



**HAL**  
open science

## Binding of $\alpha$ -toxin from in the nervous system

Jonatan Dorca-Arévalo, Alex Soler-Jover, Maryse Gibert, Michel R. Popoff,  
Mireia Martín-Satué, Juan Blasi

► **To cite this version:**

Jonatan Dorca-Arévalo, Alex Soler-Jover, Maryse Gibert, Michel R. Popoff, Mireia Martín-Satué, et al.. Binding of  $\alpha$ -toxin from in the nervous system. *Veterinary Microbiology*, 2008, 131 (1-2), pp.14. 10.1016/j.vetmic.2008.02.015 . hal-00532401

**HAL Id: hal-00532401**

**<https://hal.science/hal-00532401>**

Submitted on 4 Nov 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: Binding of  $\epsilon$ -toxin from *Clostridium perfringens* in the nervous system

Authors: Jonatan Dorca-Arévalo, Alex Soler-Jover, Maryse Gibert, Michel R. Popoff, Mireia Martín-Satué, Juan Blasi



PII: S0378-1135(08)00069-2  
DOI: doi:10.1016/j.vetmic.2008.02.015  
Reference: VETMIC 3967

To appear in: *VETMIC*

Received date: 9-1-2008  
Revised date: 18-2-2008  
Accepted date: 20-2-2008

Please cite this article as: Dorca-Arévalo, J., Soler-Jover, A., Gibert, M., Popoff, M.R., Martín-Satué, M., Blasi, J., Binding of  $\epsilon$ -toxin from *Clostridium perfringens* in the nervous system, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.02.015

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 BINDING OF  $\epsilon$ -TOXIN FROM *Clostridium perfringens* IN THE NERVOUS  
2 SYSTEM

3

4 Jonatan Dorca-Arévalo<sup>a</sup>, Alex Soler-Jover<sup>a,+</sup>, Maryse Gibert<sup>b</sup>, Michel R. Popoff<sup>b</sup>, Mireia  
5 Martín-Satué<sup>a</sup> and Juan Blasi<sup>a\*</sup>

6 <sup>a</sup>Laboratori de Neurobiologia Cel·lular i Molecular, Departament de Patologia i  
7 Terapèutica Experimental, Campus de Bellvitge, Universitat de Barcelona-IDIBELL,  
8 c/Feixa Llarga s/n, 08907 L'Hospitalet de Llobregat. Spain

9 <sup>b</sup>CNR Anaérobies, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris cedex, France

10

11 \* Corresponding author:

12 Tel. (34) 934024279; FAX. (34) 934035810; email: [blasi@ub.edu](mailto:blasi@ub.edu)

13

14 <sup>+</sup> Present address: Dept. of Medicine, University of California, San Diego, 9500 Gilman  
15 Dr. 0838, La Jolla, CA 92093-0838

16

17 Proofs should be sent to:

18 Juan Blasi

19 Departament de Patologia i Terapèutica Experimental

20 Campus de Bellvitge. Universitat de Barcelona

21 c/Feixa Llarga s/n

22 08907 L'Hospitalet de Llobregat

23 Spain

24 email: [blasi@ub.edu](mailto:blasi@ub.edu)

25

## 1 ABSTRACT

2

3 Epsilon-toxin ( $\epsilon$ -toxin), produced by *Clostridium perfringens* type D, is the main agent  
4 responsible for enterotoxaemia in livestock. Neurological disorders are a characteristic  
5 of the onset of toxin poisoning.  $\epsilon$ -Toxin accumulates specifically in the central nervous  
6 system, where it produces a glutamatergic-mediated excitotoxic effect. However, no  
7 detailed study of putative binding structures in the nervous tissue has been carried out to  
8 date. Here we attempt to identify specific acceptor moieties and cell targets for  $\epsilon$ -toxin,  
9 mainly in the mouse nervous system but also in the brains of sheep and cattle. An  $\epsilon$ -  
10 toxin-GFP fusion protein was produced and used to incubate brain sections, which were  
11 then analyzed by confocal microscopy. The results clearly show specific binding of  $\epsilon$ -  
12 toxin to myelin structures.  $\epsilon$ -Prototoxin-GFP and  $\epsilon$ -toxin-GFP, the inactive and active  
13 forms of the toxin respectively, showed identical results. By means of pronase E  
14 treatment, we found that the binding was mainly associated to a protein component of  
15 the myelin. Myelinated peripheral nerve fibres were also stained by  $\epsilon$ -toxin. Moreover,  
16 the binding to myelin was not restricted to rodents, but was also found in humans, sheep  
17 and cattle. Curiously, in the brains of both sheep and cattle, the toxin strongly stained  
18 the vascular endothelium, a result that may explain the differences in potency and effect  
19 between species. Although the binding of  $\epsilon$ -toxin to myelin does not directly explain its  
20 neurotoxic effect, this feature opens up a new line of enquiry into its mechanism of  
21 toxicity and establishes the usefulness of this toxin for the study of the mammalian  
22 nervous system.

23

24 Keywords:  $\epsilon$ -toxin, *clostridium perfringens*, myelin, glutamate release

25

## 1 INTRODUCTION

2 Epsilon-toxin ( $\epsilon$ -toxin), a protein synthesized by the B and D strains of the anaerobic  
3 bacteria *Clostridium perfringens*, causes fatal enterotoxaemia in livestock, characterized  
4 by acute neurological signs and sudden death (Finnie, 2004). The toxin is produced as a  
5 non-toxic precursor molecule that is activated upon proteolytic cleavage of amino and  
6 carboxy terminal peptides (Minami et al., 1997). Although non-active, the prototoxin  
7 molecule presumably binds to the same surface cell receptors as the full active molecule  
8 and can prevent its binding and further toxicity. The active toxin produces diffuse  
9 vasogenic oedema in organs such as the lungs, kidneys and brain and causes  
10 neurological disorders such as opisthotonus, convulsions and agonal struggling, leading  
11 rapidly to death (Finnie, 2004; Smedley et al., 2004).

12 Mice have been widely used to study the effect of  $\epsilon$ -toxin and provide a useful model  
13 for laboratory controlled intoxication studies (Finnie, 1984a, b; Fernandez-Miyakawa et  
14 al., 2007a, b and c). After i.v. or i.p. injections into mice the toxin accumulates in  
15 several organs, above all in the kidneys and in the nervous system: the binding to the  
16 nervous system is specific and saturable (Nagahama and Sakurai, 1991). In mice, as in  
17 sheep and other naturally sensitive species, the toxin has the capacity to cross the blood  
18 brain barrier (BBB) and enter the brain parenchyma (Soler-Jover et al., 2007). However,  
19 there is little information about the final location of the toxin in the brain once it crosses  
20 the BBB. Previous results have shown the binding of  $\epsilon$ -toxin to a synaptosomal fraction  
21 (Nagahama and Sakurai, 1992) and glial cells (Soler-Jover et al., 2007), suggesting  
22 possible targets. Based on the variety of symptoms and on the distribution of the toxin,  
23 we decided to examine the toxin's potential targets by studying its binding to the  
24 nervous system in mice. We then extended the study to include sheep, one of  $\epsilon$ -toxin's  
25 principal natural targets.

## 1 MATERIALS AND METHODS

2

### 3 **Expression of the recombinant protein $\epsilon$ -prototoxin-GFP**

4  $\epsilon$ -Prototoxin and  $\epsilon$ -prototoxin-GFP were produced and purified as previously  
5 described (Soler-Jover et al., 2007). Briefly, the expression of either  $\epsilon$ -prototoxin or  $\epsilon$ -  
6 prototoxin-GFP was induced overnight with 0.4 mM isopropyl beta-D-  
7 thiogalactopyranoside (IPTG) at room temperature (RT), in 250 ml LB medium  
8 cultures. Cells were pelleted and resuspended in ice cold phosphate buffer (PB) 20 mM  
9 pH 7.5 and NaCl 250 mM, sonicated and centrifuged at 15000 x g for 20 min. The  
10 resultant supernatant was incubated with 0.7 ml of Glutathione Sepharose<sup>TM</sup> 4B beads  
11 (Amersham Pharmacia Biotech, Freiburg, Germany) for 1 hour at 4°C. Finally, the  
12 recombinant proteins were eluted by thrombin cleavage in 20 mM PB pH 7.5 containing  
13 250 mM NaCl, 2.5 mM CaCl<sub>2</sub>.

14 When required,  $\epsilon$ -prototoxin and  $\epsilon$ -prototoxin-GFP were fully activated to  $\epsilon$ -toxin  
15 and  $\epsilon$ -toxin-GFP respectively by incubation with trypsin-coated agarose beads for 30  
16 min at RT (Sigma-Aldrich, Madrid, Spain), following the manufacturer's instructions.

17

### 18 **Mouse and rat synaptosomes**

19 Animals were housed under conventional conditions in climatized rooms with free  
20 access to standard pelleted food and tap water. All animal experiments were carried out  
21 in accordance with EU guidelines (86/609/EU), and Spanish regulations (BOE 67/8509-  
22 12, 1988) for the use of laboratory animals, and approved by the Ethics and Scientific  
23 Committee at the University of Barcelona. Sheep and bovine brain samples were  
24 obtained from the Mercabarna slaughterhouse in Barcelona. Human peripheral nerve  
25 fibres from *cauda equina* were obtained from spinal cord samples from the Brain Bank

1 of the Institute of Neuropathology, following the guidelines of the local Ethics  
2 Committee.

3 For each experiment, 3 brains from male OF1 Swiss mice weighing 20 g, or one  
4 brain from a Sprague Dawley rat, were extracted and homogenized in sucrose 320 mM  
5 with 10 up-and-down strokes at 600 rpm in a motor driven glass homogenizer with a  
6 teflon piston (B. Braun Biotech International). The brain homogenates were centrifuged  
7 at 3000 x g in a Beckman J2-HS centrifuge for 2 min at 4°C and the supernatants were  
8 centrifuged again at 14500 x g for 12 min at 4°C. The pellet (P2) was resuspended in 4  
9 ml of sucrose 320 mM, loaded on a discontinuous Ficoll gradient (4ml of Ficoll at 12%,  
10 1 ml at 9% and 4 ml at 5%) and centrifuged at 22500 rpm in a SW41TI Beckman rotor  
11 for 35 min at 4°C. The synaptosomal enriched fractions were collected at the two  
12 interfaces, between 5% and 9%, and 9% and 12% of Ficoll concentration.

13 After protein content quantification (Bradford, 1976), the synaptosomal fraction was  
14 distributed in aliquots of 0.5 mg, centrifuged at 20800 x g for 12 min at 4°C and kept on  
15 ice until used. Under these conditions, synaptosomes can be kept for several hours  
16 without losing their capacity to release glutamate.

17

### 18 **$\epsilon$ -Prototoxin-GFP and $\epsilon$ -toxin-GFP incubations on synaptosomal fractions**

19 Mouse or rat synaptosomal fractions were attached to polylysinated cover slips to  
20 analyze the binding of  $\epsilon$ -prototoxin-GFP and  $\epsilon$ -toxin-GFP to isolated nerve terminals  
21 and their possible colocalization with a neuronal marker.

22 After fixation of the synaptosomes in 4% paraformaldehyde and three washes with  
23 PBS, the non-specific binding was blocked by incubating the cover slips in PBS  
24 containing 0.2% gelatin and 20% of normal goat serum (NGS) for 1 hour at RT. The

1 cover slips were then incubated with 500 nM  $\epsilon$ -prototoxin-GFP or  $\epsilon$ -toxin-GFP in PBS  
2 containing 0.2% gelatin and 1% NGS for 1 hour at RT.

3 For the immunofluorescent analysis, synaptosomes bound to cover slips were  
4 blocked for 1h as previously described and incubated overnight at 4°C with antibodies  
5 against synaptic proteins, SNAP-25 (rabbit serum 1:100 dilution; Binz et al., 1994)  
6 VAMP-2 (1:300 dilution, monoclonal antibody, clone 69.1, Synaptic Systems), against  
7 the myelin basic protein (MBP, 1:300 dilution, monoclonal antibody, Chemicon  
8 MAB387) or against neurofilament 200kDa (1:100 dilution, monoclonal antibody, clone  
9 RT97, Chemicon). After three washes with PBS, the cover slips were incubated with the  
10 appropriate secondary antibodies coupled to Alexa Fluor 488 or 546. After three washes  
11 with PBS, the cover slips were mounted with the Aqueous Mounting Medium  
12 (GEL/MOUNT™, Biomedica corp.) and further examined under a Leica TCS 4D  
13 confocal microscope (Serveis Científico-Tècnics, UB-IDIBELL). In the double  
14 fluorescence experiments the  $\epsilon$ -prototoxin-GFP was added together with the  
15 corresponding secondary antibody and the samples were processed as described above.

16

#### 17 **$\epsilon$ -Prototoxin-GFP and $\epsilon$ -toxin-GFP incubations on slices**

18 To study the morphological binding of  $\epsilon$ -prototoxin-GFP and  $\epsilon$ -toxin-GFP to the  
19 nervous system, mouse, sheep and cow brains and mouse vagus nerve samples were  
20 fixed immediately after extraction by immersion in 4% paraformaldehyde in PB (100  
21 mM, pH7.2) for 12 hours. Samples were cryoprotected by immersion in 30% sucrose  
22 and snap frozen in precooled isobutanol. Cryostat sections of 10  $\mu$ m were obtained,  
23 mounted onto poly-L-lysine coated glass slides and stored at -20 °C until used for  $\epsilon$ -  
24 prototoxin-GFP and  $\epsilon$ -toxin-GFP incubations.

1 The non-specific binding was blocked by incubating the sections in PBS containing  
2 0.2% gelatin and 20% NGS for 1 hour at RT. Sections were then incubated with 300  
3 nM of  $\epsilon$ -prototoxin-GFP or  $\epsilon$ -toxin-GFP in PBS containing 0.2% gelatin and 1% NGS  
4 for 1 hour at RT. After 3 washes with PBS, the samples were stained with TO-PRO-3  
5 (1:1000 dilution, Molecular Probes, Invitrogen) for 7 min, washed again and fixed in  
6 4% paraformaldehyde for 10 minutes, rinsed and mounted with an Aqueous Mounting  
7 Medium (GEL/MOUNT™, Biomedica corp.) and further examined under a Leica TCS  
8 4D confocal microscope (Serveis Científic-Tècnics, UB-IDIBELL). In double  
9 fluorescence experiments the sections were incubated for 1 h at RT with PBS containing  
10 0.2% gelatin and 20% NGS followed by the primary antibody overnight at 4°C. After  
11 washing three times with PBS,  $\epsilon$ -prototoxin-GFP was added to the sections together  
12 with the corresponding secondary antibody and processed as stated above.

13 In the special case of mouse peripheral nerves, the mouse sciatic nerve was dissected  
14 and teased to obtain isolated nerve fibres. Fibres were incubated with  $\epsilon$ -prototoxin-GFP  
15 or  $\epsilon$ -toxin-GFP as previously described in slices. Isolated nerve fibres from human  
16 *cauda equina* (spinal cord horse's tail) were obtained from the Institut de  
17 Neuropatologia, Hospital Universitari de Bellvitge-IDIBELL.

18 The specificity of the binding was verified by co-incubating the sections with native  
19  $\epsilon$ -prototoxin or  $\epsilon$ -toxin together with  $\epsilon$ -prototoxin-GFP or  $\epsilon$ -toxin-GFP respectively in a  
20 20:1 molar ratio.

21 Similar experiments were performed with brain samples from sheep and cattle. In  
22 this case, samples were fixed by immersion in 4% paraformaldehyde for 12 hours and  
23 processed as described for mouse brain in order to obtain cryostat slices and to perform  
24 the  $\epsilon$ -prototoxin-GFP and  $\epsilon$ -toxin-GFP incubations.

25

## 1 Treatment with Pronase E, N-glycosidase F and detergents

2 In a series of experiments, tissue sections were incubated with pronase E, N-  
3 glycosidase F or detergents to assess the nature of the  $\epsilon$ -prototoxin-GFP and  $\epsilon$ -toxin-  
4 GFP binding.

5 To determine whether a protein was directly involved in the binding of  $\epsilon$ -toxin, tissue  
6 sections were incubated with different concentrations of pronase E in PBS 0.1 M (from  
7 0.1 to 1 mg/ml) at 37°C for different times (5, 15, 30 and 60 min).

8 To verify the possible participation of N-glycidic residues in the binding of  $\epsilon$ -toxin,  
9 N-glycosidase F was used to hydrolyze N-glycan chains from glycoproteins. Sections  
10 were incubated with 10 U/ml of N-glycosidase F overnight at 37°C in 20 mM PB, pH 8  
11 with 10 mM EDTA.

12 To assess the sensitivity of  $\epsilon$ -prototoxin-GFP to detergents, the sections were  
13 incubated with Triton X-100, Nonidet p-40 or CHAPS at 0.5% or 2% in 20 mM PB pH  
14 8 with 10 mM EDTA for 1 hour at 4°C.

15 After the treatments, the sections were washed three times with PBS at RT and  
16 incubated with  $\epsilon$ -prototoxin-GFP as described above. In the case of detergent  
17 incubations, the first two PBS washes were done at 4°C. Depending on the treatment,  
18 controls were performed by omitting pronase E, N-glycosidase F or detergents in the  
19 incubation buffer.

20

## 21 RESULTS

22

### 23 Glutamate release from brain synaptosomes

24 Isolated nerve terminal preparations (synaptosomes) have been widely used to  
25 analyze the effect of numerous substances, including excitatory and inhibitory

1 neurotoxins, on neurotransmitter release. Synaptosomes were isolated from rat or mouse  
2 brains and a specific fluorometric assay was performed to detect “on line” glutamate  
3 release (Nicholls and Sihra, 1986), to study the possible excitatory effect of  $\epsilon$ -toxin on  
4 glutamate release directly from nerve terminals. No glutamate release from rat or mouse  
5 brain synaptosomes could be detected when different concentrations of  $\epsilon$ -toxin were  
6 added immediately before the neurotransmitter release assay, in contrast to the calcium-  
7 dependent release of glutamate induced by chemical depolarization (Figure 1).  $\epsilon$ -Toxin  
8 did not interfere with the fluorometric assay by itself or with the potassium stimulated  
9 glutamate secretion, even after one hour of preincubation with the active  $\epsilon$ -toxin before  
10 the glutamate release was tested. The decrease in fluorescence in the presence of the  
11 toxin but in the absence of glutamate in the extracellular medium (a symptom of non-  
12 stimulation) is due to the slow reoxidation of NADH by the small fraction of damaged  
13 mitochondria present in the incubation media (Nicholls and Sihra, 1986). These results  
14 indicate that  $\epsilon$ -toxin does not directly promote glutamate release from nerve terminals.

15

#### 16 **Binding of $\epsilon$ -toxin to the mouse brain synaptosomal fraction**

17 The absence of  $\epsilon$ -toxin-dependent direct neurotransmitter release prompted us to  
18 check the putative binding of  $\epsilon$ -toxin to the brain synaptosomal fraction. In this case, the  
19 fusion protein  $\epsilon$ -prototoxin coupled to GFP was used to directly visualize its binding to  
20 a mouse brain synaptosomal preparation immobilized on glass cover slips. Confocal  
21 images of the synaptosomal preparation incubated with the  $\epsilon$ -prototoxin-GFP showed  
22 specifically stained small round structures (Figure 2 A, D and G). Similar results were  
23 obtained when synaptosomes were incubated with  $\epsilon$ -toxin for 1 h at RT followed by a  
24 specific anti- $\epsilon$ -toxin rabbit serum and Alexa Fluor 546 coupled goat anti-rabbit  
25 secondary antibody (not shown). In order to confirm the binding of  $\epsilon$ -prototoxin-GFP to

1 isolated nerve terminals, antibodies against synaptic proteins were used as synaptosomal  
2 markers and co-incubated with the  $\epsilon$ -prototoxin-GFP to perform double fluorescence  
3 staining assays. Surprisingly, no colocalization was observed when any of the  
4 antibodies against synaptic proteins (SNAP-25, synaptobrevin/VAMP2, Figures 2 B and  
5 2 E respectively) were used to stain isolated nerve terminals. In a few cases, when  
6 synaptic membrane proteins such as SNAP25 were used as synaptic markers, some  
7 stained structures appeared surrounded by  $\epsilon$ -toxin-GFP staining (insert Figure 2 C). This  
8 particular pattern was not observed when synaptobrevin/VAMP 2 staining was used as a  
9 synaptic vesicle marker (insert Figure 2 F). However, a complete colocalization was  
10 evident when anti-myelin basic protein (MBP) antibody was used together with  $\epsilon$ -  
11 prototoxin-GFP (Figures 2 G, H and I). These results strongly suggest that  $\epsilon$ -toxin binds  
12 to myelinic structures present as debris in the synaptosomal preparations together with  
13 the isolated nerve terminals.

14

#### 15 **Binding of $\epsilon$ -toxin to myelin in central nervous system**

16 To confirm the binding of  $\epsilon$ -toxin to myelinic structures, mouse brain slices were  
17 obtained and cerebellar sections were incubated with  $\epsilon$ -toxin-GFP (Figure 3 A and C).  
18  $\epsilon$ -Toxin-GFP visibly stained myelinic structures in the mouse cerebellum. Moreover, no  
19 differences between the  $\epsilon$ -prototoxin and the activated form were observed (not shown).  
20 This binding to myelinic structures was specific, since excess amounts of  $\epsilon$ -prototoxin  
21 (non-GFP coupled) prevented the staining of cerebellar slices by either the  $\epsilon$ -prototoxin-  
22 GFP or the full activated  $\epsilon$ -toxin-GFP (data not shown). Moreover, cerebellar slices  
23 were incubated with  $\epsilon$ -prototoxin followed by a specific anti- $\epsilon$ -prototoxin serum to  
24 detect the non-labelled toxin bound to the sections. After incubation with the Alexa  
25 Fluor 488 coupled goat anti-rabbit secondary antibody, the staining pattern was similar

1 to those found with  $\epsilon$ -prototoxin-GFP or  $\epsilon$ -toxin-GFP (not shown). Additionally, no  
2 staining was found when sections were incubated with GFP alone (not shown). A  
3 counterstaining with the nuclear marker TO-PRO-3 was used (Figure 3 B and C) to  
4 clearly differentiate the cerebellar granular layer from the white matter.

5 As in the experiments on synaptosomes described above, neuronal and myelin  
6 markers were used to fully demonstrate the binding of  $\epsilon$ -toxin to myelinic structures.  
7 Cerebellar sections were incubated with  $\epsilon$ -prototoxin-GFP (Figure 3 D and G) together  
8 with anti-MBP or anti-neurofilament (Figures 3 E and respectively). As expected,  
9 neither the neuronal marker (anti-neurofilament RT97 antibody) nor synaptic markers  
10 (anti-SNAP25 antibody) colocalized with the  $\epsilon$ -prototoxin-GFP staining (Figure 3 I and  
11 not shown). The neurofilament staining of the cerebellar white matter revealed RT97  
12 stained structures, possibly corresponding to neuronal axons, surrounded by  $\epsilon$ -  
13 prototoxin-GFP positive structures corresponding to myelinic sheaths (Figure 3 I).  
14 Conversely, anti-MBP antibody labelling fully colocalized with the  $\epsilon$ -prototoxin-GFP  
15 staining (Figure 3 F).

16 After confirming the specific binding of  $\epsilon$ -prototoxin-GFP and  $\epsilon$ -toxin-GFP to  
17 myelinic structures, a series of experiments were performed to further characterize the  
18 nature of this interaction (Figure 4). To identify a possible protein component in the  $\epsilon$ -  
19 prototoxin-GFP binding to myelin, brain slices were previously incubated with pronase  
20 E in order to enzymatically remove a possible protein receptor component for the toxin.  
21 In this case, the binding of  $\epsilon$ -prototoxin-GFP to cerebellar slices was clearly decreased  
22 (Figure 4 B) compared to the control non-treated cerebellar slices (Figure 4 A).  
23 Treatment with N-glycosidase F, to remove N-glycidic moieties on myelin, did not  
24 modify the binding pattern (Figure 4 C), indicating that the binding of  $\epsilon$ -prototoxin-GFP  
25 to myelin was independent of a glycidic environment. Curiously, detergent pre-

1 treatments of cerebellar slices did not show any evident decrease in  $\epsilon$ -prototoxin-GFP  
2 binding under these experimental conditions (Triton X-100 and CHAPS, Figures 4 D  
3 and not shown respectively). Moreover, the label was even more intense after detergent  
4 treatments, suggesting that the detergents used (Triton X-100, CHAPS) unmasked  
5 previously inaccessible binding sites to the toxin.

6

### 7 **Binding of $\epsilon$ -toxin to myelinated peripheral nerve fibres**

8 The binding was not exclusive to myelin in the central nervous system. To check the  
9 possible binding of the toxin to myelin in the peripheral nervous system, sections from  
10 mouse vagus nerve were incubated with  $\epsilon$ -prototoxin-GFP in order to localize and  
11 identify myelinic nerve fibres (Figure 5 A). Furthermore, isolated myelinic nerve fibres  
12 were obtained by teasing mouse sciatic nerve and incubated with  $\epsilon$ -prototoxin-GFP.  
13 Isolated fibres showed a clear surface staining, more accentuated at the Ranvier nodes  
14 (Figure 5 B). Similar results were obtained incubating the nerve fibres with  $\epsilon$ -toxin-GFP  
15 (not shown). To further examine the possibility that  $\epsilon$ -toxin could bind to the myelinic  
16 structures in other species, isolated nerve fibres from the human spinal cord *cauda*  
17 *equina* were incubated under the same conditions as described above for mice. A similar  
18 binding pattern was obtained (Figure 5 C), suggesting that the binding of  $\epsilon$ -toxin to  
19 myelin may be a common attribute among mammalian species.

20 To investigate this possibility, frozen sections of the striatum and cerebellum from  
21 the brains of lambs and cattle, two animal species that are specially sensitive to  $\epsilon$ -toxin,  
22 were obtained and processed in order to analyze the possible binding of  $\epsilon$ -prototoxin-  
23 GFP to myelinic structures. As expected,  $\epsilon$ -prototoxin-GFP stained myelin in both lamb  
24 and cow brains (Figure 6 A, B and D) though with different levels of intensity,  
25 suggesting differences in the affinity of  $\epsilon$ -prototoxin-GFP to the different myelin

1 species. Curiously, in addition to myelin staining,  $\epsilon$ -prototoxin-GFP labelled endothelial  
2 cells from blood brain microvessels in both lamb and cow brains with high intensity and  
3 specificity (Figure 6 A, B, C and D); this does not occur in rodent species.

4

## 5 DISCUSSION

6

7 Some of the neurological disorders produced by  $\epsilon$ -toxin in experimental and  
8 naturally poisoned animals are due to the “massive” secretion of glutamate from  
9 glutamatergic nerve terminals, triggering an excitotoxic episode characterized by  
10 convulsions (seizures) and neuronal cell death. This effect is an established  
11 characteristic of  $\epsilon$ -toxin, observed in laboratory animals when lethal and sublethal doses  
12 of the toxin were injected (Miyamoto et al., 1998; 2000). The massive glutamate release  
13 may be achieved by a direct effect of the toxin on nerve terminals, acting as a  
14 stimulatory toxin, or by acting on neuronal soma or glial cells. Previous results showing  
15 the binding of  $\epsilon$ -toxin to a rat brain synaptosomal fraction (Nagahama and Sakurai,  
16 1992) strongly suggested a direct action of  $\epsilon$ -toxin on nerve terminals, probably via the  
17 formation of a pore in the plasma membrane as a consequence of its oligomerization  
18 (Miyata et al., 2001; 2002). By using a well-established fluorometric assay to detect the  
19 glutamate release from brain synaptosomes “on line” (Nicholls and Sihra, 1986) we  
20 observed that the toxin does not act directly on nerve terminals, suggesting that its  
21 capacity to elicit glutamate release may act through another mechanism, either on  
22 neuronal soma or axons, or on glial or other cells present in the nervous tissue.

23 Taking advantage of the fusion proteins  $\epsilon$ -prototoxin-GFP and  $\epsilon$ -toxin-GFP which  
24 retain the full binding activity of the native molecules, we performed a series of  
25 experiments to examine the binding of  $\epsilon$ -toxin to nervous tissue. We have previously

1 found that  $\epsilon$ -toxin-GFP and  $\epsilon$ -prototoxin-GFP fusion proteins are valuable tools for  
2 detecting the binding of the toxin to sensitive cells and tissues (Soler-Jover et al., 2004;  
3 2007). These studies demonstrated that the  $\epsilon$ -toxin-GFP molecules bind to particular  
4 structures and tissues, and that this binding was specific, since previous incubation with  
5 non-labelled  $\epsilon$ -prototoxin prevented  $\epsilon$ -prototoxin-GFP binding.

6 As noted above, a previous report showed the binding of  $\epsilon$ -toxin to plasma  
7 membranes prepared from a synaptosomal fraction composed mainly of isolated nerve  
8 terminals (Nagahama and Sakurai, 1992). Here, combining the  $\epsilon$ -prototoxin-GFP fusion  
9 protein and confocal microscopy we show that the myelin structures present in the  
10 synaptosomal preparation are the main source of  $\epsilon$ -toxin binding sites. Moreover, the  
11 use of synaptic and myelinic markers confirmed the specific labelling of myelinic  
12 structures by  $\epsilon$ -prototoxin-GFP. Myelin components, considered as contaminants, have  
13 been detected in variable amounts along the different fraction obtained from brain  
14 synaptosomal and synaptic vesicle preparations (Takamori et al., 2006). In fact, the  
15 presence of myelin contamination in crude synaptosomal fraction has been one of the  
16 major problems in obtaining pure nerve terminal preparations. Further steps in  
17 synaptosomal fraction purification, such as the Ficoll gradient centrifugation used here,  
18 can remove most of the myelin contamination, but a slight presence of myelinic markers  
19 remains in the synaptosomal fraction.

20 In mice, myelin specifically bound  $\epsilon$ -toxin in both the central and peripheral nervous  
21 systems. The binding was specific and sensitive to proteases. These results are in partial  
22 agreement with previous data on the binding of  $\epsilon$ -toxin to rat brain homogenates and  
23 synaptosomal fractions, where the binding was partially sensitive to pronase E and  
24 neuraminidase but completely abolished by lipase (Nagahama and Sakurai, 1992). The  
25 differences between that report and ours, regarding the glycidic component of  $\epsilon$ -toxin

1 receptor and on the effect of detergent treatments, may be due to the different  
2 experimental approaches used. Pronase E treatment did not completely abolish the  
3 binding of  $\epsilon$ -prototoxin-GFP to myelinic structures. This may be due to an incomplete  
4 action of the enzyme in the preparations used here or to the presence of two binding  
5 sites, one a protein binding site and the other with a main glucidic or lipidic moiety. In  
6 either case, both elements would be components of the plasma membrane and sensitive  
7 to detergent treatment.

8 The binding of the  $\epsilon$ -toxin to myelin might not correspond to a natural target of the  
9 toxin in its toxic pathway. In fact, the binding and effect of injected  $\epsilon$ -toxin has not been  
10 correlated either with myelin-containing structures or with its synthesizing cells (Finnie,  
11 2004; Soler-Jover et al., 2007). Moreover, these results do not directly identify myelin  
12 as the main or a potential biological target of  $\epsilon$ -toxin, but they evidence the toxin's  
13 specific binding to a neural structure and suggest potential applications of the toxin as a  
14 specific marker in the study of the nervous system, in particular in myelinic structures.  
15 Indeed, the toxin and its GFP forms may be useful tools to identify, quantify and  
16 analyze myelinic structures. This potential use of the  $\epsilon$ -toxin-GFP in myelin binding and  
17 detection may, however, be limited by its capacity to bind to myelin from different  
18 species. The binding is clear in myelin from mice and rat origin and in myelin from  
19 human peripheral nerves, but it is slightly diminished in cow and sheep brains.

20 The binding of  $\epsilon$ -toxin to brain endothelial microvasculature also varies according to  
21 the species studied. Although previous evidence demonstrated the effect of  $\epsilon$ -toxin on  
22 endothelial permeabilization and blood brain barrier translocation in mice (Finnie, 2004;  
23 Soler-Jover et al., 2007), the binding to mouse brain endothelia could barely be detected  
24 by direct incubation. This characteristic of  $\epsilon$ -toxin contrasts with the *in vivo* binding of  
25 the toxin to brain blood vessels upon its injection in mice (Soler-Jover et al., 2007).

1 Thus, although the effect and the binding to brain endothelia are required for the toxin's  
2 neurological effects in mice, its binding probably requires intact endothelia, which, in  
3 mice, may be sensitive to the fixation and/or further manipulation for fluorescence  
4 observation. This is not the case in the rest of species studied here, which in fact are the  
5  $\epsilon$ -toxin's natural targets; in these cases the binding to the brain microvasculature was  
6 evident.

7 Finally, it would be reasonable to suggest a double binding capacity of  $\epsilon$ -toxin in  
8 nervous system: one to the endothelium, where it performs at least part of its noxious  
9 function (endothelial permeabilization and oedema) and the other to one or more myelin  
10 components with an as yet undefined function.

11

## 12 CONCLUSIONS

13

14  $\epsilon$ -Toxin binds specifically to myelin in both the central and peripheral nerve systems.  
15 The binding is mediated, at least in part, by a protein component of myelin. This  
16 characteristic of the toxin is not limited to mouse or rat myelin, the animal models used  
17 here, but is also evident in ovine, bovine and human myelin, though with different  
18 levels of intensity. In both ovine and bovine brains, at least, the toxin binds strongly to  
19 vascular endothelia. The functional importance of the  $\epsilon$ -toxin feature to bind myelin is  
20 still unknown. The identification of the myelin component that binds to  $\epsilon$ -toxin is  
21 currently under study and its identification will broaden our understanding of the  
22 molecular interaction of  $\epsilon$ -toxin with its cellular targets.

23

24

25

## 1 ACKNOWLEDGEMENTS

2

3 We are grateful to Serveis Científicotècnics of the University of Barcelona (Bellvitge  
4 Campus) for their assistance with the confocal microscopy tests. We would also like to  
5 thank Inmaculada Gómez de Aranda and Benjamín Torrejón for their excellent  
6 technical assistance, Xenia Grandes and Ezequiel Mas for their help obtaining isolated  
7 nerve fibres, Dr. Isidre Ferrer and the Brain Bank of the Institute of Neuropathology for  
8 providing the human nerve fibres and Serveis Lingüístics (University of Barcelona) for  
9 the careful reading of the manuscript and linguistic help. J.D-A. is a recipient of a  
10 predoctoral fellowship from IDIBELL. This work has been supported by Ministerio de  
11 Educación y Ciencia of Spain (BFU2005-02202).

12

## 13 BIBLIOGRAPHY

14

15 Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Sudhof, T.C., Jahn, R.,  
16 Niemann, H., 1994, Proteolysis of SNAP-25 by types E and A botulinical  
17 neurotoxins. *J Biol Chem* 269, 1617-1620.

18 Bradford, M.M., 1976, A rapid and sensitive method for the quantitation of microgram  
19 quantities of protein utilizing the principle of protein-dye binding. *Anal*  
20 *Biochem* 72, 248-254.

21 Fernandez-Miyakawa, M.E., Fisher, D.J., Poon, R., Sayeed, S., Adams, V., Rood, J.I.,  
22 McClane, B.A., Uzal, F.A., 2007a, Both epsilon-toxin and beta-toxin are  
23 important for the lethal properties of *Clostridium perfringens* type B isolates in  
24 the mouse intravenous injection model. *Infect Immun* 75, 1443-1452.

- 1 Fernandez-Miyakawa, M.E., Jost, B.H., Billington, S.J., Uzal, F.A., 2007b, Lethal  
2 effects of *Clostridium perfringens* epsilon toxin are potentiated by alpha and  
3 perfringolysin-O toxins in a mouse model. Vet Microbiol  
4 doi:10.1016/j.vetmic.2007.09.013
- 5 Fernandez-Miyakawa, M.E., Sayeed, S., Fisher, D.J., Poon, R., Adams, V., Rood, J.I.,  
6 McClane, B.A., Saputo, J., Uzal, F.A., 2007c, Development and application of  
7 an oral challenge mouse model for studying *Clostridium perfringens* Type D  
8 Infection. Infect Immun 75, 4282-4288.
- 9 Finnie, J.W., 1984a, Histopathological changes in the brain of mice given *Clostridium*  
10 *perfringens* type D epsilon toxin. J Comp Pathol 94, 363-370.
- 11 Finnie, J.W., 1984b, Ultrastructural changes in the brain of mice given *Clostridium*  
12 *perfringens* type D epsilon toxin. J Comp Pathol 94, 445-452.
- 13 Finnie, J.W., 2004, Neurological disorders produced by *Clostridium perfringens* type D  
14 epsilon toxin. Anaerobe 10, 145-150.
- 15 Minami, J., Katayama, S., Matsushita, O., Matsushita, C., Okabe, A., 1997, Lambda-  
16 toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by  
17 releasing its N- and C-terminal peptides. Microbiol Immunol 41, 527-535.
- 18 Miyamoto, O., Minami, J., Toyoshima, T., Nakamura, T., Masada, T., Nagao, S., Negi,  
19 T., Itano, T., Okabe, A., 1998, Neurotoxicity of *Clostridium perfringens* epsilon-  
20 toxin for the rat hippocampus via the glutamatergic system. Infect Immun 66,  
21 2501-2508.
- 22 Miyamoto, O., Sumitani, K., Nakamura, T., Yamagami, S., Miyata, S., Itano, T., Negi,  
23 T., Okabe, A., 2000, *Clostridium perfringens* epsilon toxin causes excessive  
24 release of glutamate in the mouse hippocampus. FEMS Microbiol Lett 189, 109-  
25 113.

- 1 Miyata, S., Matsushita, O., Minami, J., Katayama, S., Shimamoto, S., Okabe, A., 2001,  
2 Cleavage of a C-terminal peptide is essential for heptamerization of *Clostridium*  
3 *perfringens* epsilon-toxin in the synaptosomal membrane. J Biol Chem 276,  
4 13778-13783.
- 5 Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S., Okabe, A., 2002,  
6 *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the  
7 detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat  
8 synaptosomes. J Biol Chem 277, 39463-39468.
- 9 Nagahama, M., Sakurai, J., 1991, Distribution of labeled *Clostridium perfringens*  
10 epsilon toxin in mice. Toxicon 29, 211-217.
- 11 Nagahama, M., Sakurai, J., 1992, High-affinity binding of *Clostridium perfringens*  
12 epsilon-toxin to rat brain. Infect Immun 60, 1237-1240.
- 13 Nicholls, D.G., Sihra, T.S., 1986, Synaptosomes possess an exocytotic pool of  
14 glutamate. Nature 321, 772-773.
- 15 Smedley, J.G., 3rd, Fisher, D.J., Sayeed, S., Chakrabarti, G., McClane, B.A., 2004, The  
16 enteric toxins of *Clostridium perfringens*. Rev Physiol Biochem Pharmacol 152,  
17 183-204.
- 18 Soler-Jover, A., Blasi, J., Gomez de Aranda, I., Navarro, P., Gibert, M., Popoff, M.R.,  
19 Martin-Satue, M., 2004, Effect of epsilon toxin-GFP on MDCK cells and renal  
20 tubules in vivo. J Histochem Cytochem 52, 931-942.
- 21 Soler-Jover, A., Dorca, J., Popoff, M.R., Gibert, M., Saura, J., Tusell, J.M., Serratos,  
22 J., Blasi, J., Martin-Satue, M., 2007, Distribution of *Clostridium perfringens*  
23 epsilon toxin in the brains of acutely intoxicated mice and its effect upon glial  
24 cells. Toxicon 50, 530-540.

1 Takamori, S., Holt, M., Stenius, K., Lemke, E.A., Grønborg, M., Riedel, D., Urlaub, H.,  
2 Schenck, S., Brügger, B., Ringler, P., Müller, S.A., Rammner, B., Gräter, F.,  
3 Hub, J.S., De Groot, B.L., Mieskes, G., Moriyama, Y., Klingauf, J., Grubmüller,  
4 H., Heuser, J., Wieland, F., Jahn, R., 2006, Molecular anatomy of a trafficking  
5 organelle. Cell 127, 831-846.

6

## 7 FIGURE CAPTIONS

8

9 **Figure 1.- Glutamate release from mouse brain synaptosomes.** Glutamate release  
10 from mouse brain synaptosomes was measured on line by a spectrofluorimetric method  
11 (see material and methods). After reaching a stable base line, synaptosomes were  
12 challenged (arrow) with chemical depolarization (KCl 50 mM final concentration) or  
13 with  $\epsilon$ -toxin at different concentrations (50 or 100 nM). At the end of each  
14 measurement, a now standard concentration of sodium glutamate (10 nmols) was added  
15 to the reaction cuvette to quantify the amount of glutamate released from synaptosomes.

16

17 **Figure 2.- Colocalization of  $\epsilon$ -prototoxin-GFP with myelin in a mouse brain**  
18 **synaptosomal fraction.** Synaptosomal fraction from mouse brains were fixed on glass  
19 cover slips and incubated with  $\epsilon$ -prototoxin-GFP (green in A, C, D, F, G and I) after  
20 preincubation with antibody against SNAP-25 (B, C) antibody against  
21 synaptobrevin/VAMP2 (E, F) or antibody against Myelin Basic Protein (H, I). The  
22 polyclonal antibody against SNAP-25 and the monoclonal antibodies against  
23 synaptobrevin/VAMP2 and Myelin Basic Protein (MBP) were revealed with Alexa  
24 Fluor 546 coupled goat anti-rabbit or goat anti-mouse secondary antibodies respectively  
25 (in red). C, F and I are merged images of  $\epsilon$ -prototoxin-GFP with the respective

1 markers. Inserts show detailed colocalization (I) or non-colocalization (C and F) of  $\epsilon$ -  
2 prototoxin-GFP with MBP or SNAP-25 and synaptobrevin/VAMP2 respectively. Note  
3 the SNAP-25 staining pattern surrounded by  $\epsilon$ -prototoxin-GFP in the insert of C. Bars  
4 in C and I represent 20  $\mu\text{m}$ . Bar in F represents 10  $\mu\text{m}$ . Bars in the figure-inserts  
5 represent 1  $\mu\text{m}$ .

6  
7 **Figure 3.- Binding of  $\epsilon$ -toxin to myelinic structures in mouse brain.** Sections from  
8 mouse cerebellum were incubated with  $\epsilon$ -prototoxin-GFP (A and C). The sections were  
9 counterstained with the nuclear marker TO-PRO-3 for convenient cerebellar layer  
10 identification (B and C). C is a merged image of TO-PRO-3 labelling with  $\epsilon$ -  
11 prototoxin-GFP. To demonstrate the colocalization of  $\epsilon$ -prototoxin-GFP staining with  
12 myelinic components, sections from mouse cerebellum were stained with  $\epsilon$ -prototoxin-  
13 GFP (D, F, G and I) together with MBP (E, F) or neurofilament (RT97, H, I)  
14 monoclonal antibodies. Primary antibodies were detected with Alexa Fluor 546 coupled  
15 goat anti-mouse secondary antibodies (in red). Note the colocalization pattern of  $\epsilon$ -  
16 prototoxin-GFP staining with the MBP antibody (F). Note that the RT97 stained  
17 structures on the cerebellar white matter, which basically correspond to neuronal axons,  
18 are surrounded by the  $\epsilon$ -prototoxin-GFP staining (I). F and I are merged images of  $\epsilon$ -  
19 prototoxin-GFP staining with the respective antibodies. GL: granular layer; WM: white  
20 matter. Scale bars: C, 75  $\mu\text{m}$ ; F, 50  $\mu\text{m}$  and I, 15  $\mu\text{m}$ .

21  
22 **Figure 4.- Effect of pronase E, N-glycosidase F and Triton X-100 on the  $\epsilon$ -**  
23 **prototoxin-GFP binding to myelin.** Sections from mouse cerebellum were incubated  
24 directly with  $\epsilon$ -prototoxin-GFP as a control (A), or after treatment of the sections with  
25 pronase E (1 mg/ml at 37°C for 30 min, B), N-glycosidase F (10 U O/N at 37°C, C) or

1 2% Triton X-100 (1 h at 4°C, D). Note the reduced labelling of myelin by  $\epsilon$ -prototoxin-  
2 GFP after the pronase E treatment (B). Bars represent 40  $\mu$ m.

3

4 **Figure 5.- Binding of  $\epsilon$ -toxin to peripheral nerve fibres.** (A) Cross section of mouse  
5 left vagus nerve incubated with  $\epsilon$ -prototoxin-GFP. (B) Teased fibre from mouse sciatic  
6 nerve incubated with  $\epsilon$ -prototoxin-GFP. (C) Teased nerve fibre from human *cauda*  
7 *equina* incubated with  $\epsilon$ -prototoxin-GFP. Note the staining of  $\epsilon$ -prototoxin-GFP to  
8 isolated nerve fibres, mainly at the Ranvier nodes (arrows). Scale bars: A, 40  $\mu$ m; B and  
9 C, 15  $\mu$ m.

10

11 **Figure 6.- Binding of  $\epsilon$ -toxin to sheep and bovine nervous system.** Sections from  
12 sheep (A, B and C) and bovine (D) brains were incubated with  $\epsilon$ -prototoxin-GFP. (A)  
13 Cerebellar section showing the binding of  $\epsilon$ -prototoxin-GFP to the white matter (WM)  
14 and blood vessels (arrows). (B) Detail of a section from sheep striatum showing the  
15 binding of  $\epsilon$ -prototoxin-GFP to myelin structures (WM) and blood vessels (arrow). (C)  
16 Blood vessels (arrows) from the molecular layer of the sheep cerebellum stained with  
17 the  $\epsilon$ -prototoxin-GFP. (D) A section of the bovine cerebellum showing the binding of  $\epsilon$ -  
18 prototoxin-GFP to myelin structures (WM) and blood vessels (arrow). WM: white  
19 matter; GL: granular layer; PL: Purkinje cell layer and ML: molecular layer. Scale bars:  
20 A and C, 75  $\mu$ m; B: 40  $\mu$ m; D, 25  $\mu$ m.











