation of the autophagic cascade. Transient activation of PI3-K/Akt and p21ras/MAPK pathways has been correlated with a moderate activation of autophagy, which leads to cytoprotective autophagy (Wang *et al.* 2009). Although the molecular cell death pathway involved in motoneuronal death after spinal cord excitotoxic damage is not completely understood, apoptosis, necrosis, and autophagy have been proposed (Kwon *et al.* 2004; Matyja *et al.* 2005, 2008; Kanno *et al.* 2009a, b). Mutated glutamate receptor causes continuous ion flow in Lucher mice in which cell death is not caused by autophagy, but rather by necrosis with autophagic features (Nishiyama and Yuzaki 2010). Furthermore, autophagy induction after lithium treatment of chick SCOCs lesioned by kainic acid revealed motoneuron survival (Caldero *et al.* 2010). Moderate and

early autophagy induced by Hc-TeTx after excitotoxic damage could be a crucial role to restore cellular homeostasis. Several studies have suggested a mechanism of cross-talk between autophagy and apoptosis (Maiuri *et al.* 2007a,b; Levine *et al.* 2008; Rubinstein *et al.* 2011). Hc-TeTx exerts an anti-apoptotic effect on several neuronal types in culture (Chaib-Oukadour *et al.* 2004, 2009), and the present results suggest that it also activates the autophagic response in MNs.

In summary, we report the potential effect of Hc-TeTx in protecting spinal MNs against glutamate excitotoxicity. We confirm that Hc-TeTx exerts this protective effect through the transient and early activation of p21ras/MAPK and PI3-K/Akt pathways, and consequently the cytoprotective autophagy cascade. All these findings suggest a possible therapeutical role of Hc-TeTx to enhance MN survival after SCIs, which should be assessed by consequent *in vivo* studies.

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