



ELSEVIER

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Original Contribution

Neuroglobin expression and oxidant/antioxidant balance after graded traumatic brain injury in the rat

Valentina Di Pietro^a, Giacomo Lazzarino^b, Angela Maria Amorini^b, Barbara Tavazzi^b, Serafina D'Urso^c, Salvatore Longo^b, Roberto Vagnozzi^d, Stefano Signoretti^e, Elisabetta Clementi^f, Bruno Giardina^b, Giuseppe Lazzarino^{c,*}, Antonio Belli^a^a Neurotrauma and Neurodegeneration Section, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK^b Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome, Rome, Italy^c Department of Biology, Geology, and Environmental Sciences, Division of Biochemistry and Molecular Biology, University of Catania, 95125 Catania, Italy^d Department of Biomedicine and Prevention, Section of Neurosurgery, University of Rome Tor Vergata, Rome, Italy^e Division of Neurosurgery, Department of Neurosciences, Head and Neck Surgery, S. Camillo Hospital, Rome, Italy^f CNR Institute of "Chimica del riconoscimento molecolare," Catholic University of Rome, Rome, Italy

ARTICLE INFO

Article history:

Received 3 December 2013

Received in revised form

7 January 2014

Accepted 24 January 2014

Available online 31 January 2014

Keywords:

Brain antioxidants

Neuroglobin

Neuroprotection

Nitrosative stress

Oxidative stress

Traumatic brain injury

Free radicals

ABSTRACT

Neuroglobin is a neuron-specific hexacoordinated globin capable of binding various ligands, including O₂, NO, and CO, the biological function of which is still uncertain. Various studies seem to indicate that neuroglobin is a neuroprotective agent when overexpressed, acting as a potent inhibitor of oxidative and nitrosative stress. In this study, we evaluated the pathophysiological response of the neuroglobin gene and protein expression in the cerebral tissue of rats sustaining traumatic brain injury of differing severity, while simultaneously measuring the oxidant/antioxidant balance. Two levels of trauma (mild and severe) were induced in anesthetized animals using the weight-drop model of diffuse axonal injury. Rats were then sacrificed at 6, 12, 24, 48, and 120 h after traumatic brain injury, and the gene and protein expression of neuroglobin and the concentrations of malondialdehyde (as a parameter representative of reactive oxygen species-mediated damage), nitrite + nitrate (indicative of NO metabolism), ascorbate, and glutathione (GSH) were determined in the brain tissue. Results indicated that mild traumatic brain injury, although causing a reversible increase in oxidative/nitrosative stress (increase in malondialdehyde and nitrite + nitrate) and an imbalance in antioxidants (decrease in ascorbate and GSH), did not induce any change in neuroglobin. Conversely, severe traumatic brain injury caused an over nine- and a fivefold increase in neuroglobin gene and protein expression, respectively, as well as a remarkable increase in oxidative/nitrosative stress and depletion of antioxidants. The results of this study, showing a lack of effect in mild traumatic brain injury as well as asynchronous time course changes in neuroglobin expression, oxidative/nitrosative stress, and antioxidants in severe traumatic brain injury, do not seem to support the role of neuroglobin as an endogenous neuroprotective antioxidant agent, at least under pathophysiological conditions.

© 2014 The Authors. Published by Elsevier Inc. Open access under [CC BY](http://creativecommons.org/licenses/by/4.0/) license.

Neuroglobin (Ngb)¹ is a hemoprotein that is considered a "hexacoordinated" globin, for the characteristics of the binding of the heme prosthetic group to the protein [1]. Although the six coordination bonds of its iron atom are engaged in coordinating the nitrogen of the tetrapyrrole ring (four bonds) and two histidines

of the heme pocket (two bonds), Ngb is capable of binding various ligands, including molecular oxygen, carbon monoxide, and nitric oxide (NO) [2], as well as forming a homodimer through the specific oxidation of two cysteine residues and changing the oxidation state of its iron atom [1,2]. Ngb has very recently been discovered [3] and has the peculiarity of being located in the brain [3], specifically in particular brain regions [4] and more specifically in neurons and not in the other cerebral cell types [4]. Because of the multiplicity of ligands with similar affinity [2], it is not yet clear what the biochemical role of Ngb is under physiological conditions, although several hypotheses have been formulated

Abbreviations: Ngb, neuroglobin; TBI, traumatic brain injury; mTBI, mild TBI; sTBI, severe TBI; ROS, reactive oxygen species; RNS, reactive nitrogen species; GSH, reduced glutathione

* Corresponding author. Fax: +39 095337036.

E-mail address: lazzarig@uniuc.it (G. Lazzarino).

<http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.032>

0891-5849 © 2014 The Authors. Published by Elsevier Inc. Open access under [CC BY](http://creativecommons.org/licenses/by/4.0/) license.

in the past decade [5]. In particular, the interest in this protein significantly increased when several experimental studies evidenced that the gene expression and the levels of Ngb were positively affected by cerebral ischemia [6]. Furthermore, it was indicated that Ngb overexpression was capable of decreasing damage induced by oxygen deprivation in cellular and animal models of ischemia [7,8]. Such beneficial effects were attributed to the capacity of Ngb to modulate NO formation [9,10] and generally to decrease the toxic effects of reactive oxygen (ROS) and reactive nitrogen species (RNS) [9–12]. Therefore, in the constant endeavor to find new targets for effective therapies, as well as to find biomarkers of clinical utility, Ngb has been considered as a highly promising macromolecule in studies of acute and chronic neurodegeneration [13,14].

At present, very little is known about the effect of traumatic brain injury (TBI) on Ngb expression, with a few studies indicating Ngb overexpression after mechanical injury [15] and neuroprotection from TBI-associated damages in the Ngb-overexpressing animals [16]. TBI can be considered a peculiar type of acute neurodegeneration with molecular mechanisms of cell damage strictly related to the severity of injury [17,18]. TBI is the leading cause of death under 45 years of age in Western countries [19] and carries an enormous socioeconomic burden: for example, in the United States, the annual aggregated direct medical costs and indirect costs of work loss and lost quality of life are estimated to range from \$60.4 billion to \$221 billion for the civilian population alone [20]. Its incidence is on the rise and by 2020 the World Health Organization estimates that TBI will be the largest cause of disability worldwide. Although our knowledge of the pathobiological mechanisms of TBI-associated damage has increased tremendously in recent years, there are no current valid therapies to counteract the adverse effects of TBI, nor are there validated biomarkers to prognosticate its outcome [21]. Thanks to hundreds of studies using different models of experimental TBI, it has been possible to show evidence that TBI affects an incredibly vast number of neuronal functions, including ionic balance [18,22], signal transduction [23], mitochondrial functions [24], and gene expression [25,26].

Using the weight-drop model of closed TBI, characterized by diffuse axonal injury, it was possible to show that the changes in parameters representative of energy metabolism, oxidative stress, and cerebral antioxidant defenses were deeply influenced by the severity of TBI [27]. Furthermore, it was observed that the mitochondrial phosphorylating capacity, concentrations of the nicotinic coenzyme pool, and oxidative/nitrosative stress were in close correlation with the severity of TBI [27,28]. Particular relevance has been attributed in the past few years to the implications that the onset of oxidative/nitrosative stress after a TBI may have on the apoptotic process [29] and, more in general, on the irreversible damage of the postinjured brain tissue [30]. Interrupting the vicious circle linking mitochondrial malfunctioning, induction of ROS and RNS, damage to macromolecules, and induction of apoptosis is considered a highly valuable target for new pharmacological therapies in TBI [31].

As the pathophysiological role of Ngb and its interplay with the oxidant/antioxidant balance after TBI have not yet been clearly established, in this study we evaluated how diffuse mild TBI (mTBI) and severe TBI (sTBI) affect gene expression and protein levels of Ngb at various times after injury. We also assessed the potential relationships of Ngb modulation with ROS, RNS, and brain low-molecular-weight antioxidants.

Materials and methods

Experimental protocol

The experimental protocol was approved by the ethics committee of the Catholic University of Rome, according to international standards and guidelines for animal care. Male Wistar rats

of 250–300 g were used in this study. They were randomly divided into three groups: (1) sham-operated (control group); (2) mild diffuse TBI (mTBI group); (3) severe diffuse TBI (sTBI group). As for the anesthetic mixture, animals received 35 mg/kg bw ketamine and 0.25 mg/kg bw midazolam by intraperitoneal injection. Mild or severe TBI was induced by dropping a 450-g weight from 1 or 2 m height, respectively, according to the weight-drop impact acceleration model [32]. Rats that suffered from skull fracture, seizures, or nasal bleeding or that did not survive the impact were excluded from the study. Six, 12, 24, 48, and 120 h after brain injury, the rats ($n = 9$ for each time point in each of the two groups of injured animals) were again anesthetized and then immediately sacrificed. Control animals were sacrificed 12 h after the initial anesthesia ($n = 9$).

Selective preparation of tissue for molecular and biochemical analyses

An *in vivo* craniectomy to minimize metabolite loss was performed in all animals during anesthesia. The rat skull was carefully removed, the brain was exposed and sharply cut along the sagittal fissure, and the two hemispheres were freeze-clamped by aluminum tongs, precooled in liquid nitrogen, and immediately immersed in liquid nitrogen to accelerate freezing of the tissue and increase the recovery of metabolites [33]. To obtain total RNA, one hemisphere was extracted in Trizol (Invitrogen Life Technologies), using the Ultra-Turrax homogenizer (Janke Kunkel, Staufen, Germany) at 24,000 rpm/min, producing a final 10% homogenate (wt/vol).

To perform Ngb quantification, one brain hemisphere was homogenized in 15 mM KCl + 1 mM KH_2PO_4 , pH 7.4, at 24,000 rpm/min for 90 s in the cold, followed by centrifugation at 18,690 g for 15 min at 4 °C.

Efficient deproteinization of the tissue for metabolite analysis, ensuring no loss of oxidizable compounds, was achieved according to the organic solvent deproteinizing procedure described in detail elsewhere [34].

To obtain simultaneous measurements of the parameters of interest in six animals at each time point and for both levels of injury, the hemispheres of three animals were processed for RNA and protein extraction, the hemispheres of three animals were processed for RNA and metabolite extraction, and the hemispheres of three animals were processed for metabolite and protein extraction. Using this protocol for tissue manipulation and properly mixing the different processing of the right and left hemispheres, the concomitant evaluation of parameters reflecting oxidative/nitrosative stress, tissue antioxidant defenses, and Ngb gene expression and quantification was obtained in six hemispheres (three right + three left hemispheres) using nine animals for each time point.

Gene expression of Ngb

RNA extracted from brain samples was reverse transcribed to cDNA using a Superscript II reverse transcriptase kit (Invitrogen). From each sample, 1 μg of total RNA, 500 ng of oligo(dT) primers (Roche Molecular Biochemicals, UK), and 200 U of Superscript II, in a total volume of 20 μl , were incubated at 42 °C for 60 min. The concentration and purity of the resulting cDNA were then determined with a ND-1000 UV–Vis spectrophotometer (NanoDrop). RT-qPCR with melting curve analysis was performed in a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad, USA). In each reaction, 100 ng of cDNA was mixed with 25 μl $2 \times$ SYBR Green PCR Master Mix (Applied Biosystems) and proper primers (300 nM final concentration) in a final volume of 50 μl . The thermal profile began with incubation at 95 °C for 10 min, followed by 40 amplification cycles alternating 94 °C for 15 s and 60 °C for 60 s. To confirm the specificity of reactions, a melting curve

was produced by conducting 81 melting cycles of 30 s every 0.5 °C, from 55 to 95 °C. Data were analyzed by the iQ5 Optical System software (Bio-Rad).

Primers were designed with the 0.2 version of the Primer3 Input software developed by the Whitehead Institute for Biomedical Research (Cambridge, MA, USA) and using as template the sequences of *Rattus norvegicus* Ngb (NM_033359.3) published by the National Center for Biotechnology Information. The following primer sequences were used: Ngb forward, 5'-CCTGTCTCCA-CAGCCTCTTC-3'; Ngb reverse, 5'-ACGGCACAGAGCAAGAAAGT-3'. For accurate gene expression measurements with RT-qPCR, results were normalized to the housekeeping gene ubiquitin C of *R. norvegicus* (Ubc NM_017314.1), selected using the geNorm Housekeeping Gene Selection Kit (Primer Design Ltd.) from 12 candidate reference genes. Changes in transcript abundance of Ngb were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen [35].

Quantification of Ngb

Ngb was quantified using the immunoenzymatic ELISA kit (Cusabio Biotech, Wuhan, China) according to the instructions of the manufacturer. Briefly, 100 μ l of standards and supernatants of brain homogenates were incubated in microplate wells precoated with antibody specific for Ngb. After incubation, biotinylation, and conjugation with streptavidin–horseradish peroxidase, plates were incubated for 30 min at 37 °C with 3,3',5,5'-tetramethylbenzidine. The reaction was stopped by addition of 50 μ l of acidic solution and absorbance of the resulting yellow product was measured spectrophotometrically at 450 nm (Molecular Devices, Sunnyvale, CA, USA). Using this protocol, the standard curves for Ngb were linear in the range 15.6–1000 pg/ml.

Determination by HPLC of malondialdehyde, ascorbate, GSH, nitrite, and nitrate

Malondialdehyde (MDA), ascorbate, GSH, nitrite, and nitrate were measured in 20 μ l of deproteinized brain extracts by high-performance liquid chromatography (HPLC). When referring to ascorbate and GSH, it is intended throughout the text that only the fully reduced forms of ascorbate and GSH were considered, separated, quantified, and calculated in samples of brain extracts. The various compounds were separated and quantified according to an ion-pairing method formerly set up in our laboratory [34,36] and using a Hypersil C-18, 250 4.6-mm, 5- μ m particle size column, provided with its own guard column (Thermo Fisher Scientific, Rodano, Milan, Italy). The HPLC apparatus consisted of a Spectra-System P4000 pump system (Thermo Fisher Scientific) and a highly sensitive UV6000LP diode array detector (Thermo Fisher Scientific), equipped with 5-cm light-path flow cell and set up between 200 and 300 nm wavelengths. The method allows the direct determination of the aforementioned compounds in deproteinized tissue extracts, with no sample derivatization [34,36,37]. MDA and ascorbate were detected at 266 nm wavelength, whereas GSH, nitrite, and nitrate were revealed at 206 nm wavelength. The suitability of these chromatographic conditions for nitrite and nitrate quantifications, as well as the comparison with other analytical techniques, has been fully described in a previous study [38]. Assignment and calculation of the various compounds in chromatographic runs of brain samples were performed by comparing retention times, absorption spectra, and area of the peaks to those of chromatographic runs of mixtures containing known concentrations of true MDA, ascorbate, GSH, nitrite, and nitrate.

Statistical analysis

The within-group comparison at each time was performed by one-way analysis of variance. Differences across groups were estimated by two-way analysis of variance for repeated measures. Fisher's protected least square was used as the post hoc test. Differences were regarded as statistically significant at a value of $p < 0.05$.

Results

Effects of graded TBI on gene and protein expression of Ngb

Data reported in Fig. 1 illustrate the time course changes in gene (Fig. 1A) and protein expression (Fig. 1B) of Ngb after mild or severe TBI. A strikingly different postinjury gene modulation was recorded in mTBI rats compared to what we observed in animals subjected to sTBI. After an mTBI, the expression of the gene encoding Ngb significantly increased only at 48 h postinjury (+79%, $p < 0.01$ with respect to controls), with no differences compared to the preimpact value at either the earlier or the later time points after trauma. When animals experienced an sTBI, a dramatic increase in the gene expression of Ngb (+929%, $p < 0.0001$ with respect to controls) was already evident at 6 h postinjury. Increases over the preimpact value of +490, +171, and +133% ($p < 0.001$ compared to controls) occurred at 12, 24, and 48 h, respectively. At 120 h after sTBI, a decrease of 60% in the gene expression of Ngb was recorded ($p < 0.01$ with respect to controls). Comparing the gene responses after mTBI and sTBI (Fig. 1A), it was observed that Ngb gene expression was significantly higher in sTBI rats at 6, 12, and 24 h ($p < 0.001$ compared to mTBI-injured rats), whereas no difference or lower values ($p < 0.01$ compared to mTBI-injured rats) were observed at 48 and 120 h postimpact, respectively.

As expected, mTBI-injured rats showed no significant changes in the Ngb levels at any time point postinjury, even though a tendency to increase at longer times postimpact was observed (Fig. 1B). Conversely, sTBI caused Ngb to increase to 522, 334, 270, and 210 pg/mg protein ($p < 0.001$ compared to controls) at 12, 24, 48, and 120 h after trauma, respectively, thereby indicating that the increase in gene expression was accompanied by an increased synthesis of Ngb (Fig. 1B). Comparing the two levels of injury, it was possible to observe that Ngb was significantly higher in sTBI rats at 12, 24, 48, and 120 h after trauma ($p < 0.001$ compared to mTBI rats), suggesting a differential neuronal response as a function of the severity of injury.

Oxidants and antioxidants after graded TBI

Because one of the possible biological roles of Ngb is to protect neurons from damage mediated by increased oxidative/nitrosative stress [11,12], we measured parameters related to overproduction of ROS (MDA) and RNS (nitrite and nitrate) to evaluate the effect of excess oxidants. The simultaneous determination of ascorbate and GSH allowed the evaluation of changes in low-molecular-weight antioxidants after graded TBI.

In Fig. 2, the time course changes of MDA (Fig. 2A) and of nitrite + nitrate (Fig. 2B) are illustrated. Mild TBI produced a significant increase in MDA from 3.6 ± 2.9 (value of controls) to 31.1 ± 8.5 and 11.4 ± 4.9 nmol/g wet wt, respectively, at 6 and 24 h postinjury ($p < 0.001$ compared to controls). At the subsequent time points after mTBI, MDA levels returned to preimpact values and no differences were observed compared to sham-operated rats. In contrast, sTBI caused a much more evident increase in cerebral MDA, with values significantly higher than those of controls at any time postinjury.

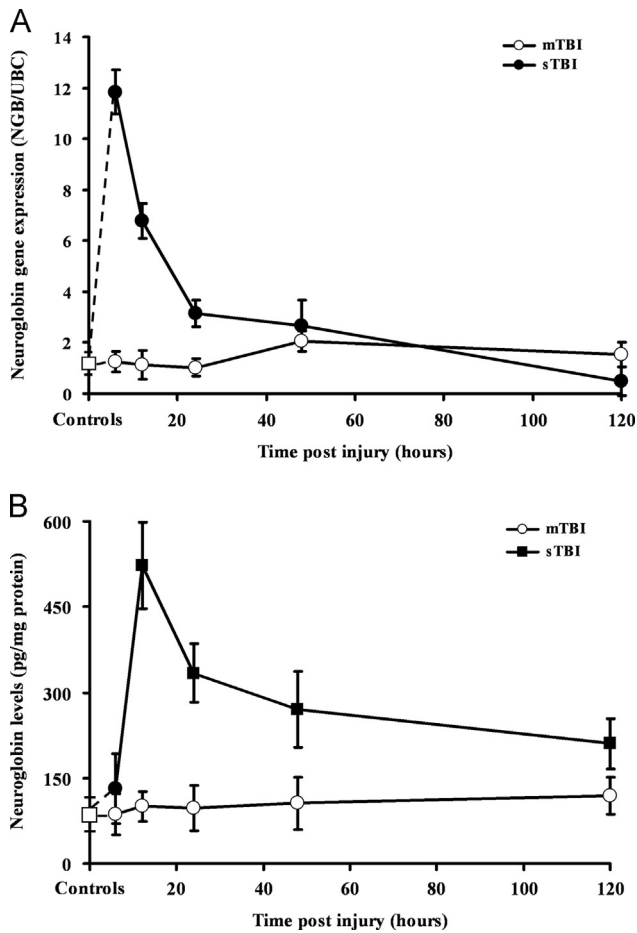


Fig. 1. Time course of the (A) gene and (B) protein expression of neuroglobin in brain homogenates of rats subjected to mild or severe traumatic brain injury. Each point represents the mean value obtained from six different brain samples (three left + three right hemispheres). Standard deviations are represented by vertical bars. Significances have been omitted for the sake of clarity and are described in the text.

In particular, maximal concentration of MDA was recorded at 12 h postinjury (74.1 ± 11.2 nmol/g wet wt; $p < 0.0001$ with respect to controls); at longer times after impact, MDA slowly decreased, showing a concentration of 30.8 ± 7.5 nmol/g wet wt even at 120 h after trauma ($p < 0.0001$ compared to controls). It is worth emphasizing that mildly injured animals had significantly lower MDA levels compared to those measured in severely injured rats at any time point after trauma occurrence ($p < 0.0001$).

As shown in Fig. 2B, the increase in oxidative stress was accompanied by an increase in RNS production causing a concomitant sustained nitrosative stress. Therefore, although a significant rise in the sum of nitrite + nitrate in mTBI rats with respect to control values was recorded only at 12 (+30%) and 24 (+25.9%) h postinjury ($p < 0.01$), the concentration of these ions deriving from metabolism of NO in sTBI rats was significantly higher than both the preimpact value ($p < 0.0001$) and any of the values recorded in mTBI rats after trauma ($p < 0.0001$).

As expected, the differential effect of graded TBI on excess oxidants caused different changes in brain antioxidants. The time course changes of ascorbate in mTBI rats (Fig. 3A) were characterized by a significant decline at 6 h (−9.2%; $p < 0.05$ compared to controls) and 12 h postinjury (−16.2%; $p < 0.01$ with respect to controls), followed by a rapid normalization up to values of the sham-operated rats. On the other hand, sTBI induced a dramatic and irreversible depletion of cerebral ascorbate, which was significantly decreased ($p < 0.0001$ compared to controls) at 6

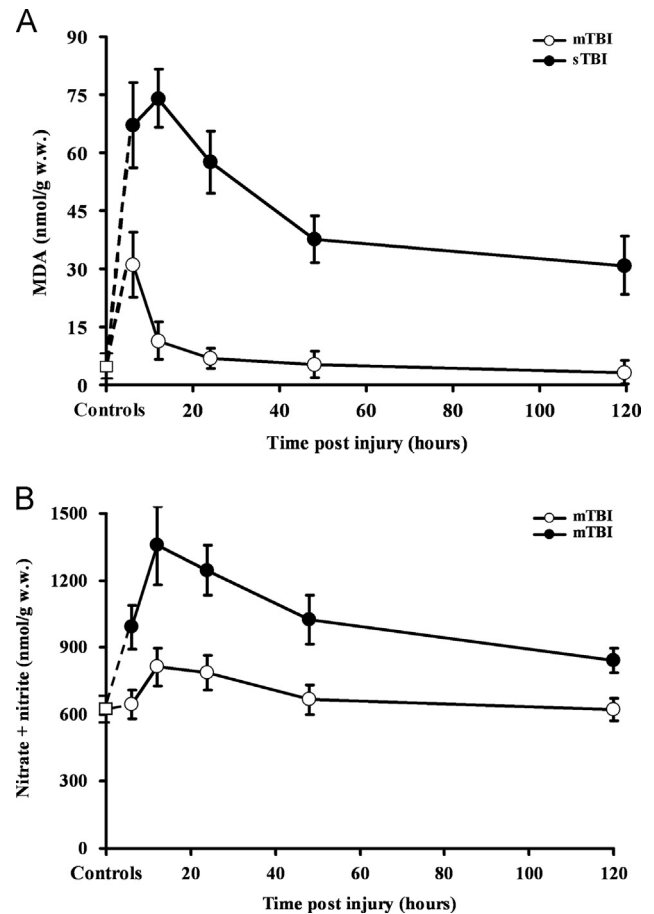


Fig. 2. Concentrations of (A) malondialdehyde (MDA) and (B) nitrite + nitrate recorded in deproteinized brain homogenates of rats at various times after a mild or a severe traumatic brain injury. Each point represents the mean value obtained from six different brain samples (three left + three right hemispheres). Standard deviations are represented by vertical bars. Significances have been omitted for the sake of clarity and are described in the text.

(−34%), 12 (−47.7%), 24 (−50.8%), 48 (−54.1%), and 120 h (−51.3%) after trauma induction. These significant differences were observed at any time postinjury comparing ascorbate values for mTBI and sTBI ($p < 0.0001$).

Similar effects were recorded for GSH, the concentrations of which were reversibly depleted in mTBI only. In fact, rats experiencing this level of injury had significantly depleted GSH values only at 24 h after injury, when a 13.2% decrease with respect to controls was recorded ($p < 0.01$). A steady decline in the cerebral GSH content was instead measured in sTBI, starting from the early times postinjury. In these animals, GSH levels at any time postinjury were significantly lower than both control values ($p < 0.0001$) and those determined in mTBI rats at corresponding times ($p < 0.0001$).

Discussion

Since its recent discovery [3], NgB has been hypothesized to act as a neuroprotector in view of the fact that its overexpression significantly reduces cerebral tissue injury in experimental models of brain ischemia [7,8]. These conclusions were drawn despite the unclear physiological function of the protein, which is present only in neurons [39], is not uniformly distributed throughout the brain [40], and is found in relatively modest concentrations [4,5]. However, its characteristics of binding reversibly not only to oxygen, but also to

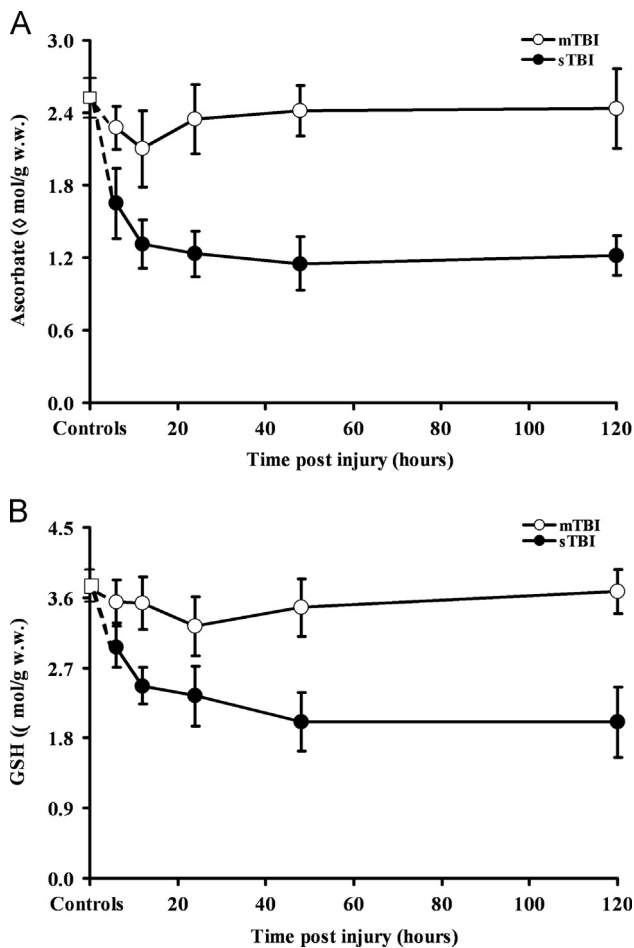


Fig. 3. Time course changes in the low-molecular-weight antioxidants (A) ascorbate and (B) GSH occurring in rats after a mild or a severe traumatic brain injury. Each point represents the mean value obtained from six different brain samples (three left + three right hemispheres). Standard deviations are represented by vertical bars. Significances have been omitted for the sake of clarity and are described in the text.

NO [2]; being located within mitochondria [41,42]; and interacting with cytochrome *c* during apoptosis [43,44] allowed one to postulate that the neuroprotection exerted by NgB may be due to its capacity to intervene, directly or indirectly, in the mechanisms modulating oxidative/nitrosative stress, mitochondrial functions, and apoptosis [9,44].

Data reported in this study clearly showed that TBI affects the gene and protein expression of NgB in a manner related to both the severity of the injury and the time postinjury, thereby suggesting that the modulation of this protein might play a significant role in the pathobiology of traumatic brain injury. According to our results, however, it is unclear what the biochemical meaning of the observed TBI-mediated NgB modulation might be. In fact, we found that in our mTBI rats a mild injury provoked a transient, yet conspicuous, increase in MDA due to ROS-mediated lipid peroxidation (Fig. 2A), increase in nitrite + nitrate due to elevated NO and RNS generation (Fig. 2B), and decrease in the low-molecular-weight antioxidants ascorbate and GSH (Fig. 3A and B). This was accompanied by almost no changes in NgB gene and protein expression (Fig. 1A and B), suggesting that this protein does not play a significant role in the transient changes related to oxidant/antioxidant balance after an mTBI, i.e., NgB has no neuroprotective effects toward ROS- and RNS-mediated damage.

On the other hand, even the results that we obtained in sTBI rats do not seem to support the notion that links NgB to neuroprotection:

in fact, induction of a severe diffuse TBI caused a relevant modulation of NgB gene and protein expression, with an evident increase in gene expression after 6–24 h and remarkably high protein levels 24–120 h after impact (Fig. 1A and B), which coincided with the maximal concentrations of MDA (deriving from sustained ROS-mediated lipid peroxidation) and nitrite + nitrate (originating from protracted NO overproduction). Furthermore, notwithstanding the increase in NgB in the 24–120 h period after injury, the two most abundant hydrophilic antioxidants, ascorbate and GSH, showed a steady and irreversible depletion. If NgB had a significant neuroprotective effect, we would have expected either an asynchrony between increase in NgB and parameters of oxidative/nitrosative stress or a recovery of antioxidants.

Numerous experimental studies demonstrated that overexpression of NgB effectively counteracted tissue injury induced by experimental ischemia [7,8]; however, recent data showed that even NgB-null mice have a reduced infarct size compared to control wild type [45]. Paradoxically, the same authors recently reported that mice overexpressing NgB had reduced brain infarct size caused by permanent middle artery occlusion [40]. Therefore, apart from the NgB-null mice study [45], it seems to be generally accepted that NgB overexpression is beneficial in the case of experimental ischemia [7,8,40]. Even in the case of TBI, NgB overexpression has been associated with neuroprotection [15,16]. Clinically, it was demonstrated that NgB polymorphisms influenced the outcome of TBI patients [46] and that NgB immunoreactivity increased in the cortical peri-infarct region of patients with ischemic stroke [47]. These data contributed to reinforcing the concept that NgB may represent a novel target for the pharmacological therapy of various neurological disorders [48], even though neither the physiological nor the pathological roles of NgB have been fully understood. Anyhow, when demonstrated, NgB overexpression after brain injury is interpreted as an attempt to decrease neuronal damage mainly associated with increased oxidative/nitrosative stress.

The neuronal modulation of NgB after ischemia [6] is similar to the positive modulation of NgB occurring after graded TBI recorded in our experiments; however, the data referring to the oxidant/antioxidant balance recorded in both mTBI- and sTBI-injured rats, and connected to ROS and RNS overproduction, do not seem to corroborate the notion that an increase in NgB equates to reduced brain injury. Although the severe level of TBI is associated with irreversible brain injury [27,28,49], mTBI causes only transient alterations in the absence of visible histological damage [27,50]. Therefore, mTBI would have been a suitable grade of injury to observe a time course of eventual NgB overexpression mirroring the recovery period from the transitory oxidant/antioxidant imbalance. Conversely, our mTBI rats did not show any change in NgB gene or protein expression.

Our results indicate that NgB overexpression is proportional to the level of injury, the biological meaning of which is unclear. In comparison with the experiments conducted under conditions of NgB overexpression [7,8,10,40,51,52], our results reflect a more realistic pathophysiological response of neurons after a traumatic brain injury. It is also worth emphasizing that the NgB overexpression we measured in sTBI animals was at the levels observed in experiments with overexpressing animals [40], despite the fact that we used a commercial antibody for NgB quantification. In fact, previous data have indicated that the use of commercial NgB antibodies might represent a significant source of variability when trying to obtain absolute NgB concentration [40,45,53].

Notwithstanding, in the present model of brain injury, these levels of NgB overexpression neither produced a decrease in NO metabolites (nitrite + nitrate), nor decreased ROS-mediated lipid peroxidation, nor improved the level of brain antioxidants. This discrepancy with previous observations might be due either to a

transitory and not constitutive increase in protein levels occurring in our animals or to additional roles of Ngb other than that of ROS and RNS scavenger/modulator. The latter would be supported by the notion that Ngb can transfer an electron to cytochrome *c* (Cyt *c*) at a high bimolecular rate, comparable to other mechanisms of interprotein electron transfer in mitochondria [54], where the protein is in part localized [41,42,54].

In consideration of the very low oxygen tension found within neurons, the relatively low Ngb concentration, and the Ngb oxygen binding affinity [2], the protein should mainly be found in vivo in its hexacoordinated form and might actively participate in various redox reactions, including that with Cyt *c*. In this case, Ngb would efficiently operate as an antiapoptotic molecule [44,55] mainly through the reduction of Cyt *c*, rather than through a direct ROS- and RNS-scavenging effect. This would also explain the minimal Ngb changes we found after an mTBI, a type of injury characterized by no evident neuronal loss due to apoptosis/necrosis [50], and the remarkable increase after an sTBI, an injury associated with relevant loss of neurons caused by both apoptosis and necrosis [56].

In conclusion, our results evidenced that, depending on the level of injury and on the time interval after injury, Ngb gene and protein expressions are affected by TBI with no apparent link with oxidative/nitrosative stress, suggesting that the role of this protein under physiological and pathological conditions might possibly be related to other mechanisms of neuroprotection, including antiapoptotic effects.

Acknowledgment

This work has been supported, in part, by funds from the Catholic University of Rome, the University of Catania, and the University of Birmingham.

References

- [1] Kakar, S.; Hoffman, F. G.; Storz, J. F.; Fabian, M.; Hargrove, M. S. Structure and reactivity of hexacoordinate hemoglobins. *Biophys. Chem.* **152**:1–14; 2010.
- [2] Dewilde, S.; Kiger, L.; Burmester, T.; Hankeln, T.; Baudin-Creux, V.; Aerts, T.; Marden, M. C.; Caubergs, R.; Moens, L. Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. *J. Biol. Chem.* **276**:38949–38955; 2001.
- [3] Trent 3rd J. T.; Watts, R. A.; Hargrove, M. S. Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *J. Biol. Chem.* **276**:30106–30110; 2001.
- [4] Hundahl, C. A.; Kelsen, J.; Dewilde, S.; Hay-Schmidt, A. Neuroglobin in the rat brain (II): co-localisation with neurotransmitters. *Neuroendocrinology* **88**:183–198; 2008.
- [5] Hua, S.; Antao, S. T.; Corbett, A.; Witting, P. K. The significance of neuroglobin in the brain. *Curr. Med. Chem.* **17**:160–172; 2010.
- [6] Shang, A.; Zhou, D.; Wang, L.; Gao, Y.; Fan, M.; Wang, X.; Zhou, R.; Zhang, C. Increased neuroglobin levels in the cerebral cortex and serum after ischemia-reperfusion insults. *Brain Res.* **1078**:219–226; 2006.
- [7] Yu, Z.; Liu, J.; Guo, S.; Xing, C.; Fan, X.; Ning, M.; Yuan, J. C.; Lo, E. H.; Wang, X. Neuroglobin-overexpression alters hypoxic response gene expression in primary neuron culture following oxygen glucose deprivation. *Neuroscience* **162**:396–403; 2009.
- [8] Khan, A. A.; Wang, Y.; Sun, Y.; Mao, X. O.; Xie, L.; Miles, E.; Graboski, J.; Chen, S.; Ellerby, L. M.; Jin, K.; Greenberg, D. A. Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. *Proc. Natl. Acad. Sci. USA* **103**:17944–17948; 2006.
- [9] Tiso, M.; Tejero, J.; Basu, S.; Azarov, I.; Wang, X.; Simplaceanu, V.; Frizzell, S.; Jayaraman, T.; Geary, L.; Shapiro, C.; Ho, C.; Shiva, S.; Kim-Shapiro, D. B.; Gladwin, M. T. Human neuroglobin functions as a redox-regulated nitrite reductase. *J. Biol. Chem.* **286**:18277–18289; 2011.
- [10] Singh, S.; Zhuo, M.; Gorgun, F. M.; Englander, E. W. Overexpressed neuroglobin raises threshold for nitric oxide-induced impairment of mitochondrial respiratory activities and stress signaling in primary cortical neurons. *Nitric Oxide* **32**:21–28; 2013.
- [11] Li, W.; Wu, Y.; Ren, C.; Lu, Y.; Gao, Y.; Zheng, X.; Zhang, C. The activity of recombinant human neuroglobin as an antioxidant and free radical scavenger. *Proteins* **79**:115–125; 2011.
- [12] Li, R. C.; Morris, M. W.; Lee, S. K.; Pouranfar, F.; Wang, Y.; Gozal, D. Neuroglobin protects PC12 cells against oxidative stress. *Brain Res.* **1190**:159–166; 2008.
- [13] Yu, Z.; Liu, N.; Liu, J.; Yang, K.; Wang, X. Neuroglobin, a novel target for endogenous neuroprotection against stroke and neurodegenerative disorders. *Int. J. Mol. Sci.* **13**:6995–7014; 2012.
- [14] Li, S. Q.; Li, W. B.; Zhang, M.; Wu, Y. Z.; Hu, Y. Y. The role of neuroglobin in the neuroprotection of limb ischemic preconditioning in rats. *Mol. Neurobiol.* **47**:197–208; 2013.
- [15] Shang, A.; Liu, K.; Wang, H.; Wang, J.; Hang, X.; Yang, Y.; Wang, Z.; Zhang, C.; Zhou, D. Neuroprotective effects of neuroglobin after mechanical injury. *Neural. Res.* **33**:551–558; 2012.
- [16] Shang, A.; Feng, X.; Wang, H.; Wang, J.; Hang, X.; Yang, Y.; Wang, Z.; Zhou, D. Neuroglobin upregulation offers neuroprotection in traumatic brain injury. *Neural. Res.* **34**:588–594; 2012.
- [17] Signoretti, S.; Lazzarino, G.; Tavazzi, B.; Vagnozzi, R. The pathophysiology of concussion. *PM R* **3**(10 Suppl. 2):S359–S368; 2011.
- [18] Giza, C. C.; Hovda, D. A. The neurometabolic cascade of concussion. *J. Athl. Train.* **36**:228–235; 2001.
- [19] Coronado, V. G.; Xu, L.; Basavaraju, S. V.; McGuire, L. C.; Wald, M. M.; Faul, M. D.; Guzman, B. R.; Hemphill, J. D. Surveillance for traumatic brain injury-related deaths—United States, 1997–2007. *MMWR Surveill. Summ* **60**:1–32; 2011.
- [20] Coronado, V. G.; McGuire, L. C.; Sarmiento, K.; Bell, J.; Lionbarger, M. R.; Jones, C. D.; Geller, A. I.; Khoury, N.; Xu, L. Trends in traumatic brain injury in the U.S. and the public health response: 1995–2009. *J. Saf. Res* **43**:299–307; 2012.
- [21] Sharma, R.; Laskowitz, D. T. Biomarkers in traumatic brain injury. *Curr. Neurol. Neurosci. Rep.* **12**:560–569; 2012.
- [22] Reinert, M.; Hoelper, B.; Doppenberg, E.; Zauner, A.; Bullock, R. Substrate delivery and ionic balance disturbance after severe human head injury. *Acta Neurochir. Suppl.* **76**:439–444; 2000.
- [23] Titus, D. J.; Sakurai, A.; Kang, Y.; Furonos, C.; Jergova, S.; Santos, R.; Sick, T. J.; Atkins, C. M. Phosphodiesterase inhibition rescues chronic cognitive deficits induced by traumatic brain injury. *J. Neurosci.* **33**:5216–5226; 2013.
- [24] Balan, I. S.; Saladino, A. J.; Aarabi, B.; Castellani, R. J.; Wade, C.; Stein, D. M.; Eisenberg, H. M.; Chen, H. H.; Fiskum, G. Cellular alterations in human traumatic brain injury: changes in mitochondrial morphology reflect regional levels of injury severity. *J. Neurotrauma* **30**:367–381; 2013.
- [25] Di Pietro, V.; Amin, D.; Pernagallo, S.; Lazzarino, G.; Tavazzi, B.; Vagnozzi, R.; Pringle, A.; Belli, A. Transcriptomics of traumatic brain injury gene expression and molecular pathways of different grades of insult in a rat organotypic hippocampal culture model. *J. Neurotrauma* **27**:349–359; 2010.
- [26] Di Pietro, V.; Amorini, A. M.; Tavazzi, B.; Hovda, D. A.; Signoretti, S.; Giza, C. C.; Lazzarino, G.; Vagnozzi, R.; Lazzarino, G.; Belli, A. Potentially neuroprotective gene modulation in an in vitro model of mild traumatic brain injury. *Mol. Cell. Biochem.* **375**:185–198; 2013.
- [27] Tavazzi, B.; Signoretti, S.; Lazzarino, G.; Amorini, A. M.; Delfini, R.; Cimatti, M.; Marmarou, A.; Vagnozzi, R. Cerebral oxidative stress and depression of energy metabolism correlate with severity of diffuse brain injury in rats. *Neurosurgery* **56**:582–589; 2005.
- [28] Tavazzi, B.; Vagnozzi, R.; Signoretti, S.; Amorini, A. M.; Belli, A.; Cimatti, M.; Delfini, R.; Di Pietro, V.; Finocchiaro, A.; Lazzarino, G. Temporal window of metabolic brain vulnerability to concussions: oxidative and nitrosative stresses—part II. *Neurosurgery* **61**:390–396; 2007.
- [29] Yamada, K. H.; Kozlowski, D. A.; Seidl, S. E.; Lance, S.; Wieschhaus, A. J.; Sundivakkam, P.; Tirupathi, C.; Chishti, I.; Herman, I. M.; Kuchay, S. M.; Chishti, A. H. Targeted gene inactivation of calpain-1 suppresses cortical degeneration due to traumatic brain injury and neuronal apoptosis induced by oxidative stress. *J. Biol. Chem.* **287**:13182–13193; 2012.
- [30] Abdul-Muneer, P. M.; Schuetz, H.; Wang, F.; Skotak, M.; Jones, J.; Gorantla, S.; Zimmerman, M. C.; Chandra, N.; Haorah, J. Induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast. *Free Radic. Biol. Med.* **60**:282–291; 2013.
- [31] Hall, E. D.; Vaishnav, R. A.; Mustafa, A. G. Antioxidant therapies for traumatic brain injury. *Neurotherapeutics* **7**:51–61; 2010.
- [32] Marmarou, A.; Foda, M. A.; van den Brink, W.; Campbell, J.; Kita, H.; Demetriadou, K. A new model of diffuse brain injury in rats. Part I. Pathophysiology and biomechanics. *J. Neurosurg.* **80**:291–300; 1994.
- [33] Lazzarino, G.; Nuutinen, M.; Tavazzi, B.; Di Pietro, D.; Giardina, B. A method for preparing freeze-clamped tissue samples for metabolite analyses. *Anal. Biochem.* **181**:239–241; 1989.
- [34] Lazzarino, G.; Amorini, A. M.; Fazzina, G.; Vagnozzi, R.; Signoretti, S.; Donzelli, S.; Di Stasio, E.; Giardina, B.; Tavazzi, B. Single-sample preparation for simultaneous cellular redox and energy state determination. *Anal. Biochem.* **322**:51–59; 2003.
- [35] Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**:402–408; 2001.
- [36] Tavazzi, B.; Lazzarino, G.; Leone, P.; Amorini, A. M.; Bellia, F.; Janson, C. G.; Di Pietro, V.; Ceccarelli, L.; Donzelli, S.; Francis, J. S.; Giardina, B. Simultaneous high performance liquid chromatographic separation of purines, pyrimidines, N-acetylated amino acids, and dicarboxylic acids for the chemical diagnosis of inborn errors of metabolism. *Clin. Biochem.* **38**:997–1008; 2005.
- [37] Lazzarino, G.; Di Pietro, D.; Tavazzi, B.; Cerroni, L.; Giardina, B. Simultaneous separation of malondialdehyde, ascorbic acid, and adenine nucleotide derivatives from biological samples by ion-pairing high-performance liquid chromatography. *Anal. Biochem.* **197**:191–196; 1991.

- [38] Romitelli, F.; Santini, S. A.; Chierici, E.; Pitocco, D.; Tavazzi, B.; Amorini, A. M.; Lazzarino, G.; Di Stasio, E. Comparison of nitrite/nitrate concentration in human plasma and serum samples measured by the enzymatic batch Griess assay, ion-pairing HPLC and ion-trap GC–MS: the importance of a correct removal of proteins in the Griess assay. *J. Chromatogr. B* **851**:257–267; 2007.
- [39] Haines, B.; Mao, X.; Xie, L.; Spusta, S.; Zeng, X.; Jin, K.; Greenberg, D. A. Neuroglobin expression in neurogenesis. *Neurosci. Lett.* **549**:3–6; 2013.
- [40] Raida, Z.; Hundahl, C. A.; Nyengaard, J. R.; Hay-Schmidt, A. Neuroglobin over expressing mice: expression pattern and effect on brain ischemic infarct size. *PLoS One* **8**:e76565; 2013.
- [41] Lechauve, C.; Augustin, S.; Cwerman-Thibault, H.; Bouaita, A.; Forster, V.; Céliier, C.; Rustin, P.; Marden, M. C.; Sahel, J. A.; Corral-Debrinski, M. Neuroglobin involvement in respiratory chain function and retinal ganglion cell integrity. *Biochim. Biophys. Acta* **1823**:2261–2273; 2012.
- [42] Yu, Z.; Xu, J.; Liu, N.; Wang, Y.; Li, X.; Pallast, S.; van Leyen, K.; Wang, X. Mitochondrial distribution of neuroglobin and its response to oxygen–glucose deprivation in primary-cultured mouse cortical neurons. *Neuroscience* **218**:235–242; 2012.
- [43] Bønding, S. H.; Henty, K.; Dingley, A. J.; Brittain, T. The binding of cytochrome c to neuroglobin: a docking and surface plasmon resonance study. *Int. J. Biol. Macromol.* **43**:295–299; 2008.
- [44] Brittain, T.; Skommer, J.; Henty, K.; Birch, N.; Raychaudhuri, S. A role for human neuroglobin in apoptosis. *IUBMB Life* **62**:878–885; 2010.
- [45] Raida, Z.; Hundahl, C. A.; Kelsen, J.; Nyengaard, J. R.; Hay-Schmidt, A. Reduced infarct size in neuroglobin-null mice after experimental stroke in vivo. *Exp. Transl. Stroke Med.* **4**:15; 2012.
- [46] Chuang, P. Y.; Conley, Y. P.; Poloyac, S. M.; Okonkwo, D. O.; Ren, D.; Sherwood, P. R.; Hrvanek, M.; Alexander, S. A. Neuroglobin genetic polymorphisms and their relationship to functional outcomes after traumatic brain injury. *J. Neurotrauma* **27**:999–1006; 2010.
- [47] Jin, K.; Mao, Y.; Mao, X.; Xie, L.; Greenberg, D. A. Neuroglobin expression in ischemic stroke. *Stroke* **41**:557–559; 2010.
- [48] Jin, K.; Mao, X. O.; Xie, L.; Greenberg, D. A. Pharmacological induction of neuroglobin expression. *Pharmacology* **87**:81–84; 2011.
- [49] Lusardi, T. A.; Lytle, N. K.; Szybala, C.; Boison, D. Caffeine prevents acute mortality after TBI in rats without increased morbidity. *Exp. Neurol.* **234**:161–168; 2012.
- [50] Eakin, K.; Miller, J. P. Mild traumatic brain injury is associated with impaired hippocampal spatiotemporal representation in the absence of histological changes. *J. Neurotrauma* **29**:1180–1187; 2012.
- [51] Yu, Z.; Liu, N.; Li, Y.; Xu, J.; Wang, X. Neuroglobin overexpression inhibits oxygen–glucose deprivation-induced mitochondrial permeability transition pore opening in primary cultured mouse cortical neurons. *Neurobiol. Dis.* **56**:95–103; 2013.
- [52] Van Leuven, W.; Van Dam, D.; Moens, L.; De Deyn, P. P.; Dewilde, S. A behavioural study of neuroglobin-overexpressing mice under normoxic and hypoxic conditions. *Biochim. Biophys. Acta* **1834**:1764–1771; 2013.
- [53] Hundahl, C. A.; Fahrenkrug, J.; Luuk, H.; Hay-Schmidt, A.; Hannibal, J. Restricted expression of neuroglobin in the mouse retina and co-localization with melanopsin and tyrosine hydroxylase. *Biochem. Biophys. Res. Commun.* **425**:100–106; 2012.
- [54] Kiger, L.; Tilleman, L.; Geuens, E.; Hoogewijs, D.; Lechauve, C.; Moens, L.; Dewilde, S.; Marden, M. C. Electron transfer function versus oxygen delivery: a comparative study for several hexacoordinated globins across the animal kingdom. *PLoS One* **6**:e20478; 2011.
- [55] Yu, Z.; Poppe, J. L.; Wang, X. Mitochondrial mechanisms of neuroglobin's neuroprotection. *Oxid. Med. Cell. Longevity* **2013**:756989; 2013.
- [56] Miñambres, E.; Ballesteros, M. A.; Mayorga, M.; Marin, M. J.; Muñoz, P.; Figols, J.; López-Hoyos, M. Cerebral apoptosis in severe traumatic brain injury patients: an in vitro, in vivo, and postmortem study. *J. Neurotrauma* **25**:581–591; 2008.