Anti-inflammatory agents reduce microglial response, demyelinating process and neuronal toxin uptake in a model of encephalopathy produced by Shiga Toxin 2

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Abstract

Infections by Enterohemorrhagic Escherichia coli may cause in addition to hemolytic uremic syndrome neurological disorders which may lead to fatal outcomes in patients. The brain striatum is usually affected during this outcome. The aim of this study was to
determine in this area the role of the microglia in pro-inflammatory events that may occur
during Shiga toxin 2 intoxication and consequently to this, whether oligodendrocytes were
being affected. In the present paper we demonstrated that anti-inflammatory treatments
reduced deleterious effects in brain striatal cells exposed to Shiga toxin 2 and LPS. While
dexamethasone treatment decreased microglial activation and recovered myelin integrity in
the mice striatum, etanercept treatment decreased neuronal uptake of Stx2 in rat striatal
neurons, improving the affected area from toxin-derived injury. In conclusion, microglial
activation is related to pro-inflammatory events that may deteriorate the brain function
during intoxication with Stx2 and LPS. Consequently, the role of anti-inflammatory agents
in the treatment of EHEC-derived encephalopathy should be studied in clinical trials.

**Key words:** Encephalopathy; Dexamethasone; Etanercept; Neuroprotection; HUS;
Inflammation.

1. Introduction

Many enterohemorrhagic Escherichia coli (EHEC) produce and release Shiga toxin 2
(Stx2), which causes ribotoxic stress and may lead the cells (who express its receptor
globotriaosylceramide – Gb3) to apoptosis. Infection with EHEC may produce hemorrhagic
colitis and Hemolytic Uremic Syndrome (HUS) in patients (Karmali, 2004). About 9–15%
of them are affected neurologically, with signs that include decerebrate posturing,
hemiparesis, ataxia, cranial nerve palsy, ophthalmological dysfunction, hallucination,
seizure and changes in the level of consciousness (from lethargy to coma). When
neurological symptoms are involved the case-fatality-ratio may rise up to 40%. (Bale et al.,
1980; Cimolai et al., 1992; Gianantonio et al., 1973; Hamano et al., 1993; Tapper et al., 1995).

We have previously demonstrated in a murine model of encephalopathy that Stx2 affects, at the cellular and behavioral level, motor areas of the central nervous system (CNS) including the striatum, the motor cortex and the cerebellum. And that a pro-inflammatory involvement compromises the neurovascular unit including neurons, endothelial and glial cells (D'Alessio et al., 2016; Pinto et al., 2017; Pinto et al., 2013).

Glial cells make up more than 90% of brain cells and are divided into two populations: macroglia (astrocytes and oligodendrocytes) and the microglia. Microglial cells are the resident mononuclear phagocytes of the CNS and they are widely distributed throughout the CNS parenchyma, which ranges between 5 to 20% of total glial cell population and an average of 10% of adult CNS cell population (Lawson et al., 1990). These cells play a crucial role on the homeostasis of the CNS in health and even in disease. When activated by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) microglial cells respond by releasing a variety of cytotoxic mediators such as reactive oxygen species (ROS), proinflammatory cytokines (TNF-α, IL-1β, IL-6), adenosine triphosphate (ATP), and derivatives of arachidonic acid (eicosanoids). All these factors are involved in multiple CNS disorders including demyelinating diseases (Kawai and Akira, 2007; Lehnardt, 2010; Xiang et al., 2015).

As it is known, as a Gram negative bacterium, EHEC not only secrets Stx2 but also releases the outer membrane component lipopolysaccharide (LPS), an endotoxin that induces tissular production of a variety of inflammatory mediators by binding the toll-like receptor 4 (TLR4) (Alexander and Rietschel, 2001).
As it has been reported the inflammatory involvement fulfills a decisive role in patients suffering from HUS (Lee et al., 2013), in the current paper we demonstrate in two animal models that pharmacological treatment with different anti-inflammatory agents succeeds to reduce the encephalopathy depending on the microglia status and on the availability, and neuronal uptake of Stx2. The aim of this work was to determine: (i) the participation of the microglia in the encephalopathy; (ii) the contribution of LPS to Stx2 pathogenicity; (iii) whether Stx2 and/or Stx2 together with LPS trigger a demyelinating process; (iv) the effect of dexametasone on microglia and oligodendrocytes status, and of etanercept on neuronal Stx2 uptake.

2. Materials and methods

2.1 Dexamethasone protection assay

Adult male Swiss mice (20 g) were subjected to the following intravenous (i.v.) treatments: vehicle (saline solution); LPS (800ng); Stx2 (1ng); Stx2+LPS (1ng of Stx2+800ng of LPS). The i.v. treatment was administered by the lateral tail vein (100 ul of total solution volume) only once to each mouse. They were then divided into eight groups (n=4): two groups treated with vehicle, two with LPS, two with Stx2 and two with Stx2+LPS as described before. Half of these groups were treated with an intraperitoneal injection (i.p.) with 7.5 mg/kg dexamethasone (100 μl per dose) twice a day for 4 days, starting when they received their respective i.v. treatment (vehicle, LPS, Stx2 or Stx2+LPS), and the other half received 100 μl of i.p. saline solution twice a day, also for four days. All animals were perfused on the fourth day (day of the first injection counted as day 0). The animals were housed in an
air conditioned and light-controlled (lights on between 06:00 am and 06:00 pm) animal facility. Food and water were provided ad libitum. At the fourth day after the treatment, mice were perfused transcardially as described before (Pinto at al. 2013) and subsequently processed for immunofluorescence microscopy.

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 N°. 046/2017). All the procedures were performed in accordance with the EEC guidelines for care and use of experimental animals (EEC Council 86/609).

2.2 Ionized Calcium-Binding Adapter Molecule 1 (IBA1) and Myelin Basic Protein (MBP) Immunofluorescence

Twenty µm sections were incubated with 10mM PBS Triton X-100 0.1% for one hour (After several rinses with 1.0 mM PBS), followed by normal goat serum (Sigma, St. Louis, MO, USA) 10% with 0.3% Triton X-100 for one hour in the same solution. Sections were then subsequently incubated with mice anti-IBA1 antibody (Millipore, Temecula, Ca, USA) or with rabbit anti-MBP antibody (Dako, Glostrup, Denmark), both diluted at 1:500 in 10mM PBS with Triton X-100 0.3% at 4°C for 48 hours. After several rinses with 10mM PBS Triton X-100 0.025% sections were incubated with goat IgG anti-mouse coupled to Texas Red (Amersham, GE, Piscataway, NJ, USA) or goat IgG anti-Rabbit Alexa Fluor 555 (Invitrogen Molecular Probes, Carlsbad, California, USA) respectively, both diluted at 1:500 in the same buffer with Triton X-100 0.3% for 2 hours. Finally, sections were rinsed with 10mM PBS and mounted on slides. Controls were performed using the same
procedure but without adding the primary antibody. A red fluorescence filter was used for visualization of both immunofluorescences.

Hoescht 33342 (Sigma, St. Louis, MO, USA) was used (1µg/ml 10min) to show the cell nuclei of the brain striatum and the internal capsule after at the end of all immunofluorescences. All analyses were carried out in the same comparable areas.

Twelve different sections per treatment from the dorsal striatum were analyzed to determine microglial activation (IBA1) and in the internal capsule to determine demyelinated process (MBP). A confocal laser scanning microscope (Olympus FV10-ASW) with a 40x objective lens was used. The images obtained were analyzed using the Fiji ImageJ software (NIH). The criteria used to determine microglial activation depended in the number and in the expression levels of IBA1 immunopositive cells, and to determine demyelinated process depended in the expression level of MBP.

### 2.3 Etanercept protection assay

For this study, male Sprague-Dawley rats (300g) were housed in an air conditioned and light-controlled animal facility (lights on between 06:00 am and 06:00 pm). Food and water were provided ad libitum. Anaesthetized rats (ketamine 50 mg/kg – diazepam 0.35 mg/kg, i.p.) were stereotaxically implanted in the interventricular foramina (foramina of Monro) with a stainless steel guide cannula (Plastic One, Roanoke, VA). The placement coordinates were anteroposterior: − 0.96 mm; lateral: 2 mm and vertical: 3.2 mm (Paxinos and Watson, 2005). 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 that extends 0.5 mm below the guide cannula was used for the injections. Correct placement of the ventricle cannulae
was verified at the end of the experiment, followed by postmortem brain fixation and cut on a cryostat; data obtained from improperly implanted animals were excluded from analysis. Cannulae were fixed to the skull surface with three screws and dental acrylic cement and temporarily occluded with dummy cannulae. After surgery, animals were caged individually.

Rats were randomly assigned to different experimental groups and each rat was used only once. One week after the day of the experiment, the intracerebroventricular (i.c.v.) injection was made in freely moving animals through a 30-gauge needle connected by polyethylene tubing to a 10 μl Hamilton syringe. The needle was left in place for 30 seconds to prevent back flow of the injected solution. The rats were i.c.v. injected with 1 μl of vehicle, 1 μl of Stx2 (1 ng/μl) or 1 μl of Stx2 (1 ng/μl)+Etanercept (2,1 mmoles). After 4 days, the rats were sacrificed for ultrastructural studies.

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)]. Brains were removed from skull, post-fixed in the same fixative solution for 2 h. Brain sections were cut on a cryostat. Serial 40-μm-thick coronal sections were obtained and collected in 0.1 M phosphate buffer. Brain floating sections obtained were processed either for light microscope blue toluidine staining or for ultrathin slices to immunoelectron microscope studies.

2.4 Nissl staining
A light microscope with a 4x objective lens was employed to observe the rat brains with Nissl staining. The images obtained were analyzed using the Fiji ImageJ software (NIH). The criterion used to determine a brain damage produced by Stx2 and the possible protected effect of etanercept, was the ratio expressed in percent between the paled staining region (damaged area) and the total striatum. For this purpose, the “wand tool” in the ImageJ software was employed to select and measured the paled stained area and the entire striatal area.

2.5 Immunogold-electron microscopy technique

A primary antibody against Stx2 and a secondary antibody coupled to a gold particle that recognized the primary antibody were used for immunogold-electron microscopy to detect the presence of Stx2 in striatal neurons as described: brain floating sections were rinsed in 10 mM PBS, and then incubated for 20 min in PBS containing 5% normal donkey serum. Sections were incubated for 48 h at 4 °C with the monoclonal anti-Stx2B antibody (Biodesign International) (1:100). Controls were performed by an isotype control using the monoclonal anti-BrdU (clone DU 33 #B 2531, Sigma, St. Louis, MO, USA). After several rinses in PBS, the sections were incubated with a secondary donkey anti-mouse antibody (1:100) coupled with gold particles (Vector Laboratories, Burlingame, CA, USA) diluted in PBS for 1 h. The sections were subsequently processed for transmission electron microscope (TEM) observation. The samples were first assessed by light microscopy with blue toluidine to select the areas for TEM studies. Ultrathin sections were cut from selected areas and contrasted with 1% OsO4 and 1% uranyl acetate, dehydrated and flat-embedded in Durcupan (Sigma, St. Louis, MO, USA). Five brain sections from 5 independent
experiments of each condition were used to immunolocalize Stx2. The sections were contrasted with lead citrate, 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).

2.6 Statistical analysis

The data are presented as mean ± SEM. In the dexamethasone protection assay, statistical significance was performed using a one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test between the four i.v. treatments (vehicle, LPS, Stx2 and Stx2+LPS). In the case of comparison of different treatment groups and their respective controls when challenged with i.p. dexamethasone or saline solution, a two-way analysis of ANOVA was used followed by Bonferroni post hoc test (GraphPad Prism 4, GraphPad Software, Inc.). In addition, in the etanercept protection assay, statistical significance was performed using a t-test (Wilcoxon matched pair test) with a confidence interval of 95% when Stx2 treatment were compare to Stx2+etanercept, and a two-way analysis of ANOVA was used followed by Bonferroni post hoc test when another variable were added (contralateral vs ipsilateral) (GraphPad Prism 4, GraphPad Software, Inc.). The criterion for significance was p<0.05 for all the experiments.

3. Results
3.1 Intravenous administration of a sublethal dose of Stx2 activates the microglia, LPS exacerbates it and dexamethasone decreases the microglia activation in the brain striatum.

Ionized calcium binding adaptor molecule 1 (IBA1) is a microglia/macrophage-specific calcium-binding protein and a marker for microglial activation. An anti-IBA1 antibody was used to detect whether the toxins can induce microglia activation after four days of treatment. Activated microglial cells were defined by an increase in the number (hyperplasia) and/or the expression levels of the microglial marker IBA1. Accordingly, the number of immunopositive cells for IBA1 was counted, and the integral optical density (IOD) that represents its expression levels was also analyzed. A significant increase in the number of IBA1 positive cells and its expression (activated microglia) were found after 4 days of treatment with LPS, Stx2 and Stx2+LPS treatments compared with the control one (20.19 ± 2.37 control, 32.476 ± 2.178 LPS, 43.36 ± 3.17 Stx2, 58.21 ± 3.76 Stx2+LPS cells); (11.72 ± 1.21 control, 14.73 ± 1.13 LPS, 19.69 ± 1.72 Stx2, 25.18 ± 2.18 Stx2+LPS AU in IOD) (Figure 1 A-D, J-K). Moreover, the maximal increase on IBA1 immunopositive cells and its expression was observed following Stx2+LPS (Figure 1 D). Treatment with dexamethasone significantly reduced the number of IBA1 positive cells as well as its expression in Stx2 and Stx2+LPS treated-mice (Figure 1 E-H, L-M).

3.2 Intravenous administration of a sublethal dose of Stx2 reduces the expression of MBP, LPS exacerbates its effect and dexamethasone partially recovers its normal expression in the brain striatum.
An anti-MBP antibody was employed to identify myelin sheath to detect demyelinating process by the deleterious actions of the toxins after four days of treatment. The expression of MBP was measured by IOD. A significant decrease on its expression was found following 4 days of treatment with LPS, Stx2 and Stx2+LPS treatments (42.41 ± 3.84 control, 26.8 ± 2.52 LPS, 20.65 ± 0.88 Stx2, 17.79 ± 1.64 Stx2+LPS AU in IOD) (Figure 2 A-D, J). Moreover, the maximal decrease was observed following Stx2+LPS treatment as compared to the Stx2 or LPS treatments (Figure 2 B-D). Treatment with dexamethasone partially, but significantly rescued the MBP expression from deleterious action of both toxins (Figure 2 E-H, K).

3.3 Etanercept restores Nissl staining loss in the rat striatal nuclei treated with Stx2.

Nissl staining was employed to determine the effect of etanercept on striatal integrity. I.c.v. administration of Stx2 caused loss of white and grey matters from ipsilateral external globus pallidus and caudate putamen of the dorsal striatum (Fig. 3B), compared with the contralateral untreated side (Fig. 3A), (23.80% ± 1.94 vs 7.10% ± 1.63 respectively, p<0.05, Fig. 3F). This loss was reversed when the soluble TNF-α receptor etanercept was administered together with Stx2 (Fig. 3D), (23.80 ± 1.94 % vs 6.30% ± 1.13 respectively, p<0.05, Fig. 3E), and as compared with its contralateral side (Fig. 3C) (6.30% ± 1.13 vs 3.91% ± 0.55 respectively, p<0.05, Fig. 3F).

3.4 Etanercept decreased the number of immunoelectron-gold particles to Stx2 in rat striatal neurons treated with Stx2.
It was determined whether the i.c.v administration of the soluble receptor etanercept decreased Stx2 uptake in neurons by the immunogold technique in a TEM. A Stx2 antibody conjugated with 10 nm colloidal gold particles was employed to determine the effect of etanercept in Stx2 neuronal uptake. Anti-Stx2 B immuno-gold particles were observed in cytoplasms and nuclei of neurons from the rat brain striatum (Figure 4D and 5D). The etanercept treatment significantly decreased the number of Stx2B immuno-gold particles in both cytoplasm (Figure 4E) and nuclei (Figure 5 E) of neurons (64.22 ± 7.32 Stx2, 7.04 ± 1.99 Stx2+etanercept, number of immune-gold particles and 86.75 ± 10.57 Stx2, 23.27 ± 2.43 Stx2+etanercept, number of immune-gold particles, respectively).

4. Discussion

The present results of the murine model of encephalopathy developed by sub lethal administration of Stx2 showed an activated microglia state accompanying demyelination. These events were enhanced when Stx2 was co-administered with LPS respect normal mice (vehicles). The present results demonstrate that dexamethasone and etanercept, two different anti-inflammatory agents used in two independent experiments manage to reduce the deleterious effects caused by Stx2 and / or Stx2 together with LPS in the brain striatum. Therefore, in this model of encephalopathy, a pro-inflammatory state is observed that significantly contributes to the observed damage. Treatment with dexamethasone tends to return the microglial state and the demyelinating process to normal values, while etanercept prevented the uptake of Stx2 in neurons.

Microglial cells are constantly scanning and sensing their environment for disruptions in homeostasis and, as immune sentinels and effectors they participate in many functions such
as synaptic organization, control of neuronal excitability, phagocytic removal of debris and trophic support leading to brain protection and repair (Denes et al., 2007; Nimmerjahn et al., 2005). Paradoxically, deleterious effects of microglial activation are associated with some neurodegenerative diseases, infections, stroke, tumors or acute and chronic demyelinating encephalomyelitis such as multiple sclerosis (MS). Indeed, myelin destruction is associated with activated microglia (Lassmann et al., 2007; Trapp et al., 1998). In addition, they produce ROS and NO radicals, both important sources of oxidative damage observed in the pathogenesis of demyelinating diseases (Torre-Fuentes et al., 2017), which may have important implications in the pathogenesis of the encephalopathy produced by Stx2, as demonstrated in our model.

Oligodendrocytes produce the myelin sheath that covers CNS axons; they are able to keep axonal integrity, support axonal metabolism and neuronal survival (Baumann and Pham-Dinh, 2001). As microglial cells express pro-inflammatory cytokines such as TNF-α and/or IL-1β, they may damage oligodendrocytes and/or the myelin (Beck et al., 1988; Sharief and Hentges, 1991), an event that occurred in the present model.

Etanercept, the soluble receptor of TNF-α inhibits the biological action of TNF-α (Liao and Xu, 2017). In the present work, the presence of pro-inflammatory cytokines such as TNF-α involved in neuronal Stx2 uptake is evidenced as the administration of etanercept decreased the uptake of Stx2 in neurons and concomitantly prevented from striatal white and grey matter loss. Stx2 uptake in neurons may occur through globothriaosylceramide (Gb3) receptor. The presence of this neuronal receptor was demonstrated by previous works from our laboratory and from others (Obata et al., 2008; Tironi-Farinati et al., 2010).
Immunoelectron microscope analysis revealed the presence of Stx2 in neurons that coincided with an abnormal neuronal phenotype. This is consistent with previous published results of neuronal damage by Stx2 (Goldstein et al., 2007).

The beneficial action of etanercept may target Stx2-Gb3-mediated neuronal upregulation and/or the blood-brain barrier (BBB) permeability. This neutralizing agent may have decreased the uptake of Stx2 in neurons by reducing TNF-α availability and Gb3 upregulation, as it was reported that TNF-α increased Gb3 expression and sensitivity to Stx2. However, this mechanism was observed in human cerebral endothelial cells (Eisenhauer et al., 2001). Similarly, our laboratory observed LPS treatment upregulated neuronal Gb3 expression (Pinto et al., 2017). Then, it is inferred that Microglial cells may respond to LPS by secreting high amounts of TNF-α, and this cytokine may target neurons contributing to the observed upregulated Gb3. Alternatively, the deleterious effect of TNF-α in the brain may be due to an increase in BBB permeability (Ahishali et al., 2005), or other events that have not been elucidated yet.

In a previous work we reported that dexamethasone was able to prevent mice death when they were systemically treated with two lethal dose50 of Stx2 (3.2 ng per mouse), improving neurological and clinical signs. It was observed that this drug rescued the striatal microvasculature, vascular endothelial growth factor (VEGF) expression and reactive astrocytes to normal parameters, though reducing the BBB permeability. Also, as a neuroprotectant drug it reduced the number of neurons with aberrant phenotype, and finally it succeeded to reduce the expression of the Stx-Gb3 receptor in neurons by which consequently, the presence of Stx2 in neurons was reduced (Pinto et al., 2017). Similar findings were observed in the motor cortex (Pinto et al., 2013). In light of these data the anti-inflammatory dexamethasone probably reduced the deleterious effects of Stx2 and
LPS, by diminishing the presence of pro-inflammatory cytokines, or even by strengthening the BBB. Therefore, dexamethasone may be beneficial in patients suffering from EHEC-derived encephalopathy. Indeed, steroid pulse therapy was administered to a Japanese patient intoxicated with EHEC and improved her neurological condition (Ito et al., 2015).

In summary, the present results confirm that sublethal administration of Stx2 and Stx2 together with LPS damaged oligodendrocytes and neurons. Also, activated microglia and release of TNF-α were demonstrated to be involved in the pathogenesis of myelin sheath damage and neurodegeneration. Anti-inflammatory agents are beneficial candidates in improving the neurological state and they should seriously be taken into account in clinical trials for EHEC-derived encephalopathy treatment.

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5. REFERENCES


6. FIGURE LEGENDS

**Figure 1: Changes in IBA1 expression.** Immunofluorescence using an anti-IBA1 antibody was employed to show the microglia. A, E: vehicle; B, F: LPS; C, G: Stx2; D, H: Stx2+LPS. I: negative control by not adding the primary antibody. The scale bar in “I” it does apply for all micrographs. The toxins increased the number of IBA1 positive cells (J) and the expression level of IBA1 (K). Dexamethasone treatment (E-H) decreased the number of IBA1 positive cells (L) and its expression (M). Data are expressed as mean ± SEM, with n=4 mouse per group. J, K: data were analyzed by one-way ANOVA followed by post hoc Tukey. L, M: data were analyzed by two-way ANOVA followed by post hoc Bonferroni. *: significant differences (p <0.05).

**Figure 2: Changes in MBP expression.** Micrographs show immunofluorescence staining for MBP in the internal capsule. A, E: vehicle; B, F: LPS; C, G: Stx2; D, H: Stx2+LPS. Dexamethasone treatment (E through H). I: negative control by not adding the primary antibody. The scale bar in “I” it does apply for all micrographs. The toxins decreased the levels of MBP immunofluorescence (J). Dexamethasone partially restored the levels of MBP immunofluorescence expression (K). Data are expressed as mean ± SEM, with n=4 mouse per group. J: data were analyzed by one-way ANOVA followed by post hoc Tukey. K: data were analyzed by two-way ANOVA followed by post hoc Bonferroni. *: significant differences (p <0.05). *: significant differences (p <0.05).
**Figure 3:** Nissl staining micrographs corresponding to ipsilateral and contralateral striatum subjected to i.c.v. administration of Stx2. A: contralateral striatum from i.c.v. treatment with Stx2. B: ipsilateral striatum i.c.v. treated with Stx2. C: contralateral striatum from i.c.v. treatment with etanercept and Stx2. D: ipsilateral striatum i.c.v. treated with etanercept and Stx2. The scale bar in D applies for A-D micrographs. G: The i.c.v. treatment site (1- the ipsilateral striatum; 2- the contralateral striatum). E: The toxin produced a decreased in the nissl staining coloration and etanercept partially restored the nissl staining coloration. F: Comparison between the ipsilateral and contralateral striatum. Data are expressed as mean ± SEM, with n=6 rats per group. F: data were analyzed by t-test (Wilcoxon matched pair test). *: significant differences (p <0.05).

**Figure 4:** Immuno-gold localization and quantification of Stx2 on striatal cell cytoplasm. TEM micrographs show immune-gold localization of Stx2 on striatal cells cytoplasm. A: Isotype control; B (in lower magnification), D (in higher magnification): Stx2 treated rats; C (in lower magnification), E (in higher magnification): Stx2+Etanercept treated rats. The scale bar in “A” applies for B and C micrographs. The scale bar in “D” applies for “E”. Data are expressed as mean ± SEM, with n=6 rats per group. F: data were analyzed by t-test (Wilcoxon matched pair test). *: significant differences (p <0.05).

**Figure 5:** Immuno-gold localization and quantification of Stx2 on striatal cell nuclei. TEM micrographs show immune-gold localization of Stx2 on striatal cells nuclei. A: Isotype control; B (in lower magnification), D (in higher magnification): Stx2 treated rats; C (in lower magnification), E (in higher magnification): Stx2+Etanercept treated rats. The scale bar in “A” applies for B and C micrographs. The scale bar in “D” applies for “E”.
Data are expressed as mean ± SEM, with n=6 rats per group. F: data were analyzed by t-test (Wilcoxon matched pair test). *: significant differences (p <0.05).