

## RESEARCH ARTICLE

# Long-Term Effects of Hypoxia-Reoxygenation on Thioredoxins in Rat Central Nervous System

Otero-Losada M<sup>1</sup>, Canepa L<sup>2</sup>, Udovin L<sup>1</sup>, Kobiec T<sup>1,3</sup>, Toro-Urrego N<sup>1</sup>, Kolliker Frers RA<sup>1</sup> and Capani F<sup>1,\*</sup>

<sup>1</sup>Institute of Cardiological Research, University of Buenos Aires, National Research Council, ININCA.UBA-CONICET, Buenos Aires, Argentina; <sup>2</sup>Departamento de Biología, Universidad Argentina John Kennedy (UAJK), Buenos Aires, Argentina; <sup>3</sup>Centro de Investigaciones en Psicología y Psicopedagogía (CIPP), Pontificia Universidad Católica Argentina, Buenos Aires, Argentina

## ARTICLE HISTORY

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**Abstract:** Oxidative stress induced by the oxidative pathway dysregulation following ischemia/reperfusion has been proposed as an important cause of neuronal death and brain damage. The proteins of the thioredoxin (Trx) family are crucial mediators of protein function regulating the intracellular hydrogen peroxide levels and redox-sensitive post-translational protein changes. This study evaluates the long-term effects of common carotid artery ligation-induced ischemia/reperfusion on the protein expression and distribution of fourteen members of the Trx family and related proteins (Grx1, Grx2, Grx3, Grx5, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, Trx1, Trx2, TrxR1, TrxR2) in the most hypoxia susceptible rat brain areas, namely, cerebellum, corpus striatum, and the hippocampus. The thioredoxin proteins displayed a complex, cell-type, and tissue-specific expression pattern following ischemia/reperfusion. Even 60 days after ischemia/reperfusion, Western blot analysis showed a persistent expression of Trx1 and Grx2 in several brain areas. Thioredoxins might participate in the long-term restoration of redox signaling, and the recovery of the affected tissues.

**Keywords:** Common carotid artery occlusion, thioredoxin family, CNS, hypoxia-ischemia.

## 1. INTRODUCTION

Brain damage resulting from an ischemic event in the fetus or newborn infant (also known as perinatal asphyxia, PA) remains a major cause of neonatal death and neurological deficits in children. Cerebral palsy, mental retardation, and epilepsy are among the most common complications of perinatal asphyxia [1-4]. The incidence of severe perinatal asphyxia is estimated to about 1:1000 live births in developed countries, and 5-10:1000 live births in developing countries [5]. This clinical picture has been largely reproduced, exposing rodents to an oxygen-deprived atmosphere on post-natal day seven after the right common carotid artery (CCA) ligation [3, 6-9]. By post-natal day seven, the rat brain is histologically similar to that of a 32-34 week gestation human fetus or a newborn infant, e.g., the cerebrocortical neuronal layering is complete, the germinal matrix shows involution, and the white matter has undergone little myelination [10].

Reactive oxygen species (ROS) are a likely cause of neuronal death and brain damage after ischemia/reperfusion [11]. Aerobic metabolism renders oxygen free radicals in the cytoplasm and mitochondria [12], physiologically involved in specific signalling processes such as developmental regulation, cell proliferation, differentiation, and apoptosis. Locally produced, and swiftly degraded by specific enzymes, ROS interact with specific target molecules [12]. However, excessive production of ROS can lead to oxidative and irreversible damage to macromolecules and has been linked to various pathological conditions like ischemia-reperfusion [11, 13, 14].

Several therapeutic approaches have been proposed to neutralize ischemia-induced ROS like the administration of degrading

substances to reduce their chemical reactivity, free radical inhibitors, and scavengers [5]. However, no neuroprotective agent has been proved safe and effective in protecting ischemic-insult neonates from neurological sequels. It urges deepening our insight into biochemical and cellular mechanisms of neuronal injury induced by perinatal asphyxia to identify potential therapeutic targets and compounds [15].

Thioredoxin (Trx) family members are small proteins typically bearing the unique Trx folding and the cysteine-containing active site motif, crucial for electron transfer and oxidoreductase activity [16, 17]. Thioredoxins and glutaredoxins (Grx) were first identified as electron donors to ribonucleotide reductase, and later described as cellular antioxidants protecting cells from reactive oxygen species-induced cell death. Peroxiredoxins (Prx), involved in peroxide decomposition, were identified as one of the major cellular peroxidases [16]. Currently, the thioredoxin family is recognized as comprising regulatory proteins crucially affecting protein function through oxidative post-translational changes or involved in the local and specific hydrogen peroxide degradation [17].

This study analyzes the expression and distribution of fourteen members of the Trx family, potentially essential for the regeneration upon long-term brain damage, in a perinatal hypoxia-ischemia rat model induced by a common carotid artery ligation.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Pregnant Sprague-Dawley rats from the central bioterium of the School of Veterinary Sciences of the University of Buenos Aires were kept in a controlled environment at 21 ± 2 °C and 65 ± 5% humidity on a 12h light-dark cycle (lights on at 8 a.m.) with free access to Purina chow food and tap water. The experiments were conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), and approved by the Institutional Animal Care and Use

\*Address correspondence to this author at the Institute of Cardiological Research, University of Buenos Aires, National Research Council, ININCA.UBA-CONICET, Buenos Aires, Argentina Marcelo T. de Alvear 2270, C1122, Buenos Aires, Argentina; Tel/Fax: +5411 52852760; E-mail: [fcapani@fmed.uba.ar](mailto:fcapani@fmed.uba.ar)

Committee at the School of Medicine of the University of Buenos Aires. All efforts were made to reduce the number of animals used and minimize suffering.

## 2.2. Common Carotid Artery Ligation Procedure

On day seven after birth (P7), male offsprings were i.p. anesthetized with ketamine 40 mg/kg-xylazine 4 mg/kg, and placed on a heat plate to keep body temperature constant at 37 °C. The right common carotid artery (CCA) was exposed by an incision on the right side of the neck, isolated from nerve and vein, and permanently ligated with 6-0 surgical silk (Carotid group). After wound suture, the animals were placed back to their cages for 4-5 h. After recovery, the pups were placed in a stoppered 1L glass jar partially submerged in a 37 °C water bath for thermal constancy, and exposed to 100 % N<sub>2</sub> (3L/min N<sub>2</sub> flow) for 3 min to induce anoxia. Sham-surgery rats (Sham) underwent right CCA surgical exposure only but no ligation or N<sub>2</sub> exposure. Euthanasia was administered to all rats at 30, 60, and 90 days of age.

## 2.3. Western Blotting and Enzyme-linked Immunosorbent Assay (ELISA)

The expression pattern of fourteen Trx proteins was analyzed in the cerebellum, corpus striatum, and hippocampus using Western blot or in the case of Grx2 by ELISA.

Western blot analysis was performed slightly modified from Godoy *et al.* (2011). Rat brains dissected after euthanasia by decapitation were homogenized in ice-cold cell lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.1% NP-40 non-ionic detergent), fast-frozen in liquid nitrogen and kept at -20 °C. For analysis, the samples were thawed on ice and centrifuged at 13,500 x g for 20 min at 4 °C. After discarding the pellets, the supernatants were analyzed in 96-well plates for total protein concentration using the Bradford solution (Bio-Rad, Munich, Germany) and bovine serum albumin (BSA) as the standard in at least triplicates. Total protein (10–20 µg) was diluted in sample buffer (0.3 M Tris/HCl, pH 7, 50% glycerol, 5% SDS, 1 mM EDTA, 0.1% bromophenol blue), and subjected to SDS-PAGE using the Novex minicell (Invitrogen, Carlsbad, CA, USA) with precast 4–20% Precise gels (Pierce–Thermo Fisher). Protein was transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Germany) following the manufacturer's instructions. Membranes blocked with 5% nonfat milk powder and 1% BSA in Tris-buffer saline containing 0.05% Tween 20 were incubated with specific primary antibodies at 4 °C overnight. Antigen-antibody complexes were stained using horseradish peroxidase (HRP)-coupled anti-rabbit antibody (Bio-Rad, Richmond CA, USA). Enhanced chemiluminescence was recorded using a gel documentation system from Intas (Göttingen, Germany). A specific sandwich ELISA technique allowed quantifying glutaredoxin-2 level as described by Lundberg *et al.* (2004) and Hanschmann *et al.* (2010). The source and generation of the antibodies used in this study are described by Godoy *et al.* (2011).

## 2.4. Immunohistochemistry

Immunohistochemistry analysis was performed as described by Aon-Bertolino *et al.* (2011) and Godoy *et al.* (2011) with slight modifications. Animals were anesthetized with 0.1 mL of a 28% w/v chloral hydrate solution/100 g body weight, and intracardially perfused with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) freshly prepared in phosphate buffer 0.1 M, pH 7.4. Following brain dissection and a 2-h fixation in 4% paraformaldehyde solution, coronal sections (40 µm thick) cut on an Oxford vibratome for light microscopy were incubated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidases. After a 3-step PBS washing, nonspecific antibody binding sites were blocked with 10% normal goat serum (Invitrogen Corporation, Camarillo, CA, USA) in PBS, and sections were incubated overnight with the primary antibodies at 4 °C. After the 3-step PBS washing and room

temperature incubation with a biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) for 60 min, the Extravidin-Peroxidase detection system (Sigma-Aldrich, St. Louis, MO, USA) was used for antigen staining following the manufacturer's recommendations. Sections were incubated at room temperature with the substrate Diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) for 5 min, mounted with Canada balsam (Sigma-Aldrich, St. Louis, MO, USA), and were examined by light microscopy using a Leitz Laborlux S microscope (Heidelberg, Germany) equipped with a CCD video camera (Canon). Images were analyzed and compiled using Adobe Photoshop 11.0 CS4.

## 2.5. Statistical Analysis

Western-blot bands intensity was quantified using Gel-Pro® Analyzer 3.1 and expressed as the percentage of that observed in sham-operated rats. Results were expressed as the mean ± SEM of at least four independent determinations of 6 sham-operated or 8 CCA-ligation rats. Between-group differences in biochemical parameters and protein levels were analyzed using the two-tailed Student's t-test. The level of significance was conventionally set at 5 %. All analyses were performed using SPSS 15.0 (Chicago, IL, USA).

## 3. RESULTS

### 3.1. Changes in Protein Expression Following Ischemia-Reperfusion

Figs. (1) and (6) illustrate the expression pattern of the fourteen Trx family proteins in the cerebellum, corpus striatum, and hippocampus.

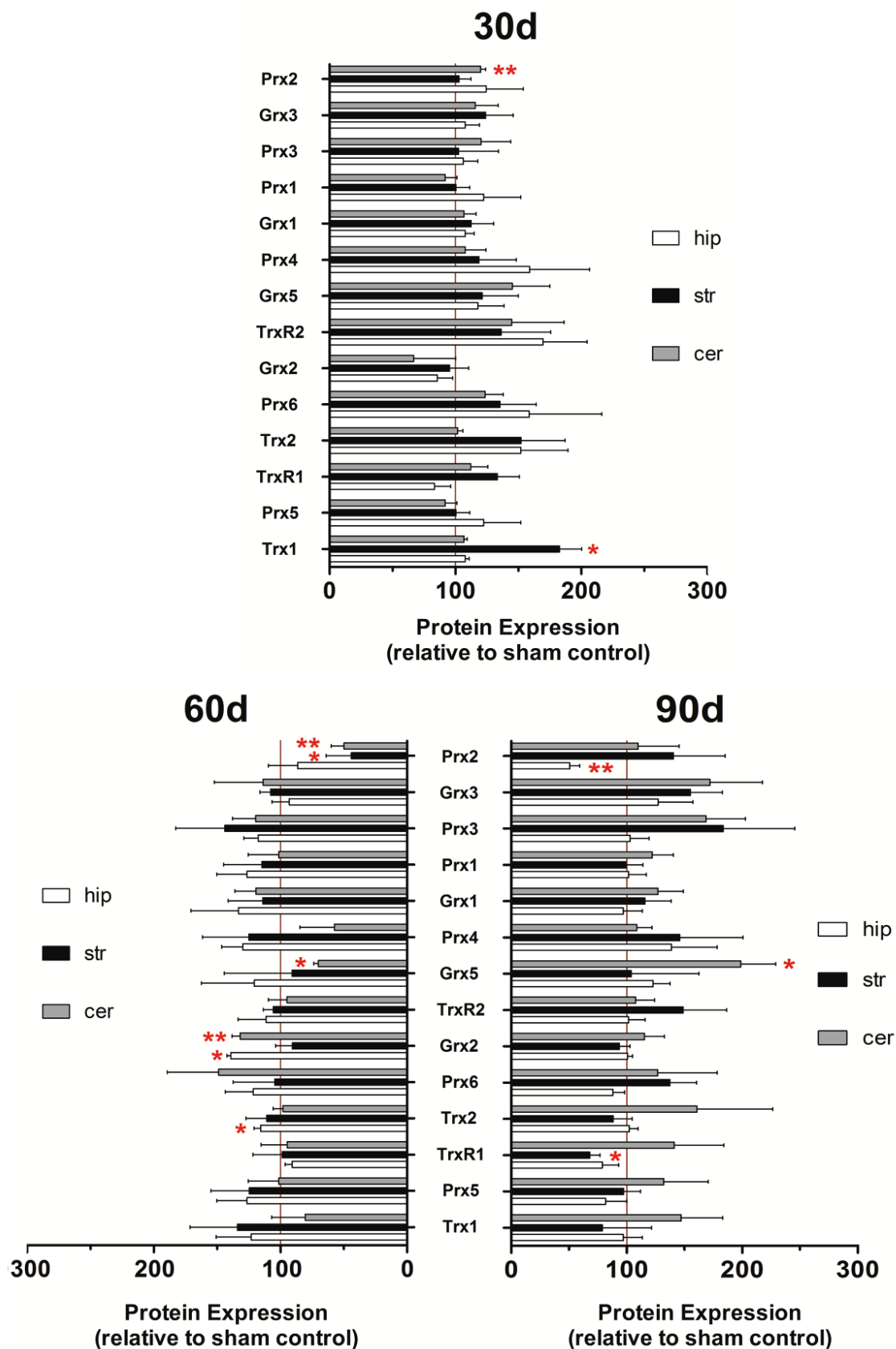
Thirty-day-old rats with CCA ligation showed an increase in Prx2 and Trx1 in the cerebellum and corpus striatum, respectively (Fig. 2a). Rats with CCA ligation 60 days old showed decreased expression of Prx2 and Grx5 in the cerebellum, and also in the corpus striatum for the former (Fig. 2b). The expression of Trx2 and Grx2 increased in the hippocampus and the latter in the cerebellum as well (Figure 2 b). On post-natal day 90, the Grx5 protein level increased in the cerebellum, and Prx2 and TrxR1 expressions decreased in the hippocampus and corpus striatum, respectively (Fig. 2c).

### 3.2. Distribution of the Trx family and related proteins in the cerebellum, hippocampus and corpus striatum

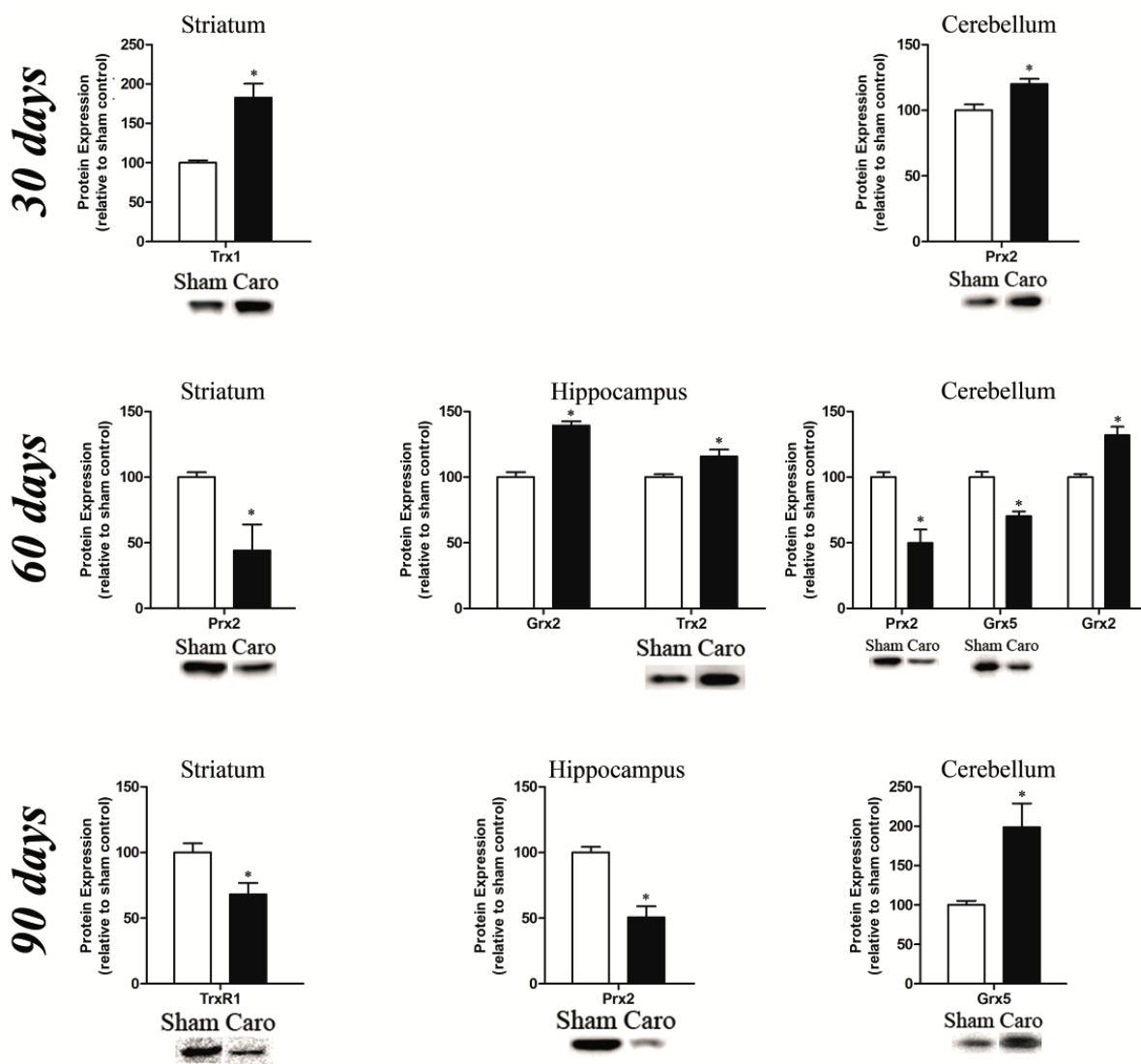
In agreement with western blot analyses, Trx1 staining increased in cellular bodies of the dorsal striatum (Fig. 3a) and the CA1 region of the hippocampus in 30-day old rats with CCA ligation (Fig. 4b) and conversely decreased in the axons of the CA1 pyramidal cells as compared with the sham-operated group. Both rat groups showed Prx2 cytosolic distribution in the cerebellum with increased immunoreactivity in the axonal projections of Purkinje cells in 30-day old rats with CCA ligation (Fig. 3c).

Sixty-day-old rats with CCA ligation showed a higher number of Grx2-immunostained cellular bodies in both the hippocampus and cerebellum (Figs. 4a and 4b). Similarly, a higher number of Trx2-positive cellular bodies (Fig. 4c) and potential astrocytes (Fig. 4c, insert) were observed in the hippocampus in 60-day old rats with CCA ligation as compared with sham-operated rats. While all 60-day-old rats showed sustained Prx2 cytosolic distribution, Prx2 immunoreactivity decreased in the corpus striatum and cerebellum in rats with CCA ligation as compared with the sham-operated group (Figs. 4d and 4e).

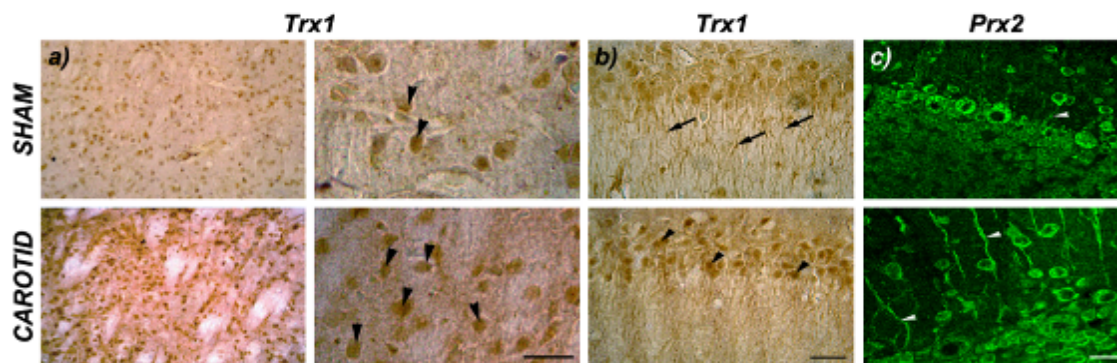
Notwithstanding the increase in nuclear Prx2 immunostaining found in 90-day-old rats with CCA ligation (Fig. 5a), a decrease was observed in the CA1 area of the hippocampus as compared with their sham-operated counterparts. Similarly, nuclear Grx5 immunostaining increased in the cerebellum Purkinje layer in 90-day-old rats with CCA ligation (Fig. 5b). At the same time, TrxR1



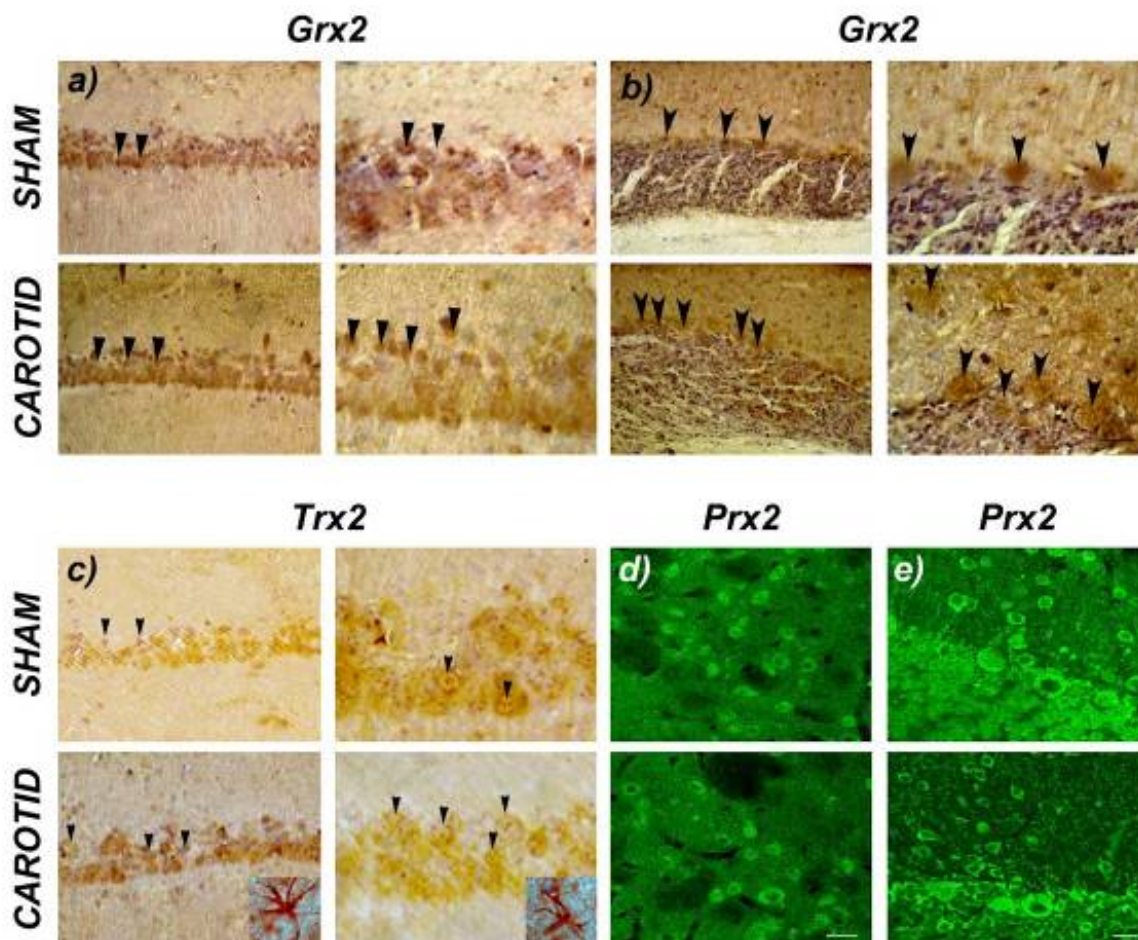
**Fig. (1).** Expression of thioredoxins and related proteins in CCA-ligation rats (n=8) and sham-operated (n=6) rats. The hippocampus (hip), corpus striatum (str), and cerebellum (cer) were isolated and analyzed for protein expression using Western blot, or a specific sandwich ELISA for Grx2. The diagram depicts protein expression as the relative percentage of that observed in sham-operated rats. Values represent the mean  $\pm$  SEM. \* $p \leq 0.05$ . (A higher resolution / colour version of this figure is available in the electronic copy of the article).



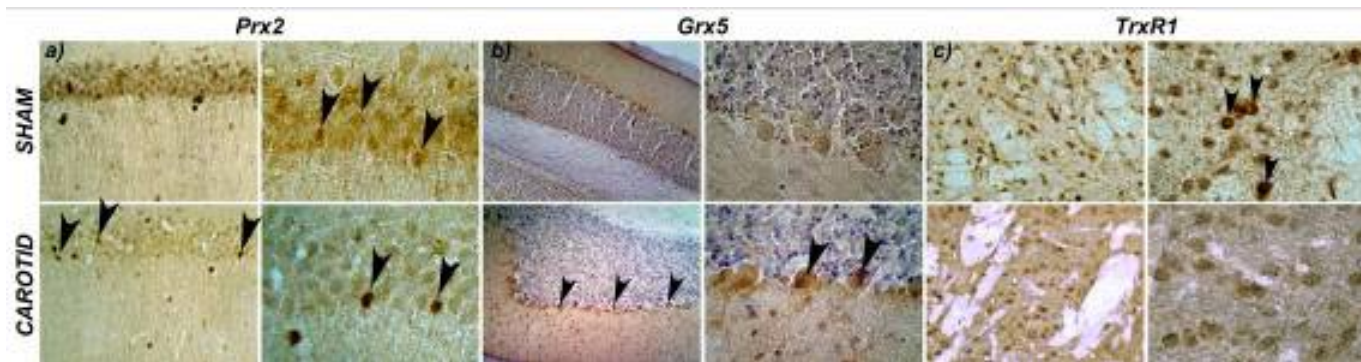
**Fig. (2).** Distinctive expression pattern of specific redoxins in CCA-ligation rats (n=8) and control sham-operated rats (n=6) overtime after the ischemic insult. (b) striatum (left) and cerebellum (right) at 30 days of age; (c) striatum (left), hippocampus (middle) and cerebellum (right) at 60 days of age; (d) striatum (left), hippocampus (middle) and cerebellum (right) at 90 days of age. Protein expression is shown as the relative percentage to that observed in sham-operated rats. Representative Western blots are also illustrated. Values represent the mean ± SEM of 5 independent determinations at least. \* p ≤ 0.05. (A higher resolution / colour version of this figure is available in the electronic copy of the article).



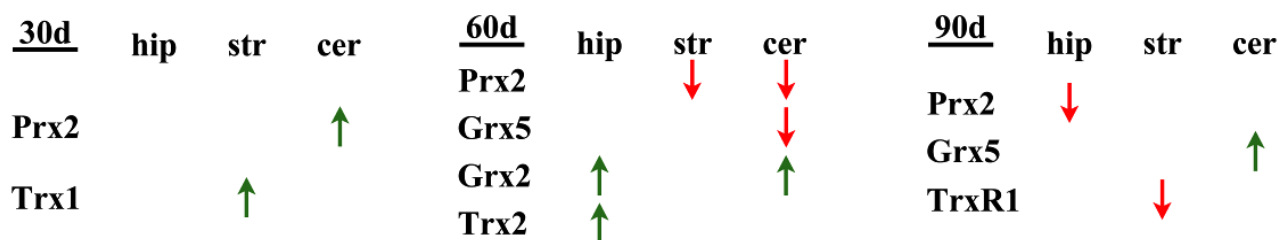
**Fig. (3).** Immunohistochemical analysis of the expression pattern of the thioredoxins family of proteins in sham-operated (n=6) and CCA-ligation (n=6) 30-day old rats. a) Trx1 representative pictures of dorsal striatum: arrowheads show an increase in the number of immunopositive positive cellular bodies; b) Trx1 representative pictures of the CA1 area of the hippocampus: arrowheads show the change in immunopositive cellular bodies in CCA-ligation rats, whereas arrows show the large increase in immunopositive pyramidal cells projections in sham-operated rats; c) Prx2 representative pictures of the cerebellum: disruption of the Purkinje cell layer is observed in the CCA-ligation group (bottom panel), arrowheads show a large increase in immunopositive axonal projections of Purkinje cells in CCA-ligation rats. Scale bar: 50 µm. (A higher resolution / colour version of this figure is available in the electronic copy of the article).



**Fig. (4).** Immunohistochemical analysis of thioredoxins expression in sham-operated (n=6) and CCA-ligation (n=6) rats 60 days after the ischemic insult. a) Grx2 immunostaining in the CA1 area of the hippocampus: arrowheads indicate an increased number of immunopositive cellular bodies in CCA-ligation rats; b) Grx2 localization in the cerebellum: arrowheads show an increased number of immunopositive cellular bodies and Purkinje cell layer is disrupted in CCA-ligation rats; c) Trx2 expression pattern in the CA1 area of the hippocampus: arrowheads show an increased number of immunopositive cellular bodies in CCA-ligation rats, which bear a strong immunoreactivity in what appear to be astrocytes (Insert); d) Prx2 representative pictures in the striatum: cytosolic distribution is observed in both rat groups; e) Prx2 localization in the cerebellum: immunopositivity is decreased in the granular layer in CCA-ligation rat. Scale bar: 50  $\mu$ m. (A higher resolution / colour version of this figure is available in the electronic copy of the article).



**Fig. (5).** Immunohistochemical analysis of the expression pattern of thioredoxin family of proteins in sham-operated (n=6) and CCA-ligation (n=6) rats 90 days after the ischemic insult. a) Prx2 representative pictures from CA1 area of the hippocampus: arrowheads show an increased number of immunopositive cellular bodies in the CCA-ligation group regardless of the overall immunostaining decrease; b) Grx5 representative pictures from the cerebellum: arrowheads show an increase in nuclear staining in the Purkinje cell layer in CCA-ligation rats; c) TrxR1 representative pictures from the striatum: arrowheads show an increase in immunopositive cellular bodies in the sham-operated group. Scale bar: 50  $\mu$ m. Bar diagrams depict the mean of at least four independent measures in sham-operated (n = 6) and CCA-ligation (n = 8) animals  $\pm$  SEM. (A higher resolution / colour version of this figure is available in the electronic copy of the article).



**Fig. (6).** Differential expression of thioredoxins in the brain of CCA-ligation rats. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

nuclear immunostaining decreased in the dorsal striatum in 90-day-old rats with CCA ligation (Fig. 5c).

#### 4. DISCUSSION

Reperfusion following an ischemic event boosts free radicals production with ensuing oxidative stress [18, 19], which has been consistently presumed as an important cause of ischemia/reperfusion-induced neuronal death [11, 19-21].

Previously, we reported the complex expression pattern of thioredoxins and related proteins in the rat central nervous system (CNS) [22]. It confirmed tissue- and cell type-specific distribution of these proteins, as an expression of specific functions and family members' complex crosstalk (Fig 6).

Presently, we examined the expression and distribution of 14 proteins of the thioredoxin family in a murine model of perinatal asphyxia aiming to analyze and understand their specific role in the immediate and long-term effects triggered by a hypoxic insult. We observed complex tissue-specific changes in the expression and distribution of distinct proteins following oxygen deprivation. Interestingly, the analyzed proteins seemed differently affected, depending on the time elapsed after the hypoxic insult. Although there are no available reports on Trx family proteins in perinatal asphyxia, both NO (nitric oxide) and ROS release has been implicated in this condition [11], potentially leading to redox signaling dysregulation, which might be responsible for inducing apoptosis in the CNS and long-term neurological deficits. Much like perinatal asphyxia pathology, redox control disruption or overall ROS generation [13, 14] by mitochondrial chain uncoupling [23] and inflammation [24, 25] were described as the key destructive downstream mechanisms underlying ischemia/reperfusion injury.

Our study shows sustained Trx1 expression as late as 30 days after ischemia/reperfusion (Fig. 1). Notwithstanding severe hypoxia effects on cytosolic antioxidant Trx1 expression in the brain have been shown in preconditioned rats 24 h and 72 h after reperfusion [17, 26, 27], no study has been conducted on CNS long-term alterations. The increased expression of Grx2 lasts for as long as 60 days after ischemia/reperfusion in the CCA ligated rats (Fig. 1). Oxidoreductase expression induction was also observed in a renal ischemia/reperfusion injury model [28]. Glutaredoxin 2 was overexpressed in proximal tubule cells, which can recover after an ischemic insult. Hypoxia-reoxygenation exposed HEK293, and HeLa cells overexpressing Grx2 showed less oxidative damage and increased survival and proliferation rates as compared with the wild-type counterparts [28]. All in all, the Grx2 over-expression might represent the system's attempt to recovering from the hypoxic-ischemic insult. As well as the anti-apoptotic effect of ROS-inducing drugs such as doxorubicin [17, 29], Grx2 has been recently proved essential for embryonic brain development [30]. Oxidoreductase-silenced Zebrafish typically showed overall neuronal loss due to apoptosis and lack of a developed axonal scaffold [30]. The findings suggest that these proteins could be involved in long-term regeneration of physiological redox signaling with the recovery of affected tissues.

Carotid artery ligation reduced Prx2 expression in rats 60 and 90 days old. Peroxiredoxin 2 exhibited potent pro-survival effects in ischemic neurons by keeping Trx reduced [31]. Thereby, this reduction could be an effect of the depletion of the protein while maintaining the high levels of Trx1 expression.

The Trx family of proteins has been reported as contributing to early brain tolerance after stress exposure [27, 32]. Here we report brain protection as long as 90 days after the insult. These changes presumably contributed to an adaptive response to the adverse conditions.

The superior brainstem, cerebellum, white matter and subcortical structures supplied by the distal branches of deep and superficial penetrating blood vessels, cerebral white matter between the major cerebral artery territories, the CA1 region of the hippocampus, and neocortical layers 3, 5, and 6 are some of the most hypoxia-ischemia vulnerable brain areas [33-35]. The modified pattern of the Trx family protein expression found in the most vulnerable areas of the brain, particularly in the hippocampus and cerebellum endorses the idea of the therapeutic use of these proteins to counteract brain damage following an ischemia/reperfusion insult. Notwithstanding the beneficial effects of Trx administration in adult stroke models [36, 37] and the implication of several Trx family members in cell survival, proliferation, and regeneration processes [38, 39], to our knowledge, thioredoxins have not been so far tested in a neonatal model of asphyxia.

#### CONCLUSION

Present findings suggest that the Trx family of proteins might contribute to long-term survival and recovery supporting their therapeutic use to curtail ischemic brain oxidative damage following an ischemia/reperfusion insult. Characterization of ischemia/reperfusion oxidative brain damage and analysis of the involved mechanisms are required to understand the underneath processes triggered by ischemia/reperfusion and to what extent and in what way thioredoxins contribute to recovery from brain hypoxic stress.

#### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

#### HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

#### CONSENT FOR PUBLICATION

Not applicable.

#### AVAILABILITY OF DATA AND MATERIALS

Not applicable.

#### FUNDING

None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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