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Exogenous T3 administration provides neuroprotection in a murine model of traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) induces primary and secondary damage in both the endothelium and the brain parenchyma. While neurons die quickly by necrosis, a vicious cycle of secondary injury in endothelial cells exacerbates the initial injury. Thyroid hormones are reported to be decreased in patients with brain injury. Controlled cortical impact injury (CCI) is a widely used, clinically relevant model of TBI. Here, using CCI in adult male mice, we set to determine whether 3,5,3′-triiodothyronine (T3) attenuates posttraumatic neurodegeneration and neuroinflammation in an experimental model of TBI. Treatment with T3 (1.2 μg/100 g body weight, i.p.) 1 h after TBI resulted in a significant improvement in motor and cognitive recovery after CCI, as well as in marked reduction of lesion volumes. Mouse model for brain injury showed reactive astrocytes with increased glial fibrillary acidic protein, and formation of inducible nitric oxide synthase (iNOS). Western blot analysis revealed the ability of T3 to reduce brain trauma through modulation of cytoplasmic-nuclear shuttling of nuclear factor-κB (NF-κB). Twenty-four hours after brain trauma, T3-treated mice also showed significantly lower number of TUNEL(+) apoptotic neurons and curtailed induction of Bax, compared to vehicle control. In addition, T3 significantly enhanced the post-TBI expression of the neuroprotective neurotrophins (BDNF and GDNF) compared to vehicle. Our data provide an additional mechanism for the anti-inflammatory effects of thyroid hormone with critical implications in immunopathology at the cross-roads of the immune-endocrine circuits.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide [1]. The diagnosis of TBI includes a broad range of short and long-term physical, cognitive, and emotional impairments, depending on the severity of the injury [2,3]. The initial mechanical damage on the brain leads to blood–brain barrier (BBB) disruption, development of cerebral edema, and subsequent increase of intracranial pressure [4]. The secondary injury is accompanied by an inflammatory response and intrathecal cellular release of cytokines, which results in additional delayed neuronal cell death, and finally leading to secondary expansion of the primary lesion [5,6]. Thyroid hormones are reported to be decreased in patients with brain injury [7]. Thyroid hormones play an essential role in brain development, are necessary for optimal cellular repair after injury and for normal homeostasis. Severe TBI associated with basilar skull fracture, hypothalamic edema, prolonged unresponsiveness, hyponatremia, and/or hypotension is associated with a higher occurrence of endocrinopathy [8]. Endocrine failure may produce clinically important consequences during acute and convalescent care after TBI, and may be caused by direct injury to the hypothalamic–pituitary axis (HPA), neuroendocrinological effects from catecholamines and cytokines, or from systemic infection/inflammation. Thyroid hormone metabolism has been reported to be abnormal in patients with TBI, with an impairment of L-thyroxine (T4)-to-3,5,3′-triiodothyronine (T3) conversion resulting in a low T3 state.

Thyroid hormone has a broad spectrum of actions on the nervous system. These relate to brain development and morphogenesis and to a variety of brain functions and to behaviors of the intact organism. The molecular basis for such actions includes modulation of expression of specific genes, regulation of energetics in the mitochondrion and a variety of non-genomic actions on ion transport systems, on the cytoskeleton, intracellular

Abbreviations: BDNF, brain deriverd neurotrophic factor; CCI, controlled cortical impact; EBST, elevated body swing test; GDNF, glial cell line derived neurotrophic factor; GFAP, glial fibrillary acidic protein; HPA, hypothalamic–pituitary axis; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappaB; NO, nitric oxide; T4, L-thyroxine; T3, 3,5,3′-triiodothyronine; TBI, traumatic brain injury; TTC, 2,3,5-triphenyltetrazolium chloride.

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protein trafficking, on specific kinase activation and specific protein phosphorylation, and on cell migration and angiogenesis [9,10].

T3 can be derived from (i) conversion of the prohormone T4 by outer ring (5′-) deiodination in the peripheral tissues, (ii) by T4 to T3 conversion within the thyroid gland, and (iii) by direct secretion of de novo synthesized thyroidal T3. Estimates of the contribution of extrathyroidal T4–T3 conversion to the total T3 pool vary from 20% to 100% in the rat [11–13].

Both hypothyroidism [14–17] and thyroid hormone administration [18] have been reported to be protective in the setting of brain injury. However, it is presently impractical clinically to induce hypothyroidism acutely. Reduction of hippocampal neuronal damage from ischemia with repeated daily T4 administration was shown by Rami and Krieglstein [18], with approximately a 50% increase in neuronal density attributable to hormone treatment.

Several studies by Mendes-de-Aguiar et al. [19] reported that T3 at 10^{-8} M eliminated the "gliotoxic" effect of glutamate on cultured cerebellar astrocytes from newborn rats and ensured viability of astrocytes and of neurons co-cultured with the astrocytes. The mechanism involved in these observations included increased astrocyte uptake of glutamate. Moreover, Losi et al. [20] showed that T3 protected rat hippocampal neurons against glutamate toxicity by a non-genomic mechanism. Thus, the defense against glutamate toxicity in neurons and glial cells imposed by T3 may be both genomic and non-genomic in mechanism [9,10].

The pathophysiology of TBI has been a focus of extensive studies in recent years; animal models have proved to be important tools in this field, and are employed to investigate the mechanisms of primary and secondary injury. In the CCI model, the injury to the brain initially presents as necrotic cell death in the underlying tissue and white matter axonal injury, both reminiscent of the clinical TBI pathology, but also followed by apoptotic cell death in surrounding tissue due to multiple subsequent events such as edema, ischemia, excitotoxicity and altered gene expression [21]. The failure of therapies targeted only to neuronal protection is attributable, in part, to the lack of concomitant protection of cerebral blood vessels from the secondary injury of inflammation and reactive oxygen species/reactive nitrogen species stress. Several studies report that "low-T3 syndrome" occurs during the acute phase of TBI [22–24].

To counteract the damage produced by TBI, we used T3 1 h after TBI as a new therapeutic approach. In particular, we examined the following endpoints: (1) behavioral alteration (motor as well as anxiety activity), (2) edema and brain infarctions, (3) glial fibrillary acidic protein (GFAP) expression, (4) inducible nitric oxide synthase (iNOS) expression, (5) neurotrophins, (6) NF-κB activation, and (7) apoptotic pathway.

2. Materials and methods

2.1. Animals

Male CD1 mice (6–7 weeks old) weighing 25–30 g were kept five per cage under a constant 12-h light/dark cycle, at room temperature (23 °C). Food and water were available *ad libitum*. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. Controlled cortical impact (CCI) experimental TBI

TBI was induced in mice ($n = 30$ per group) by controlled cortical impactor. A craniotomy was made in the right hemisphere encompassing bregma and lambda and between the sagittal suture and the coronal ridge with a Micro motor hand piece and drill (UGO Basile S.R.L., Comerio VA, Italy). The resulting bone flap was removed, and the craniotomy enlarged further with cranial rongeurs. A cortical contusion was produced on the exposed cortex using a controlled impactor device Impact One™ Stereotaxic impactor for CCI (myNeurolab.com, Richmond), as described by Ahmad et al. [25]. Briefly, 4-mm craniotomy was performed on the right side of the skull to expose the dura, with the center of the opening located 3 mm posterior to the bregma and 2.5 mm lateral to the midline. The duration of impact was 50 ms. The impact tip was advanced farther to produce a brain injury of moderate severity for mice (tip diameter, 4 mm; cortical contusion depth, 3 mm; impact velocity, 1.5 m/s).

Immediately after injury, the skin incision was closed with nylon sutures, and 2% lidocaine jelly was applied to the lesion site to minimize any possible discomfort.

Mice were administered T3 (1.2 μg/100 g body weight) 1 h after TBI, or vehicle. T3 was purchased from EMD Biosciences (San Diego, CA). The dose and the administration route of T3, used in this study, were based on previous *in vivo* study [26]. The timing of ip T3 injection at 1 h after TBI, between the primary and secondary injury, has the potential to either prevent or to reduce the final neurological deficit. Several recent results illustrate the importance of initiating therapeutic interventions as soon as possible following TBI, preferably within 4 h post-injury, to achieve the best possible neuroprotective effect [27]. Sham mice underwent the same surgical procedure including anesthesia and craniotomy but were not injured.

Two sets of experiments were performed. The first set was to investigate the protective effects of T3 treatment. All animals were randomized into 1 of 4 groups:

(i) *TBI + vehicle*: mice were subjected to CCI and vehicle was administered at 1 h after TBI;
(ii) *TBI + T3 group*: mice were subjected to CCI and T3 (1.2 μg/100 g body weight) was administered at 1 h after TBI;
(iii) *Sham + vehicle group*: mice were subjected to the surgical procedures as above group (anesthesia and craniotomy) except that the impact tip was not applied and vehicle was administered at 1 h after craniotomy;
(iv) *Sham + T3 group*: mice were subjected to the surgical procedures as above group (anesthesia and craniotomy) except that the impact tip was not applied and T3 (1.2 μg/100 g body weight) was administered at 1 h after craniotomy.

Testing at 24 h following TBI was completed as follows: (1) TTC staining ($n = 10$ mice/group); (2) mast cells staining ($n = 10$ mice/group); (3) TUNEL assay ($n = 5$ mice/group); and (4) Western blot analysis ($n = 5$ mice/group).

In the second set of experiments, additional animals were used for Elevated Plus Maze (1 day, 2 days, 3 days, 6 days and 10 days post-TBI), rotarod test (at 24 h after TBI), brain water content (at 24 h after TBI), and swing test (EBST, at 24 h after TBI).

2.3. Neurological function evaluation

2.3.1. Elevated Plus Maze (EPM)

Since anxiety tends to be one the most prevalent neurobehavioral conditions after TBI, anxiety deficits in a TBI model of neurotrauma were evaluated using Elevated Plus Maze system at 1 day, 2 days, 3 days, 6 days and 10 days post-TBI, and compared with Sham injured controls. In this test, anxiety is reflected by the avoidance of the open arms of the maze [28]. The EPM is made of dark gray plastic and consists of two opposing open (30 cm × 5 cm, 300 lux) and two opposing closed arms (30 cm × 5 cm × 15 cm, 10 lux) connected by a central platform (5 cm × 5 cm, 90 lux). The EPM was located 50 cm above the floor and surrounded by a black curtain. Each trial lasted for 5 min and the apparatus was cleaned before

each test session. The mouse was placed on the central platform facing an open arm. Behavior was videotaped with a camera fixed above the EPM. The percentage of time spent on the open arms was scored by a researcher blind of the animal treatment.

2.3.2. Rotarod test

An accelerating rotarod was used to measure motor function and balance. The rotarod treadmill (Accuscan, Inc., Columbus, OH, USA) provided a motor balance and coordination assessment. Each animal was placed in a neutral position on a cylinder (1 cm diameter for mice), then the rod was rotated with the speed accelerated linearly from 0 rpm to 24 rpm within 60 s, and the time spent on the rotarod was recorded automatically. The maximum score given to an animal was fixed to 60. For testing, animals were given 3 trials and the average score on these 3 trials was used as the individual rotarod score.

2.3.3. Swing test (EBST)

The elevated body swing test (EBST) provided a motor asymmetry parameter and involved handling the animal by its tail and recording the direction of the biased body swings. The EBST consisted of 20 trials with the number of swings ipsilateral and contralateral to the injured hemisphere recorded and expressed in percentage to determine the biased swing activity [29].

2.3.4. Measurement of edema (brain water content)

At 24 h following TBI, animals were euthanized to determine brain water content (edema), as previously reported [25]. The cortices, excluding the cerebellum, were quickly removed, and the contralateral and ipsilateral hemispheres separately weighed. Each hemisphere was dried at 60 °C for 72 h, and the dry weight was determined. Water content was calculated in ipsilateral hemisphere as:

Water content (%) = (wet weight − dry weight)/wet weight × 100.

2.4. Evaluation of infarction using TTC staining

To evaluate infarct, the 2,3,5-triphenyltetrazolium chloride (TTC) staining technique was used. Briefly, the mice were killed by decapitation at 24 h following TBI. The brains were quickly removed and placed in ice cold Saline for 5 min. Six serial sections from each brain were cut at 2-mm intervals from the frontal pole using rodent brain matrix (ASI Instrument Inc., Warren, MI). The sections were incubated in 2% TTC saline solution for 30 min at 37 °C. The stained brain sections were stored in 10% formalin and refrigerated at 4 °C for further processing and have taken picture.

2.5. Staining of mast cells

Brain sections were cut 5 mm thick and stained with 0.25% toluidine blue, pH 2.5, for 45 min at room temperature. The sections were then dehydrated and mounted in xylene-based medium for viewing. Three non-sequential sections were chosen from one random block from each brain for examination. All sections were evaluated at 200×, while some sections were photographed at 400× using a Nikon inverted microscope. Identification of mast cells and their cytoplasmic granules was carried out following the application of toluidine blue staining protocols.

2.6. Terminal deoxynucleotidyltrasferase-mediated UTP end labeling (TUNEL) assay

TUNEL staining was quantified on stained sections from the injury core at the level 0.74 mm from the bregma. TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag, HRP kit DBA, Milan, Italy). Sections were incubated with 15 mg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37 °C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL positive cells/high-power field was counted in 5–10 fields for each coded slide.

2.7. Western blot analysis for GFAP, Bax, Bcl-2, NF-κB, iNOS, BDNF and GDNF

Brains from injured or Sham animals were collected 1 day post CCI or Sham surgery, and a 4 mm coronal section from the injured area over the parietal cortex and ipsilateral hippocampus were collected. Cytosolic and nuclear extracts were prepared as previously described [30] with slight modifications. Tissues from each mouse were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 mM pepstatin A, 20 mM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1000 × *g* for 10 min at 4 C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 mm leupeptin, 0.2 mM sodium orthovanadate. After centrifugation 30 min at 15,000 × *g* at 4 C, the supernatants containing the nuclear protein were stored at −80 C for further analysis. NF-κB p65 translocation was quantified in nuclear fraction. The filters were blocked with 1× PBS, 5% (w/v) non fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs anti-Bax (1:500; Santa Cruz Biotechnology), anti-Bcl-2 (1:500; Santa Cruz Biotechnology), anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology), anti-iNOS (1:200; BD transduction), anti-phospho-P38 (1:1000; Cell Signaling), anti-GDNF (1:500; Santa Cruz Biotechnology), anti-BDNF (1:500; Santa Cruz Biotechnology), anti-GFAP (1:500; Santa Cruz Biotechnology), in 1× PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT) at 4 °C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of tissue lysates, they were also incubated in the presence of the antibody against β-actin (1:10,000; Santa Cruz Biotechnology). The relative expression of the protein bands of Bax (∼23 kDa), Bcl-2 (26 kDa), GFAP (50 kDa), NF-κB p65 (∼65 kDa) and iNOS (∼130 kDa), BDNF (14 kDa), and GDNF (15 kDa) was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM).

2.8. Statistical methods

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of *N* observations. For the *in vivo* studies *N* represents the number of animals studied. In the experiments involving histology, the figures shown are representative of at least three experiments performed on different experimental days. A *P*-value of less than 0.05 was considered significant. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparison.

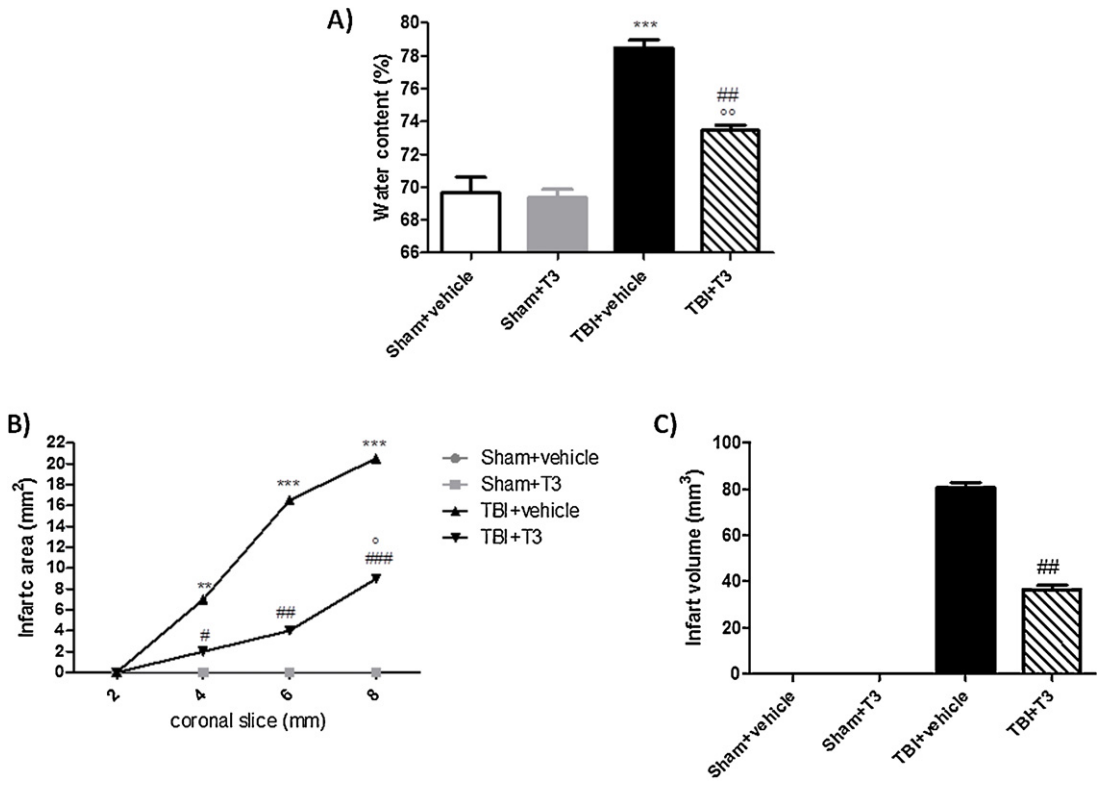

Fig. 1. T3 reduces both edema and brain infarctions following TBI. Brain edema was assessed by brain water content in the ipsilateral hemisphere. At 24 h after TBI showed increase in levels of water content in the TBI brain (A), while the treatment with T3 decreased the water content in the TBI brain (A). Moreover, TTC stained brain section (3 out of the six consecutive sections from cranial to caudate region) corresponding to largest infraction (data not show) from each group. Brain sections (2 mm thick) were stained with TTC at 24 h after TBI to show significant reduction infarct area (B) and volume (C). The figures are representative of at least three experiments performed on different experimental days. Each data are expressed as mean ± SEM from $N = 10$ male CD mice for each group. A P-value of less than 0.05 was considered significant. $^{***}P < 0.001$ vs. Sham + veh, $^{\#\#}P < 0.01$ vs. TBI + vehicle, and $^{\circ}P < 0.05$ and $^{\circ\circ}P < 0.01$ vs. Sham + T3.

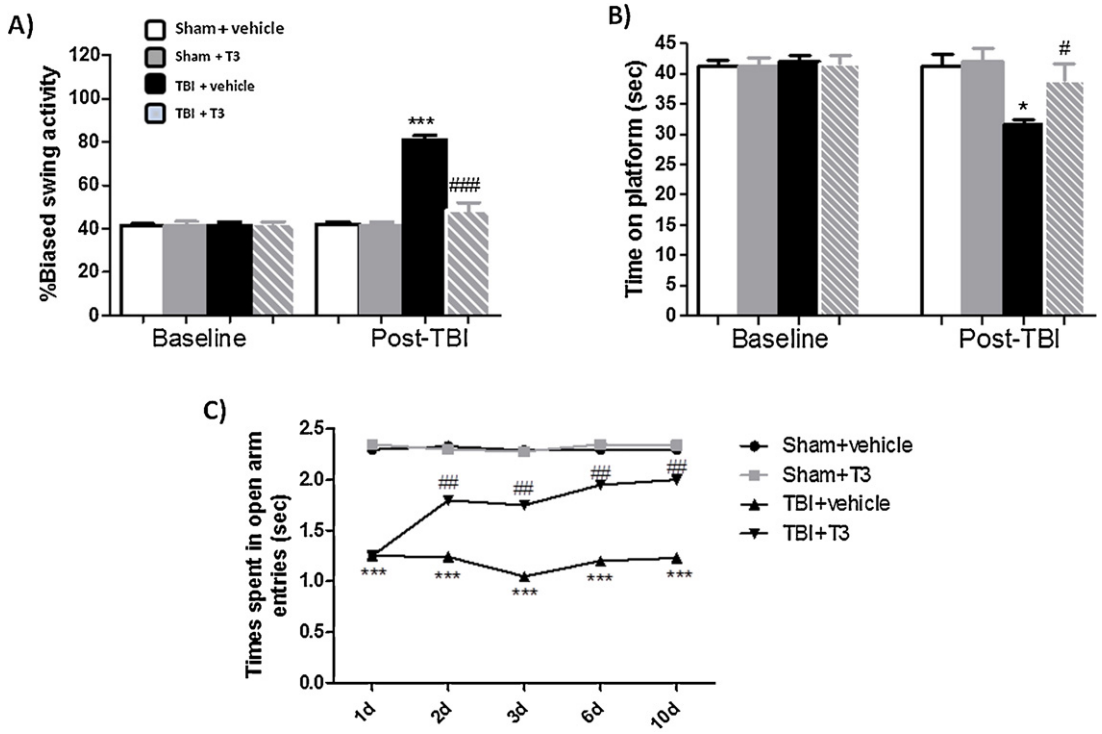

Fig. 2. T3 aids recovery and improves behavioral function. At 24 h after TBI, animals showed significant impairments in motor deficits as revealed by significantly biased swing activity (A) and shortened time to stay on rotarod (B). On the contrary treatment with T3 1 h post-TBI significantly improved motor function evaluated by EBST (A) and rotarod task (B). Moreover, T3 improves cognitive function (C) as evaluated by Elevated Plus Maze test at 1, 2, 3, 6 and 10 days after TBI. Each data are expressed as mean ± SEM from $N = 10$ male CD mice for each group. A P value of less than 0.05 was considered significant. $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. Sham + veh, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. TBI + veh, and $^{\circ\circ}P < 0.001$ vs. Sham + T3.

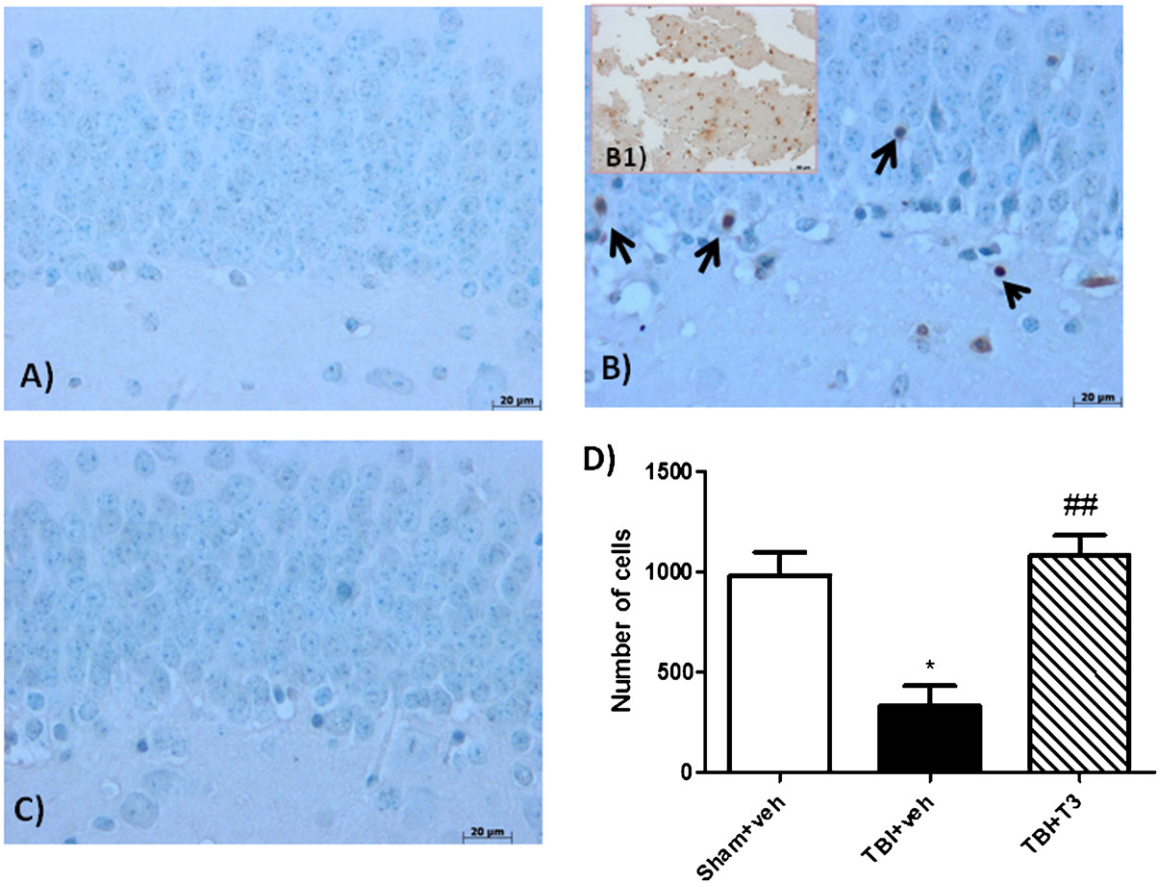

Fig. 3. T3 reduces MCs infiltration. In order to identify the presence of mast cells the tissues slides were also stained with acidified toluidine blue. No granules were found in the brain tissues from control mice (A). On the contrary, a significant presence of MCs, mainly localized in the perivascular area, have been observed in the brain tissues collected at 24 h after injury (B, B1). T3 treatment significantly reduced MC infiltration in the brain tissue after TBI (C). The figures are representative of at least three experiments performed on different experimental days. Quantitative evaluation of the number of MCs was performed, and graphic representation of the number of toluidine blue-stained MCs was shown in panel D. $^{*}P < 0.05$ Sham + veh, $^{\#\#}P < 0.01$ vs. TBI + veh. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Reduction in both edema and brain infarctions following TBI

Brain water content is a sensitive measure of cerebral edema. This measure indicates pathology associated with endothelial cell activation and endothelial dysfunction. As shown in Fig. 1A water content was significantly different between groups overall with levels significantly higher in animals subjected to TBI compared to Sham + T3 group. The increased water content in the ipsilateral brain induced by TBI was significantly decreased by T3 treatment at 24 h post-injury. Directly related to overall brain injury, measurement of brain infarctions is a standard method to evaluate ischemic injury after stroke. To evaluate the effect of T3 on brain infarctions in the TBI brain, we performed TTC staining. The infarction area (Fig. 1B), and infarct volume (Fig. 1C) were significantly reduced by treatment with T3.

3.2. Recovery and improvement in behavioral function

Mice subjected to moderate severe injury showed significant hippocampal damage, behavioral deficits and low mortality. Mice were sacrificed 24 h after TBI. To investigate the relationship between neurological deficits in the setting TBI, we used two different tests in two different steps (Fig. 2). In the first step to assess motor function mice were subjected 24 h after TBI to the EBST, rotarod test, considered the most sensitive vestibulomotor measure. CCI-injured mice displayed a range of impairments in locomotor tasks. T3 treatment significantly improved latency compared to TBI + veh group. In the second step, since anxiety is considered a critical component of behavioral change after brain injury, CCI injured mice were subjected to the EPM at different time: 1 day, 2 days, 3 days, 6 days and 10 days and mice were administered T3 treatment every day. The daily treatment of T3 after TBI significantly improved latency compared to TBI + veh group (Fig 2).

3.3. T3 reduces MCs infiltration

In the brain tissues, collected 24 h after TBI (Fig. 3B and B1), MCs cells were observed in the perivascular area. Please note that in Fig. 3B the arrow indicates the presence of mast cell and arrowhead showed the presence of MCs in degranulation phase. Cells showing to have metachromatic granules when stained with acidified toluidine blue were identified as mast cells. On the contrary, significant less MCs density and degranulation were observed in the brain tissues after TBI collected from mice which have been treated with T3 (Fig. 3C). No granules were found in the brain tissues from Sham-operated mice plus T3 (Fig. 3A). There is no difference between T3-treated Sham animals and Sham animals + vehicle (data not shown).

3.4. T3 reduces reactive astrocytes with increased GFAP

In order to better study the cellular changes in the brain, we investigate the expression of GFAP, which has been implicated in

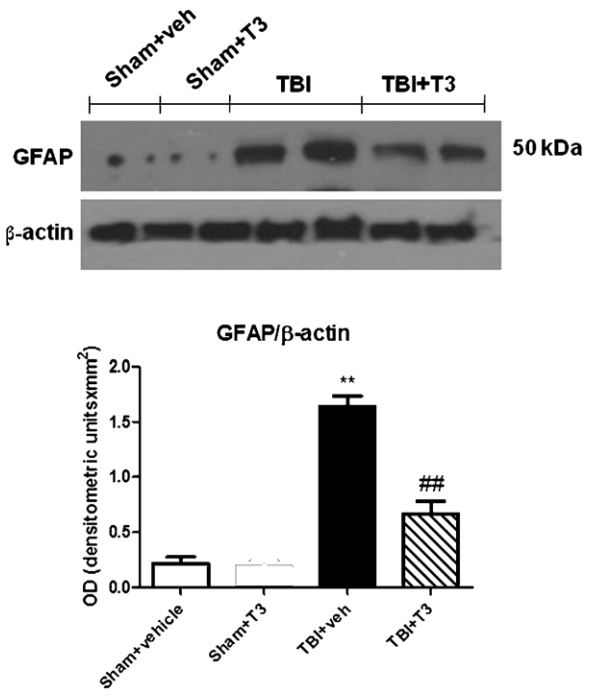

Fig. 4. Effect of T3 on GFAP expression after TBI. GFAP is an intermediate filament protein that is expressed by numerous cell types of the central nervous system (CNS) including astrocytes. GFAP has also been found to be increased by TBI. T3 treatment reduced the expression of GFAP (50 kDa) in the brain tissue. Densitometry analysis GFAP from brain tissues was assessed. $^{**}P < 0.01$ vs. Sham + veh and $^{\#\#}P < 0.01$ vs. TBI + veh.

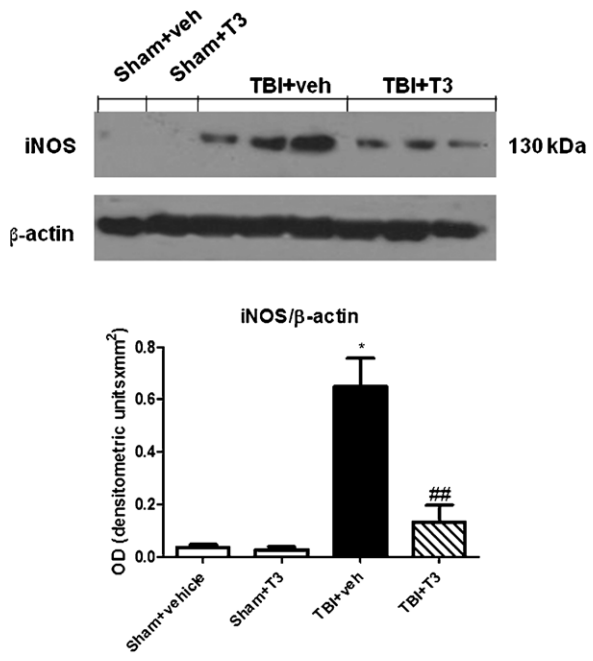

Fig. 5. Effect of T3 on iNOS expression. By Western blot analysis, a significant increase in iNOS expression was observed in the brain from mice subjected to TBI. On the contrary, T3 treatment prevented the TBI-induced iNOS expression (A). β-actin was used as internal control. A representative blot of lysates obtained from each group is shown, and densitometry analysis of all animals is reported ($n = 10$ mice from each group). A P-value of less than 0.05 was considered significant. $^{*}P < 0.05$ vs. Sham + veh and $^{\#\#}P < 0.01$ vs. TBI + veh.

the pathogenesis of TBI. As shown in Fig. 4 (see also densitometric analysis), there was a strong induction of GFAP localized in the brain tissues from mice subjected to TBI. T3 treatment (1.2 μg/100 g body weight) is able to reduce the expression of GFAP (Fig. 4, see densitometric analysis) in the injured brain. T3 did not modify the expression of GFAP in Sham animals

3.5. T3 modulates the expression of iNOS after TBI

To determine the role of NO produced during TBI, iNOS expression was evaluated by Western blot (Fig. 5). A significant increase in iNOS expression was observed in the brain from mice subjected to TBI. On the contrary, T3 treatment blunted TBI-induced iNOS expression (Fig. 5, see densitometry analysis).

3.6. Effects of T3 on apoptosis in brain after TBI

To test whether brain damage was associated with cell death by apoptosis 24 h after TBI, the appearance of effectors of canonical mitochondrial apoptosis, such as pro-apoptotic and anti-apoptotic proteins, was investigated by Western blot. Bax expression was appreciably increased in the brain from mice subjected to TBI (Fig. 6A). On the contrary, T3 treatment (1.2 μg/100 g body weight) reduced the TBI-induced Bax expression (see densitometry analysis). Moreover, T3 treatment attenuated the loss of expression for Bcl-2 in brain from TBI subjected mice (Fig. 6C, see densitometry analysis). Moreover, TUNEL-like staining in the perilesional brain tissue was performed. Almost no apoptotic cells were detected in the brain from Sham-operated mice (see positive cell count, Fig. 7). Twenty-four hours after the trauma, brain tissues from mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (see positive cell count, Fig. 7). In contrast, tissues obtained from mice treated with T3 demonstrated POCHE no apoptotic cells or fragments.

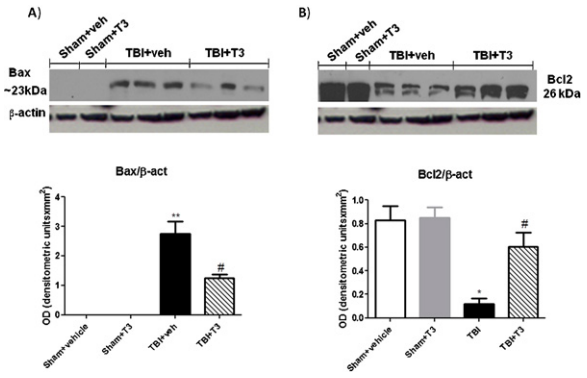

Fig. 6. Effects of T3 on Bax and Bcl-2 in brain tissue after TBI. Western blot analysis shows that TBI caused a significant increase in Bax expression (A) in the brain tissues and T3 treatment significantly reduced the propatotic Bax expression in the brain at 24 h after injury. In addition, Bcl-2 expression was significantly reduced in TBI-injured mice at 24 h (B). T3 treatment significantly restored Bcl-2 expression in the brain. A P-value of less than 0.05 was considered significant. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham + veh, and $^{\#}P < 0.05$ vs. TBI + veh.

3.7. T3 treatment restored BDNF and GDNF expression

BDNF and GDNF expression decreased at 24 h after TBI. T3 significantly enhanced the post-TBI expression of the neuroprotective neurotrophins BDNF (Fig. 8A), and GDNF (Fig. 8B) compared to vehicle.

3.8. Effect of T3 treatment on NF-κB p65 translocation

To investigate the cellular mechanisms whereby treatment with T3 attenuates the development of TBI, and since NF-κB pathway is directly involved in TRβ1 expression, we evaluated the translocation of p65 by Western blot analysis. NF-κB p65 translocation in the brain nuclear fractions was significantly increased 24 h after

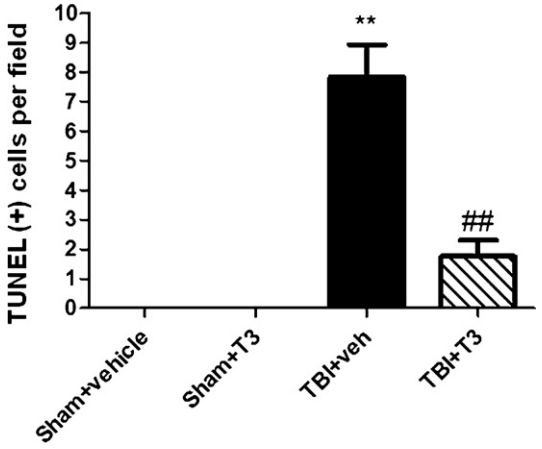

Fig. 7. Effect of T3 on the last phase of apoptosis. TBI induced DNA fragmentation as shown by labeling the terminal end of nucleic acids. Mice treated with T3 demonstrated less appearance of dark brown apoptotic cells and intercellular apoptotic fragments. $^{**}P < 0.01$ vs. Sham + veh and $^{\#\#}P < 0.01$ vs. TBI + veh.

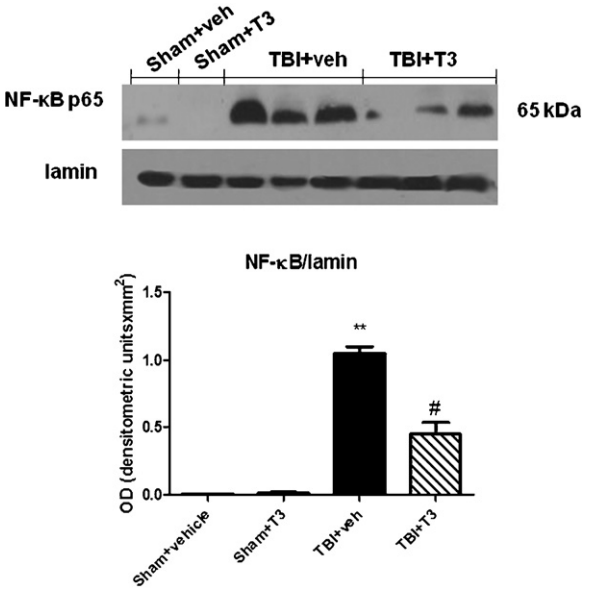

Fig. 9. Effect of T3 on p65 translocation. TBI caused a significant increase in translocation of p65 into the nucleus compared to the control tissue. T3 treatment significantly reduced the translocation of p65. Lamin was used as internal control for nuclear extract. A representative blot of lysates obtained from each group is shown. The relative expression of the protein bands from three separated experiments was standardized for densitometric analysis to housekeeping genes. A *P*-value of less than 0.05 was considered significant. $^{**}P < 0.01$ vs. CTR and $^{\#}P < 0.05$ vs. TBI + vehicle.

TBI compared with control mice (Fig. 9). T3 treatment (1.2 μg/100 g body weight) significantly reduced the translocation of p65 in the nucleus (Fig. 9).

4. Discussion

TBI is a major health and socioeconomic problem that affects all societies. TBI is more common in young adults, particularly men (75%), which causes high costs to society because of life years lost due to death and disability [31]

Factors influencing the outcome of brain injury are numerous and part of a complex network.

Current treatment of acute TBI includes surgical intervention and supportive care therapies. Treatment of elevated intracranial pressure and optimizing cerebral perfusion are cornerstones of current therapy. These approaches do not directly address the secondary neurological sequelae that lead to continued brain injury

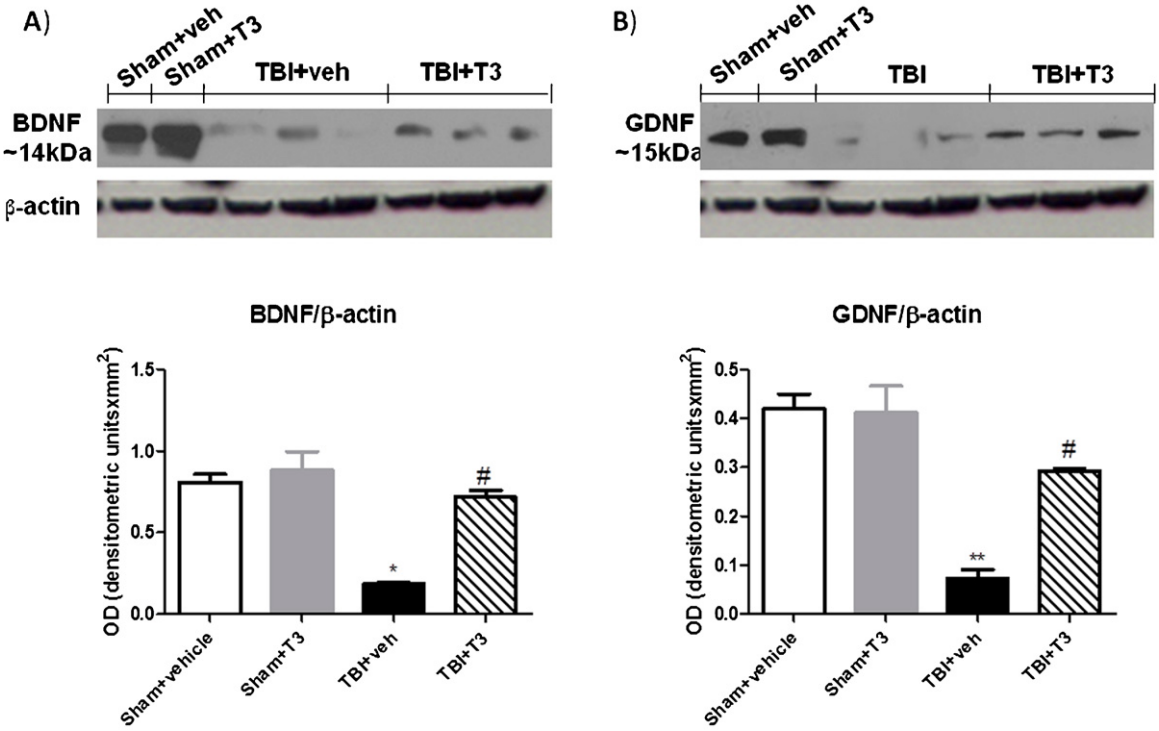

Fig. 8. Western blot analysis for GDNF and BDNF expression. By Western blot analysis, a basal level of BDNF (A) and GDNF (B) expression was detected in brain samples from controls. BDNF and GDNF levels were significantly reduced in brain samples from TBI-vehicle mice. T3 treatment significantly reduced the TBI-induced inhibition of GDNF and BDNF expression. The results in panel a1 and b1 are expressed as mean ± SEM from $n = 5/6$ brain for each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham + veh, and $^{\#}P < 0.05$ vs. TBI + veh.

after TBI. Depending on injury severity, a complex cascade of processes are activated and generate continued endogenous changes affecting cellular systems and overall outcome from the initial insult to the brain. Homeostatic cellular processes governing calcium influx, mitochondrial function, membrane stability, redox balance, blood flow and cytoskeletal structure often become dysfunctional after TBI. Interruption of this cascade may be the target of numerous pharmacotherapeutic agents. While progesterone and ciclosporin have shown promise in phase II studies, success in larger phase III, randomized, multicentre, clinical trials are pending. Consequently, no neuroprotective treatment options currently exist that improve neurological outcome after TBI. For this reason a CCI injury methods were employed to mimic the TBI deficits. Animal models of TBI using the CCI technique are physiologically relevant to TBI in humans. CCI reproduces many of the features of brain injuries, including motor deficits, memory and neuron loss [21,32–34]. The severity of injury can be controlled by altering the velocity and depth of the impact and the size of the impactor tip [35]. It is recognized that very severe injury involves several pathways, thus making it difficult to delineate the critical ones. Within minutes or hours following a traumatic event, a dramatic increase in free radical saturates endogenous scavenging mechanisms leading to the breakdown of membrane lipids, essential proteins, and DNA ultimately leading to cell death [36]. However, even greater apoptotic neuronal loss occurs hours and days later, caused by secondary injury from cerebral ischemia/hypoxia as well as inflammatory and oxidative stress. Evidence for the existence of a 'traumatic penumbra' (tissue that is most at risk of secondary ischemic injury and that will be most affected by changes in physiology or therapeutic interventions) has been shown in human traumatic head injury [37]. Cerebral blood flow is approximately half normal following TBI in the first 24 h [38], and alterations in vascular functions are mainly due to endothelial dysfunction and reduced NO bioavailability, which leads to oxidative exacerbations and blood–brain barrier leakage. In the current study we hypothesize an efficacy of T3 in ameliorating the secondary injury components of TBI. We therefore used a moderate TBI model in mice to test its therapeutic potential [21,33,39]. In addition, major depression and anxiety are common comorbidity observed in TBI patients [40]. The neurological evaluation was performed using two different tests, respectively in acute and chronic phase. In particular, in the acute phase rotarod test revealed that T3 improved latency compared to the TBI animals; in chronic EPM showed increased anxiety-like behavior in TBI mice as demonstrated by Baratz et al. [41]. Moreover, here we demonstrate that T3 treatment exerts beneficial effects, improves neurobehavioral functions, reduces apoptotic cell death, and also decreases edema as well as contusion volume. Although hypopituitarism is a known complication of traumatic head injury, it may be under-recognized due to its subtle clinical manifestations. The critical contributions of thyroid hormone to development of the nervous system [42,43], to mature and immature brain glial cell [42,44], and neuronal function [45] and to behavior [46] are well-known. While hypothyroidism has been reported by some observers to be neuroprotective [14,16], repeated administration of T4 has also been shown to protect against brain injury [18] and thyroid hormone is known to support the integrity of brain vasculature [47].

Apoptosis is an important mediator of secondary damage after brain injury. It incurs its affects through at least two phases: an initial phase, in which apoptosis accompanies necrosis in the degeneration of multiple cell types and a later phase, which predominantly involves oligodendrocytes and microglia [48]. In an effort to prevent or diminish levels of apoptosis, we have identified in TBI animals apoptotic transcriptional changes, including upregulation of proapoptotic Bax and down regulation of anti-apoptotic Bcl-2 protein. T3 treatment significantly reduced Bax expression, while on the contrary, restored Bcl-2 expression.

In this study, protein analysis indicated that GDNF and BDNF expressions were down-regulated by TBI in mice, while a local and sustained increase in the GDNF and BDNF expression in the perilesioned tissue following intraperitoneally administration of T3 was shown. Moreover, here we show the inhibitory effect of T3 on the severity of brain injury appears to be mediated by glial cells.

In summary, our data suggest that T3 represents an interesting tool to develop new approaches to the management of brain injury and may be useful in the therapy of conditions associated with TBI.

Thyroid hormone has both genomic [9,49,50] and nongenomic [51,52] effects on the activity of the Na^+/H^+ exchanger and the amino acid transport system A [53]. Both transport systems are activated through a transduction pathway involving PKC, phosphatidyl inositol 3-kinase, and the MAPK pathway [54]. Some of these actions rapidly lead to posttranslational modification of nucleoproteins, *e.g.* serine phosphorylation of the nuclear thyroid hormone receptor TRβ1 [55], estrogen receptor [56], and p53 [57]. These actions on nucleoproteins are mediated by MAPK. Upstream of MAPK, PKC and the phosphatidylinositol pathway may be activated by iodothyronines [58].

Edema formation was clearly visible 24 h after TBI. Brain edema as a result of secondary injury following TBI is a major clinical concern, and T3 treatment reduces the secondary neuronal death by lowering inflammation and edema formation.

TBI mice show astrogliosis. Astrocytes are viewed as playing a role in neuronal support [59]. This is especially important when the brain is challenged with an insult. Their distribution around the infarct forms a physical barrier called the glial scar, which impedes axonal regeneration and accelerates neuronal damage [60].

Although this model only affects neurons in the cortex, hippocampal neurons are vulnerable and may be susceptible to pathological events occurring in neighboring areas [61].

The manifestation of cognitive and behavioral deficits following brain trauma confounds rehabilitation and contribute to a poor quality of life. Remediation of learning and memory deficits remains a significant goal of TBI research; unfortunately, many promising therapies for TBI are limited by a narrow therapeutic window. Standardization of functional outcome parameters in TBI animal models should facilitate the validation and direct comparison between research studies.

Several recent results illustrate the importance of initiating therapeutic interventions as soon as possible following TBI, preferably within 4 h post-injury, to achieve the best possible neuroprotective effect; for this reason we choose to inject T3 at 1 h after TBI, between the primary and secondary injury, in order to reduce the final neurological deficit. The failure of therapies targeted only to neuronal protection is, in part, attributable to the lack of concomitant protection of cerebral blood vessels from the secondary injury of inflammation and accumulating oxidative exacerbations. An inhibition of inflammation and reduction of oxidative exacerbations by a post-stroke injury treatment with the NO-modulating effect of T3 resulted in an increased neurovascular protection, leading to improved neurological symptoms.

Among the inflammatory mediators, iNOS induction has been implicated in TBI [62]. Expression of iNOS has been found near necrotic and inflammatory areas mainly in neutrophils/macrophages, where it plays a crucial role in secondary brain damage subsequent to TBI in humans, and inhibition of iNOS has protected against injury in TBI animal models [63]. We observed that T3 inhibited the expression of iNOS after injury, indicating that this hormone is able to protect the brain against iNOS-mediated neurodegeneration in TBI. The inhibition of NF-κB by T3 indicates that T3 may exert an iNOS inhibitory effect in an NF-κB dependent pathway.

5. Conclusion

Taken together our results suggest that T3 could represent an interesting approach for the management of secondary damage following TBI counteracting behavioral changes and inflammatory process.

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