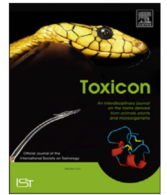
Toxicon 130 (2017) 19–28

Contents lists available at ScienceDirect

Toxicon

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



Clostridium perfringens epsilon toxin induces permanent neuronal degeneration and behavioral changes

Winston E. Morris [a], Jorge Goldstein [b, f], Leandro M. Redondo [a, f], Adriana Cangelosi [c], Patricia Geoghegan [c], Marcela Brocco [d, f], Fabián C. Loidl [e, f], Mariano E. Fernandez-Miyakawa [a, f, *]

[a] *Instituto de Patobiología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25 (1686), Hurlingham, Buenos Aires, Argentina*
[b] *Departamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Ciudad Autónoma de Buenos Aires, Argentina*
[c] *Centro Nacional de Control de Calidad de Biológicos, ANLIS "Dr. Carlos G. Malbrán", Av. Vélez Sarsfield 563, C1282AFF, Ciudad Autónoma de Buenos Aires, Argentina*
[d] *Instituto de Investigaciones Biotecnológicas, "Dr. Rodolfo A. Ugalde" IIB-INTECH UNSAM-CONICET, Av. 25 de Mayo y Francia, Campus Miguelete UNSAM, Edificio IIB-INTECH San Martín, Buenos Aires, Argentina*
[e] *Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Ciudad Autónoma de Buenos Aires, Argentina*
[f] *Consejo Nacional de Investigaciones Científicas y Técnicas, Rivadavia 1917 (1033), Ciudad Autónoma de Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 20 September 2016
Received in revised form
18 February 2017
Accepted 20 February 2017
Available online 22 February 2017

Keywords:
Clostridium perfringens
Epsilon toxin
Brain
Enterotoxemia
Behavior
Neurodegeneration

ABSTRACT

Clostridium perfringens epsilon toxin (ETX), the most potent toxin produced by this bacteria, plays a key role in the pathogenesis of enterotoxaemia in ruminants, causing brain edema and encephalomalacia. Studies of animals suffering from ETX intoxication describe severe neurological disorders that are thought to be the result of vasogenic brain edemas and indirect neuronal toxicity, killing oligodendrocytes but not astrocytes, microglia, or neurons *in vitro*. In this study, by means of intravenous and intracerebroventricular delivery of sub-lethal concentrations of ETX, the histological and ultrastructural changes of the brain were studied in rats and mice. Histological analysis showed degenerative changes in neurons from the cortex, hippocampus, striatum and hypothalamus. Ultrastructurally, necrotic neurons and apoptotic cells were observed in these same areas, among axons with accumulation of neurofilaments and demyelination as well as synaptic stripping. Lesions observed in the brain after sub-lethal exposure to ETX, result in permanent behavioral changes in animals surviving ETX exposure, as observed individually in several animals and assessed in the Inclined Plane Test and the Wire Hang Test. Pharmacological studies showed that dexamethasone and reserpine but not ketamine or riluzole were able to reduce the brain lesions and the lethality of ETX. Cytotoxicity was not observed upon neuronal primary cultures *in vitro*. Therefore, we hypothesize that ETX can affect the brain of animals independently of death, producing changes on neurons or glia as the result of complex interactions, independently of ETX-BBB interactions.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Clostridium perfringens is a spore-forming bacteria, often found as a normal inhabitant of the intestine of most animal species and humans (Fernandez-Miyakawa and Redondo, 2016; Kohn and Warrack, 1955; Mantis, 2005; Meer and Songer, 1997; Murrell et al., 1986). However, when the intestinal environment is altered by sudden changes in diet or other factors, *C. perfringens* proliferates and produces potent toxins that act locally or are absorbed into the general circulation (Fernandez-Miyakawa and Redondo,

* Corresponding author. Instituto de Patobiología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25 (1686), Hurlingham, Buenos Aires, Argentina.

E-mail addresses: morris.winston@inta.gob.ar (W.E. Morris), jogol@fmed.uba.ar (J. Goldstein), redondo.leandro@inta.gob.ar (L.M. Redondo), acangelosi@anlis.gov.ar (A. Cangelosi), pgeoghegan@anlis.gov.ar (P. Geoghegan), mbrocco@iib.unsam.edu.ar (M. Brocco), floidl@fmed.uba.ar (F.C. Loidl), fernandezmiyakawa.m@inta.gob.ar (M.E. Fernandez-Miyakawa).

http://dx.doi.org/10.1016/j.toxicon.2017.02.019
0041-0101/© 2017 Elsevier Ltd. All rights reserved.

2016; Niilo, 1980). Epsilon toxin (ETX) is considered the most potent toxin produced by this bacteria and it has been also considered a category B toxin by the Centre of Disease Control (CDC) for its potential use in bioterrorism (Mantis, 2005). This toxin is produced by *C. perfringens* type B and D as a prototoxin, activated through proteolytic cleavage by the animal's trypsin, quimotrypsin or *C. perfringens* zinc-metalloprotease and then absorbed into the blood stream affecting other organs such as the brain (Fernandez-Miyakawa and Redondo, 2016; Minami et al., 1997).

ETX plays a key role in the pathogenesis of enterotoxaemia in ruminants (Uzal and Songer, 2008). In sheep, this disease is acute and fatal, characterized predominantly by nervous signs such as opisthotonus, convulsions, muscular incoordination and struggling (Miyamoto et al., 1998; Uzal et al., 2002, 2004). In the brain of sheep, edema surrounding blood vessels in the internal capsule, thalamus, cerebellar peduncles and/or cerebellum, is a common feature; however focal symmetric encephalomalacia can occur as well (Finnie, 2003, 2004; Ghabriel et al., 2000; Miyamoto et al., 1998; Miyata et al., 2002; Zhu et al., 2001). In rat and mouse experimental models of enterotoxaemia, the described affected areas of the central nervous system (CNS) are the basal ganglia, internal capsule, thalamus, subcortical white matter, substantia nigra, hippocampus and cerebral peduncles (Finnie, 1984, 1984b, 2004). The nature and distribution of the lesions in mice CNS are comparable to those observed in lambs suffering from *C. perfringens* type D enterotoxemia, therefore rats and mice models have frequently been chosen to study the pathogenesis of the ovine disease (Finnie, 2003). In goats, on the other hand, the disease is most commonly an acute or sub-acute enterocolitis, though lung and brain edema can occur as well, the latter even progressing to focal symmetric encephalomalacia (Uzal and Songer, 2008; Garcia et al., 2013).

Various authors have studied the neuropathology of *C. perfringens* ETX in rat, mouse, sheep, goat or bovine experimental models (reviewed in Garcia et al., 2013). However, these studies were unable to determine if ETX had a direct effect on glia and neurons or if these changes were secondary to changes on blood-brain barrier's (BBB) (Finnie, 1984, 1984b; Miyamoto et al., 1998; Uzal and Kelly, 1997; Uzal et al., 2002). Miyamoto et al. (1998, 2000) have found evidence that suggests that ETX acts directly or indirectly on the neurons through the glutamatergic system (Nagahama and Sakurai, 1992). However, Dorca-Arevalo et al (2008) described no glutamate release from nerve terminals when incubating them with GFP-ETX. This study suggests that the massive glutamate release is due to other mechanisms such as the binding of the toxin to the cell-soma, axons or to the glia or other cells. In a subsequent study, Lonchamp et al. (2010) observed that a subset of neurons can be directly affected by ETX-induced glutamate release. In the same work, the authors also report that ETX binds to oligodendrocytes suggesting that these cells also can be a target for ETX, and for this reason the involvement of oligodendrocytes in glutamate release cannot be ruled out (Lonchamp et al., 2010). Recent studies proposed the oligodendrocytes as the cellular target of ETX in the central nervous system (Linden et al., 2015; Wioland et al., 2015). Although these studies described different alterations in oligodendrocytes caused by ETX, both groups described demyelination with preservation of neurons, astrocytes, and microglia (Linden et al., 2015; Wioland et al., 2015).

The effects of sub-lethal ETX challenge have also been studied (Finnie, 1984, 1984b) and yet there are -to our knowledge-no studies on the behavioral changes that sub-lethal ETX challenge may induce in the surviving animal. In this study, we provide evidence to support the hypothesis that ETX can affect with some severity the brain of animals independently of death, producing changes on neurons or glia separately of BBB alteration. These effects in the brain, after sub-lethal exposure of the animal to ETX, may cause permanent behavioral alterations. On the other hand, lethal effects of ETX could be prevented by pharmacological interventions. These results provide innovative insights into the pathophysiology of *C. perfringens* type B or D enterotoxemia and potential side effects affecting productive parameters.

2. Materials and methods

2.1. Animals

Male Sprague - Dawley rats (250–300 g) and outbreed NIH Swiss-white mice of either sex (18–20 g) were used in this study. The animals were housed under standard housing conditions, with air conditioned and 12 h light-dark cycles (from 6 a.m. to 6 p.m. approx.), with food and water provided *ad libitum*. The experimental protocols and euthanasia procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Universidad de Buenos Aires, ANLIS- Malbrán and the Comité Institucional para el cuidado y uso de animales de Experimentación -CICVyA-INTA under protocols 32/2010 and 27/2011.

2.2. ETX purification

Purified epsilon prototoxin was prepared from an overnight culture of *C. perfringens* type D (strain NCTC 8346) in Trypticase-yeast-glucose medium, under anaerobic conditions at 37 °C. Overnight cultures were centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant containing ETX was saved for toxin purification. The toxin was then precipitated by ammonium sulfate. Two columns were prepared with DEAE and CM Sepharose (Pharmacia, Sweden), respectively, equilibrated in 10 mM Tris, pH 7.5. The toxin was applied to the DEAE column, and the effluent was monitored at 220 nm. The initially eluted peak was saved and applied to the CM column. Again the effluent was monitored at 220 nm, and the first peak was collected, dialyzed against phosphate buffer solution (PBS), and freeze-dried. A single band corresponding to epsilon prototoxin on SDS-PAGE was visualized with Coomasie blue.

2.3. Sub-lethal dose determination (LD_{50})

Prior to its use in the experiments, ETX was reconstituted at an initial concentration of 40 μg/ml and activated by incubation at 37 °C during 30 min with 0.05% trypsin (Sigma). To determine LD_{50}, groups of 4 randomly selected NIH mice of either sex and weighing 18–20 g, were intravenously (i.v.) inoculated with 0.01, 0.005 and 0.0025 μg/ml of activated ETX diluted in saline solution. A similar group was inoculated with saline alone and used as control. After ETX injection, mice were observed for up to 96 h to monitor for lethality. Because of the rapid action of the toxin, spontaneous death was considered as a potential outcome of the experiments and it was discussed and approved by the ANLIS-Malbrán IACUC. The assay endpoints were defined as spontaneous death, development of severe clinical signs necessitating euthanasia, or survival without clinical alterations during a set period of time. The LD_{50} was determined as the reciprocal of the concentration of toxin that killed 50% of the inoculated animals and was calculated according Reed and Muench method (1938). The dose in which the mice did not die within a period of 96 h, were considered sub-lethal and selected to use for the present work.

2.4. Histology

After ETX inoculation, brains of spontaneously dead animals was taken immediately and fixed in paraformaldehyde solution. Animals surviving sub-lethal ETX doses together with the control animals were put under deep anesthesia (100 mg/kg sodium pentobarbital, i.p.), without delay, they were perfused intracardially with a paraformaldehyde (4%) solution. The brains were removed at the end of perfusion and embedded in paraffin wax, sectioned at 4 μm and stained with haematoxylin-eosin or Fluoro-jade B stains. Neuronal damage was considered when neurons display chromatolysis or retracted cytoplasm. Neuronal damage was determined and expressed as a percentage of the total number analyzed neurons.

2.5. Behavioral performance

2.5.1. Inclined plane test

This test assesses an animal's ability to maintain its position on a board which is raised progressively on an inclined plane system, which uses a stepping motor that changes the angle of the inclined plane, from 0 to 90°. This task constitutes a quantitative measurement test of the limb motor function of laboratory animals (Chang et al., 2008; Wells et al., 2003). In the present study, groups of 5 mice were tested after treatment with either peptone water (Control), ETX 0.75 LD_{50} or ETX 0.37 LD_{50}. The inclined plane test was performed at day 0, 1, 7 and 14. Each time, the inclination angle in which the mouse loses its grip was recorded. The inclined plane score was defined as the maximum angle at which a mouse is able to maintain its position. The scores were determined 3 times per animal and averaged.

2.5.2. Wire hang test

This test also evaluated motor performance (Karl et al., 2003; Kumon et al., 2010). In this test, the animals are allowed to hold by the forelimbs from a horizontal bar attached to two vertical poles. In the present study, groups of 5 mice were tested after treatment with either peptone water (Control), ETX 0.75 LD_{50} or ETX 0.37 LD_{50}. The test was performed at day 0, 1, 7 and 14 and in each occasion, the time in seconds spent by the animals hanging from the bar until they fell down was recorded, determinations were performed 3 times per animal and averaged. The endpoint for this test was set to 10 s.

2.6. Intracerebroventricular inoculation (i.c.v.)

Under anesthesia (ketamine 50 mg/kg - diazepam 0.35 mg/kg, i.p.), rats were stereotaxically implanted into the lateral ventricle with a stainless steel guide cannula (Plastic One, Roanoke, VA). The placement coordinates were anteroposterior: −1.80 mm; lateral; 2.4 mm and vertical: 3.2 mm (Paxinos and Watson, 2008). To reach the ventricle area and minimize the damage of tissue, a 21-gauge guide cannula was implanted at this point. Then, a 30-gauge needle extending 0.5 mm below the guide cannula was used for the injections. Correct placement of the ventricle cannula was verified at the end of the experiment. The cannula were fixed to the skull surface with three screws and dental acrylic cement and temporarily occluded with a dummy cannula. After surgery, animals were caged individually. There were randomly assigned to different experimental groups and each rat was used only once. One week after the experiment, freely moving animals were i.c.v. injected through a 30-gauge needle connected by polyethylene tubing to a 20-μl Hamilton syringe. The needle was left in place for 30 s to prevent backflow of the injected solution. Two rats were i.c.v. injected with 6 μl of vehicle (control) and another two rats were injected with ETX 0.05 LD_{50}. Two hours after i.c.v. infusions, the rats were anesthetized with sodium pentobarbital (60 mg/kg) and perfused transcardially with 0.9% NaCl solution followed by 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (PB) [fixative per animal weight (ml/g)]. The brains were removed from skull, post-fixed in the same fixative solution for 2 h. Samples were embedded in paraffin wax. Sections were cut at 4 mm and stained with haematoxylin-eosin and examined by light microscopy. All these procedures were performed according to regular protocols (Boccoli et al., 2008; Goldstein et al., 2007).

2.7. Transmission electron microscopy (TEM) of i.c.v brains

Tissue samples from i.c.v. rat cortex, hypothalamus and striatum were processed for transmission electron microscopy. Tissue sections were stained with toluidine blue and examined by light microscopy to select the areas for TEM studies. Ultrathin sections were then cut from selected areas, and contrasted with 1% OsO4 and 1% uranyl acetate, dehydrated and embedded in Durcupan. The sections were contrasted with lead citrate, examined and photographed on a Zeiss 109 electron microscope. Observed neuronal and myelin sheath alterations were described. To quantify ETX induced demyelination; 10 electron micrographs with an estimated area of 3721 μm^2 were taken at different parts of the brain, in these micrographs axon diameter and myelin sheath thickness was measured directly at locations where it was sectioned transversely rather than obliquely, and a myelin ratio was calculated for each myelinated axon by dividing myelin sheath thickness by total axon diameter, and then a mean value was calculated for each brain region in ETX treated and control groups.

2.8. Neuron primary cell cultures

Neuron primary cell cultures were established from 19-day-old fetal Sprague Dawley rat hippocampus as described previously (Brocco et al., 2010). Tissue was treated with 0.25% trypsin in Hank's solution for 15 min at 37° C. A single-cell solution was prepared by dissociation in 10% horse serum supplemented Neurobasal medium containing 4.5 g/l glucose, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded on coverslips coated with 0.1 mg/ml poly-L-lysine hydrobromide (Sigma, St. Louis, MO) and 20 μg/μl laminin (Gibco, Carlsbad, CA) at a density of 30,000 cells/cm^2. After 2 h, medium was changed to Neurobasal/N2 (Neurobasal supplemented with 4.5 g/l glucose, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, 1 g/l ovalbumin and N2 and B27 serum-free supplements from Gibco). According with previous reports (Brewer and Cotman, 1989; Liu et al., 2000), the use of a defined media without serum and polylysine and laminin substrate contributes to maintain neurons (>90–95%) and do not support proliferation or survival of non-neuronal cells (i.e. glial cells, fibroblast).

2.9. ETX in cell cultures

Neuron primary cultures were treated with different concentrations of activated ETX. After toxin activation, the mix containing ETX and trypsin was supplemented with fetal bovine serum to inactivate trypsin that otherwise would affect cell viability. Control cells were treated with the mix without ETX but containing the inactivated trypsin. After incubating treated and control cells with ETX for 48 h, neurons were stained with Trypan-blue dye to assess viability. To evaluate changes in cell morphology, another set of cells (treated vs. control) was fixed with 4% (w/v) paraformaldehyde, 4% sacarose in PBS, and stained with May Grünwald Giemsa. Cells were observed under a light microscope. Additional

changes in cell structure were assessed using immunofluorescence techniques. After ETX treatment, cells were fixed with 4% paraformaldehyde in PBS, washed three times with PBS, blocked with 3% (w/v) bovine serum albumin for 1 h and washed three times with PBS. Cells were incubated overnight at 4 °C with anti-tubulin (1:2000; Clone B-5-1, Sigma), washed and then incubated with rodhamine-conjugated phalloidin to stain F-actin, and with the appropriate secondary conjugated antibodies at 37 °C for 1 h. Next, cells were washed twice with PBS and then incubated with DAPI to label nuclei. Finally, cells were washed with distilled water and mounted with FluorSave reagent (Calbiochem, San Diego, CA) as anti-fading reagent. Fluorescent images were obtained using a Nikon E600 microscope equipped with epifluorescence illumination Nikon, Japan) with 40× and 100× oil-immersion lens.

2.10. Pharmacological modulation of ETX effects

In a first set of experiments, groups of 7 mice were i.p. inoculated with peptone water, riluzole (10 mg/kg in saline), reserpine (10 mg/kg in saline), dexamethasone (15 mg/kg) or ketamine (50 mg/kg) and challenged i.v. with 1.25 LD_{50} of ETX. Additionally, groups of 5 mice were inoculated with peptone water, riluzole, reserpine, dexamethasone or ketamine but not challenged with ETX and used as controls. Toxin challenge was performed one hour after saline or riluzole injections, 30 min after ketamine, and 24 h after dexamethasone or reserpine injection. Based on obtained results, a second set of experiments were performed. Groups of 7 mice were inoculated with reserpine and dexamethasone as described before, and challenged i.v. with 1.5, 1.8 and 2 LD_{50} of ETX. In all the experiments, mice were observed before and after ETX injection and the time of death post ETX injection (PTI) was recorded. Immediately after death, the brains of two mice of each ETX group were removed and immersion fixed in 10% buffered formalin, together with the brains of two euthanized mice of each control group. The tissues were embedded in paraffin wax, sectioned at 4 μm and stained with haematoxylin-eosin or Fluoro-jade B stains.

2.11. Statistical analysis

Physiological data were analyzed with repeated-measures ANOVA for differences between time points and groups. Differences in neuron degeneration and demyelination between ETX treated and control animals were analyzed using Mann-Whitney test (GraphPad Prism4, GraphPad Software, Inc.). Significance was set at a value of $P < 0.05$. Data are expressed as mean ± standard deviation of the mean.

3. Results

3.1. Effects of systemic ETX on the brain

To study the effects of ETX on cells of the central nervous system, mice were injected with different doses of ETX. According with the results from lethality tests, the intravenous LD_{50} of the ETX was determined to be 0.005 μg/ml per mouse. Mice receiving ≥1 LD50 ETX lethal doses, died within the first 48 h, and no obvious histological changes were observed in these animals, while mice receiving sub-lethal ETX doses showed neurons with chromatolysis and retracted cytoplasm compatible with degenerative changes. Compared with control mice, injected only with vehicle solution, in ETX-injected mice neuronal degeneration was more frequent in the cortex (68% vs. 10%; $P < 0.05$), cerebellum (84% vs. 3%; $P < 0.01$), hippocampus (56% vs. 3%; $P < 0.01$) and striatum (65% vs. 7%; $P < 0.01$), and hypothalamus (77% vs. 8%; $P < 0.001$) (Fig. 1). In the cortex, neurons from the 3rd and 5th layers were particularly affected (laminar necrosis) (Fig. 2A–B). In the cerebellum, shrunken degenerative Purkinje cells were seen interspersed with other apparently normal cells (Fig. 2C–D). In the hippocampus CA2 pyramidal neurons with chromatolysis and retracted cytoplasm were evident (Fig. 2E–F). No evidence of perivascular edema was detected in the any of the ETX challenged mice. No significant histological changes were observed in the brains of any of the control mice. Degenerative neurons from the cortex, hippocampus, striatum, thalamus and hypothalamus were detected using Fluoro-jade B stain, in the control group no Fluoro-jade B stained neurons were observed (supplementary data Fig. S1).

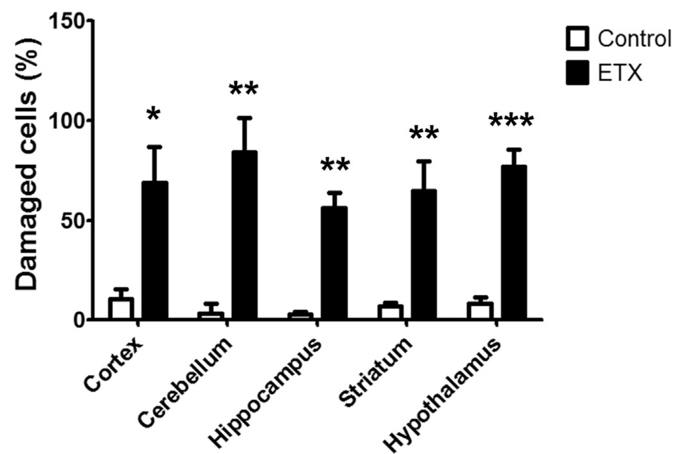


Fig. 1. Sub-lethal dose of ETX causes neurodegenerative changes in mice brain. Quantification of the percentage of damaged neurons in different parts of the brain in mice inoculated with one sub-lethal dose of ETX (0.5 LD50) compared with mice inoculated with vehicle solution. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

3.2. Effect of sub-lethal doses of ETX on mice behavior

In mice treated with sub-lethal doses of ETX, behavioral changes were noted. Some of these mice exhibited obvious alterations, characterized by uncoordinated movements and convulsion in one mouse, and hyperactivity and intense aggressiveness in two other ETX treated animals. The inclined plane scores following treatment with the toxin and/or vehicle are resumed in Fig. 3A. The control group showed a greater score than both ETX treated groups during all the period. However, significant differences ($P < 0.05$) in the inclined plane score were observed from day 1 until day 7 when comparing control vs. ETX (0.75 LD50) and (0.37 LD50) mice. No significant differences were observed between control and any of the treated groups in the following days; although ETX treated animals had the worst profile in the test. In the Wire Hang Test, statistically significant differences between control and ETX treated mice were detected in days 1 ($P < 0.05$), 7 ($P < 0.001$) and 14 ($P < 0.001$) PTI. These differences were more obvious at day 7 and 14 PTI (Fig. 3B). These results suggest that animals can be permanently affected at least, in their motor function by sub-lethal exposure to ETX.

3.3. Effects of intracerebroventricular ETX on the brain

To test the hypothesis that ETX can induce brain alterations independently of toxin passage through BBB, ETX was directly injected into the brain of rats via intracerebroventricular inoculation (i.c.v.). Ultrastructure analysis of brain from ETX i.c.v. inoculated rats shows neurodegenerative changes in the cortex, striatum,

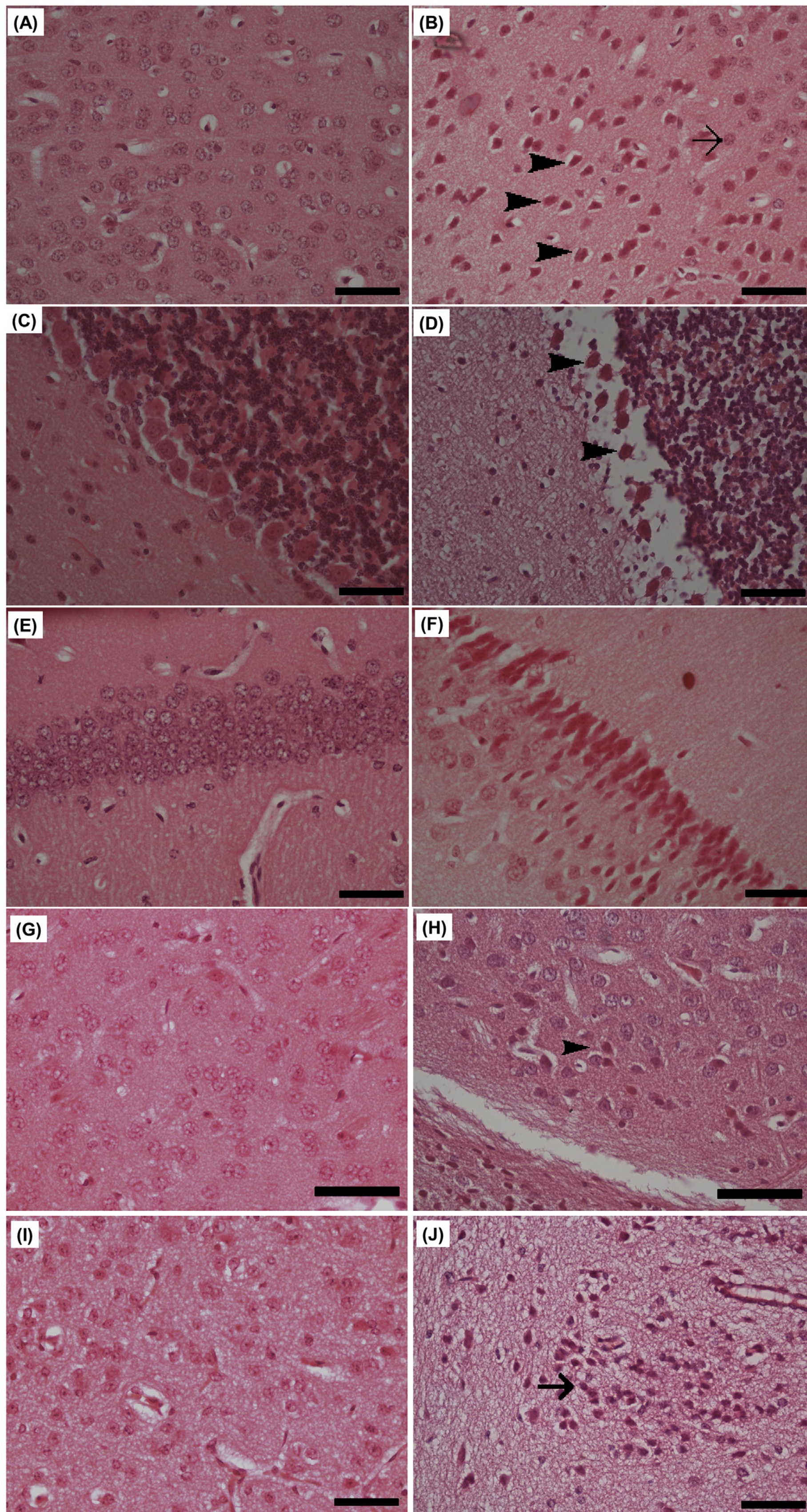


Fig. 2. Neurodegenerative effects of systemic ETX in i.v. challenged mice (H/E). (A) Sections from cortex of mice inoculated with vehicle exhibiting well preserved neurons. (B) Section from cortex of ETX i.v. inoculated mice in which laminar necrosis (arrowhead) can be observed, adjacent to areas of well preserved neurons (arrow). (C) Sections from cerebellum of mice inoculated with vehicle exhibiting well preserved Purkinje cells. (D) In the cerebellum of ETX treated mice, the Purkinje cells appear degenerated (arrows) and the molecular layer is vacuolated. Sections from hippocampus from control mice (E) and from ETX i.v. challenged mice (F) degenerated neurons can also be seen (F). Bar = 50 μm. Neurodegenerative effects of systemic ETX in i.v. challenged mice (H/E). In mice inoculated with vehicle solution it is possible to observed well preserved neurons in striatum (G) and hypothalamus (I), while in ETX treated animals in which neurons displaying degenerative changes can be seen striatum (H) (arrowhead) and hypothalamus (arrow) (J). Bar = 50 μm.

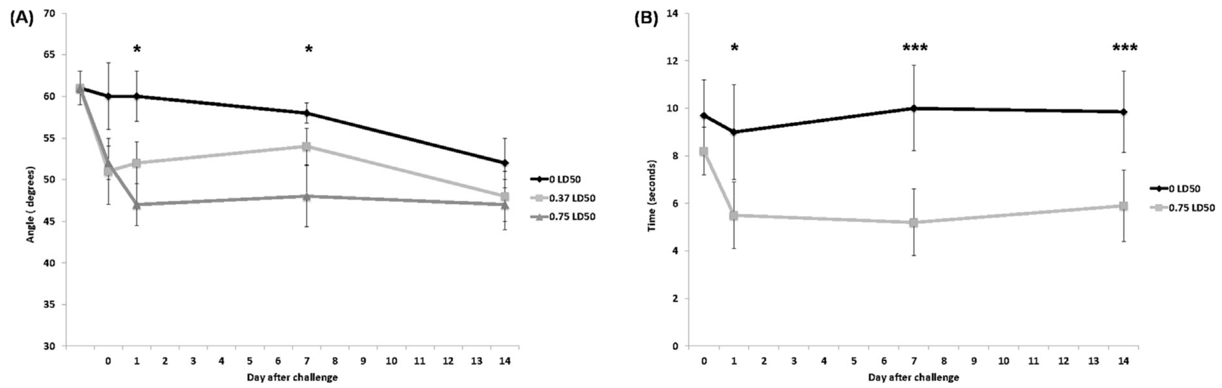


Fig. 3. Behavioral changes in ETX treated mice. (A) Inclined plane test scores from control and ETX (0.37 and 0.75 LD50) treated mice, values represent mean and standard deviation of the inclination angle in which the mouse loses its grip. (B) Wire hang test scores from control and ETX (0.75 LD50) treated mice, values represent mean and standard deviation the time in seconds spent by the animals hanging from the bar until they fell down. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

hippocampus and hypothalamus. In these areas, necrotic neurons and apoptotic cells were seen. Less severe changes were also seen, including neurons and astrocytes exhibiting with nuclear indentation, vacuolated cytoplasm, rough endoplasmic reticulum disorganization, abundant free ribosomes, mitochondrial degeneration and often intracellular edema were also observed (Fig. 4A–D). Glial processes between pre- and post-synapses, showing synaptic stripping were also seen (Fig. 4E–G). Intracellular edema was notable in some glial cells at the proximity of blood vessels, although no perivascular edema was observed and the vascular endothelium appeared well preserved (Fig. 4H). Axons exhibiting accumulation of neurofilaments and demyelination together with some dendrites with hypertrophy were often found. Myelin quantification reveals significant differences in striatum ($P < 0.05$) and hippocampus ($P < 0.01$); in the rest of the brain no significant differences were observed in myelin sheath thickness, although ultrastructural abnormalities were evident in ETX treated rats (Fig. 5). These findings suggest that ETX can induce direct neuronal damage. No ultrastructural changes were seen in any of the control rat brains.

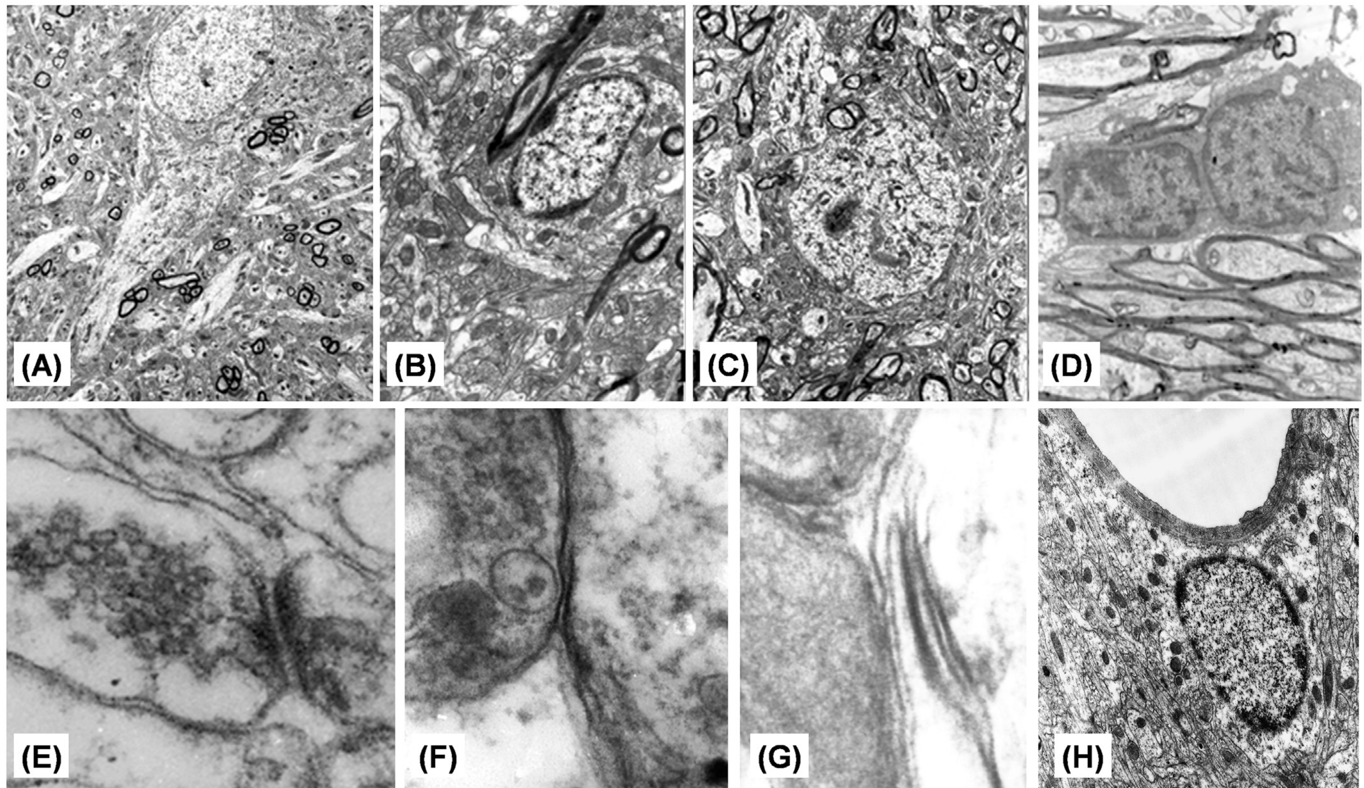


Fig. 4. Transmission electron microscopy of rat brains inoculated i.c.v with vehicle or ETX (0.05 LD50). Neuron (A) and glia (B) from control rat displaying normal appearance with well preserved cytoplasm nucleus and organelles are shown. A neuron (C) from ETX treated rat showing with lytic necrosis, displaying organelle disaggregation, swollen nucleus and loss of the chromatin integrity. Apoptotic cell (D) being phagocytised. (E) Neuronal synapses from a control rat brain. (F and G) Glial processes between pre- and post-synapses, showing synaptic stripping were seen in ETX treated rats. (H) Blood vessels from i.c.v. ETX challenged rat, no perivascular edema was observed and the vascular endothelium appeared well preserved.

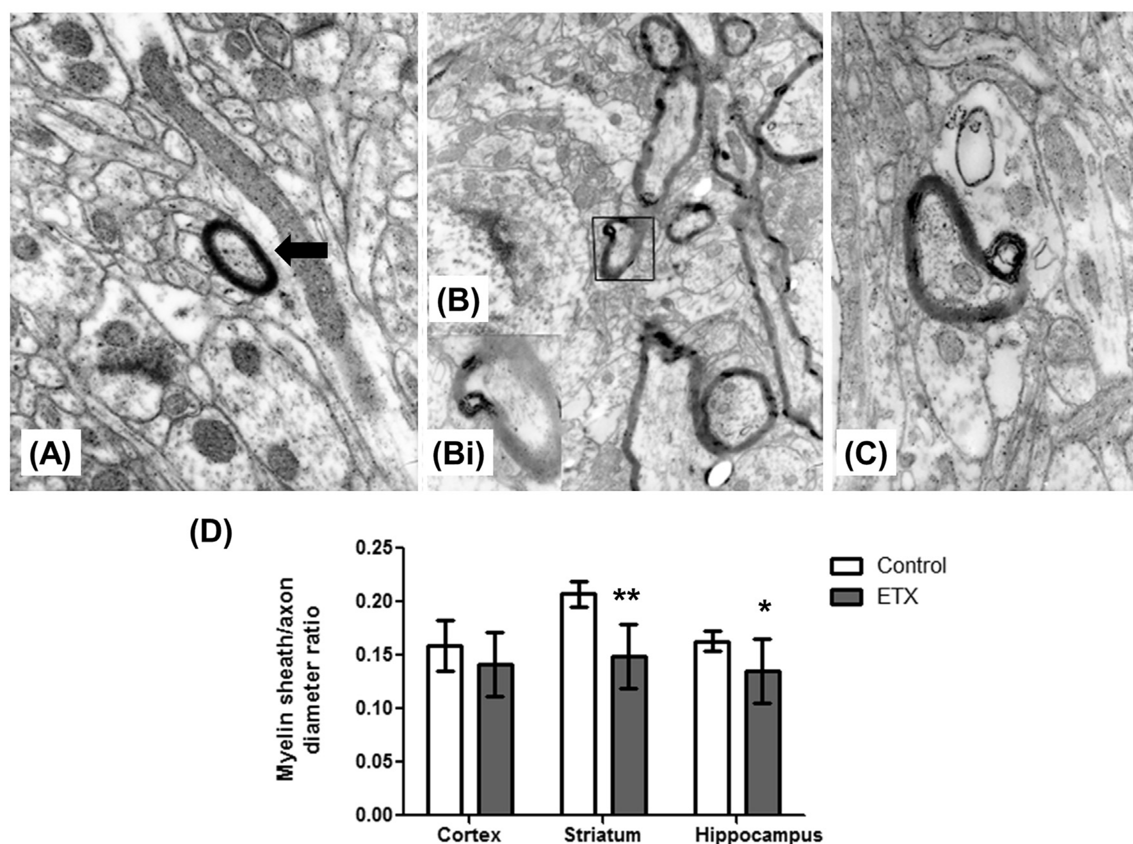


Fig. 5. Transmission electron microscopy of rat brains inoculated i.c.v with vehicle or ETX (0.05 LD50). In control rats (A), axons and myelin sheath show normal appearance (arrow). In ETX treated rats demyelization (B, Bi and C) was frequently seen. (D) Morphometric data derived from ultrastructural measurements in myelin sheath in ETX i.c.v. challenged and control rats. Bars shows myelin sheath thickness-axon diameter ratio. (*) $P < 0.05$; (**) $P < 0.01$.

3.4. ETX toxicity in neurons cultures

To further evaluate if ETX induced neurodegenerative changes observed in i.c.v. rats could be produced directly by ETX in neurons, cytotoxicity was evaluated in neuron primary cultures established from rat hippocampus. Neurons did not exhibited any cytotoxic changes in either the control or ETX challenged cells, even with high concentrations of ETX (60 LD50). No differences in the viability of these cells were observed when cells were stained with the vital dye Trypan blue, and no alterations were noticed in cytoskeleton structures or nucleus (supplementary data Fig. S2). These results suggest that most of the cytotoxic effects of ETX in different cellular types of the brain *in vivo* are not associated with direct cytotoxicity.

3.5. Pharmacological modulation of ETX effects in the brain

According with previous studies related with neuropathology of *C. perfringens* ETX, we evaluated if riluzole, ketamine, reserpine and dexamethasone can prevent lethality or neurodegeneration induced by ETX. In a first series of experiments, ETX dose was selected to induce lethality within a period of 48 h i.e. 1.25 LD_{50}. Under this condition, toxin-inoculated -but no control-mice exhibited convulsive episodes and death. Pre-treating mice with reserpine or dexamethasone before administration of 1.25 LD_{50} of ETX led to neuroprotection in 100% of the challenged mice. No death was observed and histological and Fluoro-jade B analyses of different regions of the brain did not reveal neurological damage. Administration of riluzole and ketamine before ETX challenged fail to protect mice, in the ketamine pre-treated groups even a slight increase in lethality was observed. The results of the experimental challenge of mice with 1.25 LD50 of ETX, injected or not with the different drugs, are summarized in Fig. 6. We also evaluated whether reserpine and dexamethasone can prevent lethality or neurodegeneration at higher ETX-doses challenge. However, the tested drugs fail to prevent death when mice were challenged with 2, 1.8 and 1.5 LD50. Although reserpine pretreated mice showed longer survival times even with 2 LD50, these differences were not significant ($P > 0.05$).

4. Discussion

In recent years, appreciation of the importance of intestinal microbiota in the regulation of the gut-brain axis was increased (Chen et al., 2013; Dinan and Cryan, 2013). It is known that the microbiota "communicates" with the CNS via the neural, endocrine or immune systems, affecting host's behavior (Chen et al., 2013; Dinan and Cryan, 2013). Although *C. perfringens* is part of the microbiota in most animal species, little is known about the effect of this bacteria or its toxins can have on the host's behavior. Miyamoto et al. (2000) have reported seizure in mice receiving either 2 or 4 LD_{50} ETX i.v. injections; however, there are no reports on the long term effects of sub-lethal ETX injections in animal behavior. In preliminary studies, we have observed that mice surviving ETX challenge (either intravenous or intragastric toxin administration) developed alterations in their motor functions as well as their social interaction. Some of these mice were observed up to one year after ETX and no recovery was noticed (data not published). In order to study ETX induced behavioral changes in

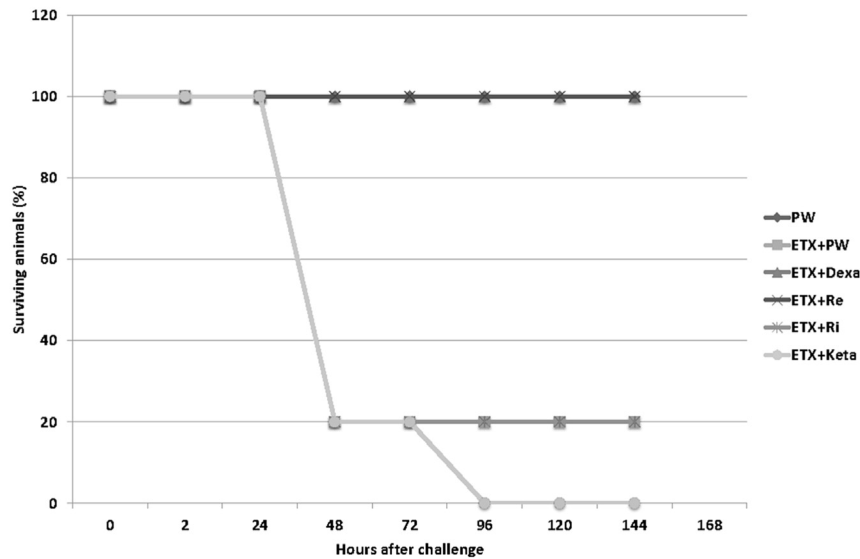


Fig. 6. Pharmacological modulation of ETX effects. Mice were pretreated with selected drugs and challenged with 1.25 LD50 ETX. One group of mice were injected with peptone water without ETX (PW, negative control); another group was challenge i.v. with ETX after receive peptone water (ETX + PW; positive control), mice challenged i.v. with ETX after receive selected drugs. Reserpine (ETX + Re), riluzole (ETX + Ri), ketamine (ETX + Ke) or dexamethasone (ETX + De). Control groups pretreated with selected drugs but without ETX challenged were omitted in the figure.

mice, a protocol with sub-lethal challenge was performed. After toxin injection, treated animals had a drop in the angle in which they could cling to when tested in an inclined plane test. The histological analysis of the brains of the intoxicated mice shows degenerative changes in neurons from different organs including cortex, cerebellum, hippocampus and striatum. These structural alterations are in agreement with previous studies which describes that the drop in the angle in the inclined plane test are associated with brain injury in rats predominantly in the hippocampus (Chang et al., 2008 Chio et al., 2007) but also to alterations in the striatum, cerebellum and cortex. In the Wire Hang Test, which measures neuromuscular strength (Karl et al., 2003) statistical differences between control and ETX group were maintained until the termination of the experiment on day 14. These results shows that mice surviving sub-lethal ETX exposure may become handicapped, developing permanent motor dysfunction and behavioral changes.

Although *C. perfringens* ETX enteroxemia is generally associated with ruminants (Niilo, 1980; Fernandez-Miyakawa and Redondo, 2016), previous works, reported a positive correlation between sheep contact and the chronic form of multiple sclerosis (MS) in humans (Murrell et al., 1986). In recent years, Rumah and coworkers reported the isolation of *C. perfringens* type B from a human patient at first clinical presentation of MS and also found 10% immunoreactivity to ETX in other MS patients compared to 1% in control subjects (Rumah et al., 2013). In concordance with the suggested role of ETX in human MS, the neurological signs -such as dyscoordination and imbalance-described in the present work in ETX challenged mice, may be analogous to those described in human patients with MS (Murrell et al., 1986; Rumah et al., 2013). Although there seems to be a strong association between ETX and MS, the role of this toxin in MS or other human neurodegenerative disorders needs further investigation.

In the present study, mice challenged with sub-lethal doses of ETX develop nervous signs and behavior alterations and histological alterations compatible with neuronal degeneration. Described histological analysis was in concordance with previous reports (Finnie, 1984), in which animals challenged with lethal doses of ETX showed no histological alterations in brain parenchyma, while mice receiving sub-lethal doses showed histological alterations compatible with neurodegenerative changes. In these animals no evident edema neither structural alteration were observed in blood vessels and BBB. Although perivascular edema may not be histologically visible during the first hours PTI, subtle changes of the BBB permeability cannot be discarded. Previous studies have described that in i.v. ETX challenged mice, the toxin was able not only to induce brain edema but to cross the BBB and bind to glial cells (Dorca-Arevalo et al., 2008; Soler-Jover et al., 2007). To test if the ETX-BBB interaction is essential in ETX induced neuropathology, sub-lethal doses of toxin were inoculated in rats directly into the brain parenchyma by i.c.v. injection. In i.c.v. challenged rats, neurodegenerative changes were seen as early as 4 hs PTI, characterized by necrotic and apoptotic-like changes in glia and neurons together with demyelination in the cortex, striatum and thalamus. Similar observations were reported by Finnie (1984b) after i.v. injections. However, in the present study, either perivascular edema or changes in the vascular endothelium were not observed, suggesting that ETX also affects directly the brain parenchyma regardless of edema.

To further evaluate the susceptibility of neurons to ETX cytotoxicity, neuronal primary cultures were established from rat hippocampus and incubated with ETX. The lack of cytotoxic effects of ETX on isolated neurons may be suggestive of an indirect mechanism involved in *in vivo* observed ETX induced neurodegeneration. The described alterations in glial cells and the results obtained in pharmacological modulation experiments may reinforce this idea. In previous works using GFP-targeted ETX (Soler-Jover et al., 2007); the authors describe toxin binding and alterations of the viability of astrocytes. Lonchamp et al. (2010) observed no binding of ETX to astrocytes from the cerebellar cortex and suggested that the GFP-tag could influence this binding. In contrast, these same authors reported binding of the toxin to oligodendrocytes and granule cells from the cerebellum. Recent reports have described binding of ETX to granule cells and oligodendrocytes and demyelination induction in the CNS (Linden et al., 2015; Wioland et al., 2015). In the present study, neuron cells were obtained from the hippocampus, and it is possible that cells from this brain area are not directly targeted by ETX. Wioland et al (2015) reported that ETX binds to oligodendrocytes and acts in a pore-forming independent manner (Wioland

et al., 2015). In the present study, i.c.v. ETX inoculated rats exhibited demyelination in some of the axons but it was not possible to determine if this is a direct effect of ETX on these oligodendrocytes or the result of a more intricate reaction.

Currently, besides immunotherapy or husbandry methods, there are not effective and adequate treatment options for enterotoxemia. Based in previous reports and the results of our studies, we evaluated the potential of pharmacological interventions to moderate the lethal consequences of the disease. As most reports propose a major role for BBB alterations in ETX neurotoxicity (Zhu et al., 2001; Garcia et al., 2013), we proposed that reducing BBB permeability by pharmacological treatment would affect the subtle change observed in animal models and decrease ETX lethality. Dexamethasone was selected as it is known to prevent tight junction protein reorganization and degradation, decreasing the BBB permeability after injury (Tenenbaum et al., 2008). In mice pretreated with dexamethasone and then challenged with 1.25 LD_{50} of ETX mortality could be prevented and no histological damage was observed. After challenge with higher ETX doses, it was not possible to protect mice with dexamethasone, suggesting that other marked toxin activities are related to ETX neuronal damage. Based on the results reported in the present work, we also focused in ETX damage to brain parenchyma. However search for neuroprotective compounds is a difficult task, particularly for ETX, since the mechanism of damage is complex and most likely multifactorial. One of the most remarkable ultrastructural changes observed in i.c.v. treated rats include synaptic stripping. This change has been associated with brain injury in inflammation or as a mechanism of neuroprotection, mostly via an increased uptake of excessive glutamate in a number of different conditions (Perry and O'Connor, 2010; Yamada et al., 2011). The role of glutamate in ETX neurotoxicity has also been reported (Lonchamp et al., 2010; Miyamoto et al., 1998, 2000). In a previous study (Lonchamp et al., 2010); the authors reported glutamate release in granule cells directly targeted by ETX and stimulation of the glutamatergic transmission at the granule cell-Purkinje cell synapse. This activation may propagate throughout the brain inducing glutamate excitotoxicity, since glutamate is present in most cells in the brain. It has also been reported that mice and rats receiving riluzole, a glutamate release inhibitor, prior to the injection of 2 or 4 LD_{50} of ETX produced minor histological changes and even absence of brain edema, particularly in the hippocampus (Miyamoto et al., 1998, 2000; Nagahama and Sakurai, 1993). However, Nagahama and Sakurai reported that mice challenged with ETX survived longer when they were previously treated with reserpine, a noradrenaline release inhibitor, but not with riluzole (Nagahama and Sakurai, 1993). In the present study, mice mortality rate after i.v. challenge with 1.25 LD_{50} of ETX was initially reduced by pre-treatment with reserpine more than with riluzole. However, further attempts to produce reserpine protection after challenge with higher ETX doses, revealed inconsistent results and in all cases ETX challenged mice showed behavioral changes and death. Administration of ketamine (antagonist of the NMDA receptor) before ETX challenged fail to protect mice, and even a slight increase in lethality was observed. NMDA receptor antagonists may increase the release of endogenous excitatory aminoacids glutamate and aspartate (Liu and Moghaddam, 1995) activating glutamatergic neurotransmission at non-NMDA receptors. These conflicting results suggest that although glutamatergic system activation could be partially responsible for neuronal damage produced by ETX under certain undefined conditions, other mechanisms must be implicated. Indeed, mortality rate after ETX challenge with 1.25 LD_{50} was reduced by treating mice with reserpine, corresponding with other studies (Nagahama and Sakurai, 1993). This result suggests that the catecholaminergic system may be involved in ETX neurodegenerative pathogenesis. Further research is necessary to understand if some particular circumstances of animal and the brain physiology, like husbandry stressful situations, can affect the fate of ETX and the impact of brain intoxication in a previously overstimulated CNS. Furthermore, it is necessary to search for other potential drugs as well as combination of some of them to neutralize ETX induced neuronal damage.

The results of the present report support the hypothesis that ETX induced BBB changes are not the only mechanism behind ETX brain damage. It may affect some subsets of neurons (i.e. granular cells in the cerebellum) though not all glia (Dorca-Arevalo et al., 2008; Soler-Jover et al., 2007) or neurons are susceptible. It also over-activates the glutamatergic and catecholaminergic systems, causing further disarrangements and damage to the entire brain. Also, it seems that ETX associated neurological disease in animal would be more complex than previously supposed. It is highly probable that the exposure of an animal to low levels of ETX that do not produce an obvious clinical sign could produce permanent changes in the CNS enough to alter the normal behavior. Monogastric animals in which *C. perfringens* producing ETX has been isolated (i. e. humans) but lethal disease has not been described, could be likewise affected. Although enterotoxemia occurs in ruminants and it is usually considered a deadly disease with great impact in animal production, further research evaluating the productive influence of no lethal ETX encounter in domestic animal would be necessary to understand other potential impact of this enterotoxic infection.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgments

This research was supported by Instituto Nacional de Tecnología Agropecuaria Grant INTA PNBIO-1131032. We also thank the Consejo Nacional de Investigaciones Científicas y Tecnologicas - Argentina for support.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2017.02.019.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2017.02.019.

References

Boccoli, J., Loidl, C.F., Lopez-Costa, J.J., Creydt, V.P., Ibarra, C., Goldstein, J., 2008. Intracerebroventricular administration of Shiga toxin type 2 altered the expression levels of neuronal nitric oxide synthase and glial fibrillary acidic protein in rat brains. Brain Res. 1230, 320–333.

Brewer, G.J., Cotman, C.W., 1989. Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. Brain Res. 494 (1), 65–74.

Brocco, M.A., Fernandez, M.E., Frasch, A.C., 2010. Filopodial protrusions induced by glycoprotein M6a exhibit high motility and aids synapse formation. Eur. J. Neurosci. 31, 195–202.

Chio, C.C., Kuo, J.R., Hsiao, S.H., Chang, C.P., Lin, M.T., 2007. Effect of brain cooling on brain ischemia and damage markers after fluid percussion brain injury in rats. Shock 28, 284–290.

Chen, X., D'Souza, R., Hong, S.T., 2013. The role of gut microbiota in the gut-brain axis: current challenges and perspectives. Protein Cell. 4, 403–414.

Chang, M.W., Young, M.S., Lin, M.T., 2008. An inclined plane system with microcontroller to determine limb motor function of laboratory animals. J. Neurosci. Methods 168, 186–194.

Dinan, T.G., Cryan, J.F., 2013. Melancholic microbes: a link between gut microbiota and depression? Neurogastroenterol. Motil. 25, 713–719.
Dorca-Arevalo, J., Soler-Jover, A., Gibert, M., Popoff, M.R., Martin-Satue, M., Blasi, J., 2008. Binding of epsilon-toxin from *Clostridium perfringens* in the nervous system. Veterinary Microbiol. 131, 14–25.
Fernandez-Miyakawa, M.E., Redondo, L.M., 2016. Role of *Clostridium perfringens* alpha, beta, epsilon and iota toxins in enterotoxemia of monogastrics and ruminants. In Microb. Toxins, Toxinol. 1–26.
Finnie, J.W., 1984. Histopathological changes in the brain of mice given *Clostridium perfringens* type D epsilon toxin. J. Comp. Pathol. 94, 363–370.
Finnie, J.W., 1984b. Ultrastructural changes in the brain of mice given *Clostridium perfringens* type D epsilon toxin. J. Comp. Pathol. 94, 445–452.
Finnie, J.W., 2003. Pathogenesis of brain damage produced in sheep by *Clostridium perfringens* type D epsilon toxin: a review. Aust. Veterinary J. 81, 219–221.
Finnie, J.W., 2004. Neurological disorders produced by *Clostridium perfringens* type D epsilon toxin. Anaerobe 10, 145–150.
Garcia, J.P., Adams, V., Beingesser, J., Hughes, M.L., Poon, R., Lyras, D., Hill, A., McClane, B.A., Rood, J.I., Uzal, F.A., 2013. Epsilon toxin is essential for the virulence of *Clostridium perfringens* type D infection in sheep, goats, and mice. Infect. Immun. 81 (7), 2405–2414.
Ghabriel, M.N., Zhu, C., Reilly, P.L., Blumbergs, P.C., Manavis, J., Finnie, J.W., 2000. Toxin-induced vasogenic cerebral oedema in a rat model. Acta Neurochir. Suppl. 76, 231–236.
Goldstein, J., Loidl, C.F., Creydt, V.P., Boccoli, J., Ibarra, C., 2007. Intracerebroventricular administration of Shiga toxin type 2 induces striatal neuronal death and glial alterations: an ultrastructural study. Brain Res. 1161, 106–115.
Karl, T., Pabst, R., von Horsten, S., 2003. Behavioral phenotyping of mice in pharmacological and toxicological research. Exp. Toxicol. Pathol. 55, 69–83.
Kohn, J., Warrack, G.H., 1955. Recovery of *Clostridium welchii* type D from man. Lancet 268–385.
Kumon, M., Yamamoto, K., Takahashi, A., Wada, K., Wada, E., 2010. Maternal dietary restriction during lactation influences postnatal growth and behavior in the offspring of mice. Neurochem. Int. 57, 43–50.
Linden, J.R., Ma, Y., Zhao, B., Harris, J.M., Rumah, K.R., Wiemers, N.S., Vartanian, T., 2015. *Clostridium perfringens* Epsilon toxin causes selective death of mature oligodendrocytes and central nervous system demyelination. MBio 6, e02513.
Liu, J., Moghaddam, B., 1995. Regulation of glutamate efflux by excitatory aminoacid receptors: evidence for tonic inhibitory and phasic excitatory regulation. J. Pharmacol. Exp. Ther. 274, 1209–1215.
Liu, Q.Y., Schaffner, A.E., Chang, Y.H., Maric, D., Barker, J.L., 2000. Persistent activation of GABA (A) receptor/Cl (-) channels by astrocyte-derived GABA in cultured embryonic rat hippocampal neurons. J. Neurophysiol. 84 (0022–3077), 1392–1403.
Lonchamp, E., Dupont, J.L., Wioland, L., Courjaret, R., Mbebi-Liegeois, C., Jover, E., Doussau, F., Popoff, M.R., Bossu, J.L., de Barry, J., Poulain, B., 2010. *Clostridium perfringens* epsilon toxin targets granule cells in the mouse cerebellum and stimulates glutamate release. PLoS ONE 5 (9), e13046. http://dx.doi.org/10.1371/journal.pone.0013046.
Mantis, N.J., 2005. Vaccines against the category B toxins: staphylococcal enterotoxin B, epsilon toxin and ricin. Adv. Drug Deliv. Rev. 57, 1424–1439.
Meer, R.R., Songer, J.G., 1997. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. Am. J. Vet. Res. 58, 702–705.
Minami, J., Katayama, S., Matsushita, O., Matsushita, C., Okabe, A., 1997. Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. Microbiol. Immunol. 41, 527–535.
Miyamoto, O., Minami, J., Toyoshima, T., Nakamura, T., Masada, T., Nagao, S., Itano, T., Okabe, A., 1998. Neurotoxicity of *Clostridium perfringens* epsilon-toxin for the rat hippocampus via the glutamatergic system. Infect. Immun. 66, 2501–2508.
Miyamoto, O., Sumitani, K., Nakamura, T., Yamagami, S., Miyata, S., Itano, T., Negi, T., Okabe, A., 2000. *Clostridium perfringens* epsilon toxin causes excessive release of glutamate in the mouse hippocampus. FEMS Microbiol. Lett. 189, 109–113.
Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S., Okabe, A., 2002. *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. J. Biol. Chem. 277, 39463–39468.
Murrell, T.G., O'Donoghue, P.J., Ellis, T., 1986. A review of the sheep-multiple sclerosis connection. Med. Hypotheses 19, 27–39.
Nagahama, M., Sakurai, J., 1992. High-affinity binding of *Clostridium perfringens* epsilon-toxin to rat brain. Infect. Immun. 60, 1237–1240.
Nagahama, M., Sakurai, J., 1993. Effect of drugs acting on the central nervous system on the lethality in mice of *Clostridium perfringens* epsilon toxin. Toxicon 31, 427–435.
Niilo, L., 1980. *Clostridium perfringens* in animal disease: a review of current knowledge. Can. Veterinary J. 21, 141–148.
Paxinos, G., Watson, C., 2008. The Rat Brain in Stereotaxic Coordinates. Compact: Academic Press, p. 400.
Perry, V.H., O'Connor, V., 2010. The role of microglia in synaptic stripping and synaptic degeneration: a revised perspective. ASN Neuro 2, e00047.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoint. Am. J. Hyg. 27 (3), 493–497.
Rumah, R.K., Linden, J., Fischetti, V., Vartanian, T., 2013. Isolation of *Clostridium perfringens* type B in an individual at first clinical presentation of multiple sclerosis provides clues for environmental triggers of the disease. PLoS ONE 8 (10), e76359. http://dx.doi.org/10.1371/journal.pone.0076359.
Soler-Jover, A., Dorca, J., Popoff, M.R., Gibert, M., Saura, J., Tusell, J.M., Serratosa, J., Blasi, J., Martin-Saute, M., 2007. Distribution of *Clostridium perfringens* epsilon toxin in the brains of acutely intoxicated mice and its effect upon glial cells. Toxicon 50, 530–540.
Tenenbaum, T., Matalon, D., Adam, R., Seibt, A., Wewer, C., Schwerk, C., Galla, H.J., Schroten, H., 2008. Dexamethasone prevents alteration of tight junction-associated proteins and barrier function in porcine choroid plexus epithelial cells after infection with *Streptococcus suis in vitro*. Brain Res. 1229, 1–17.
Uzal, F.A., Kelly, W.R., 1997. Effects of the intravenous administration of *Clostridium perfringens* type D epsilon toxin on young goats and lambs. J. Comp. Pathol. 116, 63–71.
Uzal, F.A., Kelly, W.R., Morris, W.E., Assis, R.A., 2002. Effects of intravenous injection of *Clostridium perfringens* type D epsilon toxin in calves. J. Comp. Pathol. 126, 71–75.
Uzal, F.A., Kelly, W.R., Morris, W.E., Bermudez, J., Baison, M., 2004. The pathology of peracute experimental *Clostridium perfringens* type D enterotoxemia in sheep. J. Veterinary Diagn. Investig. 16, 403–411.
Uzal, F.A., Songer, J.G., 2008. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. J. Veterinary Diagn. Investig. 20, 253–265.
Wells, J.E., Rice, T.K., Nuttall, R.K., Edwards, D.R., Zekki, H., Rivest, S., Yong, V.W., 2003. An adverse role for matrix metalloproteinase 12 after spinal cord injury in mice. J. Neurosci. 23, 10107–10115.
Wioland, L., Dupont, J.L., Doussau, F., Gaillard, S., Heid, F., Isope, P., Pauillac, S., Popoff, M.R., Bossu, J.L., Poulain, B., 2015. Epsilon toxin from *Clostridium perfringens* acts on oligodendrocytes without forming pores, and causes demyelination. Cell. Microbiol. 17 (3), 369–388.
Yamada, J., Nakanishi, H., Jinno, S., 2011. Differential involvement of perineuronal astrocytes and microglia in synaptic stripping after hypoglossal axotomy. Neuroscience 182, 1–10.
Zhu, C., Ghabriel, M.N., Blumbergs, P.C., Reilly, P.L., Manavis, J., Youssef, J., Hatami, S., Finnie, J.W., 2001. *Clostridium perfringens* prototoxin-induced alteration of endothelial barrier antigen (EBA) immunoreactivity at the blood-brain barrier (BBB). Exp. Neurol. 169, 72–82.