Biochimica et Biophysica Acta 1850 (2015) 1274–1285

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Thioredoxin 1 and glutaredoxin 2 contribute to maintain the phenotype and integrity of neurons following perinatal asphyxia

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ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form 14 February 2015
Accepted 24 February 2015
Available online 28 February 2015

Keywords:
Common carotid artery occlusion
Thioredoxin family of proteins
Hypoxia
Reoxygenation
Perinatal asphyxia

ABSTRACT

Background: Thioredoxin (Trx) family proteins are crucial mediators of cell functions via regulation of the thiol redox state of various key proteins and the levels of the intracellular second messenger hydrogen peroxide. Their expression, localization and functions are altered in various pathologies. Here, we have analyzed the impact of Trx family proteins in neuronal development and recovery, following hypoxia/ischemia and reperfusion.
Methods: We have analyzed the regulation and potential functions of Trx family proteins during hypoxia/ischemia and reoxygenation of the developing brain in both an animal and a cellular model of perinatal asphyxia. We have analyzed the distribution of 14 Trx family and related proteins in the cerebellum, striatum, and hippocampus, three areas of the rat brain that are especially susceptible to hypoxia. Using SH-SY5Y cells subjected to hypoxia and reoxygenation, we have analyzed the functions of some redoxins suggested by the animal experiment.
Results and conclusions: We have described/discovered a complex, cell-type and tissue-specific expression pattern following the hypoxia/ischemia and reoxygenation. Particularly, Grx2 and Trx1 showed distinct changes during tissue recovery following hypoxia/ischemia and reoxygenation. Silencing of these proteins in SH-SY5Y cells subjected to hypoxia-reoxygenation confirmed that these proteins are required to maintain the normal neuronal phenotype.
General significance: These findings demonstrate the significance of redox signaling in cellular pathways. Grx2 and Trx1 contribute significantly to neuronal integrity and could be clinically relevant in neuronal damage following perinatal asphyxia and other neuronal disorders.

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 PA [1–4]. The incidence of severe PA is estimated to be about 1/1000 live births in developed countries, and 5–10/1000 live births in developing countries [5]. This clinical picture has been extensively reproduced in murine models of PA consisting of the ligation of the right common carotid artery followed by an exposure to an oxygen deprived environment at postnatal day 7 [3,6–10]. The model for common carotid artery ligation used in the present study has been previously developed and validated by Lopez-Aguilera et al., 2012 [7]. It has been extensively investigated and is generally accepted that the rat brain at the age of 7 days is histologically similar to that of a 32–34 week gestation human fetus or newborn infant, i.e., the cerebral cortical neuronal layering is complete, the germinal matrix is involuting, and white matter has undergone some myelination [11–13].

Damage caused by the effects of reactive oxygen species (ROS) has been proposed as an important cause of neuronal death and consequently brain damage after hypoxia-ischemia [14,15]. In aerobic cells, ROS are produced within the cytoplasm and mitochondria [16]. Under physiological conditions, they are part of

Abbreviations: CCA, Common carotid artery; cer, Cerebellum; CNS, Central nervous system; ELISA, Enzyme-linked immunosorbent assay; Grx, Glutaredoxins; hip, Hippocampus; P7, Postnatal day 7; PA, Perinatal asphyxia; PBS, Phosphate buffer saline; Prx, Peroxiredoxins; ROS, Reactive oxygen species; SEM, Standard error of the mean; str, Striatum; Trx, Thioredoxins; TrxR, Thioredoxin reductase

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http://dx.doi.org/10.1016/j.bbagen.2015.02.015
0304-4165/

specific signaling processes, regulating for instance developmental processes, cell proliferation, differentiation and apoptosis [16–18]. They are locally produced by specific enzymes and are rapidly degraded by others, affecting specific target molecules and signaling pathways [16,18]. However, excessive production of specific ROS, e.g. hydroxyl radicals, can lead to oxidative and irreversible damage to macromolecules and has been linked to various pathological conditions, including hypoxia/ischemia and reoxygenation [14,19,20]. Several therapeutic approaches to counteract the effects induced by ROS during hypoxia/ischemia and especially reoxygenation have been proposed. However, no neuroprotective agent has been proven safe and effective in the protection of neonates from neurological sequels following an ischemic insult beside hypothermia in some specific cases [21]. Therefore, it is essential to gain more insights into biochemical and cellular mechanisms of neuronal injury induced by PA to identify potential therapeutic targets, compounds and strategies [22].

The members of the thioredoxin (Trx) family are small proteins that present the characteristic Trx fold, and the cysteine(s)-containing active site motif, which is crucial for the transfer of electrons and the general oxidoreductase activity [23,24]. The Trx superfamily of proteins includes thioredoxins (Trxs) glutaredoxins (Grxs) and peroxiredoxins (Prxs) [25]. These proteins share a common structural motif, the Trx fold, consisting of a central core of four-stranded β-sheets surrounded by three or more α-helices. The thiol-disulfide oxidoreductases Trx and Grx also share the conserved active site Cys-X-X-Cys that enables them to catalyze thiol-disulfide exchange reactions [25]. Peroxiredoxins are thiol-dependent peroxidases [23,24]. Trx was first described as a hydrogen donor for ribonucleotide reductase from *Escherichia coli* [26]. Later on, these proteins were recognized as key regulators in the cell response to redox signals [24]. Mammals possess two principal Trx isoforms, the cytosolic Trx1 and the mitochondrial Trx2. These proteins are reduced by thioredoxin reductases [27,28], the cytosolic TrxR1 and the mitochondrial TrxR2, respectively. TrxRs are homodimeric flavo- and seleno-enzymes which are able to reduce Trxs, as well as peroxides and other compounds [29–31]. Mammalian genomes encode various Grxs, among them the cytosolic Grx1 and Grx3, the mitochondrial Grx5 and Grx2a, and the least frequent nuclear/cytosolic isoforms of Grx2 (Grx2b and Grx2c, respectively) [24,32]. Grxs are reduced by glutathione, with electrons from glutathione reductase and NADPH [32]. Peroxiredoxins (Prxs) reduce different peroxides and peroxynitrite [33]. Prx1 to Prx4 belong to the typical 2-Cys Prx class, Prx5 is an atypical 2-Cys Prx, Prx6 is a 1-Cys Prx [34,35]. Prx1, Prx2 and Prx5 can be found in the nucleus and cytoplasm, whereas Prx3 and Prx5 are localized in mitochondria. Prx4 and Prx6 can be found in the cytoplasm. In addition, Prx1, Prx2 and Prx4 were shown to be secreted from the cell [34,36].

Here, we have analyzed the regulation and potential functions of Trx family proteins on the effects of hypoxia/ischemia and reoxygenation on the developing brain both *in vivo* and in a neurological cell culture model.

2. Experimental procedures

2.1. Animals

All 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 Committee at the University of Buenos Aires (School of Medicine). All efforts were made to reduce the number of animals used and to minimize suffering. Pregnant rats were obtained from the School of Veterinary Sciences' central *vivarium* at the University of Buenos Aires. All animals were kept in a temperature (21 ± 2 °C) and humidity (65 ± 5%) controlled environment on a 12 h light/dark cycle. Animals had *ad libitum* access to food (Purina chow) and tap water.

2.2. Model for common carotid artery ligation

The model for common carotid artery ligation used in this study, has been previously developed and validated by Lopez-Aguilera et al. [7]. Seven days of age (P7) male Sprague–Dawley rats were anesthetized with a combination of ketamine (40 mg/kg) and xylazine (4 mg/kg). The animals were placed on a heat plate ensuring a constant body temperature of 37 °C. An incision on the right side of the neck was performed exposing the right common carotid artery (CCA), which was then isolated from nerve and vein and permanently ligated with a 6–0 surgical silk (carotid group n = 14). The wound was then sutured and the animals were returned to their dams for recovery for 4–5 h. Subsequently, pups were placed in a stoppered 1 L glass jar and exposed to 100% nitrogen (delivered at 3 L per minute) for 3 min to induce anoxia. The jar was partially submerged in a 37 °C water bath to maintain a constant thermal environment. In sham operated rats (sham group n = 12) the right CCA was exposed but not ligated and no nitrogen was supplied. At 21 days of age (14 days post-surgery), when the synaptic connections are well established in the rat [37], animals were sacrificed.

2.3. Brain dissection

Brains were dissected as previously described in Chiu et al. [38]. After the animals were sacrificed at 21 days of age, brains were isolated and dissected at 4 °C. Brains were cut in half into right and left hemisphere. Three cuts were performed in each hemisphere. The first cut was situated at the *Genu* of the *Corpus callosum* (~Bregma + 1.0 mm based on the rat brain atlas by Paxinos and Watson [39]), the second cut was situated at the anterior tip of the *Fornix* (approx. Bregma − 1.0 mm based on the rat brain atlas by Paxinos and Watson [39]). The third cut was situated at the 4th ventricle (~Bregma − 8.0 mm based on the rat brain atlas by Paxinos and Watson [39]). The striatum was dissected between the first and second cut with the help of two Miltex Iris Tissue Forceps, and stored at − 80 °C. The hippocampus was dissected after the second cut. The midbrain was removed to expose the hippocampus, which was then dissected from the cortex using two tissue forceps, and stored at − 80 °C. Finally, the cerebellum was dissected after the third cut, that was separated from the ponds and medulla oblongata with the help of two tissue forceps, and stored at − 80 °C.

2.4. Cell culture

SH-SY5Y cells were cultivated in MEM (PAA), supplemented with 10% FCS good (PAN), 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a 90% humidified atmosphere containing 5% CO_2. SH-SY5Y cells were transiently transfected with 15 μg specific, custom-made siRNA (Eurogentech) for Trx1 (sense: GUA GAU GUG GAU GAC UGU C, antisense: GAC AGU CAU CCA CAU CUA C) and Grx2 (sense: GGU GCA ACU GAC ACU CAU; antisense: UAU GAG UGU CAG UUG CAC). Unspecific control (scr)bled siRNA (sense: CAU UCA CUC AGG UCA UCA, antisense: CUG AUG ACC UGA GUG AAU) was used as control. Five million SH-SY5Y cells were resuspended in electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, pH 7.15), mixed with siRNA and were electroporated in a total volume of 550 μl at 230 V, 1050 microfarads and 500 ohm. FCS was immediately added to the transfected cells and they were seeded out in 1:5 conditioned medium (1 part old and 4 parts fresh medium). To sufficiently knock-down Trx1 and Grx2, cells were transfected a second time after 3 days.

Twenty-four hours following the second transfection, cells were incubated under 1% O_2 and 5% CO_2 at 37 °C (hypoxia) in a CO_2 incubator (binder) for 24 h followed by a reoxygenation period of 24 or 48 h in an atmosphere containing 20% O_2 and 5% CO_2 at 37 °C. Cells were detached by trypsin treatment, washed with PBS, lysed in lysis buffer (10 mM

Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP-40, protease inhibitors) and were frozen at −80 °C.

2.5. Western blotting and ELISA

Western blot analysis was performed essentially as previously described in Godoy et al. [40]. Animals were euthanized by decapitation, brains were dissected, homogenized in ice-cold lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP-40, protease inhibitors) and were fast frozen in liquid nitrogen. Tissue and cell lysates were thawed on ice and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatants were analyzed for total protein concentration using Bradford solution (Bio-Rad, Munich, Germany) in 96-well plates using bovine serum albumin (BSA) as standard. 10–20 μg of total protein were diluted in sample buffer (0.3 M Tris/HCl, pH 7, 50% glycerol, 5% SDS, 1 mM EDTA, 0.1% bromphenol blue). In the case of tissue homogenates, the samples were subjected to SDS-PAGE using the Novex mini-cell (Invitrogen, Carlsbad, CA, USA) with precast 4–20% Precise gels (Pierce-Thermo Fisher). Proteins were transferred to PVDF membranes (Schleicher & Schuell, Germany) according to the manufacturer's instructions. In the case of cell lysates, the samples were subjected to SDS-PAGE using the Mini-Protean TGX stain-free 4–20% precast gels (Biorad) and were transferred to PVDF membranes using the Trans-Blot Turbo Transfer System (Biorad).

Membranes were blocked with 5% nonfat milk powder and 1% BSA in Tris-buffered saline containing 0.05% Tween 20 and incubated with specific primary antibodies at 4 °C overnight. Antigen–antibody complexes were stained using horseradish peroxidase (HRP)-coupled antibodies (Bio-Rad, Richmond CA, USA) and the enhanced chemiluminescence method. Luminescence was recorded using a gel documentation system from Intas (Göttingen, Germany) or the ChemiDoc™ XRS+ System, respectively. Anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control for the tissue sample. For cell lysates, total protein in each lane of a blot was quantified based on the stain-free technology of Biorad and used for normalization of the blotting data obtained from densitometric analysis [41,42]. The generation and validation for Western blot analysis of the antibodies against the Trx family of proteins (Grx1, Grx2, Grx3, Grx5, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, Trx1, Trx2, TrxR1 and TrxR2) used in this study were described in Aon-Bertolino et al. [43] and Godoy et al. [44]. Antibodies detecting GAPDH (G9545), HSP70 (4873S), GFAP (PA3-16727), neurofilament M (2838S), NeuroD1 (sc-1084) were purchased from Sigma Aldrich (Steinheim, Germany), Santa Cruz Biotechnology Inc. (Santa Cruz, USA), Cell Signaling Technology (Danvers, USA) and Thermo Fisher Scientific Inc. (Rockford, USA). To analyze the levels of ubiquitin, polyubiquitin and ubiquitinylated proteins a specific antibody against ubiquitin (sc-9133) was used.

The more sensitive sandwich ELISA was used to quantify the levels of glutaredoxin 2 as described in Hanschmann et al. [45]; the antibodies for Grx2 were not validated for Western blot analysis.

2.6. Quantitative PCR

Cells were harvested by trypsinization, were washed once with PBS and RNA isolation was performed according to the manual provided for the NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany). The RNA concentration was determined from the absorbance at 260 nm, analyzed with a NanoDrop 2000c spectrophotometer (Thermo Scientific). First strand cDNA was prepared using the RevertAid First Strand cDNA Synthesis Kit according to the protocol provided using 1 μg RNA as template and Oligo dT_{18} primer (Thermo Scientific). 1 μl of cDNA was used as template for quantitative PCR (qPCR). The SensiMix SYBR HI-ROX contained SYBR® Green I dye, dNTPs, stabilizers, and a hot start DNA polymerase (Bioline, London, UK). The primer concentration used for the qPCR was 0.25 μM each (Grx2: 5′-CTGGTTTGGAGCAGGAGCGGCTC; 3′-GCCTATGAGTGTCAGTTGCACC; Trx1: 5′-GGTGAAGCAGATCGAGAGCAAG; 3′-CACACTCTGAAGCAACATCCTG) and the volume was adjusted to 20 μl with ddH_2O. All qPCRs were performed using the CFX96 Real Time System from BioRad. For optimization, a gradient qPCR from 55 °C to 65 °C was performed and samples were analyzed by agarose gel electrophoresis. At 58 °C annealing temperature, the reaction resulted in a specific product at the expected size. GAPDH was used as a reference (primer pair: 5′-CAAGGTCATCCATGACAACTTTG; 3′-GTCCACCACCCTGTTGCTGTAG) in the ΔΔCq mode. Calculations were performed with the Biorad software.

2.7. Immunohistochemistry

Immunohistochemistry analysis was performed as previously described in Aon-Bertolino et al. [43] and Godoy et al. [44] with slight modifications. Animals were anesthetized with 28% (w/v) chloral hydrate, 0.1 ml/100 g of body weight, and intracardially perfused with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) freshly prepared in 0.1 M phosphate buffer, pH 7.4. Brains were dissected and post-fixed in the same solution for 2 h. Coronal brain sections (40 μm thick) were cut on an Oxford vibratome and then recovered for light microscopic studies. Prior to staining, sections were incubated in 3% hydrogen peroxide for 10 min to quench endogenous peroxidases. After three washing steps in PBS, 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. The generation and validation for IHC of the antibodies against the Trx family of proteins (Grx1, Grx2, Grx3, Grx5, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, Trx1, Trx2, TrxR1 and TrxR2) used in this study were described in Aon-Bertolino et al. [43] and Godoy et al. [44]. Sections were washed three times with PBS and subsequently incubated with a biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) for 60 min at room temperature. The Extravidin-Peroxidase detection system (Sigma-Aldrich, St. Louis, MO, USA) was used for antigen staining according to the manufacturer's recommendations. Sections were incubated with the substrate Diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature. Samples were counterstained for 1 min at RT with hematoxilin and were mounted with Canada balsam (Sigma-Aldrich, St. Louis, MO, USA). Sections without incubation with the primary antibody were used as control to verify the specificity of the secondary antibody. Sections 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. Note that for each protein staining all samples (both sham and carotid groups) were processed together in the same batch, using the same antibody dilutions and the same time for DAB development. Intensity assessment was carried out as a blind test with a 0 to +++ score used as a qualitative measurement.

2.8. Immunocytochemistry

Transiently transfected SH-SY5Y cells were seeded on 100 μg/ml fibronectin-coated coverslips in 24-well plates. After induction of hypoxia-reoxygenation, the cells were fixed with 4% paraformaldehyde for 20 min and permeabilized and blocked (0.3% Triton X-100, 3% (w/v) BSA, 10 mM HEPES in PBS). Cells were incubated overnight with the primary antibody, diluted in 3% (w/v) BSA in PBS at 4 °C and with Alexa-488 labeled secondary antibody (Invitrogen) for 1 h at RT. In addition, F-actin was stained for 1 h at RT using Phalloidin (Invitrogen) and nuclei for 10 min at RT using 100 ng/ml DAPI (Sigma). Coverslips were mounted with Mowiol and processed for confocal microscopy. Cells were analyzed using a Leica TCS SP5 microscope with a 63-fold/1.4 oil lens.

2.9. Cell proliferation and viability (MTT assay)

Cell proliferation and viability were analyzed using the tetrazolium dye MTT (thiazolyl blue tetrazolium bromide, Roth) in a colorimetric assay. 20,000–35,000 cells per well were seeded in a 96 well plate. Following 24 h incubation at 20% oxygen, cells were cultured for 24 h at 1% oxygen with or without subsequent reoxygenation for 24 h or 48 h. For these three different conditions, control cells were seeded out at the same density and were grown for the same duration at 20% oxygen. Cells were washed once with PBS and were incubated with 500 μg/ml of the yellow MTT dye solved in cell culture medium for up to 4 h at 37 °C. The formation of the insoluble, purple formazan via the reduction of MTT was determined by light microscopy. The MTT dye was removed and the plate was stored at −80 °C over night. Cells were lysed in lysis reagent (346 mM SDS, 0.2% HCl in DMSO) for up to 30 min at RT. The absorbance was measured at 550 nm in the Tecan plate reader against a blank without any cells.

2.10. Statistical analysis

At 21 days of age, animals were prepared for the sham-operated group (n = 12) as well as the carotid-ligated group (n = 14). Band intensities of Western blots were quantified using GelPro 3.1 or ImageJ and were expressed as percentage of the control levels (sham operated rats or control SH-SY5Y cells). Total protein amount, visualized using the stain-free technology of Biorad was quantified using the ImageLab 5.0 software (Biorad). Bar diagrams depict the mean of four independent quantifications of each sample of sham-operated (sham, n = 6) and ischemic (carotid, n = 8) animals ± SEM, correlated to total protein. A two way ANOVA with the factors of condition (sham, carotid) and region (hippocampus, striatum, cerebellum) or culture condition (20% O_2, 1% O_2, 24 h reox, 48 h reox) and treatment (control (scr), siGrx2, siTrx1) followed by Tukey HSD post hoc tests for pair-wise multiple comparisons were employed to analyze the statistical significance of changes in biochemical parameters and protein levels. The level of significance was set up at 5%. All analyses were performed using SPSS 15.0 (Chicago, IL, USA).

3. Results

3.1. Changes in Trx family protein expression and localisation following hypoxia/ischemia and reoxygenation induced by common carotid artery ligation

The expression pattern of the 14 Trx family proteins in carotid and sham operated animals was analyzed in cerebellum, striatum and hippocampus homogenates by Western blot or in the case of Grx2 by

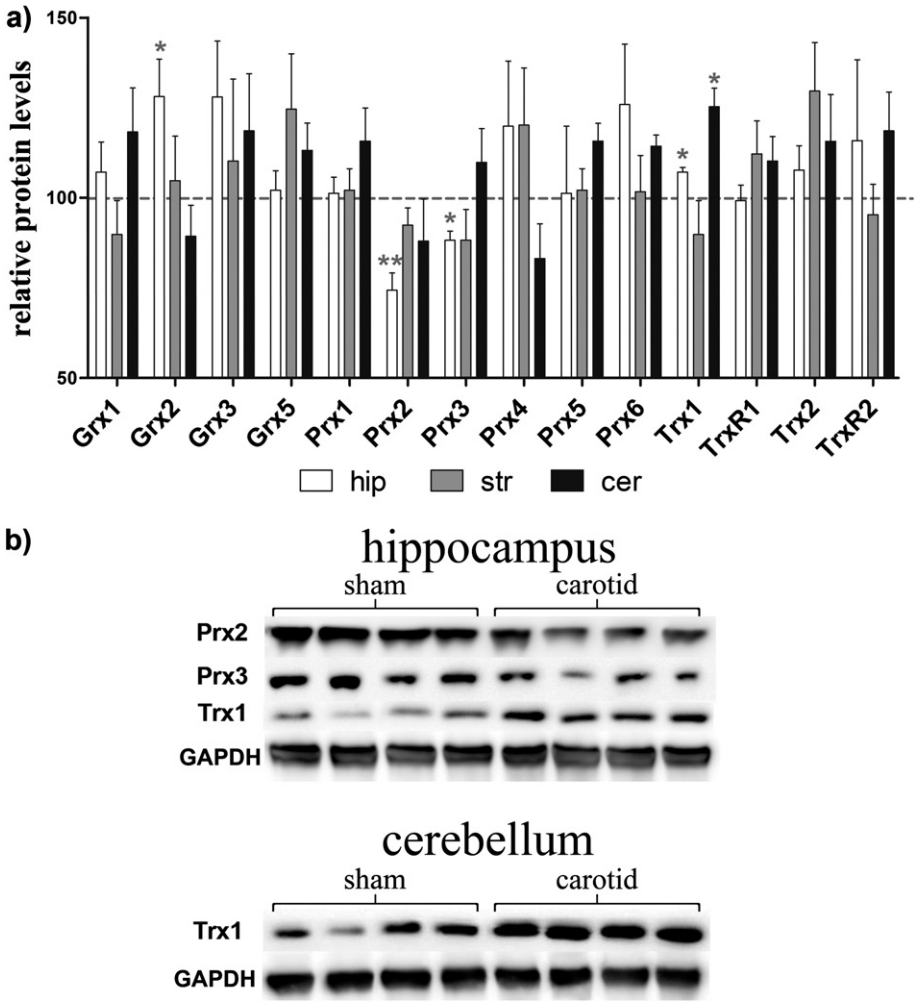

Fig. 1. Relative protein levels of Trx family and related proteins in carotid-ligated asphyxic and sham-operated control rats. Hippocampus (hip), striatum (str), and cerebellum (cer) were isolated and analyzed for protein levels by Western blot or in the case of Grx2 by a specific sandwich ELISA. a) The diagram depicts the relative protein levels of asphyxic animals stated in percent compared to sham-operated rats. b) Representative Western blots are illustrated for the groups showing a significant change. Bars represent the mean ± SEM of eight carotid and six sham rats. *$p \leq 0.05$, **$p \leq 0.01$ in respect to sham animals. Two way ANOVA [condition (sham, carotid) × brain region (hippocampus, striatum, cerebellum)] followed by Tukey HSD post hoc tests for pair-wise multiple comparisons were employed to analyze the statistical significance.

a specific sandwich ELISA (Fig. 1). All antibodies were evaluated thoroughly beforehand [43,44].

At 21 days of age (14 days post-surgery), the Western blot analysis showed increased levels of Grx2 and Trx1 and decreased levels of Prx2 and Prx3 in the hippocampus of carotid-clamped rats (Fig. 1). An increase in the protein amount of Trx1 was also observed in the cerebellum (Fig. 1). The immunohistochemical analysis of Grx2 resulted in a more intense staining throughout the entire hippocampus compared to the control group (Fig. 2a). In particular, the nuclei of the neurons displayed an increased Grx2 immunostaining. Staining for Prx2 was markedly decreased in the somata of the neurons of carotid-clamped animals in comparison to the sham group; this tendency was most pronounced in the CA1 area of the brain (Fig. 2b). Similar to Grx2, Trx1 immunostaining was increased in the extracellular matrix and possibly in the nuclei of the neurons in all areas of the hippocampus (Fig. 2c). The increased immunostaining of Grx2 and Trx1 suggested important functions in the protection and preservation of the neuronal phenotype and integrity.

3.2. The importance and effects of Trx1 and Grx2 in a cellular model of hypoxia-reoxygenation

To confirm this hypothesis and to analyze the role and effects of Trx1 and Grx2, we used a cellular model. In order to mimic hypoxic conditions, human neuroblastoma SH-SY5Y cells were cultivated for 24 h in an atmosphere containing 1% oxygen. In addition, cells were reoxygenated for 24 h or 48 h at 20% oxygen. Cells were harvested, lysed, and analyzed for Grx2 and Trx1 mRNA and protein levels by quantitative RT-PCR and Western blotting, respectively. Moreover, the levels of Hsp70 were assessed as a neuronal damage marker [46,47]. Hypoxia and reperfusion did not significantly affect the protein amounts of Grx2 or Trx1 at the analyzed time points (Fig. 3). The protein levels of HSP70, on the other hand, decreased continuously during the treatment to approx. 55% after 48 h of reoxygenation (Fig. 3).

To analyze the function of Grx2 and Trx1 during hypoxia/reoxygenation, we specifically silenced the expression of the redoxins using the RNA-interference technique (Fig. 4). We aimed at analyzing cell morphology, differentiation, proliferation and viability (Figs. 5 and 6). siRNA-silencing of Grx2c was originally developed in Hanschmann et al. [45], siRNA silencing of Trx1 was newly established here. Transfected SH-SY5Y cells were cultivated for 24 h at 1% oxygen, followed by up to 48 h of reoxygenation at 20% oxygen. The efficiency of the knock-down was assessed by quantitative RT-PCR, Western blot and ELISA. siRNA mediated silencing decreased the levels of both Trx1 and Grx2 mRNA to below 10% (Fig. 4a), Trx1 protein levels were down to below 6% (Fig. 4b) and the levels of Grx2 below 5% (quantified by

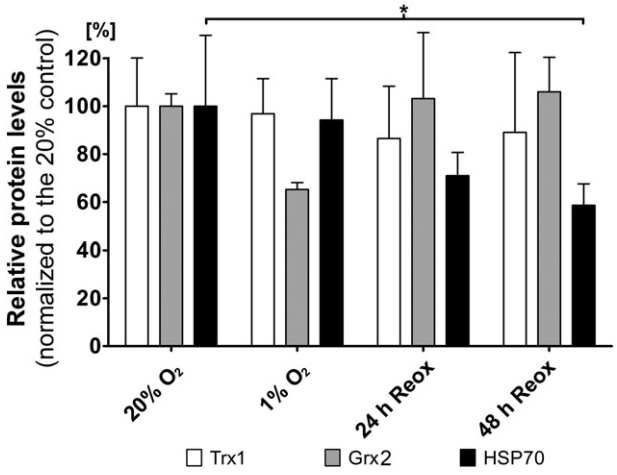

Fig. 3. Effect of hypoxia and reoxygenation on the levels of Grx2, Trx1, and HSP70 in SH-SY5Y cells. SH-SY5Y cells were cultured for 24 h in an atmosphere containing 20% O_2 or 1% O_2, respectively. Thereafter, both the normoxic and hypoxic cells were incubated for another 24 or 48 h at 20% O_2. The levels of Trx1, Grx2, and HSP70 were quantified by Western blotting and densitometric analysis. Three independent experiments were averaged, the SD is included.

ELISA as described in Hanschmann et al. [45]). In order to evaluate the differentiation state of the neuroblastoma cell line, we analyzed the protein levels of neurogenic differentiation 1 (NeuroD1), neurofilament M, and glial fibrillary acidic protein (GFAP) (Fig. 5a, b, c). NeuroD1 is a transcription factor that plays a key role in the moment in which cells acquire a specific neuronal subtype, and is essential for the differentiation and survival of neurons in the hippocampus [48]. Neurofilaments are proteins specifically expressed in neurons, where they stabilize the axon [49]. Neurofilaments M are intermediate filament proteins that play a key role in the maintenance of the neuronal phenotype [50]. GFAP is an intermediate filament protein specifically expressed in astroglia, the dominant and functionally most dynamic glial cell type [51,52]. In addition, we also analyzed the levels of HSP70 [46,47] and poly-ubiquitinylation of proteins [53–55] as markers for neuronal and protein damage, respectively (Fig. 5d, e). The knock-down cells were also analyzed for changes in their general phenotype by immunocytochemistry, staining microtubuli and F-actin (Fig. 6a). Cells transfected with an unspecific control (scrambled, scr) siRNA showed a normal SH-SY5Y phenotype. These cells appeared to increase in volume and length during the hypoxic period and maintained this phenotype during the reoxygenation phase (Fig. 6a). In parallel, viability and proliferation were analyzed using the MTT assay (Fig. 6b). After 24 h of reoxygenation, mitochondrial activity/proliferation increased and dropped again

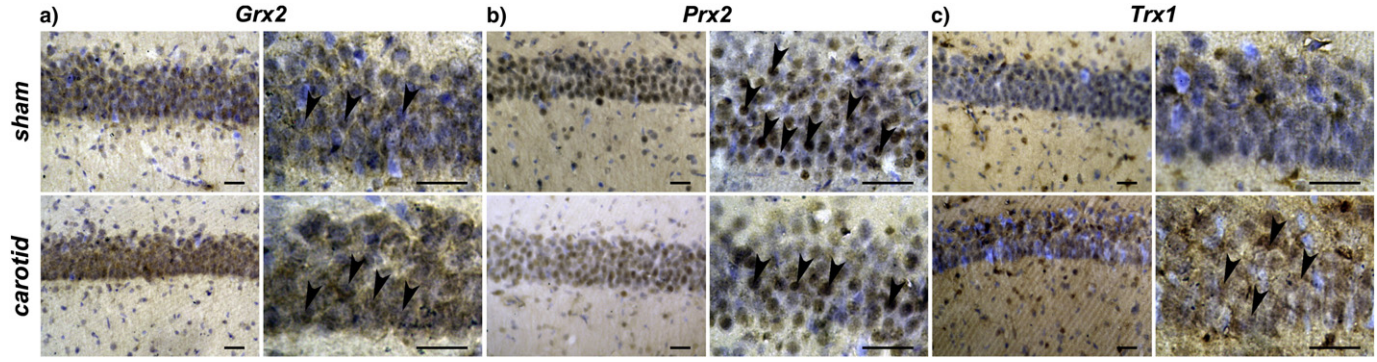

Fig. 2. Immunohistochemical analysis of the expression pattern of Trx family proteins in sham and carotid-ligated animals, 21 days after the ischemic insult. a) Representative immunostainings of Grx2 in the CA1 area of the hippocampus, arrowheads illustrate the differences in the nuclear staining following the induction of hypoxia/ischemia and reoxygenation. b) Representative pictures of Prx2 staining of the CA1 area of the hippocampus, arrowheads indicate the reduction seen in the labeling for Prx2-positive cellular bodies. c) Representative stainings for Trx1 in the CA1 area of the hippocampus, arrowheads show the faint labeling for Trx1-positive cellular bodies. Hematoxylin was used for nuclear staining. n = 6 sham and 6 carotid animals. Scale bars = 50 μm.

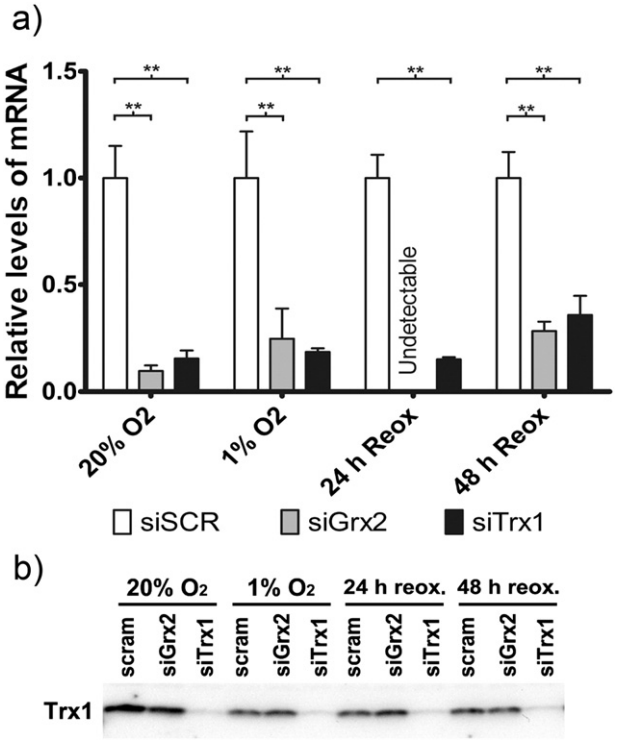

Fig. 4. Knock-down efficiency of Grx2 and Trx1 silencing. The efficiency of the knock-down of Grx2 and Trx1 was assessed by measuring of mRNA levels by means of quantitative RT-PCR (a), western Blot (b), and ELISA. Bars represent the mean ± SD. $^{**}p \leq 0.01$. Two way ANOVA [condition (20% O_2, 1% O_2, 24 h reox, 48 h reox) × treatment (control (scr), siGrx2, siTrx1)] followed by Tukey HSD post hoc tests for pair-wise multiple comparisons were employed to analyze the statistical significance.

during the extended reoxygenation phase, compared to controls that did not undergo the hypoxic treatment, but were grown for the same time lapses. NeuroD1 levels were significantly increased after 48 h of reoxygenation (Fig. 5a, f), whereas the levels of neurofilament M were slightly decreased during the reoxygenation period (Fig. 5b, f). The protein levels (see 20% O_2, siGrx2) were strongly decreased during hypoxia and reoxygenation (Fig. 5c, f). The protein amount of HSP70 was decreased by 20-30% following reoxygenation (Fig. 5d, f). Poly-ubiquitination was quantified densiometrically from Western-blots. Noteworthy, even under basal conditions, silencing of Trx1 led to an increase in poly-ubiquitinated proteins (Fig. 5e).

Grx2 silencing led to an increase in cell soma (Fig. 6a) and induced the formation of F-actin in filopodia like structures, both in the hypoxic group and the reoxygenation groups. (Fig. 6a) [55]. The lack of Grx2 increased mitochondrial activity/proliferation by approx. 25% under generally all conditions (Fig. 6b). NeuroD1 expression was increased under normal conditions as well as following reoxygenation (Fig. 5 a, f). The levels of neurofilament M were decreased under all conditions in the absence of Grx2 in comparison to the control (scr) siRNA group cultured in an atmosphere containing 20% oxygen (Fig. 5b, f). GFAP levels were decreased during hypoxia and following 48 h reoxygenation under normal cultivation conditions (control (scr) group). Grx2 silencing led to a decrease in GFAP levels both after silencing under normal conditions (see 20% O_2, siGrx2) and following reoxygenation in comparison to the 20% O_2 control (scr) group. During the hypoxic period siGrx2 cells displayed similar levels of GFAP compared to the 20% O_2 control (scr) group (Fig. 5c, f). Surprisingly, silencing of the Grx2 expression decreased HSP70 levels under all conditions analyzed for Grx2 silencing in comparison to the 20% O_2 control (scr) group (Fig. 5d, f). Moreover, the lack of Grx2 led to an increase in poly-ubiquitinated proteins, compared to the controls that were cultured under the same conditions. Interestingly, the strongest relative increase was seen at 48 h of reoxygenation (Fig. 5e).

Trx1 silencing showed some morphological alterations, most of all elongated neurites—even under normoxic conditions (Fig. 6a). Using the MTT assay, we could show that the lack of Trx1 led to a slight decrease in mitochondrial activity/proliferation of about 15% under essentially all conditions (Fig. 6b). Silencing of Trx1 did not significantly influence the levels of NeuroD1 following hypoxia and reoxygenation in comparison to the 20% O_2 control (scr) group (Fig. 5a, f), meaning that the levels of NeuroD1 increased after 48 h of reoxygenation regardless of the presence of Trx1. However, the levels of neurofilament M were clearly decreased both after Trx1 silencing in the 20% oxygen group compared to the 20% O_2 control (scr) (Fig. 5b,f), as well as after 48 h reoxygenation in both the control (scr) and the siTrx1 group in comparison with their respective 20% oxygen group. On the contrary, the levels of GFAP were more than three-fold increased following reoxygenation after Trx1 silencing (Fig. 5c, f). Trx1 silencing, by itself, had no significant effect on the levels of HSP70 in the 20% oxygen group in comparison to the 20% O_2 control (scr) group. Nonetheless, after reoxygenation Trx1 silencing led to an increase in the protein levels of HSP70 (Fig. 5d, f). In addition, the lack of Trx1 led to a general increase in poly-ubiquitinated proteins, compared to the control (scr) groups (Fig. 5e).

4. Discussion

Reperfusion after an ischemic event leads to an increased production of ROS [56,57], that have been proposed as causative agent for the death and degeneration of neurons following the insult [14,15, 57,58]. We have previously shown that Trx family (and related) proteins are distributed in a highly region and cell-type specific manner in the rat and mouse CNS [43,44], implying specific functions and a complex crosstalk between the Trx family members. Trx1 immunohistochemistry in gerbil brains during reperfusion following transient ischemia demonstrated an induction of Trx in hippocampal glial cells not seen in control animals [59]. Brain ischemia in rats, as a consequence of middle cerebral artery occlusion, leads to a decrease in Trx staining in ischemic areas, while immunoreaction and mRNA for Trx was increased in the penumbra regions [60]. Transgenic mice overexpressing human Trx1 and submitted to focal cerebral ischemia showed smaller infarct areas and fewer neuronal deficits than wild type mice [24]. In a similar manner, the loss of Grx1 correlated with neuronal damage following middle cerebral artery occlusion [61]. Trx2 overexpression protects from an oxidative insult-induced NF-κB activation and apoptosis [62]. In the present study, we examined the regulation and potential functions of 14 proteins of this family in an animal model and analyzed the function of particular redoxins in a cellular model of perinatal asphyxia, with the aim to better understand the regulation and potential roles of the proteins in the cellular pathways triggered by the hypoxic insult.

Hypoxic insults induce molecular and cellular changes, affecting cell morphology, cell polarity, osmoregulation, protein synthesis and the release of neurotransmitters [63,64]. The reoxygenation phase causes rapid changes in the redox properties of the affected tissue and an increase in the cytokine and chemokine levels, as well as the infiltration of immune cells [65]. Not surprisingly, members of the Trx family have been described to protect against ischemic injuries. Overexpression of Trx1, Grx1, Grx2, and Prx2 were shown to attenuate ischemic damage of neurons [66–68]. Moreover, Trx family proteins could function in a systemic inflammatory response, due to their versatile extra- and intracellular functions [18].

In the murine animal model, we detected complex and tissue-specific changes in the expression and distribution of distinct proteins following the hypoxic insult. Notably, the analyzed protein levels did not change uniformly, but highly specific for the region and cell type. Although no studies on Trx family proteins in PA have been published, both the release of ROS and RNS have been demonstrated under this

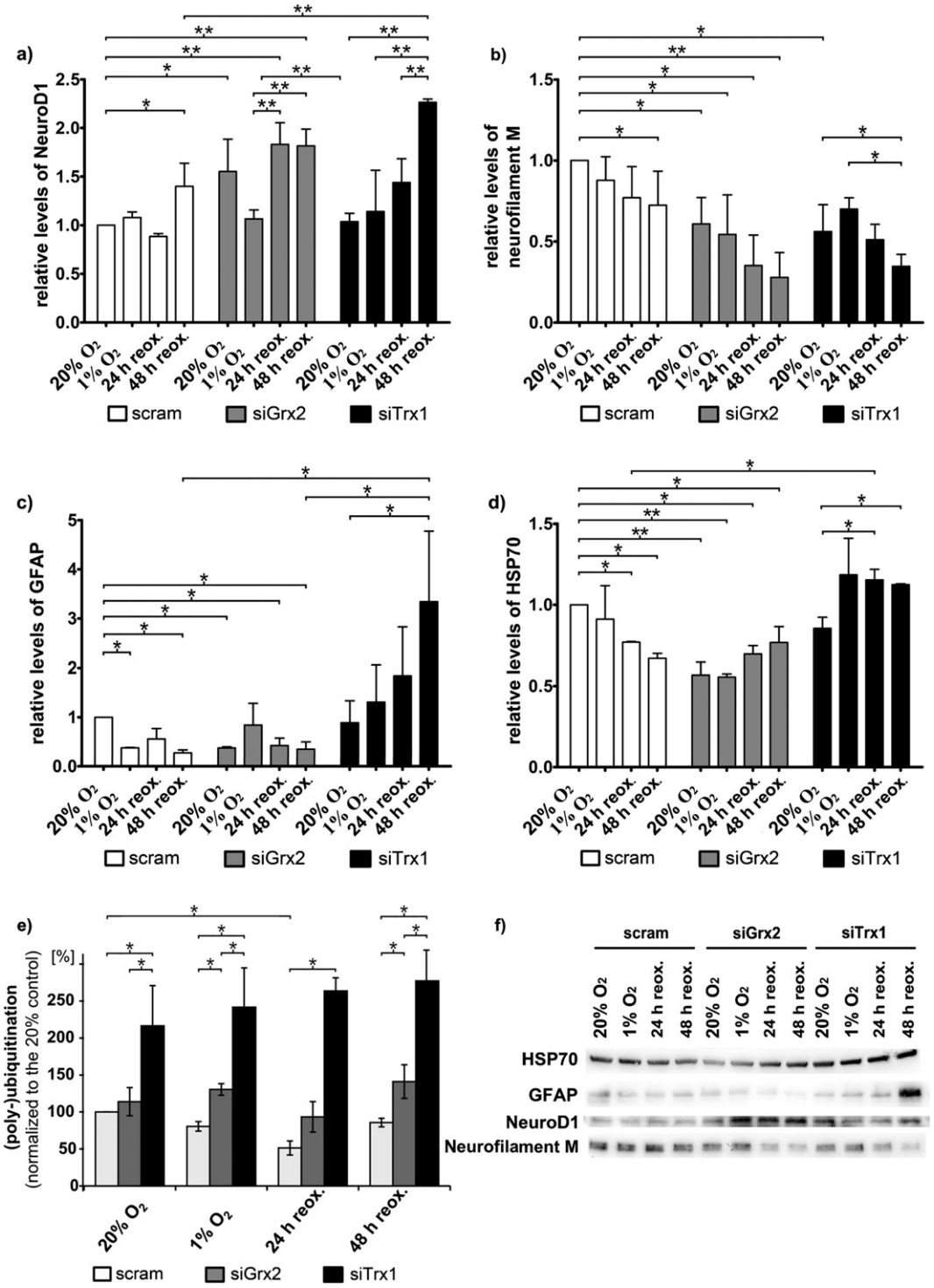

Fig. 5. Modulation of Trx1 and Grx2 expression in SH-SY5Y cells submitted to hypoxia and reoxygenation. Following transient knock-down of hTrx1 (siTrx1) and hGrx2 (siGrx2) using specific siRNAs, SH-SY5Y cells were cultured for 24 h in an atmosphere containing 20% O_2 or 1% O_2, respectively. The latter were reoxygenated at 20% O_2 for 24 h and 48 h. Cells transfected with an unspecific control siRNA (control (scr)) were used as control. The protein levels of neurogenic differentiation 1 (NeuroD1) (a, f), neurofilament M (b, f), glial fibrillary acidic protein (GFAP) (c, f), heat shock protein 70 (HSP70) (d, f), and poly-ubiquitinylation (e), were analyzed by Western Blot showing the hypoxia/reoxygenation-induced cellular damage and the state of differentiation. Densitometric quantification against total protein amount using the stain-free technology (Biorad) from three independent experiments are depicted. Relative protein levels are illustrated, comparing all treatments to control (scr) cells cultured at 20% O_2. Bars represent the mean ± SEM. $^*p \leq 0.05$, $^{**}p \leq 0.01$. Two way ANOVA [condition (20% O_2, 1% O_2, 24 h reox, 48 h reox) × treatment (control (scr), siGrx2, siTrx1)] followed by Tukey HSD post hoc tests for pair-wise multiple comparisons were employed to analyze the statistical significance.

condition [14], potentially leading to the dysregulation of redox signaling, which could be responsible for the induction of apoptosis and de-differentiation in the CNS and thus the long-term neurological deficits. The disruption of redox control or generally the generation of ROS by uncoupling of the mitochondrial chain [69] and inflammation [70,71] were described as the most destructive causes of hypoxia/ischemia and reoxygenation injury.

The thioredoxin system is a key regulator of cellular redox responses and was shown to be altered in different models of ischemia. Trx1 was shown to be up-regulated in the hippocampus of gerbil after transient

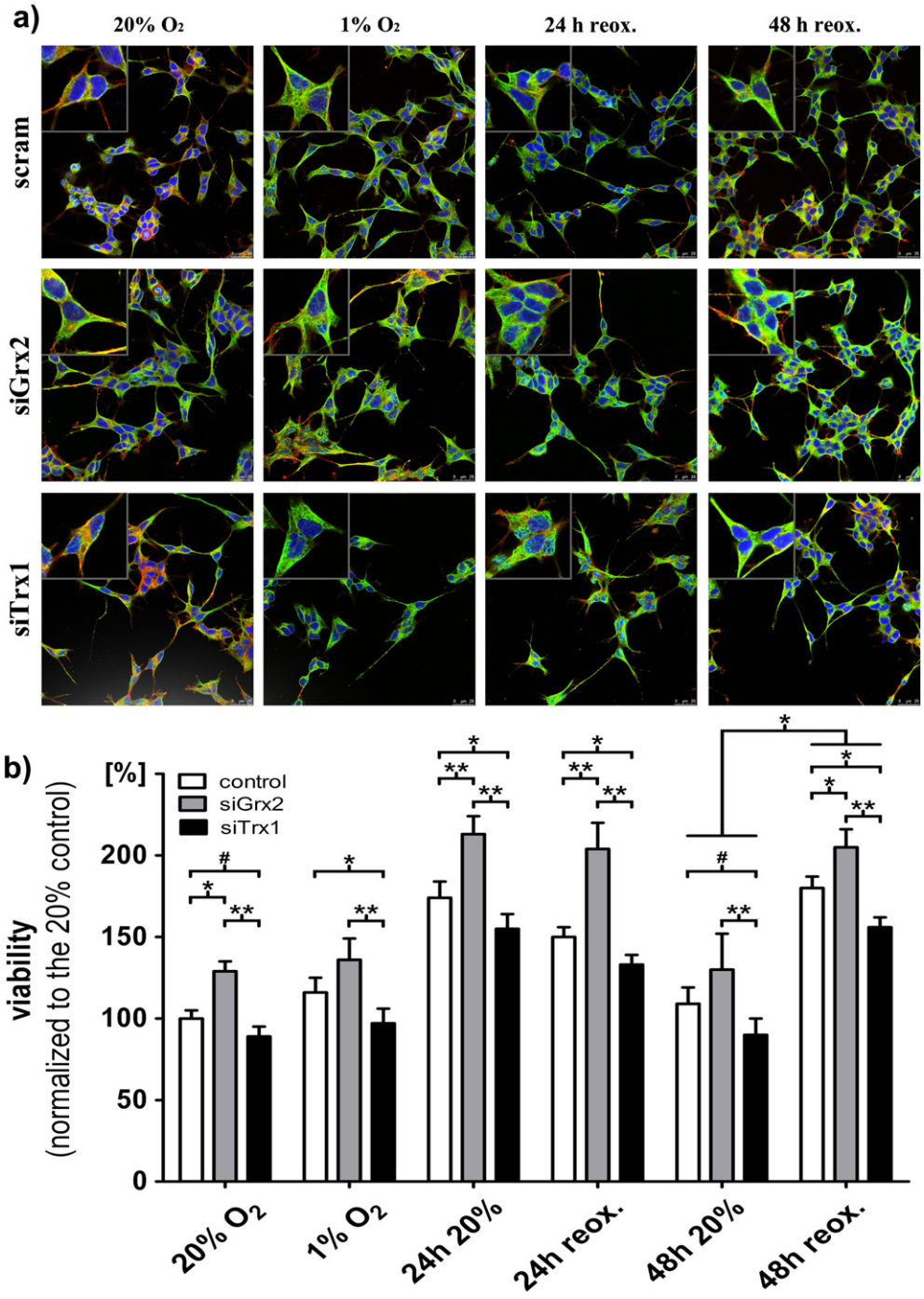

Fig. 6. Morphological evaluation of SH-SY5Y cells after modulation of Trx1 and Grx2 expression. Following transient knock-down of hTrx1 (siTrx1) and hGrx2 (siGrx2) using specific siRNAs, SH-SY5Y cells were cultured for 24 h in an atmosphere containing 20% O_2 or 1% O_2, respectively. The latter were reoxygenated at 20% O_2 for 24 h and 48 h. Cells transfected with an unspecific control (scr) siRNA were used as control. (a) Cells were fixed and analyzed for cytoskeleton changes by immunofluorescence using an antibody against tubulin and phalloidin staining for F-Actin. Five layers in the volume z = 0.5 μm were scanned by confocal microscopy. Red: phalloidin, green: tubulin, blue: DAPI. Representative pictures from n = 4 independent experiments are shown. Scale bars: 25 μm. (b) The cell number as a measure of viability and proliferation was analyzed using the MTT assay with cell cultured in 96 well plates. Three independent experiments were performed with six replicates each. The data were normalized to the 20% oxygen control at time point zero. Bars represent the mean ± SD. #$p \leq 0.06$, *$p \leq 0.05$, **$p \leq 0.01$. Two way ANOVA [condition (20% O_2, 1% O_2, 24 h 20%, 24 h reox, 48 h 20%, 48 h reox) × treatment (control (scr), siGrx2, siTrx1)] followed by Tukey HSD post hoc tests for pair-wise multiple comparisons were employed to analyze the statistical significance.

cerebral ischemia [59]. After occlusion of the middle cerebral artery, rats displayed an increased expression of Trx1 in the perifocal regions of ischemia [60]. Moreover, transgenic mice overexpressing human Trx1 that suffered a focal cerebral ischemia showed a reduction in the damage as well as lower neuronal deficits than wild type mice [24]. The MDA-MB-231 breast cancer cell line grown in hypoxia showed an increase in Trx1 expression after reoxygenation. When these cells were subjected to hypoxic cyclic preconditioning pre-treatment, the Trx1 expression levels were even higher [72]. Trx2 overexpression was shown to reduce TNF-L, normally induced by ROS, which in turn leads to NF-κB activation and apoptosis [62]. In a model for renal ischemia-reperfusion, Grx2, Prx3 and Prx6 were highly expressed in the proximal tubule cells of the kidney, which can regenerate following an ischemic insult. Interestingly, the overexpression of these proteins in

HeLa and HEK293 cells protected them from hypoxia/reoxygenation-induced oxidative damage and increased the total cell number following the ischemia [40]. Both Prx1 and Prx5 were induced after treatment with hydrogen peroxide or hypoxia. Silencing of Prx1, and Prx5 expression sensitized cells to oxidative insults [73]. All these data indicate that modulation of the Trx family of proteins is an important target for the protection of cells from an oxidative insult, but might also reveal new insights in the regenerative capacities and underlying mechanisms of specific cell types.

In our study, the immunostaining of Trx1 was increased following hypoxia/ischemia and reoxygenation in the areas of the hippocampus and cerebellum of the brain (see Fig. 1). Although the effect of severe hypoxia on the expression of the cytosolic Trx1 in the brain has been shown in preconditioned rats at 24 h and 72 h after reperfusion [61,74], no study has been performed at extended periods after reperfusion. The expression of Grx2 also increased significantly after hypoxia/ischemia and reoxygenation in the carotid-clamped group (see Fig. 1). As mentioned above, the induction of the expression of the oxidoreductase was also shown in a model for renal ischemia reperfusion injury [40]. Besides the anti-apoptotic function of mitochondrial Grx2a [75,76], cytosolic Grx2c was recently shown to be essential for embryonic brain development. Zebrafish with silenced expression of the oxidoreductase were characterized by the loss of basically all types of neurons due to apoptosis and a developed axonal scaffold [77]. Moreover, the cytosolic isoform Grx2c, has been shown to be involved in axonal outgrowth and neuronal differentiation [77,78]. We believe that Grx2 expression might be induced in an attempt to recover the system from the hypoxic-ischemic insult. Grx2 could be crucial for the long term regeneration of physiological redox signaling and the recovery of the affected tissues.

Surprisingly, Grx2 silencing had no such effect in the SH-SY5Y cells. After having been subjected to hypoxia, SH-SY5Y cells showed a reduction of the HSP70 levels under all conditions relative to the 20% O_2 control (scr) group (see Fig. 5d). Upon different stimuli, cells activate the so called unfolded protein response (UPR), allowing an initial response that leads to the restoration of the normal functions of the ER by the induction of heat shock proteins. However, this response needs to be tightly controlled; otherwise it can lead to the induction of cell death through the activation of the apoptotic pathway associated to the ER [47,79]. Therefore, high levels of the marker HSP70 (an ER chaperone), can be used as indicator of ER stress and are also generally used as neuronal damage marker [46]. In this manner, the reduction of HSP70 levels due to Grx2 silencing was unexpected and seemed like an unusual behavior considering that Grx2 has been implicated in anti-apoptotic signaling before [48,51]. However, when analyzed for poly-ubiquitination (Fig. 5 e), a marker for protein damage, the lack of Grx2 led to an increase in ubiquitination under all conditions. In comparison, Trx1 silencing had no significant effect on the levels of HSP70 under normoxic conditions. However, following reoxygenation Trx1 silencing induced an increase in the cellular HSP70 levels (Fig. 5d, f). Also, silencing of Trx1 led to an increase in poly-ubiquitinated proteins, compared to the controls (Fig. 5e). Taking all this together, the expression of Grx2 and Trx1 seems to contribute to protect neuronal cells from hypoxia-induced protein and cellular damage. Interestingly, the knock-down of Trx1 also affects cellular proliferation and viability as analyzed by the MTT assay. Following the hypoxia and reoxygenation, a decrease of approximately 15% in mitochondrial activity/proliferation was detected in Trx1 knock-down cells (Fig. 6b), indicating a reduction in cell number and/or a decreased viability which could be due to elevated levels of cellular damage. Surprisingly, Grx2 knock-down, which was shown before to sensitize cells towards distinct oxidative stimuli [76], led to an overall elevated mitochondrial activity/proliferation following the hypoxia and reoxygenation. Thus, the lower extent of cellular damage is reflected in the overall higher mitochondrial activity/proliferation of Grx2 depleted cells following the ischemic insult, compared to control cells but also compared to Trx1 depleted cells.

In our SH-SY5Y cell model we could not detect any significant changes in the protein levels of Trx1 and Grx2 when exposing the cells to hypoxia and reoxygenation (see Fig. 3). Nonetheless, when the protein levels were manipulated by RNA interference it was possible to observe functional consequences (see Figs. 5 and 6), supporting the hypothesis that both *in vivo* and *in vitro* these redoxins could have an important role in the recovery after a hypoxia and reoxygenation event.

Oxygen concentrations and ROS levels are not only known to affect cell viability, but also to affect and regulate embryonic development and cellular differentiation. NeuroD1 is a transcription factor that regulates several cells differentiation pathways, for instance, the differentiation of neuronal cells that contribute to the formation and maintenance of the cerebellum or the hippocampus [80]. While its levels were unaffected by hypoxia in our cellular model, they were increased in all samples following reoxygenation, slightly more pronounced in the absence of Trx1. Neurofilament M is an intermediate filament protein that contributes to the maintenance of the neuronal phenotype [50]; GFAP is an intermediary filament protein that is specifically expressed in astrocytes [52]. Both the lack of Grx2 and Trx1 led to a significant reduction in neurofilament M levels compared to the control siRNA 20% O_2 group as well as the siGrx2 and siTrx1 20% O_2 groups. Grx2 silencing also led to a reduction in GFAP both following silencing under normal conditions as well as following reoxygenation in comparison to the 20% O_2 control. However, silencing of the Trx1 expression led to a strong, highly significant increase in the protein levels of GFAP. SH-SY5Y cells are derived from a neuroblastoma patient and frequently used as a model for neuronal differentiation and function. These cells can be maintained in a rather undifferentiated state but can also develop a neuron-like adrenergic and dopamine-sensitive phenotype [81]. Our results suggest that in the absence of Grx2 or Trx1 the cells undergo differentiation into what appears to be a more glial-like cell type, following hypoxia and reoxygenation, as shown by the decreased neurofilament M and GFAP levels (particularly in the absence of Trx1). Grx2, for instance, was shown to be expressed in both, neurons and glia cells of the mouse brain [82]. It is tempting to speculate that both redoxins have distinct function in both neurons and glia cells and that they can also function in the development of distinct cell types within the brain. Changes in the protein levels might affect the differentiation state of a cell, and thereby also the susceptibility towards the ischemic insult.

Prx2 immunostaining was consistently reduced in the carotid-clamped animals. Depletion of Prx2 had exhibited potent pro-survival effects in ischemic neurons by indirectly maintaining Trx in its reduced state [83]. In this respect, the low levels of Prx2 found in the present study could be related to high amounts of active Trx1.

The role of Trx family proteins in hypoxia is not well understood. Previous studies have shown that the Trx family of proteins contributes to mechanisms of brain tolerance in early periods after exposure to stress [74,84]. Cells have developed response mechanisms to cope with these low oxygen concentrations. Hypoxia-inducible factors 1 and 2 constitute transcription factors that regulate the expression of more than 180 genes under hypoxic conditions [85]. The HIF-1 target ATIA (anti-TNFa-induced-apoptosis) was shown to protect cells against hypoxia-induced apoptosis via Trx2 and the generation of ROS [86]. Moreover, hypoxia-induced mitochondrial ROS are essential for HIF activation [87,88], regulate inflammatory responses via interleukin (IL)-6 [89], and apoptosis via p38 phosphorylation [90]. Other studies suggested that overexpression of Trx1 leads to elevated HIF-1α levels in cells cultured under both normoxic and hypoxic conditions, whereas inhibition of TrxR1 activity blocked the activation of HIF-1α [91]. Trx1 leads to an induction of HIF-1α, possibly by activating Akt [92]. Trx1 might also be involved in depleting HIF-1α levels upon reoxygenation [93].

Not all areas of the brain are equally vulnerable to hypoxia-ischemia. Among the most vulnerable areas are the superior brainstem, cerebellum, white matter and subcortical structures supplied by the distal

branches of deep and superficial penetrating blood vessels, cerebral white matter at the zones between the major cerebral artery territories, CA1 region of the hippocampus, and neocortical layers 3, 5, and 6 [94–96]. The expression pattern of the Trx family proteins changed in the most vulnerable areas of the brain, particularly in the hippocampus and cerebellum. Interestingly, by IHC we were able to observe an overall stronger immunostaining for Trx1 in the hippocampus, not only in the cytosol but also what appears to be a nuclear staining. Moreover, it is tempting to speculate that the overall higher background in the tissue of the asphyxic animals, compared to sham control rats, is due to Trx1 secretion into the extracellular space (see Fig. 2c). This is particularly interesting considering that Trx1 secretion [24,97–101], as well as its nuclear translocation [24,102–107] have been implicated in regulating cell proliferation, differentiation, and apoptosis. These findings imply that these proteins could be clinically applied as a therapeutic strategy to counteract the hypoxia/ischemia and reoxygenation damage. Beneficial effects of the administration of the Trx family of protein after a hypoxia-reperfusion injury in an adult model of stroke have been demonstrated already [108,109].

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SFB593-N01, LI984/3-1, and GRK1947-A1) to CHL, the Federal Ministry for Science and Education (BMBF: 01DN13023-PAREDOX) and MINCYT to CHL and FC, the German Academic Exchange Service DAAD and MINCYT (PROALAR program) to CHL and FC, the 5 (PIP 1142010010019, CONICET, Argentina) to FC, and the University of Buenos Aires (UBACYT 20020090100118) to FC. JIR and MIH are fellowship holders from the National Scientific and Technical Research Council (CONICET, Argentina). None of the authors have any conflict of interest to report related to this manuscript.

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