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Propylparaben Reduces the Long-Term Consequences in Hippocampus Induced by Traumatic Brain Injury in Rats: Its Implications as Therapeutic Strategy to Prevent Neurodegenerative Diseases

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Abstract.

Background: Severe traumatic brain injury (TBI), an important risk factor for Alzheimer's disease, induces long-term hippocampal damage and hyperexcitability. On the other hand, studies support that propylparaben (PPB) induces hippocampal neuroprotection in neurodegenerative diseases.

Objective: Experiments were designed to evaluate the effects of subchronic treatment with PPB on TBI-induced changes in the hippocampus of rats.

Methods: Severe TBI was induced using the lateral fluid percussion model. Subsequently, rats received subchronic administration with PPB (178 mg/kg, TBI+PPB) or vehicle (TBI+PEG) daily for 5 days. The following changes were examined during the experimental procedure: sensorimotor dysfunction, changes in hippocampal excitability, as well as neuronal damage and volume.

Results: TBI+PEG group showed sensorimotor dysfunction ($p < 0.001$), hyperexcitability (64.2%, $p < 0.001$), and low neuronal preservation ipsi- and contralateral to the trauma. Magnetic resonance imaging (MRI) analysis revealed lower volume (17.2%; $p < 0.01$) and great damage to the ipsilateral hippocampus. TBI+PPB group showed sensorimotor dysfunction that was partially reversed 30 days after trauma. This group showed hippocampal excitability and neuronal preservation similar to the control group. However, MRI analysis revealed lower hippocampal volume ($p < 0.05$) when compared with the control group.

Conclusion: The present study confirms that post-TBI subchronic administration with PPB reduces the long-term consequences of trauma in the hippocampus. Implications of PPB as a neuroprotective strategy to prevent the development of Alzheimer's disease as consequence of TBI are discussed.

Keywords: Alzheimer's disease, brain trauma, hippocampus, neuroprotection, propylparaben

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INTRODUCTION

Traumatic brain injury (TBI) induced by an external force causes damage to the brain and may lead to functional alterations [1, 2]. TBI is the leading cause of death and disability among individuals under the age of 45 years. Unfortunately, an increase from 64 to 74 million new TBI patients is estimated during the next years [3]. TBI is associated with cellular metabolism alterations, excitatory neurotransmitters release, ionic cell membrane pumps failure, blood-brain barrier damage, prostaglandins and leukotrienes extravasation, and proinflammatory cytokines release [4–7]. All these processes contribute to the short-term consequences induced by TBI, such as diffuse axonal injury [8, 9], neuroinflammation [10], oxidative stress [11], excitotoxicity [12, 13], and neuronal death [14]. TBI may also induce long-term consequences, such as the atrophy of brain areas [15, 16], neuropsychiatric disorders, cognitive impairment, mood disorders [17], as well as neurodegenerative diseases such as Parkinson's disease, amyotrophic lateral sclerosis, epilepsy, and Alzheimer's disease [18, 19]. It is known that TBI represents the most robust environmental risk factor for Alzheimer's disease [20–22]. Patients with symptomatic mild TBI show white matter abnormalities similar to those found in the brain of patients with early Alzheimer's disease [23]. Studies using experimental models revealed the aggregation of the tau protein and cognitive impairment, short- and long-term after a TBI [24].

The development of post-TBI pathologies is related to lesions in brain areas such as the cerebral cortex, thalamus, basal ganglia, corpus callosum, and hippocampus [6, 25–27]. The hippocampus is highly susceptible to TBI [6, 25, 27]. After TBI, the hippocampus presents a significant cell loss, particularly in hilar and CA3 neurons [28], an effect associated with hyperexcitability [29–31]. At present, there is no evidence of neuroprotective strategies to prevent the development of long-term consequences induced by TBI [32, 33].

Propylparaben (PPB) is an ester of p-hydroxybenzoic acid frequently used as an antimicrobial agent against molds and yeasts [34] and as excipient in some drugs [35]. We found that the administration of PPB in rats previously submitted to pilocarpine-induced *status epilepticus* reduced the long-term hippocampal hyperexcitability and neuronal death [36]. These effects become more evident when PPB is combined with levetiracetam [37]. This group of evidence supports that PPB induces neuroprotection, an effect explained because it lessens the excessive release of glutamate [36] as consequence of voltage-dependent sodium channel blockage [38, 39].

According to this information, it is possible to suggest that PPB will reduce the long-term consequences induced by TBI in the hippocampus. The present study focused on evaluating the effects of subchronic administration of PPB on hippocampal excitability and long-term brain damage after the induction of severe TBI in rats.

MATERIALS AND METHODS

Animals

Male adult Wistar rats (250–300 g) were maintained individually in clear acrylic boxes under controlled environmental conditions (12 h light/darkness cycles, at $22 \pm 2^{\circ}$C, and 50% humidity) with access to food and water *ad libitum*. The experimental protocol was carried out following the Official Mexican Standard (NOM-062-ZOO-1999) and the Ethics Committee of the Center for Research and Advanced Studies of the National Polytechnic Institute.

Experimental groups

Animals were randomly divided into the following groups:

a) TBI+PPB group ($n = 13$). Under general anesthesia, animals underwent severe TBI. Three hours after TBI, animals received an intraperitoneal (i.p.) injection of PPB (178 mg/kg) and subsequent i.p. injections of the same dose every 12 h for five days. This dose was chosen based on a preliminary study in our laboratory. We found that PPB at 178 mg/kg, i.p. applied as pretreatment reduced tonic-clonic convulsions in 50% of animals submitted to pilocarpine-induced status epilepticus. This treatment administered during the pilocarpine-induced status epilepticus results in lower extracellular levels of glutamate and neuronal damage in hippocampus [36]. Thirty-one days post-TBI, six rats were anesthetized and perfused. Hippocampal volume and damage were evaluated *ex vivo* using magnetic resonance imaging (MRI). The remaining animals ($n = 7$) were used to evaluate hippocampal excitability

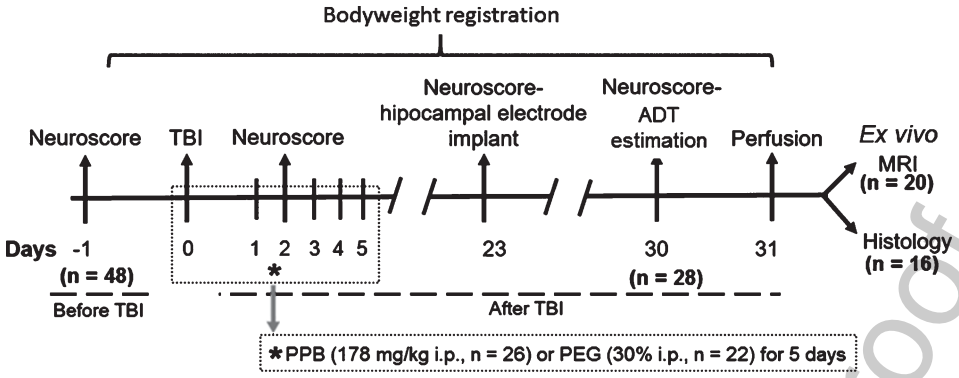

Fig. 1. Timeline and experimental design to evaluate the effects of subchronic administration of propylparaben (PPB) after severe traumatic brain injury (TBI). Bodyweight of the animals and sensorimotor function were evaluated throughout the experimental procedure. PPB or polyethylene glycol (PEG) was administered 3 h after the TBI and repeated every 12 hours for five days. Rats previously implanted with a bipolar electrode in the ventral hippocampus (day 23 post-TBI) were used to estimate the after-discharge threshold (ADT) (day 30 post-TBI). On day 31 post-TBI, these animals were perfused to perform histological analysis. Another group of animals was perfused on day 31 post-TBI for hippocampal volume and damage by *ex vivo* magnetic resonance imaging (MRI).

and neuronal damage. These rats underwent surgery to implant a bipolar electrode in the ventral hippocampus ipsilateral to the injury 23 days post-TBI. Thirty days post-TBI, hippocampal excitability was evaluated through the estimation of the after-discharge threshold (ADT). On day 31 post-TBI, rats were anesthetized and perfused. The hippocampal neuronal population (NeuN) was evaluated in different areas. Bodyweight and sensorimotor function were evaluated at different time points through the experimental procedure (Fig. 1)

b) TBI+PEG group ($n = 11$). Animals underwent the same experimental procedures as the TBI+PPB group, except for the administration of vehicle (polyethylene glycol 30%, PEG) (1 ml/kg, i.p.) instead of PPB. Four rats were used for MRI analysis. The remaining animals ($n = 7$) were used to evaluate hippocampal excitability. Four rats from this subgroup were used for a subsequent histological evaluation (Fig. 1).
c) Sham+PPB group ($n = 13$). This group underwent the same experimental procedures as the TBI+PPB group, except for the TBI. Six animals were used for MRI analysis and seven animals for the ADT evaluation (Fig. 1).
d) Sham+PEG group ($n = 11$). Animals underwent the same experimental procedures as the Sham+PPB group, except for the administration of PEG instead of PPB. Four animals were used for MRI analysis and seven animals for the ADT and histological evaluation (Fig. 1).

Evaluation of sensorimotor function

The Composite Neuroscore (NS) battery was used to evaluate sensorimotor function. The NS consists of four tests focused on evaluating the following functions: 1) ability to stand on an inclined plane at different angles (35° to 75°); 2) hindlimb and 3) forelimb counter flexion during tail suspension; and 4) ability to resist lateral pulsion to either side. Scoring for each test ranged from 0 (complete loss of function) to 4 points (normal function). A total score of 27–28 indicates a normal condition, whereas a score of 26–16 suggests mild damage, and a score ≤ 15 indicates severe sensorimotor damage [40].

Induction of severe TBI

Severe TBI was induced by the lateral fluid-percussion (LFP) injury model [41]. Rats were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (17 mg/kg, i.m.), and mounted on a stereotaxic frame. Subsequently, a circular craniotomy (5 mm diameter) was performed on the left side of the skull (anteroposterior to bregma, –5 mm; lateral, 4 mm). After the integrity of the dura mater was verified, a female Luer-lock disc was attached to the craniotomy with Vetbond 3M tissue glue (Deutschland GmbH, Germany). A stainless-steel screw was implanted lateral and anterior to bregma. All the elements were fixed to the skull with dental acrylic. TBI induction was carried out 90 min after the administration of anesthesia using a fluid percussion device (AmScien Instruments, Model FP 302, Richmond, VA, USA). TBI

was considered severe when the pulse of pressure achieved 2.6 to 3.3 atm. After TBI induction, the implant was removed, and the skin was sutured. Tramadol (20 mg/kg, s.c., NorVet) was applied 15 min after TBI induction. Animals with TBI that lost more than 30% of their initial bodyweight throughout the experimental procedure were discarded from the experiment.

Electrode implantation and evaluation of hippocampal excitability

Previously anesthetized rats (ketamine, 80 mg/kg, i.p.; xylazine, 15 mg/kg, i.m.) were placed in a stereotaxic frame. A bipolar electrode was implanted in the ventral hippocampus (anteroposterior, –5.3 mm in relation to bregma; lateral, 5.2 mm; height, 7.5 mm), ipsilateral to injury [42]. Three stainless steel screws were placed on the skull to support the implant, which was fixed with dental acrylic. ADT was estimated seven days after surgery. The procedure consisted of the application of a train of electrical stimuli (1 ms square pulses at 60 Hz for 1 s) generated with a GRASS S-48 model stimulator. The procedure was repeated every minute with an initial electric current of 10 μA, which was subsequently increased by 20% until a behavioral change or an electrographic after-discharge was induced [43]. Low values indicate neuronal hyperexcitability.

Histology and fractional counting method

Under anesthesia (pentobarbital, 70 mg/kg, i.p.), animals were perfused with 250 ml of 0.9% saline (SS) and 1 mg/l heparin (Sigma-Aldrich, Cat # H3393), followed by 250 ml of 4% paraformaldehyde (Sigma-Aldrich Cat # P6148) and 0.2% glutaraldehyde (Electron Microscopy Sci. Cat # 16210) in a phosphate buffer solution (PBS). After perfusion, the brain was dissected and kept in a 4% paraformaldehyde solution at 4°C for 168 h, and subsequently included in paraffin for further processing. Brains were sectioned in the coronal plane (5 μm thickness) into serial sections (1 of 5) through the entire dorsal hippocampus (bregma –2.5 to –4.5 mm). Slices were collected and thaw-mounted on Poly-L-lysine adhesive (Sigma-Aldrich Cat # P8920) coated glass slides.

NeuN, a neural marker, was evaluated by immunohistochemistry. For this procedure, brain sections were first incubated in an antigenic recovery solution (Diva, Biocare Medical) for 10 min at 120°C, washed in distilled water and exposed to 3% H_2O_2 for 10 min. Then, sections were incubated in goat serum (1 : 200, Vector Lab USA) for 30 min and subsequently in the primary mouse monoclonal antibody directed against NeuN (1 : 200, Millipore Cat # MAB-377). After 72 h, brain sections were incubated for 2 h with the secondary antibody (anti-mouse peroxidase) (1 : 200, Vector Lab. Cat # PI-200). Finally, the reaction was revealed with 3,3'-diaminobenzidine tetrahydrochloride (Betazoid DAB Chromogen Kit, Biocare Medical Cat # kit DB801L) and slides were coated with synthetic resin (Entellan®, Merck Millipore). Digitized images of the brain sections were obtained with a camera connected to a microscope (Nikon 10x Optical 200M) and analyzed using the Image Pro-Plus 7 software (Media Cybernetics, USA).

The fractional counting method [44] was used for the estimation of the number of neurons per volume (mm^3) in different regions of the hippocampus: dentate gyrus (DG), hilus, CA1 and CA3, ipsi- and contralateral to injury or manipulation. An experimental researcher on blind conditions assessed this estimation. The regions of interest were identified at the dorsal hippocampus (bregma –2.5 to –3.6 mm) on digital images previously obtained with a light microscope (Eclipse Ni; Nikon, Japan) and Image Pro-Plus 7 software (Media Cybernetics, USA). Three serial sections obtained every five slices (see Histology section) were evaluated for this purpose. In this case, the sampling fraction (*ssf*) corresponded to 1/5. The area sampling fraction (*asf*) was calculated [asf = area (frame)/area (x, y step)] and corresponded to the counting frame (0.460 x 0.600 mm). The thickness sampling fraction was estimated with the dissector height (*h*) relative to the section thickness *t* (*h/t*). The number of cells was calculated using the following formula:

$$N = (\Sigma Q^{-})^{*}(t/h)^{*}(1/asf)^{*}(1/ssf).$$

In this formula, Q^{-} represented the number of cells in a known volume fraction of each evaluated area.

Ex vivo magnetic resonance imaging

The perfusion protocol was conducted as previously described for the histological evaluation, except for the addition of a 2 mM gadolinium-based contrast agent (Prohance, Bracco Diagnostics Inc.) to the solution [45]. When the perfusion was finished, the head was separated from the body and immersed in 4% paraformaldehyde and 2 mM ProHance at 4°C

overnight. The following day, 0.02% sodium azide (Sigma-Aldrich Cat # S2002) was added to the buffer. The samples were stored at 4°C until imaging.

Imaging was performed at the National Laboratory for MRI (Universidad Nacional Autónoma de México) using a Bruker Pharmascan 70/16 US 7.0 T magnet and a circularly polarized rat head coil, coupled to a workstation running Paravision 6.0.1. The procedure was carried out at room temperature (21 ± 1°C). The parameters for the scans were optimized for gray- and white-matter contrast (Fast Low Angle Shot (FLASH) imaging with 3D acquisition). The resolution was 85 μm per side (TR/TE/flip angle = 30 ms/8.6 ms/20°). The acquisition time per animal was 1 h. Volumes were linearly normalized to a custom-made, unbiased, rat brain atlas, which was built by interactively registering all image volumes using a non-linear transform [46] and the Waxholm Space atlas of the Sprague Dawley rat brain [47]. The total volume of each hippocampus was calculated by manually selecting the areas of interest, using an atlas of the rat brain [42] as a reference and the ITK-SNAP software version 3.6.0 [48]. Similarly, areas corresponding to damage (hypointense voxels) were selected, and the total volumes of hippocampal injury were obtained. Brains that showed image alterations under control conditions were discarded.

Statistical analysis

A researcher blind to the experimental conditions performed each evaluation. Data are expressed as the mean ± standard error (SE) of the mean. For the analysis of parametric data, a two-way ANOVA test followed by a Tukey *post-hoc* test was used. For the analysis of nonparametric data, a Kruskal-Wallis test followed by a Dunn *post-hoc* test was used. A statistically significant difference was considered when *p-values* ≤ 0.05.

RESULTS

Histological analysis demonstrated that the electrode tips were implanted within the ventral hippocampus in all the animals used for ADT estimation.

In the Sham + PEG group, animals showed a mean bodyweight of 265 ± 5.7 g at the beginning and 358 ± 15.2 g at the end of the protocol (Fig. 2). During the experimental procedure, animals from this same group showed normal sensorimotor activity, with a score of 27–28 estimated with NS (Fig. 3).

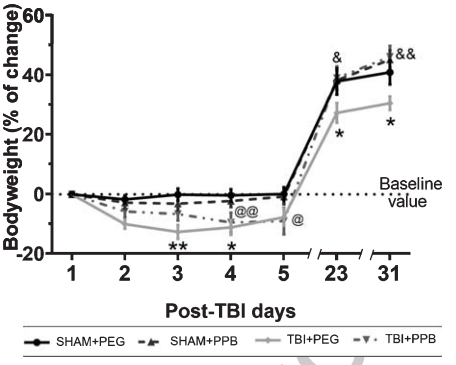

Fig. 2. Effects of subchronic administration propylparaben (PPB) on bodyweight as a consequence of a severe traumatic brain injury (TBI). Bodyweight was registered continuously for each experimental group. In comparison to the Sham + PEG and Sham + PPB groups, which showed a progressive gain in bodyweight, the TBI + PEG group showed a weight loss during the first five days after TBI. Moreover, the TBI + PEG group showed a lower weight when compared with the Sham + PEG group during the experimental protocol. Similarly, the TBI + PPB group showed a weight loss during the first five days post-TBI. However, at the end of the experimental procedure, a weight recovery similar to the Sham + PEG group was observed. The values represent the mean ± SE of the percentage of bodyweight change relative to baseline values (day 0). $^{*}p < 0.05$, $^{**}p < 0.01$ (TBI + PEG versus Sham + PEG); $^{@}p < 0.05$, $^{@@}p < 0.01$ (TBI + PPB versus Sham + PEG); $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ (TBI + PPB versus TBI + PEG).

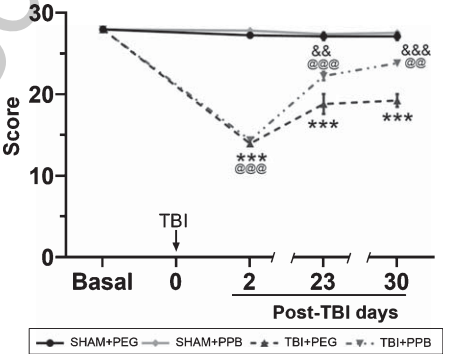

Fig. 3. Changes in the sensorimotor function induced by TBI and associated with PPB administration. The sensorimotor function was evaluated with the composite neuroscore (NS) throughout the experimental protocol. Animals from the Sham + PEG and Sham + PPB groups showed a NS rate indicating normal function (27–28). The NS estimated for the TBI + PEG group revealed a severe sensorimotor dysfunction starting 48 h after TBI and evident 23 and 30 days post-TBI. The TBI + PPB group showed a severe sensorimotor dysfunction two days after TBI. However, this effect was less evident 23 and 30 days after TBI. The values represent the mean ± SE of the scores obtained in the NS evaluations. $^{@@}p < 0.01$; $^{@@@}p < 0.001$ (TBI + PPB versus Sham + PEG); $^{***}p < 0.001$ (TBI + PEG versus Sham + PEG); $^{\&\&}p < 0.01$; $^{\&\&\&}p < 0.001$ (TBI + PPB versus TBI + PEG).

ADT was attained at 299.3 ± 22.5 μA (Fig. 4). No after-discharge was evoked as a consequence of ADT estimation (Table 1). The neuronal preservation ($NeuN^+/mm^3$) in the dorsal hippocampus ipsilateral to craniotomy was similar to the contralateral side (Fig. 5, Table 2). MRI analysis revealed a similar volume between ipsilateral (48.69 ± 2.79 mm^3) and contralateral (47.46 ± 2.61 mm^3) hippocampus. Hippocampal damage was not detected in this group (Fig. 6, Table 3). The results obtained from the Sham + PPB group showed no statistical differences when compared with those from the Sham + PEG group (Figs. 2–4, Tables 1–3).

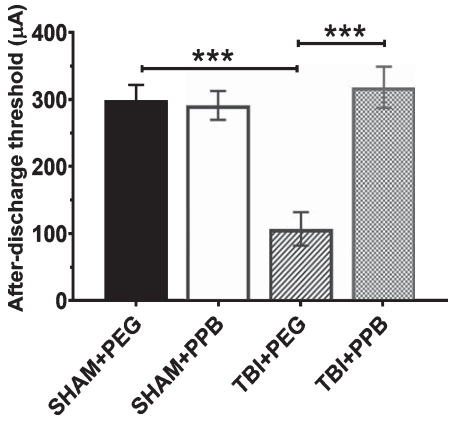

Fig. 4. Effects of subchronic administration of propylparaben (PPB) on hippocampal excitability after a severe traumatic brain injury (TBI). The estimation of the after-discharge threshold (ADT) was used to evaluate the hippocampal excitability in animals previously implanted with a bipolar electrode in the ventral hippocampus. Sham + PEG and Sham + PPB groups demonstrated similar hippocampal excitability. In contrast, animals from the TBI + PEG group achieved the ADT at lower values when compared with the Sham + PEG group, suggesting hippocampal hyperexcitability. The TBI + PPB group presented ADT values similar to Sham + PEG and Sham + PPB groups. Values are expressed as the mean ± SE of the μA required to achieve the ADT. $^{***}p < 0.001$ versus TBI + PEG.

In the TBI + PEG group, animals underwent a severe TBI with a fluid percussion of 3.28 ± 0.13 atm. A significant decrease in their bodyweight during the first 5 days post-TBI ($p < 0.01$ day 3; $p < 0.05$ at day 4 post-TBI versus Sham + PEG) was registered. From that time on, the bodyweight gradually increased, although the values were lower when compared with the Sham + PEG group (days 23 and 31 post-TBI; $p < 0.05$ versus Sham + PEG) (Fig. 2). The NS evaluation at day 2 post-TBI showed sensorimotor impairment (14 ± 0.4 points; $p < 0.001$ versus Sham + PEG), a condition that became evident at days 23 and 31 post-TBI (Fig. 3). During the evaluation of the hippocampal excitability, animals achieved the ADT at lower values (64.2%

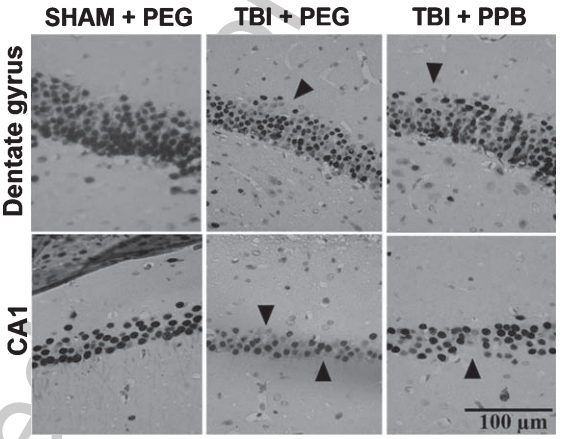

Fig. 5. Subchronic treatment with propylparaben (PPB) reduces the long-term hippocampal injury resulting from severe traumatic brain injury (TBI). Representative microphotographs of the dorsal dentate gyrus and CA1 of the Sham + PEG, TBI + PEG, and TBI + PPB groups. Brain sections were processed by immunohistochemistry to evaluate NeuN expression. Notice that sections from the TBI + PEG group show a lower number of immunoreactive cells (arrowheads) when compared with the Sham + PEG group. This effect was not observed in the TBI + PPB group.

Table 1
Behavioral and electrographic changes evoked during the after-discharge threshold estimation in animals with severe traumatic brain injury and subchronic administration of propylparaben or vehicle

Group (number of animals)	Stage (I-V) (% animals)	ADT duration (s) (% animals)	Spike frequency (Hz)
SHAM + PEG ($n = 7$)	Stage I (100%)	ND	ND
SHAM + PPB ($n = 7$)	Stage I (100%)	ND	ND
TBI + PEG ($n = 7$)	Stage I (14.3%)	85 ± 20.21 (100%)	6.3 ± 0.24
	Stage II (57.2%)		
	Stage III (28.5%)		
TBI + PPB ($n = 7$)	Stage I (71.5%)	17.83 ± 4.3 (75%) ***	3.56 ± 0.8 **
	Stage II (28.5%)	ND (25%)	

ADT, After-discharge threshold; ND, not determined; PPB, propylparaben; PEG, polyethylene glycol; TBI, traumatic brain injury. Values represent the mean ± SE of the after-discharge duration (s) and spike frequency (Hz) of the experimental groups. $^{**}p < 0.01$; $^{***}p < 0.001$ versus TBI + PEG.

Table 2
Effect of subchronic administration of propylparaben on the number of NeuN positive cells in the dorsal hippocampus, ipsi- and contralateral to severe traumatic brain injury

Group (number of rats)	Dentate Gyrus		Hilus		CA1		CA3	
	Ipsi-	Contra-	Ipsi-	Contra-	Ipsi-	Contra-	Ipsi-	Contra-
SHAM + PEG ($n=4$)	2649 ± 90.4	3057 ± 94.1	762 ± 40.9	848 ± 41	1791 ± 135.4	2672 ± 171	1329 ± 155.5	1304 ± 103
SHAM + PPB ($n=4$)	2620 ± 52.3	2953 ± 97.7	687 ± 15.1	712 ± 70.7	1534 ± 103	2444 ± 193	1180 ± 73.1	1235 ± 90
TBI + PEG ($n=4$)	$1276 \pm 42.6^{***}$	$1249 \pm 71.4^{***}$	$207 \pm 12.7^{***}$	$310 \pm 24.4^{***}$	$830 \pm 16.5^{***}$	$771 \pm 37^{***}$	$664 \pm 25^{***}$	$663 \pm 25^{***}$
TBI + PPB ($n=4$)	$2085 \pm 268^{*,\&}$	$2529 \pm 97.4^{**,\&\&\&}$	$474 \pm 42.5^{**,\&\&\&}$	$558 \pm 62.6^{*,\&}$	$1303 \pm 61.5^{**,\&\&}$	$1389 \pm 99.5^{**,\&}$	1065 ± 99	$1085 \pm 58.5^{\&}$

PPB, propylparaben; PEG, polyethylene glycol; TBI, traumatic brain injury. Values represent the mean ± SE of neural preservation (mm^3). $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ versus Sham + PEG; $^{\&}p<0.05$; $^{\&\&}p<0.01$; $^{\&\&\&}p<0.001$ versus TBI + PEG.

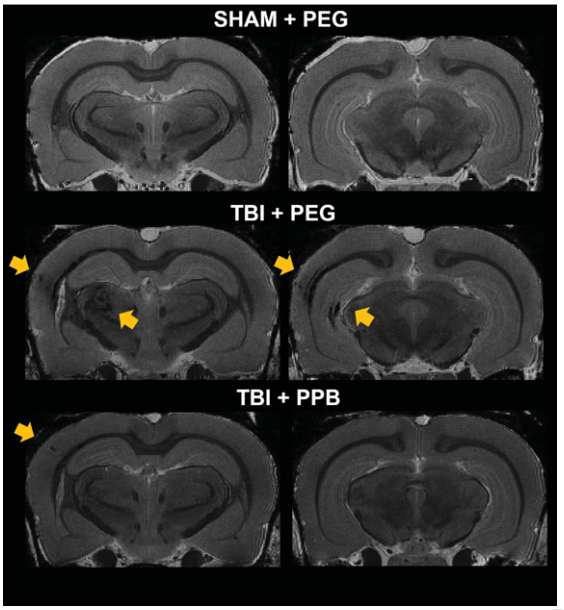

Fig. 6. Subchronic treatment with propylparaben (PPB) reduces the long-term hippocampal injury resulting from severe traumatic brain injury (TBI). Representative images of brains from Sham + PEG, TBI + PEG, and TBI + PPB groups at the level of dorsal (left) and dorso-ventral hippocampus (right) obtained with *ex vivo* magnetic resonance imaging (MRI with T2 contrast) at 31 days post TBI. Note the areas of hypointensity (arrowheads) suggestive of focal lesions in cortex, thalamus and hippocampus in the images from the TBI + PEG group. The areas of lesion in these brain areas were less evident in the images from the TBI + PPB group.

less versus Sham + PEG group, $p<0.001$), suggesting hyperexcitability (Fig. 4). As a consequence of the ADT estimation, the animals (100%) showed an after-discharge with a duration of 85 ± 20.2.s and a spike frequency of 6.3 ± 0.24 Hz (Table 1). Regarding histology evaluation, the TBI + PEG group presented a decrease in neuronal preservation in all the areas evaluated, ipsi- (DG, 52%, $p<0.001$; hilus, 73%, $p<0.001$; CA1, 54%, $p<0.001$ and CA3, 50%, $p<0.001$ versus Sham + PEG group) and contralateral (DG, 59%, $p<0.001$; hilus, 63%, $p<0.001$; CA1, 71%, $p<0.001$ and CA3, 49%, $p<0.001$ versus Sham + PEG group) to the trauma (Fig. 5, Table 2). MRI analysis revealed a decrease in hippocampal volume (17.2%; $p<0.01$ versus Sham + PEG group), as well as increased damage in the ipsilateral hippocampus ($p<0.05$ versus Sham + PEG group), changes which were less evident in the contralateral hippocampus (volume $p=0.191$, damage $p>0.99$ versus Sham + PEG group) (Table 3).

In the TBI + PPB group, rats received a fluid percussion of 3.31 ± 0.11 atm that induced a severe TBI. A decrease in bodyweight was observed in the animals on days 4 and 5 post-TBI ($p<0.01$, $p<0.05$ versus Sham + PEG). However, a noticeable bodyweight recovery was detected afterward. Indeed, animals from the TBI + PPB group showed higher bodyweight on days 23 ($p<0.05$) and 31 ($p<0.01$) post-TBI, when compared with TBI + PEG group (Fig. 2). Two days after trauma, rats showed NS of 14.4 ± 0.2 ($p<0.001$ versus Sham + PEG), indicating a severe TBI. However, a partial sensorimotor function recovery was observed on days 23 and 31 post-TBI ($p<0.01$ and $p<0.001$ versus TBI + PEG groups, respectively) (Fig. 3). Concerning ADT estimation, the TBI + PPB group achieved similar ADT values as those from the Sham + PEG group ($p=0.906$) (Fig. 4). During the ADT estimation, animals from TBI + PPB group showed a shorter after-discharge ($p<0.001$ versus TBI + PEG) (Table 1).

Immunohistochemistry showed lower neuronal preservation in all the hippocampal areas evaluated, ipsilateral (hilus, $p<0.01$; DG, $p<0.05$; CA1,

Table 3
Effect of subchronic administration of propylparaben (PPB) in traumatic brain injury (TBI)-induced changes in the total volume and volume of injury in the hippocampus, ipsi- and contralateral to the trauma and assessed with *ex vivo* magnetic resonance imaging (MRI)

Group (number of animals)	Hippocampal volume (mm^3)		Injury volume (mm^3)	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral
SHAM + PEG ($n = 4$)	48.6 ± 2.7	47.4 ± 2.6	0	0
SHAM + PPB ($n = 6$)	45.7 ± 1.16	47.6 ± 1.04	0	0
TBI + PEG ($n = 4$)	$40.3 \pm 0.40^{**}$	43.4 ± 0.95	$0.27 \pm 0.15^{*}$	0.006 ± 0.006
TBI + PPB ($n = 6$)	$42.6 \pm 0.87^{*}$	46.3 ± 0.96	$0.11 \pm 0.07^{*}$	0.01 ± 0.008

PPB, propylparaben; PEG, polyethylene glycol; TBI, traumatic brain injury. Values represent mean ± SE in mm^3. $^{*}p < 0.05$; $^{**}p < 0.01$ versus Sham + PEG.

$p < 0.01$ versus Sham + PEG group) and contralateral to the trauma (hilus, $p < 0.05$; DG, $p < 0.01$; CA1, $p < 0.01$ versus Sham + PEG group). When compared with the TBI + PEG group, animals from the TBI + PPB group demonstrated a high neuronal preservation in both ipsilateral (DG, $p < 0.05$; hilus, $p < 0.001$; CA1, $p < 0.01$) and contralateral to the trauma (DG, $p < 0.001$; hilus, $p < 0.05$; CA1, $p < 0.05$; CA3, $p < 0.05$) (Table 2, Fig. 5). Concerning MRI evaluation, the ipsilateral hippocampus showed lower volume (12%, $p < 0.05$ versus Sham + PEG) and less damage ($p < 0.05$ versus Sham + PEG). These changes were similar to those found in the TBI + PEG group (Table 3).

DISCUSSION

Severe TBI induced with the LFP model in rats is known to result in significant permanent motor and cognitive deficits [49, 50]. These alterations are the consequence of induced injury in different brain areas [51, 52]. In the present study, animals with severe TBI showed a significant sensorimotor deficit as well as hippocampal cell damage and hyperexcitability, conditions that were diminished when subchronic PPB was given after the trauma. These results support that PPB induces neuroprotection after TBI.

In experimental models of TBI, 17β-estradiol induces neuroprotection [53] by the reduction of oxidative stress [54], apoptosis [55], and pro-inflammatory cytokines [56]. Indeed, the administration of 17β-estradiol is used to induce neuroprotection for different neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [57]. The neuroprotection mediated by PPB can be explained due to its estrogenic effect [58]. PPB interacts with the estrogen receptor (30,000 times less potent than 17β-estradiol) [59] and exerts a stimulatory action on the expression of estrogen receptors [60].

TBI induces delayed glial activation [61], cerebral accumulation of amyloid-β (Aβ) protein, and oxidative stress [62]. In addition, TBI results in enhanced release of glutamate [13], a condition that elicits neuronal death [63], inflammation [64], and oxidative stress [65]. According to this information, TBI represents a potential risk factor for Alzheimer's disease [66]. Indeed, enhanced extracellular accumulation of glutamate is promoted by soluble oligomers of the Aβ protein accumulated in Alzheimer's disease brain [67].

The blockage of Na^+ channels is a strategy to reduce the neurodegeneration induce by glutamate [68]. The blockage of Na^+ channels can be related with a decrease in Ca^{+2} influx [69]. Moreover, the attenuation of intracellular Ca^{+2} is associated with a reduction of the TBI-induced diffuse axonal injury, thus avoiding the aberrant connections between different brain areas [70]. PPB is a voltage-dependent sodium channel blocker [38] that induces neuroprotective effects in experimental models of *status epilepticus* [36, 37]. The neuroprotective effect induced by PPB in TBI is similar to that achieved with the administration of other voltage-dependent Na^+ channel blockers [71]. Recently it was described that PPB is a blocker of $hNa_V1.2$ channels, sharing the mechanism of action of most of sodium channel blocking antiseizure drugs [39]. The neuroprotection induced by PPB can be associated with the blockage of $hNa_V1.2$ channels that inhibit glutamate-induced apoptosis through the modulation of the Bcl-2/Bax-dependent cell death pathways [68]. This mechanism explains the reduced extracellular levels of glutamate and neuroprotection found in the hippocampus of rats with status epilepticus and administered with PPB [36].

Some studies indicate that severe TBI can cause hippocampal hyperexcitability [29–31]. Our experiments showed hippocampal hyperexcitability (low ADT values), as well as the spreading of the

ADT-induced after-discharge to other brain areas as a result of TBI. Consistent with the histological evaluation, as well as with previous studies [36], these effects were not observed when PPB was subchronically administered after TBI. These results can be explained by the neuroprotective effect induced by PPB. However, the MRI evaluation of the hippocampal volume and damage showed no significant differences in the TBI groups, with and without PPB. These findings suggest that although PPB reduces the TBI-induced neuronal damage, changes in other cellular components—such as axons and dendrites, which may be involved in the cerebral volume—are not prevented [72, 73]. Therefore, additional studies are necessary for a better understanding of this implication.

Our findings are in agreement with other studies indicating that parabens induce neuroprotective effects. Methylparaben applied i.p. reduces the neurotoxicity and cognitive impairment induced by 6-hydroxydopamine in rats [74]. However, further research is essential to determine the optimal regimen of administration. In humans, PPB applied v.o. would achieve low concentrations in the brain as consequence of its faster metabolism in liver and short half-life (2.9 h) [75]. In rats, PPB administered v.o, presents a half-life of 90 min. However, beneficial effects in central nervous system (CNS) have been obtained when PPB is administered i.p. Talevi et al. (2007) found that PPB applied i,p, induces anticonvulsant effects in mice [76]. We demonstrated that PPB administered i.p. induces neuroprotection in rats submitted to status epilepticus [36, 37]. Pharmacokinetic studies of PPB applied i.p. are essential to clarify how it induces effects in CNS.

According to the data obtained in the present study, it is possible to support that PPB represents a drug with a potential therapeutic effect in subjects with TBI to prevent the development of long-term disorders such as Alzheimer's disease, which is highly associated to hippocampal damage [77]. Different mechanisms can be involved in the PPB-induced neuroprotection (Fig. 7). However, findings suggest that PPB chronically applied induces endocrine-disrupting potential that can contribute to breast cancer progression, tumors, birth defects, adverse reproductive outcomes, and developmental disorders due to its estrogenic effects [78, 79, 59, 60]. Studies also indicate that PPB can be toxic to liver cells due to the increased production of superoxide anions in liver cells [80]. In contrast, other studies point out that the toxicity of parabens

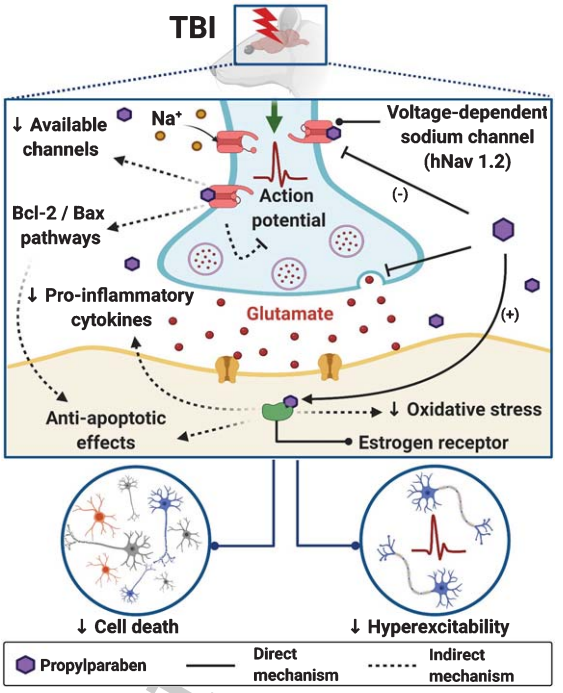

Fig. 7. Proposed direct and indirect mechanisms of neuroprotective effects of propylparaben (PPB) in severe traumatic brain injury (TBI). The blockage of hNaV1.2 channels by PPB may avoid the enhanced glutamate release that results as consequence of brain damage or hyperexcitability. The blockage of hNaV1.2 channels can also reduce the subsequent apoptosis through the modulation of Bcl-2/Bax pathways. PPB may also induce neuroprotection by activation estrogen receptors. All these mechanisms could induce neuroprotection and avoid the TBI-induced long-term consequences.

in humans has not been established and these drugs would need to be tested rigorously for safety [81]. Future studies using experimental models are essential to confirm that subchronic administration of PPB after severe TBI prevents the development of cognitive and memory impairment, as well as neurological disorders (post-traumatic epilepsy, Parkinson's disease and Alzheimer's disease) without side effects [18, 19, 82].

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