A Translational Murine Model of Sub-Lethal Intoxication with Shiga Toxin 2 Reveals Novel Ultrastructural Findings in the Brain Striatum

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Abstract

Infection by Shiga toxin-producing *Escherichia coli* causes hemorrhagic colitis, hemolytic uremic syndrome (HUS), acute renal failure, and also central nervous system complications in around 30% of the children affected. Besides, neurological deficits are one of the most unrepairable and untreatable outcomes of HUS. Study of the striatum is relevant because basal ganglia are one of the brain areas most commonly affected in patients that have suffered from HUS and since the deleterious effects of a sub-lethal dose of Shiga toxin have never been studied in the striatum, the purpose of this study was to attempt to simulate an infection by Shiga toxin-producing *E. coli* in a murine model. To this end, intravenous administration of a sub-lethal dose of Shiga toxin 2 (0.5 ng per mouse) was used and the correlation between neurological manifestations and ultrastructural changes in striatal brain cells was studied in detail. Neurological manifestations included significant motor behavior abnormalities in spontaneous motor activity, gait, pelvic elevation and hind limb activity eight days after administration of the toxin. Transmission electron microscopy revealed that the toxin caused early perivascular edema two days after administration, as well as significant damage in astrocytes four days after administration and significant damage in neurons and oligodendrocytes eight days after administration. Interrupted synapses and mast cell extravasation were also found eight days after administration of the toxin. We thus conclude that the chronological order of events observed in the striatum could explain the neurological disorders found eight days after administration of the toxin.

Introduction

Infection by Shiga toxin (Stx)-producing enterohemorrhagic *Escherichia coli* (STEC) causes hemorrhagic colitis, hemolytic uremic syndrome (HUS) [1], acute renal failure [2,3] and, less commonly, central nervous system (CNS) impairment [4]. In addition, it has been reported that the mortality rate from HUS increases from 0–5% to 7–40% when the CNS is involved [5–8]. This issue became prominent in Germany in 2011 when the infection by Shiga toxin-producing *E. coli* O104:H4 resulted in 3816 cases of gastroenteritis, 845 of which evolved to HUS and 54 to death. It was noteworthy that, although STEC strains usually develop HUS in children, in this case they affected adults, mainly women [9,10].

In addition, 48% of the 217 hospitalized patients intoxicated with STEC in Germany developed severe neurological symptoms [11]. Argentina has the highest incidence of HUS in children under 5 years old (10.5 cases per 100,000) [12,13]. Mortality and severe sequelae caused by STEC is still a persistent social and economic problem for international public health.

In children, neurological symptoms appear about eight days after the onset of hemorrhagic colitis. Symptoms include acute seizures, coma, irritability, hemiparesis, aphasia and motor alteration. Among these symptoms, seizures may potentially predict mortality or long-term neurological sequelae. It has been observed that neurological problems persist in 39% of surviving children [5]. Although the deleterious effects of STEC on the CNS are drastic, the pathogenic mechanisms whereby Stx2 causes CNS impairment still remain to be elucidated.

Several animal models have been used to study the effects of Stx on the CNS [14–18]. However, none has studied the deleterious effects of intravenous administration of Stx2 on the striatum. The neurological alterations described among models of Stx2 injection in mice include hind limb paralysis [19], lethargy, shivering, abnormal gait and spasm-like seizures [20]. Vascular and glial changes have been observed at light microscope level [19,21,22]. Intraperitoneal administration of Stx2 causes both a glial lamellipodia-like process that blocks spinal motor neuron synapses [20] and endothelial damage in the cerebral cortex, hippocampus and cerebellum [16–18,23,24]. In the rat model of...
intracerebroventricular (i.c.v.) Stx2 administration, ultrastructural observation has revealed an apoptotic form of neuronal degeneration, demyelination, astrogliosis and pathological oligodendrocytes [25,26]. Rabbit models of Stx injection have shown endothelial alterations like edema and hemorrhage [27–29], as well as alterations in the myelin sheath, neuronal degeneration, gliosis, vascular changes and hemorrhage in different brain areas [30–32]. Pigs have been found to develop edema disease characterized by CNS symptoms such as ataxia, and incoordination [33], convulsions, leg paddling and paralysis [34–37]. In baboons, seizure episodes which progressed to coma and death after i.v. administration of Stx2 have also been reported [38]. Stx1 causes ultrastructural lesions that are not observable with light microscopy [39]. More details of animal models of Stx administration can be found in a review written by Obata (2010) [40].

The study of the striatum is relevant because basal ganglia are one of the most common brain areas affected in patients that have suffered from HUS [41–43]. The striatum modulates the central motor system [44,45] and is one of the most relevant brain regions involved in neurological motor disorders in patients intoxicated with STEC [46,47]. Magnetic resonance images (MRIs) have revealed that basal ganglia are frequently involved in this pathology [42,48]. A correlation between neurological deficits and radiological images related to basal ganglia compromise in comatose patients has been frequently reported [46,47]. However, non-invasive methods such as MRI may not identify which cell type is damaged or the extent of cell damage over a certain period of the known toxin progression. In contrast, transmission electron microscopy (TEM) is a powerful technique to detect the ultrastructural state of these cells.

Thus, since the effects of sub-lethal i.v. doses of Stx2, particularly in the striatum, have never been studied in an animal model, in the present study we attempted to determine the type of ultrastructural changes produced by Stx2 in the striatum and to what extent these changes may resemble the human disease. We also describe the correlation between the neurological alterations observed and blood brain barrier disruption, mast cell extravasation, synapse interruption and striatal cell death.

Materials and Methods

Stx2 Purification

Stx2 was obtained as previously described [26]. Briefly, the toxin was purified by affinity chromatography under native conditions. Recombinant E. coli DH5α containing pStx2 were cultured overnight. The supernatant obtained was precipitated in 60% SO4 (NH4)2 1 mM PMSF, and the pellet was dialyzed overnight, resuspended in phosphate buffer solution (PBS) with a cocktail of protease inhibitors, and incubated with Globotrioside Fractogel Resin (IsoSep AB, Tullinge, Sweden). The resin was washed and the toxin eluted with MgCl2. Protein concentration was determined in all the eluates. Protein content in all the fractions was monitored by silver/Coomassie blue staining [49], and the presence of Stx2 in the eluates was confirmed by Western Blot analysis. Results showed a 7.7-kDa band corresponding to Stx2B and a 32-kDa band corresponding to Stx2A. The same batch of toxin was used for all the experiments.

The cytotoxic capacity of Stx2 was assessed in Vero cells by the neutral red assay and the cytotoxic dose 50 (CD50) found was about 1 pg/ml [50]. This effect was neutralized by means of preincubation with an anti-subunit 2B monoclonal antibody (Sifin, Berlin, Germany), and not neutralized when using an isotype antibody instead [30]. Lipopolysaccharide (LPS) was removed from the Stx2 solution by using Detoxi-gel (Pierce, Rockford, USA). This Stx2 solution contained less than 0.03 endotoxin units/ml.

Sub-lethal dose

The lethal effects of purified Stx2 were characterized in mice (n = 4 per dose). Different amounts of Stx2 (5 to 0.44 ng per animal) or vehicle were administered intravenously (i.v.) to mice weighing about 20 grams. Survival time was established when 100% of the animals survived at least 8 days after administration of 0.5 ng or less of Stx2. It was determined that with this dose mice survived even for 10 days. This amount was thus considered sub-lethal and selected for use in this study. The toxin was administered twice (once a day for two consecutive days) with time zero prior to initial administration of the toxin.

Animals

Male NIH mice (28–30 days old) were housed in an air-conditioned and light-controlled (lights on between 7:00 am and 7:00 pm) animal facility. Animals were supplied by the animal facility center of Administracion Nacional de Laboratorios e Institutos de la Salud (ANLIS) Carlos G. Malbrán, Buenos Aires, Argentina. Mice were provided with food and water ad libitum and neurological manifestations were monitored daily at the same time throughout the experiment. The experimental protocols and euthanasia procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the School of Medicine of Universidad de Buenos Aires, Argentina (Resolution No. 1099/10). All the procedures were performed in accordance with the EEC guidelines for care and use of experimental animals (EEC Council 86/609).

Behavioral motor tests

Mice were subjected to motor behavioral tests based on a primary screening using a modified version of the SHIRPA-test [51,52] 2, 4 and 8 days after i.v. administration of Stx2 or vehicle. For the purpose of clarity, non-motor SHIRPA-screen behavioral tests were not included. A total of three experiments were performed using 12 mice per treatment. Animals were placed in individual cages and the following motor behaviors were recorded over a 5-minute period, without disturbing the animal: spontaneous motor activity (i.e. whether the animals had vigorous, moderate, slow or no activity at all), gait (i.e. normal or abnormal), pelvic elevation (i.e. whether animals were able to elevate their body more than 3 mm from their horizontal axis, either normal (not deviated from the axis) or barely touching the ground), and hind limb activity (i.e. whether animals had normal or limited hind limb movements). All the animals subjected to the treatments described were given a score based on their motor capacity in the motor tests. Normal behavior (vigorous activity, normal correct posture, body not deviated from the axis and fluid hind limb activity) was given a score of 0 and abnormal behaviors were given a score of 1. The formula to calculate the SHIRPA score is:

\[
\text{SHIRPA score} = \left( \frac{\sum \text{abnormal motor behavior score per treatment time}}{N} \right) \pm \text{SD}
\]

where n (number of tests) = 4. Table 1 lists the motor behavioral tests that showed significant differences between vehicle- and Stx2-treated animals.
Table 1. Behavioral motor tests.

| Test                | Score = 0                     | Score = 1                     |
|---------------------|------------------------------|------------------------------|
| Spontaneous motor activity | Vigorous movement            | Slow movement                |
| Gait                | Normal                        | Abnormal                     |
| Pelvic Elevation    | Normal (elevation of 3 mm)    | Elevated (elevation of more than 3 mm) |
| Hind limb activity  | Normal                        | Difficulty to move           |

Transmission Electronic Microscopy (TEM)

Mice were anesthetized with Chloral hydrate (350 mg/kg) and perfused transcardially with 0.9% NaCl solution followed by 2.5% glutaraldehyde in 0.1 M PBS (fixative per animal/weight [ml/g]) for studies of Transmission Electron Microscopy (TEM). Brains were removed from the skull and post-fixed in the same fixative solution for 2 hours. Samples of dorsal striatum (3 mm² thick) were dissected and collected in 0.1 M phosphate buffer. The samples were first assessed by light microscopy with blue toluidine to select the areas for TEM. Ultrathin sections were cut from selected areas [53] and then contrasted with 1% OsO4 and 1% uranyl acetate, dehydrated and flat-embedded in Durcupan. The sections were contrasted with lead citrate and then examined and photographed on a Zeiss 109 electron microscope. Adobe Photoshop software was used in the assembly of images (Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis

Twelve animals from each treatment group were subjected to behavioral motor test studies. Four brain sections from each of four animals (i.e. a total of 16 samples for each condition) were used for TEM studies. The data are presented as mean ± SEM. Statistical significance was assessed by Student-t-test or one-way analysis of variance (ANOVA) followed by Bonferroni post tests (GraphPad Prism 4, GraphPad Software, Inc.). P values <0.05 were considered significant.

Results

To test whether sub-lethal i.v. administration of Stx2 altered motor behavioral function in mice, motor behavioral tests consisting of four neurological assessments –spontaneous motor activity, gait test, pelvic elevation and hind limb activity– were conducted 2, 4 and 8 days after administration (Fig. 1). Results evidenced motor functional deficiencies in which striatal damage could be involved. Spontaneous motor activity of Stx2-treated mice was reduced by 50% 4 days after administration and by 100% 8 days after administration. Reduced hind limb activity, abnormal gait and increased pelvic elevation were observed in 75% of the Stx2-treated mice 8 days after administration. No neurological differences were found between Stx2-treated mice and the vehicle-injected group 2 days after administration. Spontaneous motor activity, gait test, pelvic elevation and hind limb activity were normal in vehicle-treated mice throughout the eight experimental days. Behavioral changes in sensorial and learning tasks were also monitored (data not shown). Consequently, a detailed ultrastructural study was performed.

Intravenous administration of Stx2 causes necrotic and degenerative-like neurons

Conserved neurons of the striatum from the vehicle-treated group showed pale nuclei and well-dispersed chromatin, intact cytoplasmic and nuclear membranes, and intact mitochondria dispersed in the cytoplasm (Fig. 2A). In contrast, striatal neurons from the Stx2-treated group showed a progressive degenerative condition (Fig. 2B–F). Two days after administration, the cytoplasm started to become vacuolated and more contrasted than in the vehicle-injected group (2B). Two and four days after administration, nuclei in the samples from Stx2-treated mice appeared electron dense, with increased heterochromatin condensation, and resembled the early changes seen in an apoptotic state (2B, C). In these neurons, perinuclear clustering of altered mitochondria occurred within darkened and condensed cytoplasm. In addition, altered nuclear and cytoplasmic membranes were observed by ultrastructure two days after administration. Loss and/or convergence of the two membranes was observed as an earlier sign of neuronal damage. This feature could be seen only by TEM. Indentations of the nuclear membrane were frequently found 4 days after administration, compared with the nuclei from the vehicle-treated group (Fig. 2C). The changes observed over this period reflected a pathological condition as nuclear indentation would be consistent with the beginning of an apoptotic process. Eight days after administration, the predominant ultrastructural observation included neurons with cytoplasmic edema and shrunk nuclei (Fig. 2D, F), or neurons that were already in a necrotic state (Fig. 2G). Complete alteration of the endoplasmic reticulum, organelles and mitochondria were observed in neurons with edema (Fig. 2D, F). A common feature observed over the time periods analyzed was cell edema, which...
Figure 2. Intravenous administration of Stx2 causes neuronal damage. Conserved striatal neuron after i.v. vehicle (saline) administration (A); pale nucleus (N) and intact cytoplasm (c) and membranes (m); intact mitochondrion (arrow). After 2 days of treatment: vacuolated neuron with contrasted cytoplasm (c) and nucleus (N) (B); heterochromatin condensation (h). After 4 days: more contrasted nucleus (N) with increased heterochromatin (h) condensation and prominent indentation with loss of membranes (arrows) (C); vacuolated perinuclear mitochondrion (*) (C). Neuron with edema and loss of regular nuclear shape on day 8 (D). Disorganized endoplasmic reticulum (R) in the cytoplasm with edema (E) at higher magnification; loss of regular shape in the nucleus (F) of a neuron with edema (E); affected neuronal nucleus (N) with irregular shape and no apparent surrounded cytoplasm; (Ol, oligodendrocyte) (G). These features are absent in striatal neurons of the vehicle-treated group (A). Quantification of the percentage of damaged neurons with edema (H). Significant results are observed starting on day 4. Maximum number of neurons with edema in Stx2-treated mice observed on day 8 (*) (H). Results are expressed as a percentage of the total number of neurons in an area of 3721 μm². Data are mean ± SEM of 6–8 experiments (F and G). An asterisk denotes statistical significance, p<0.05. The scale bar in A applies to micrographs B–D and F–G. doi:10.1371/journal.pone.0055812.g002
increased over time (Fig. 2H) and was significantly maximum (p<0.05) 8 days after Stx administration.

**Intravenous administration of Stx2 causes edema in astrocytes and mast cell infiltration**

Normal astrocytes were observed in samples from vehicle-treated mice, usually together with mitochondria, rough endoplasmic reticulum, Golgi apparatus, dispersed small vesicles and glial filaments (Fig. 3A). In contrast, altered astrocytes were observed in Stx2-treated mice after day 2 (Fig. 3B). About 20% of the astrocytes observed exhibited edema (Fig. 3G). This was accompanied by evident disorganized endoplasmic reticulum and swollen mitochondria. However, the nuclear envelope was conserved (Fig. 3C). On day 4, perivascular astrocytic edema was also observed (Fig. 3D). Disorganized endomembranes and swollen mitochondria were found in watery cytoplasm. In contrast, nuclear integrity was not compromised (Fig. 3D). On day 8, loss of nuclear electron dense elements that migrated to the cytosol probably due to nuclear membrane rupture and swollen organelles in watery cytoplasm was evidenced (Fig. 3E). Cytoplasmic edema was the main feature observed throughout the experiment (Figs. 3B–F). The percentage of astrocytes that displayed edema increased to 60% on day 8 and was significantly higher than in vehicle- or Stx2-treated mice after 2 or 4 days (Fig. 3G). Stx2 caused astrocyte damage in the brain striatum. In addition, mast cell extravasation to the brain parenchyma was likely (Fig. 3F). Mast cells are identified by ultrastructure because they have numerous rounded non-laminated granules in comparison with basophil cells, which have larger and laminated granules. Mast cells may go through the Blood Brain Barrier (BBB) into the brain parenchyma attracted by inflammatory mechanisms after the Stx2 insult. Here, a mast cell in contact with an astrocyte was observed (Fig. 3C). Mast cells are early responders in the regulation of acute BBB changes after cerebral ischemia and hemorrhage.

**Intravenous administration of Stx2 alters BBB integrity**

Endothelial vessels from striatal brain sections of vehicle-injected mice kept their endothelial nucleus and cytoplasm conserved (Fig. 4A). Organelles were well preserved and mitochondria showed normal external and inner membranes. Since the vascular basal membrane was found covered by intact perivascular astrocytes, the BBB appeared intact. Intact perivascular astrocytes contacted dendrites and axons covered with a sheath of myelin. In contrast, a progressive intracytoplasmic edema of perivascular astrocytes was observed after i.v. administration of Stx2 starting on day 2 (Fig. 4B). An incipient edema was evidenced in perivascular astrocytes on day 2, while a complete watery edema was observed mainly on day 4 (Fig. 4C). Watery edema with collapsed and shrunk endothelial cells was observed on day 8 (Fig. 4D), although no apparent endothelial cell damage was observed. Complete watery edema was maximum on day 8 (Fig. 4E). Accordingly, perivascular edema increased over time.

**Intravenous administration of Stx2 causes interrupted synapses**

Interrupted synapses were observed in the striatum of Stx2-treated mice (Fig. 5). The number of interrupted synapses was significantly higher in Stx2-treated animals than in vehicle-treated ones. A projection of a reactive astrocyte interrupted the synaptic

![Figure 3. Intravenous administration of Stx2 causes astrocytic edema and mast cell extravasation.](image-url)
transmission of neurons by standing between the pre- and the post-
synapse (Fig. 5B, C).

Intravenous administration of Stx2 causes ultrastructural
alterations in oligodendrocytes

Normal oligodendrocytes were identified by the presence of round or oval nuclei containing heterochromatin in the periphery of the nucleus and electron-dense dark cytoplasm in vehicle-
treated mice (Fig. 6A). Oligodendrocytes were also distinguished for their cytoplasm ensheathed axons. Two days after i.v. administration of Stx2, nuclei of affected oligodendrocytes started to show an irregular shape, condensed chromatin and mild retraction (Fig. 6B). However, the nuclear envelope was still intact. In the perinucleus, an electron-dense cytoplasm with partial vacuolization was evident. The ultrastructural features observed suggest an early stage of apoptosis. In addition, disorganized myelin sheaths were observed in apparently swollen axons. On day 4, a progressive deterioration of the oligodendrocytes was observed (Fig. 6C). Nuclei became more irregular and shrunk. Further retraction of the nucleus was observed and bulked chromatin was evident. Nuclear fragments were also observed in the edematous area. At this stage, perinuclear vacuolization extended to the whole electron-dense cytoplasm and myelin appeared to be not only disorganized but also thinner than on day 2. Axons covered with myelin were irregular and electron dense contrasted. On day 8, oligodendrocytes showed contrasted nuclei and cytoplasm, and boundaries between them were difficult to differentiate (Fig. 6D). Loss of cell volume immersed in an edematous area was the main feature at this stage. However, cytoplasmic projections that resembled sheaths covering axons were still observable. Cell damage, quantified on days 2, 4, and 8 according to the criteria previously described (Fig. 6E), increased over time. Accordingly, the number of cells displaying damage was significantly higher on day 8 than on days 4 and 2.

Discussion

Sub-lethal administration of Stx2 caused motor behavioral alterations that may be related to damage of striatal cells. To our knowledge, this is the first report on the toxic effects of sub-lethal doses of i.v. administration of Stx2 in mouse brain striatum.

The striatum is part of the basal ganglia, with a relevant role in the control of voluntary movements and in the onset of disorders [44,45]. This brain area is highly affected in patients intoxicated with STEC [42,43,46–48]. Neurological complications have been found in patients with HUS and basal ganglia injury after 6–7 days of the onset of symptoms [47]. Although hind limb paralysis has been described in animal models as a disturbance caused by Stx2,
Figure 5. Intravenous administration of Stx2 causes synaptic modifications. Electron micrograph showing an apparently normal synapse (s) and pre-synaptic vesicles (v) in the striatum (A). Electron micrograph showing an increased number of pre-synaptic vesicles together with an incipient astrocytic process (*) in close proximity to a synaptic space (S) in the lower left corner (B). Electron micrograph showing full astrocytic interposition in the synaptic space (*) while the pre-synaptic terminal is full of vesicles (C). Chart illustrating the process in the synaptic space (right panel). Percentage of interrupted synapses in the striatum from Stx2-treated mice vs. vehicle-treated ones (D). An asterisk denotes statistical significance, *p<0.05. Results are expressed as a percentage of the total number of synapses in an area of 3721 μm². The scale bar in A applies to all micrographs.
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this deficit has been related only to spinal cord motor neurons and neither this nor other motor deficits have been associated with any particular brain region [19,20]. The neurological deficits found in this study on the basis of motor behavioral tests could be correlated with the damage observed in the striatum and are consistent with the mean time of onset of symptoms in patients that suffer from neurological manifestation of HUS [5]. Other brain regions associated with movement, including the motor cortex and the pyramidal tract, may also be implicated in damage by Stx2.

Neurons from vehicle-treated striatum showed a normal appearance compared with Stx2-treated ones [54,55]. In contrast, ultrastructural alterations observed in the striatum after intravenous Stx2 administration showed earlier signs of neuronal damage and an apoptotic state [54,56,57]. Pathological nuclear indentations frequently found in neurons on day 4 have also been found in Huntington’s chorea disease neurons in related animal models [50–60] or following axonal injury [61]. Overall, the altered

Figure 6. Intravenous administration of Stx2 causes pathological alterations in oligodendrocytes. Conserved oligodendrocyte in striata of brains treated with i.v. administration of vehicle; myelin formation in the cytosol (*) (A); electron-dense heterochromatin (h) mostly in the periphery of a rounded nucleus; euchromatin (e) present as electron-lucent areas of the inner nucleus. An irregular nucleus is observed 2 days after Stx2 treatment (B); vacuolated cytoplasm (v); disorganized myelin (my); Edema (E) and swollen mitochondria in the extracellular space. A shrunk and retracted nucleus (N) and perinuclear vacuolization (v) are observed on day 4 (C). Altered oligodendrocyte with a contrasted cytoplasm observed on day 8 (D); nucleus (N) and cytoplasm are difficult to differentiate; extended edema (E) and lack of myelin (L); swollen and disorganized cristae in mitochondria (m). The number of cells with edema increased with time (E). Results are expressed as a percentage of the total number of oligodendrocytes in an area of 3721 μm². Data are mean ±SEM of 6–8 experiments. An asterisk denotes statistical significance, p<0.05. The scale bar in A applies to B and C.

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changes observed in these neurons reflect a pathological condition of Stx2-treated mice.

In accordance with previous reports, normal astrocytes were observed in vehicle-treated mice [62]. Astrocytes usually cover the basement membrane of pial and vascular surfaces in the CNS, and they surround neuronal bodies, axons and other neuronal processes within the CNS. Under pathological conditions, astrocytes may show increase in volume and large quantities of filaments or watery swelling [62,63]. Failure of functionality in the nuclear envelope could be the consequence of the osmotic changes caused by the edema.

The microvasculature in the striatum from vehicle-treated mice showed a conserved BBB ultrastructure [64,65] as compared with that of Stx2-treated ones. Fujii et al. [14] found edematous endothelial cells in the cortex of a moribund mouse 2 days after injection of a lethal dose of Stx2 (4 ng per mouse). In contrast, we have shown that a sub-lethal dose of 0.5 ng per mouse was sufficient to cause a typical perivascular edema [64] and BBB disruption by altering perivascular astrocytes. While we have previously shown that intracerebral Stx2 causes vascular leakage [26], in the present study we found that the toxin disrupts the BBB.

Interposition of astrocytic processes between the pre- and postsynaptic terminals, namely astrocytic stripping, was observed in the striatum. A similar physical blocking of lamellipodia-like processes in the synapses of mouse motoneurons following Stx2 treatment has been described [20]. Synapse interruption could be a neuroprotective mechanism whereby projections from astrocytes may physically detach the synapse against excessive pre-synaptic release of glutamate [66,67]. The altered synaptic terminals may be the consequence of excessive glutamate release [20,68]. Therefore, when an astrocytic interposition between a pre- and a post-synaptic terminal occurs, post-synaptic densities may not be observed.

Mast cells were identified [69] and localized in the parenchymal striatum, probably due to the pathological condition caused by the toxin. These cells are usually located in the perivascularature [70] and migrate into the brain parenchyma by an ischemic event following brain swelling or alteration of BBB permeability [71]. Peripheral factors produced by Stx2 might contribute to mast cell infiltration and astrocytic stripping, as these findings have not been observed after local administration of Stx2 in rat striatum [26]. Nevertheless, structural differences between species might account for this difference.

The oligodendrocytic phenotype may still be observed even in the pathological condition [72]. In addition, oligodendrocytes and microglial cells might be erroneously identified, unless ultrastructural differences become evident. When microglial cells become activated by a noxious agent they display dilation of the endoplasmic reticulum channels and a great number of electron light vesicles in the cytoplasm. Also, a small number of activated cells have electron-dense lysosomes, indicating phagocytic activity [73]. As these features were not found in the injured cells, they corresponded to oligodendrocytes. Apoptosis in oligodendrocytes was observed at early stages [72], while necrosis was observed at later stages [72,74]. Damage in oligodendrocytes might cause the axonal transmission deficits observed in Stx2-treated mice, with motor alterations including hind limb paralysis. Demyelinated axons have been observed after the intracerebroventricular administration of Stx2 [26].

Stx2 has been immunolocalized in the brain parenchyma [20,26]. Although the receptor for Stx2 Gb3 is not localized in the mouse brain endothelium [20], the toxin is nevertheless able to bind to neurons. However, the mechanism whereby Stx2 enters the brain parenchyma is still unknown. The BBB must keep a healthy fluid environment to protect the brain parenchyma [13,65] from harmful elements and to allow homeostatic influx of various required substances and efflux of cell products to maintain CNS homeostasis [71,75] and our results show that vascular leakage as a result of Stx2 would alter this homeostasis. The toxin may cross the brain parenchyma through vessels of circumventricular organs that lack tight junctions, or by destroying them in the non-fenestrated endothelia, or by first crossing the blood-cerebrospinal fluid barrier to the ventricle space and then to the brain parenchyma by crossing ependymal cells. Another possibility is that the toxin crosses the BBB as a result of damage of perivascular astrocytes that constitute a part of the barrier (Fig. 4).

The possible mechanisms behind the cell damage observed include: i) cytotoxic action of Stx2 mediated by the Gb3 receptor in neurons [20,50]; ii) contribution of peripheral or locally produced pro-inflammatory cytokines and/or chemokines [76]; iii) deleterious collateral effect produced by kidney failure, which could lead to electrolyte disorders and target the brain [76]; and iv) energy depletion by lack of glucose and oxygen intake.

Regarding the second possible mechanism, it has been widely reported that TNF-α contributes to the deleterious effects of Stx2 both in vitro [77] and in vivo [78]. Also, cytokines produced in the brain have been reported [32] and their inflammatory effect could partially explain the astroglial reactivity observed [25]. Under this circumstance they can release more toxic products and target cells in the vicinity, including neurons [79].

With regard to the fourth possible mechanism, BBB rupture could prevent the incorporation of these substrates to neurons and other cells. As a consequence, energy depletion would cause membrane depolarization and the release of excitatory amino acids into the extracellular space, leading to edema and apoptosis in neurons and astrocytes [79].

In the present study, the ultrastructural lesions observed in the striatal cells could have caused the motor behavioral alterations found in Stx treated animals. However, possible lesions produced in other brain centers like the motor cortex and the pyramidal tract cannot be discarded. The main input structure of the basal ganglia is the striatum [80], which is specifically related to voluntary limb movements [81]. The mechanistic entry of Stx2 to the brain parenchyma and the subsequent events that lead to neuronal and glial degenerative conditions are a current matter of study. Other factors besides Stx2 may contribute to the described pathology, and they should also be taken into account.

In summary, a sub-lethal i.v. dose of Stx2 caused BBB disruption, astrocytic edema, neurodegeneration, oligodendrocyte death, synapse interruption and mast cell extravasation, which may explain the motor behavioral alterations and lethargy observed in mice. This damage may also reflect some clinical alterations observed in brain impairment by the toxin. Therefore, this animal model appears to be useful to compare and understand the basis of the neuropathogenic mechanisms underlying STEC intoxication in patients.

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Author Contributions

Conceived and designed the experiments: CTF PAG AC JG. Performed the experiments: CTF PAG AC AP JG. Analyzed the data: CTF JG. Contributed reagents/materials/analysis tools: PAG CFL JG. Wrote the paper: CTF JG.
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