Neuroglobin, clues to function and mechanism

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Abstract

Neuroglobin is expressed in vertebrate brain and belongs to a branch of the globin family that diverged early in evolution. Sequence conservation and presence in nervous cells of several taxa suggests a relevant role in the nervous system, with tight structural restraints. Twenty years after its discovery, a rich scientific literature provides convincing evidence of the involvement of neuroglobin in sustaining neuron viability in physiological and pathological conditions however, a full and conclusive picture of its specific function, or set of functions is still lacking.

The difficulty of unambiguously assigning a precise mechanism and biochemical role to neuroglobin might arise from the participation to one or more cell mechanism that redundantly guarantee the functioning of the highly specialized and metabolically demanding central nervous system of vertebrates.

Here we collect findings and hypotheses arising from recent biochemical, biophysical, structural, in cell and *in vivo* experimental work on neuroglobin, aiming at providing an overview of the most recent literature. Proteins are said to have jobs and hobbies, it is possible that, in the case of neuroglobin, evolution has selected for it more than one job, and support to cover for its occasional failings. Disentangling the mechanisms and roles of neuroglobin is thus a challenging task that might be achieved by considering data from different disciplines and experimental approaches.

Keywords

- heme proteins
- neuroglobin
- oxidative stress

- signaling pathways
- structure-function relationship
- neuroprotection

1. Introduction

Globins occur in all three kingdoms of life. In archeae and bacteria, their function is mostly enzymatic, although sensors globins are also present. Transport of oxygen is a function that is likely to have developed relatively recently, with the emergence of multicellular organisms (Vinogradov et al., 2006). Myoglobin (Mb) and hemoglobin (Hb) are the globins that carry out functions related to oxygen storage, transport and diffusion in higher organisms and their extensive characterization indicated that they are both endowed with physiologically relevant enzymatic properties.

In addition to Hb and Mb, six additional globin types have been discovered in vertebrates: neuroglobin (Ngb) (Burmester et al., 2000), cytoglobin (Cygb) (Burmester et al., 2002; Kawada et al., 2001; Trent and Hargrove, 2002), globin X (GbX) (Roesner et al., 2008), globin Y (GbY) (Fuchs et al., 2006), eye-globin or globin E (GbE) (Kugelstadt et al., 2004) and androglobin (Adgb) a chimaeric protein with a permutated globin fold (Hoogewijs et al., 2012).

The identification of these previously elusive globins was due to advancements in sequence analysis methods combined to an extensive work of genome sequencing. In some cases, gene identification was followed by heterologous protein expression and structural and functional characterization. The level of expression of these most recently identified globins is much lower than the one observed for Hb and Mb and they are often limited to specific cell types. Their functions have not yet been fully clarified, but there are clear indications of involvement in intracellular signaling and of enzymatic activities, especially towards nitrogen and oxygen reactive species.

Ngb, Cygb and Adgb, that emerged early in evolution and are present in most jawed vertebrates (gnathostome) are not necessarily primitive in their biological role and might carry out functions that arose in response to the functional and metabolic complexity of the specialized cell populations of multicellular organisms (Blank and Burmester, 2012). In particular, Ngb represents a puzzling and fascinating case given its main localization in the central nervous system (CNS) and

probable involvement in cell survival in pathological conditions. Several pieces of evidence reported in this review indicate that Ngb converge on the fact that this globin is an inducible protein which accumulation is required to elicit its functions.

Ngb was discovered by Burmester and colleagues who showed that it exhibits a hexacoordinated heme iron both in its ferric and ferrous forms and that it is predominantly expressed in the CNS of vertebrates (Burmester et al., 2000). The heme iron hexacoordination, which in Ngb involves two of the most conserved residues amongst globins (His(E7)64 and His(F8)96), is a specific feature of phylogenetically ancient globins. Hexacoordination had been hypothesized to be the oldest coordination scheme since this binding mode could eventually lead to a structural reorganization at the basis of a gas sensing function whereas pentacoordination, that appeared later in evolution, had been observed in globins which are endowed with a more elaborate respiratory role, such as myoglobins (Mb) and hemoglobins (Hb) (Burmester and Hankeln, 2014).

More than twenty years of scientific research on Ngb has produced an enormous amount of data and knowledge about this globin, however several aspects of its biochemistry and molecular functions are still debated. A comprehensive review of the scientific literature on Ngb can be found in (Ascenzi et al., 2016). Here, we will highlight some aspects of the biochemistry of Ngb that arises from the more recent literature, without pretending to be exhaustive and to evaluate in depth the significance of primary data. We apologize with our colleagues for the papers that we may have inadvertently missed quoting.

2. Structural aspects of neuroglobin

Crystal structures of human and murine neuroglobin in its ligand-free conformation revealed that the protein displays the same typical 3-over-3 α-helical globin fold. Although sequence alignment indicates that neuroglobin only shares 21% sequence identity with vertebrate Mb and 25% with Hb (**Fig. 1A-D**) (Burmester et al., 2000; Pesce et al., 2003; Vallone et al., 2004a). The first Ngb structures were obtained from mutated Cys-to-Ser Ngbs since cysteine residues often hamper protein crystallization. Only recently, Guimarães and colleagues provided the first structure of a true wild-type human Ngb, *i.e.* including the disulfide bridge between Cys(CD5)46 and Cys(D5)55. Although absent in murine Ngb, the disulfide bond present in human Ngb seems to tightly pack the CD loop, probably regulating its dynamics upon ligand binding and being responsible for a threefold increased affinity for exogenous ligands. (Guimarães et al., 2014).

A peculiar feature, well characterized in murine Ngb crystal structures, but also observed by NMR and resonance Raman spectroscopy in other species, is the existence of two Ngb populations in a 70%-30% proportion differing in their heme insertion orientations: the canonical heme insertion corresponds to that of Mb whereas the reversed insertion is rotated by 180° around the αγ-meso axis with respect to the canonical one The heme double insertion is attributed to the loose contacts between the porphyrin ring and the protein matrix (Du et al., 2003; Exertier et al., 2019; Milazzo et al., 2020; Pesce et al., 2003; Sebastiani et al., 2021; Vallone et al., 2004a). Another peculiarity of Ngb is its large internal cavity (120 $Å^3$ in human and 290 $Å^3$ in murine Ngb), which is an extension of the heme crevice. It has been hypothesized that the presence of this extended cavity may facilitate Ngb signaling activity by fast ligand-induced conformational changes. Additionally, a tunnel connects the bulk with the distal side of the internal cavity which may constitute an additional path with respect to direct access from the His(E7)64 gate for ligand accumulation and progression within the protein matrix. Several ligand docking pockets were identified within Ngb internal cavity that are analogous to those observed in Mb and reported to regulate the internal dynamics of small gaseous ligands (i.e., O_2, CO, and NO) (**Fig. 1C**) (Brunori and Gibson, 2001; Tilton et al., 1984). As an example, we may mention the so called “XeIV” and “XeIII” docking sites that may participate in Ngb geminate ligand rebinding, described in section “*3. Outline of Neuroglobin ligand binding kinetics and reactivity*” (Ardiccioni et al., 2019; Colloc’h et al., 2017; Moschetti et al., 2009).

Ligand binding, which occurs upon spontaneous rupture of the distal His(E7)64 and subsequent binding of a diatomic gas on the vacant sixth coordination position, triggers a rather large protein conformational reorganization, for which the most striking rearrangement regards the heme (**Fig 1B**). In fact, the crystal structure of carbon monoxide (CO) bound murine Ngb revealed that, upon dissociation of the distal axial heme iron coordination and CO ligation, the heme slides deeper inside the heme crevice, releasing its positional constraints and yielding a 2.0 Å iron displacement while causing the repositioning of Phe106 side chain (**Fig 1B inset**). The concomitant repositioning of the E-helix/EF corner/F-helix upon ligand binding reflects the relaxation of the globin frame, which most likely adopts a more stable conformation (Vallone et al., 2004b). Additionally, the CD loop on the distal side of the heme assumes a more open conformation, accompanying the swing-out movement of His(E7)64 upon dissociation from the iron as observed by crystallography and molecular dynamics simulations (Anselmi et al., 2008, 2007; Arcovito et al., 2008; Vallone et al.,

2004b). In the “Gly-loop mutant” where additional flexibility was introduced at the level of the CD loop a full swing-out movement of His(E7)64 was observed (Exertier et al., 2019). Interestingly the CD corner seems to be also closely related to the propensity of the heme iron to be hexacoordinated as shown by Boron *et al*. Indeed they observed a partial loss of hexacoordination upon graft of Mb CD loop in Ngb (Boron et al., 2015).

Another determinant regulating ligand binding parameters in Ngb is the nature of the residue in position 106. Rapid mixing experiments and X-ray crystallography performed on Ngb mutants of Phe106 substituted with bulkier tryptophan or smaller alanine showed respectively a decrease or increase in CO affinity, consistent with hindering or facilitation of the heme sliding and confirming the role of heme displacement in controlling heme availability and, consequently, reactivity (Avella et al., 2014; Exertier et al., 2019).

Moreover, the study of Ngb structural response to high pressure indicated that conformational changes upon diatomic gas ligation seems to hinge around a mechanical nucleus mainly composed of hydrophobic residues from the E-, G-, and H-helices lining the cavity and that the EF corner acts as an early sensor of the strain posed by heme sliding on Phe106 (Sacquin-Mora et al., 2017). The thermal B-factors of the EF-corner are dramatically decreased upon CO binding to murine Ngb (Vallone et al., 2004b).

Another structural aspects worth mentioning arises from the study of the interaction between human Ngb and cytochrome c (cyt c) that has been recently investigated by NMR and MD simulations. Ngb-cyt c interaction has been predicted to be mediated by heme-to-heme contacts, also involving cyt c Lys72 and Glu87 that may establish salt bridges and hydrogen bonds with Ngb (Tejero, 2020; Tiwari et al., 2018). The putative interacting residues in human Ngb (Lys72, Asp73, Thr77 and Glu87) are all conserved in mouse Ngb and are located in the E- and F-helices. This structural analysis is of relevance given the fast electron transfer occurring between the two heme proteins, leading to cyt c reduction and to a possible anti-apoptotic role for Ngb (Fago et al., 2006b).

The main contribution from structural studies, on top of the crucial information on Ngb 3D structure consist in pointing out the features that may contribute to a signaling function and to a catalytic role, such as cavity and tunnel dynamics.

3. Outline of Neuroglobin ligand binding kinetics and reactivity

Biochemical and biophysical investigations carried out on recombinant human and murine Ngb provide basic knowledge necessary to support or rule out hypotheses on functions carried out in cells and organs. Here follows an overview of the binding behavior and main redox activities that have been demonstrated and characterized *in vitro*. It must be underlined that these data need to be carefully examined considering the actual concentration of reactants or the presence of redox partners to sustain scavenging or production of reactive species by Ngb.

Ferrous neuroglobin Ngb(II) can bind diatomic gas such as O_2, CO and NO. Hexacoordination in Ngb is responsible for a peculiar binding kinetics, since exogenous ligands compete with the His(E7)64 for binding to the heme iron sixth coordination position. The rupture of His(E7)64 is the rate limit step for gas ligation (histidine dissociation rate constants vary between 0.5 and 1.2 s^{-1} for the murine globin and between 0.6 and 7.0 s^{-1} for the human form), and since ligand binding to the heme iron is strikingly fast, the pentacoordinate intermediate never accumulates (Kiger et al., 2004). His(E7)64 association and dissociation are very dependent on pH, affecting the histidine protonation state (Fago et al., 2006a; Nienhaust et al., 2004). Furthermore, CO and O_2 binding on-rates were shown to be high in comparison to the off-rates, in fact CO and O_2 association constants range from 38 to 72 $\mu M^{-1}.s^{-1}$ and from 130 to 300 $\mu M^{-1}.s^{-1}$ respectively, whereas dissociation rates vary from 0.7 x 10^{-2} to 0.5 x 10^{-1} s^{-1} for CO and from 0.3 to 0.8 s^{-1} for O_2, suggesting a rather high affinity of Ngb for these diatomic gaseous ligands (Dewilde et al., 2001). Interestingly, the disulfide bridge Cys(CD5)46-Cys(D5)55 present in human Ngb is responsible for a threefold increase in O_2 binding rates with respect to the disulphide bridge-reduced or cysteine-mutated protein, consistently with the CD loop structure and dynamics being linked to heme reactivity and ligation state (Green et al., 2003; Smagghe et al., 2006).

Binding kinetics of CO was extensively investigated using rapid mixing and flash photolysis. CO binding to ferrous deoxy hexacoordinate Ngb(II) displays CO-independent kinetics for the wild-type proteins, while CD loop mutants binding kinetics clearly show a dependence, suggesting a role for the CD loop in tuning ligand binding (Avella et al., 2014; Exertier et al., 2019; Giuffrè et al., 2008). Interestingly, murine Phe106 mutants endowed with enhanced CO binding velocities showed biphasic kinetics most likely due to a different contribution for each heme insertion mode in the absence of Phe106 constraints to the heme sliding (Exertier et al., 2019), an observation also made on human Ngb (Fago et al., 2006a).

CO rebinding to the heme iron observed by flash photolysis at near physiological temperatures enabled the identification of several kinetic steps: a geminate rebinding, an extremely fast bimolecular gaseous ligand binding, the His(E7)64 recombination and the displacement of His(E7)64 by the gaseous ligand (Kriegl et al., 2002). The geminate rebinding is the first kinetic event occurring upon ligand dissociation from the heme upon photoexcitation, it corresponds to the ultrafast rebinding of CO molecules from the immediate surrounding of the pentacoordinate heme iron at the nanosecond scale. The extremely fast bimolecular exogenous ligand binding corresponds to the rebinding of CO exploring transient docking sites in the vicinity of the heme iron that occurs at the microsecond time scale. The affinity of these docking cavities for CO explains the fast bimolecular ligand binding rates. The CO molecule escape towards the solvent allows the rebinding of His(E7)64 to the pentacoordinate heme iron (millisecond time range), however the histidine is eventually displaced by CO molecules re-entering the protein matrix on the second time scale (Abbruzzetti et al., 2009).

Given the compertition with His(E7)64, in spite of their high intrinsic affinity for the pentacoordinated heme iron, binding to Ngb(II) or Ngb(III) with heme ligands (NO, cyanide, H_2S, CO) is therefore characterized by low velocity (Bocahut et al., 2013; Brittain et al., 2008; Smagghe et al., 2006; Van Doorslaer et al., 2003). However, the relatively slow dissociation rate led to the suggestion that Ngb might represent a sink for toxic species in pathological conditions such as stroke, where their increase might allow sequestering by Ngb (Brittain et al., 2008).

A relevant property of neuroglobin as compared to Mb is the tendency of the heme iron to rapidly autoxidize (constants are 5.4 h^{-1} and 19 h^{-1}, respectively at pH 7.0 and 37.0°C) for human and murine proteins (Dewilde et al., 2001; Van Doorslaer et al., 2003). This behaviour led some authors to consider unlikely a role in dioxygen storage or diffusion even in selected cells or tissues.

The redox properties of Ngb have also been extensively investigated. These include the reactivity of Ngb(II)O_2 towards NO yielding Ngb(III) and nitrate by means of a heme-bound peroxynitrite intermediate (Brunori et al., 2005); Ngb(II)NO can react with O_2 yelding Ngb(III) and nitrate and with peroxynitrite (Herold and Fago, 2005). These reactions require a yet unidentified Ngb reductase to allow regeneration of Ngb(II).

Ngb(II) can reduce nitrite in human Ngb yielding equimolar amounts of Ngb(III) and Ngb(II)-NO, this reaction is modulated by the formation of the Cys(CD5)46-Cys(D5)55 disulphide bridge (Tiso

et al., 2011). Also murine Ngb(II) may perform nitrite reduction yielding Ngb(III) and a 30% of Ngb(II)-NO, with possible nitrosylation of Cys(D5)55 (Tejero et al., 2015). In its oxidized FeIII form Ngb binds NO leading to iron reduction and followed by oxidation by dioxygen and hydrogen peroxide (Herold et al., 2004). Other reactions include Ngb(II) oxidation by sulfur trioxide anion radicals (Gardner et al., 2015).

The biochemical characterization of Ngb activity against a number of radicals is compatible with a role in keeping under control crucial, yet potentially disruptive, species in sensitive and specialized cell types. This hypothesis awaits conclusive and specific evidence, which might be difficult to obtain, since more than one system could be operating to keep highly reactive species at bay. For a systematic description of the pseudo-enzymatic propertities of human and murine neuroglobin see (Ascenzi et al., 2016), whereas a role in oxygen binding and storage appears unlikely.

4. Clues from the world living organisms

Studies on Ngb expression and localization in organisms that experience low oxygen levels due to environmental or behavioral factors can provide indications on the involvement of this globin in coping with hypoxia and oxidative metabolic stress.

Keeping in mind the *caveat* that extreme conditions can induce adaptation by assuming new functional roles by a protein such as Ngb, it is nevertheless worthwhile to consider some notable cases where Ngb expression correlates with low oxygen conditions.

Ngb is part of a biochemical, physiological and behavioral response to an environmental challenge which exerts a very high selective pressure in freshwater fishes. Indeed two genes for myoglobin (Mb1 and Mb2) are found in carp and goldfish (*C. auratus*) and take part, together with Ngb, to adaptive and preadaptive response to low oxygen levels (Fraser et al., 2006). *C. auratus* often experiences hypoxia in its natural habitat and it seems to have developed biochemical strategies to cope with this condition (Lushchak et al., 2001). Regarding globin levels the response to hypoxia in *C. auratus* is both “adaptive” with an increase of myoglobin brain expression and “preadaptive” with levels of Ngb fivefold higher than in the model system zebrafish (Roesner et al., 2008). Interestingly, in zebrafish, Ngb protein levels increase in the brain of about 5.7 times upon severe

hypoxia. This has been interpreted as an adaptation to occasional very low oxygen levels in its warm and tropical environment (Roesner et al., 2006).

Conversely, in *Oryza latipes* (Japanese medaka) which also experiences high variability of oxygen environmental levels, Mb levels increase in the brain upon hypoxia, whereas Ngb levels are not affected (Wawrowski et al., 2011).

Altogether these data indicate a species-specific response in fishes where different proteins have been selected in response to similar selective pressure, where either Ngb or other globins can take a main role. This consideration is confirmed in the case of some antarctic fish (*Channichthiydae*, also known as “icefish”) that have lost hemoglobin, but retain Ngb, cytoglobin-1, cytoglobin-2 and globin-X, whereas other members of the antarctic Notothenioid family retain hemoglobin as well as the other members of the globin family (Cheng et al., 2009). The biophysical properties of the Ngb from the icefish *C. aceratus* parallel those of human and murine Ngb (Giordano et al., 2012). The effect of thermal and hypoxic stress in antarctic fish that are lacking (*C. hamatus*) or that retained (*T. bernacchii*) hemoglobin indicated that the expression of cellular globins (Ngb, Cygb-1, Cygb-2, Gb-X and Mb) is indeed affected in the brain, retina and gills. However, the pattern does not point to a consistent role for Ngb, but rather to different cellular globins coming into play, depending on the organ and on the species considered. As an example, hypoxia induces no increase of Ngb mRNA in *T. bernacchii* brain, but a marked increase in *C. aceratus* brain, whereas the opposite effect is observed in the gills (Giordano et al., 2021).

Among higher vertebrates, the freshwater turtle *Trachemys scripta elegans* can survive days of complete anoxia to several months during winter hibernation, due to a concerted physiological and molecular adaptation including Ngb upregulation, that was observed *in vivo* upon hypoxia and post-hypoxia reoxygenation (Nayak et al., 2009). Studies of neuronally enriched cell cultures from this reptile led to the conclusion that the greater expression of Ngb suggests a role in detoxification or reduction of ROS species, but that its role is redundant and other biochemical mechanisms seem to play a major contribution to these processes (Nayak et al., 2009).

In mammals, the analysis of Hb, Ngb and Cygb content revealed a correlation with diving behavior (Williams et al., 2008). A more detailed analysis on whale and seal species indicated that only the former adopts higher levels of Ngb as an adaptation to the diving behaviour, whereas seals rely on neurons being less reliant on oxidative metabolism, with Ngb being consistently expressed in astrocytes where ATP aerobic production is concentrated (Schneuer et al., 2012).

This finding is paralleled in the hypoxia-adapted mole *Spalax galili*, where Ngb is expressed at higher levels with respect to rat and to mole species (*Spalax judaei*) which are not environmentally exposed to oxygen deprivation (Avivi et al., 2010). The overall suggestion from the analysis of Ngb expression, cellular localization and regulation upon hypoxic or ROS/NOS stress in the above mentioned vertebrate species is that evolution has led to upregulation of its expression due to environmental aerobic respiration impairment.

Ngb is an ancient globin, arising before the Protostomia/Deuterostomia split (Burmester and Hankeln, 2009), present in many metazoan taxa including cnidaria, placozoa, choanoflagellates and sponges and it is notably absent in arthropods (Prothmann et al., 2020). Evolutionary distance between vertebrates and other taxa in which Ngb-like proteins or genes have been identified is too large to assume precise conservation of functional role and/or mechanism of action, but it certainly relevant to underline that their expression in nervous cells and involvement in oxygen sensing or radical detoxification seems to be a trait observed in all cases in which an investigation has been carried out. *In silico* searches led to the identification of Ngb-like genes in almost all metazoan lineages, Ngb-like proteins are expressed in the nervous system of the photosymbiotic acoel *Symsagittifera roscoffensis* and in the neurosensory cells of the jellyfish (cnidaria) *Clytia hemisphaerica*. Interestingly the latter is not hexacoordinated, but its three-dimensional structure finds its closest match in CO-bound murine neuroglobin (Lechauve et al., 2013).

Among the thirty three globin genes found in the model organism *C. elegans*, two (Gb5 and Gb13) are members of the Ngb clade and are associated with oxygen sensing and radical scavenging and are expressed in cells nervous cells. Remarkably the expression of human Ngb can rescue the ROS sensitivity of a Gb13 knock-out *C. elegans* strain (Persson et al., 2009; Ren et al., 2013; Zuckerman et al., 2017).

Taken together, the data on Ngb expression in vertebrates upon hypoxic stress seem to indicate that its presence may be beneficial in case of hypoxic or oxidative stress and that in some cases this property has been exploited by evolution for adaptation to constitutional or frequent hypoxia. In less complex animals Ngb-like proteins are expressed in cells of the nervous system of several taxa, even when blood or a circulatory system are absent, supporting the notion of an association of Ngb with neural cells that led to the adoption of a role in the CNS with the evolution of animal neural systems.

5. The cytoprotective role of neuroglobin: interactors and related pathways

Several pieces of evidence for the protective role of neuroglobin were obtained on different cell lines, allowing the identification of numerous molecular Ngb interactors and signaling pathways in which the globin may play a role (**Figure 2**), here we report mainly findings described in more recent literature (2016-2020).

Ngb seems to participate in anti-apoptotic/ferroptotic and antioxidant cascades (Van Acker et al., 2019b) and to promote cell survival by acting either directly against reactive oxygen species (ROS) production, via its potential scavenging action, or indirectly. As an example, Li and coworkers showed that upon oxidative stress exposition, PC12 cells transfected with a Ngb mutant, for which ligand binding is affected, displayed lower survival rate with respect to those transfected with the wild-type globin (Li et al., 2008a, 2008b). Cabezas and colleagues demonstrated that, in astrocytic T98G cells, the up-regulation of Ngb leading to ROS reduction and astrocyte protection was induced by the platelet-derived growth factor subtype BB (Cabezas et al., 2018), and it has been shown that Ngb overexpression in primary cortical neurons protects against ROS production and prevents cytoarchitectural defects (de Vidania et al., 2020).

On one hand, there are evidences that Ngb may take part in regulating cell survival through the Wnt/β-catenin pathway (Xun et al., 2018). This action appears to be mediated by an interaction with Dvl1, a crucial effector in the proliferation and differentiation of neural progenitor cells, and with ubiquitin ligases. In the latter case, the interaction between p65 and Dvl1, responsible for the activation of β-catenin, is hampered, thus inhibiting neurogenesis (Yu et al., 2018). Additionally, upon TNFα stimulation of SK-N-SH cells, the Ngb-dependent recruitment of ubiquitin ligases results in the degradation of Dvl1. The down-regulation of Dvl1 by Ngb, ultimately yields the activation of NFκB, engaging towards cell survival (Yu et al., 2012).

On the other hand, Ngb was shown to intervene in the anti-apoptotic PI3K/Akt/MAPK signalling pathway to support survival of mouse cortical astrocytes after hypoxic insults (Amri et al., 2017) and of neuroblastoma cells upon nutrient deprivation (Fiocchetti et al., 2017).

Investigations performed on PC12 cells upon oxygen-glucose deprivation indicated that Ngb may directly interact with p38 MAPK promoting axon regeneration (Xiong et al., 2018). It has also been demonstrated that, in extra-nervous tumoral tissues where ectopic Ngb expression was markedly observed (Emara et al., 2010; Fiocchetti et al., 2016, 2014; Oleksiewicz et al., 2011) such as MCF-7 breast cancer, stimulation of the ERα estrogen receptor by 17β-estradiol, led to tumor

progression through the upregulation of Ngb and the activation of the PI3K/Akt pathway (Fiocchetti et al., 2016).

Moreover, upon overexpression, Ngb is re-localized inside the mitochondria where it interacts with cyt c, blocking the activation of the down-stream cyt c dependent pro-apoptotic pathway, notably involving pro-caspases activation (Fiocchetti et al., 2018, 2014; Raychaudhuri et al., 2010; Wang et al., 2017).

17β-estradiol stimulation in MCF-7 breast cancer cells also induce the activation of NRF2, a transcription factor involved in protection against oxidative stress, through the implication of Ngb resulting in an increased tumor tolerance to ROS (Solar Fernandez et al., 2020).

Furthermore, Ngb knockdown in human glioma cells, the most common form of brain malignancy, has been correlated to lower level of phosphorylated Akt, reduced level of mTOR and Bcl-2, and increased level of Bax expression, whereas over-expression of Ngb was correlated with an increased activation of the PI3K/Akt signalling pathway (Zhang et al., 2017b). In light of these findings, Zhang and colleagues proposed Ngb as a promising biomarker of human glioma owing to its prominent expression in tumoral brain tissues with respect to normal ones (Zhang et al., 2017a).

Moreover, the interaction between Ngb and cyt c into mitochondria, where it prevents the down-stream pro-apoptotic cascade also occurs in neuronal derived cells as demonstrated by De Marinis and colleagues. Neuroglobin upregulation induced by 17β-estradiol sequesters cytochrome c in the mitochondria preventing H_2O_2-induced apoptosis of neuroblastoma cells (De Marinis et al., 2013).

These phenotypes underline the involvement in Ngb in this signalling pathway and its implication in promoting cell survival, including tumor progression. In fact, aberrant activation of the PI3K/Akt signalling pathway had been reported in various cancer types including glioma (Yang et al., 2016).

In malignant glial tissues, Ngb up-regulation upon the inhibition of PPARγ, a tumor suppressor suppressed in glioma, has also been reported, which leads to increased phosphorylation, and therefore activation, of Akt. Notably, This phenomenon has a double effect depending on the pathological/physiological context: on one side it leads to glioma cancer progression but on the other one, it could protect neural cells against oxidative stress and PPARγ pro-apoptotic action (Hu et al., 2017).

Ngb involvement in the PI3K/Akt signalling pathway plays also a part against arsenic poisoning, which increase the cellular production of ROS: while Ngb knockdown-cerebellar granule neurons

(CGN) were endowed with significantly low levels of bcl-2/bax proteins, Ngb over-expressing CGN showed higher rate of survival (Liu et al., 2021).

Although it seems that Ngb may play a central role in tumor cells defense in CNS (Emara et al., 2010), Zhang et al. suggested that Ngb may be a tumor suppressor also in hepatocellular carcinoma since the globin may interact with C-Raf-1, thus inhibiting the Raf/MEK/Erk anti-apoptotic signalling pathway (Zhang et al., 2013).

The anti-apoptotic effects of Ngb could also occur upon its direct interaction with the Pak1 kinase. Pak1 participate to the activation of the Rac1 GTPase upon phosphorylation of RhoGDI, which can detach from Rac1 and allow the latter GTPase to perform its function, resulting in the rearrangement of the actin cytoskeleton, which may constitute a cell death signal. In this pathway, Ngb seems to inhibit Pak1, ultimately leading to cell survival (Hajra and Liu, 2004; Khan et al., 2008).

Notably, Ngb neuroprotection has been shown to be induced by several steroids such as testosterone, a steroid hormone, and tibolone, a synthetic steroid used in the prevention against osteoporosis, which led to up-regulation of Ngb in T98G cell line and resulted in the conservation of mitochondrial functions and promotion of astrocyte survival upon glucose deprivation and the associated ROS production (Avila-Rodriguez et al., 2016; Toro-Urrego et al., 2016).

Excessive accumulation of amyloid beta (Aβ) is a pathological hallmark of Alzheimer disease (AD), a neurodegenerative disease, due to the progressive loss of neurons that results in the deterioration of cognitive functions. Recently, efforts have been made to understand the role of Ngb in AD. Ngb, whose expression is increased in early and moderately advanced AD stages, seems to have a protective role in this pathology, while higher levels of neuronal Ngb may potentially lower AD risk or slow AD progression (Fordel et al., 2006; Liu et al., 2018). More precisely, it was observed that Ngb silencing worsens Aβ neurotoxicity and mitochondrial dysfunction. Li and co-workers showed that, once again, the Akt pathway is probably involved in Ngb protection action upon Aβ accumulation in SH-SY5Y cells (Li et al., 2016). Notably, fucosterol, a phytosterol found in Ecklonia algae and a potential new anti-AD type of drug, increased level of Ngb mRNA in SH-SY5Y cells upon Aβ induced neurotoxicity (Gan et al., 2019).

Another neurodegenerative pathology may see an involvement of neuroglobin: the Huntington disease (HD), a degenerative disease marked by the gradual loss of neurons in discrete areas of the CNS. Indeed, the interaction between huntingtin (HTT) protein and Ngb, which over-expression

is mediated by 17β-estradiol, and their subsequent re-localization inside the mitochondria seems to protect SK-N-BE neuroblastoma cells from H_2O_2 exposure and induced apoptosis. This neuroprotective effect is lost in the presence of the pathological form of HTT, leading the Ascenzi and Marino research group to propose the 17β-estradiol/Ngb/HTT axis as a possible therapeutic target against neurodegeneration (Nuzzo et al., 2017).

Worth mentioning is the work of Watanabe and colleagues that described an alternative molecular route supporting the cytoprotective role of Ngb, the guanidine nucleotide dissociation inhibitor activity of Ngb on $G\alpha_{(i/o)}$, a subunit of the heterotrimeric G protein, identifying Ngb as an important actor promoting PC12 cell survival upon hypoxic insults (Wakasugi et al., 2003; Watanabe and Wakasugi, 2008).

The several findings reported above, strongly support the involvement of Ngb in promoting cell survival, over and above a direct role in radical scavenging and detoxification. The best characterized activity is the anti-apoptotic role, probably mediated by the interaction with cytochrome c and mitochondria. The rich literature on the induction of Ngb and of effects in cellular systems is very interesting and might constitute an indication of direct or indirect participation to signaling network/axis, however an unifying picture is still lacking given the complexity of the investigated phenomena.

6. Data from animal models

A plethora of *in vivo* animal model systems has been developed to investigate Ngb functions. Although, discrepancies may arise from the variety of models utilized, studies are again in favor of a neuroprotective effect carried out by Ngb (Luyckx et al., 2019).

A rather recent re-evaluation of Ngb age-dependent expression sites in mice revealed that although mRNA levels are low during the embryonic stage, a sudden increase occurs after birth, reaching a peak at the adult age in the cerebral cortex, cerebellum or hippocampus, which seems to be conserved among mammals (Fabrizius et al., 2016). However, it is now recognized that decreased levels with age in several rat brain regions are correlated with age-related neurodegeneration (Sun et al., 2013; Szymanski et al., 2010), it is also acknowledged that Ngb up-

regulation reduces infarct volumes and protects from ischemic insults/reperfusion, although results might differ according to the animal model utilized owing to compensation of redundant protection mechanisms (Li et al., 2010; Raida et al., 2013, 2012; Wen et al., 2018).

Ngb up-regulation and attenuated cerebral alteration have been observed in rats, notably after cardiac arrest and resuscitation (Fan et al., 2016), in obstructive sleep apnea murine models (Nair et al., 2018) and in sleep-deprived rats after a few hours of sleep recovery (Melgarejo-Gutiérrez et al., 2020). Interestingly, Ngb over-expression has been correlated to improved locomotor function upon spinal cord injury in albino Wistar rats, and researchers hypothetized that Ngb neuroprotective effect was linked to neural apoptosis inhibition through the mitochrondiral pathway (Dai et al., 2019; Lan et al., 2014). Furthermore, Yu et al demonstrated the pro-neurogenesis effect of Ngb, probably involving the activation of the Wnt signalling pathways, in mice after middle cerebral artery occlusion (Yu et al., 2018).

Correlation between neurodegenerative diseases such as Alzheimer (AD) or Huntington (HD) diseases and neuroglobin levels has been extensively investigated on animal models. Infact, Khan and coworkers observed reduced amounts of neurotoxic amyloid plaques in the brain of transgenic Ngb over-expressing mice (Khan et al., 2007). However, Ngb seems to attenuate tau protein hyperphosphorylation in AD murine models via the Akt signalling pathway, suggesting that Ngb could be a target for AD therapeutic strategies (Chen et al., 2012). Moreover, De Vidania and colleagues hypothesized that Ngb could be the first defense against accumulation and neurotoxicity of Aβ plaques during the early phase of the neurodegenerative pathology (de Vidania et al., 2020).

Population studies on neuroglobin relevance in neuroprotection confirmed observations made on animal models. Infact, lower levels of Ngb were correlated with the increased risk of AD and Ngb levels decreased with the severity of the disease (de Vidania et al., 2020; Szymanski et al., 2010). Additionally, in Alzheimer patients Ngb was localized in neurons and co-localized in site endowed with amyloid deposits (Sun et al., 2013).

Regarding other neurodegenerative diseases, colocalization of Ngb and huntingtin (HTT) has been observed in the striatum in Huntington disease mice models (Cardinale et al., 2018), while Ngb is down-regulated in SOD transgenic mice, a model widely use in the study of amyotrophic lateral sclerosis.

Several studies have also focused on Ngb protective effect against retina degeneration. In particular, it has been observed that Ngb injection increased the survival of retina ganglion cells in C57BL/6 mice after optic nerve injury, with the presence of optic axons outgrowth, absent in the control mice (Sugitani et al., 2017). The involvement of Ngb in attenuating vision impairments in retinal degeneration mice models suggests that the globin could be used as a therapeutic target against pathologies such as retinal degeneration and retinis pigmentosa (Tao et al., 2018, 2017). Cwerman-Thibault *et al.* hypothesized that Ngb could be a promising target in the treatment of glaucoma, owing to its beneficial effects on reliable glaucoma animal models (Cwerman-Thibault et al., 2017). Similarly, it has been demonstrated that depletion of Ngb in the auditory system of Ngb-knockout mice induces auditory deficits (Nowotny et al., 2017).

Effects of poisoning or exposure to neurotoxic agents on Ngb expression were widely assessed in animal models. Indeed, Azarov and colleagues demonstrated the protective effects of a distal His(E7)64 mutant of neuroglobin against carbon monoxide poisoning. Infact, the mutant, endowed with a higher affinity for CO with respect to rat hemoglobin, allows carbon monoxide exchange and trapping and promotes rat survival beyond forty minutes after poisoning. Accordingly, this variant could be envisioned as a potential biological therapeutic agent against lethal gas exposure (Azarov et al., 2016; Rose et al., 2020).

Increased level of Ngb in over-expressing mice brain also seems to counteract the negative effects of acute combustion smoke inhalation and attenuates neurobehavioral alterations (Gorgun et al., 2019). Interestingly, Male Wistar rats exposed to bisphenol A (BPA), a chemical compound considered a prototype of endocrine disruptor, displayed BPA dose-dependent increased levels of Ngb in the cortex and in the hypothalamus (da Conceição et al., 2017), whereas exposition to silver particles, causing the production of ROS, triggers the up-regulation of Ngb in the rat hippocampus and cerebellum (da Conceição et al., 2019).

Despite these observations obtained from murine models, investigations carried out on arsenic poisoning of highly exposed chinese populations point out that lower Ngb levels were measured in patients affected by arsenicosis (Liu et al., 2021).

In vivo studies animal models allowed to confirm the signalling pathways in which Ngb seems to take part. Infact, Ngb inhibits AMPK signalling, which is involved in pathway regulating anabolism and catabolism, as observed in transgenic over-expressing mice (Cai et al., 2016). Additionally, in

Sprague Dawley rats, sepsis-associated encephalopathy complications are alleviated upon Ngb-dependent activation of the PI3K/Akt signalling pathway (Deng et al., 2017).

Interestingly, Ngb seems to attenuate the neuronal injury in pregnant rats caused by sevoflurane, a general anesthesia drug that can be neurotoxic to developing brains. Infact, Ngb was shown to inhibit apoptosis through the Hif1-α signalling pathway regulating homeostasis upon low oxygen concentrations (Zhang et al., 2019).

The protective role of Ngb is not limited to the CNS but it has also been identified in ectopic sites: Ngb demonstrated positive effects on cardiomyocytes upon cardiac hypertrophy (Liu et al., 2015), evidence that was further supported by the observation that Ngb over-expressing mice have a higher survival rate after acute myocardial infarction (Luyckx et al., 2018)**.** Ngb was therefore proposed as a good candidate to target acute cardiac diseases (Van Acker et al., 2019a).

Therapies based on Ngb injection are actively explored. The use of nanoparticles to carry Ngb through the bloodstream towards nerve cells of Wistar rats showed promising results in the treatment of transient hypoxia and could be of relevance to treat stroke episodes (Blanco et al., 2020; Tun et al., 2019). Ngb has also been investigated as a possible marker for various pathologies. Several studies have proposed to use Ngb as a biomarker for the diagnosis and prognosis of glioma above mentioned, but also, retinal damage induced by light-emitting diode (LED) and traumatic brain injuries for example, owing to its significant over-expression in these pathologic situations (Vorasubin et al., 2016; Yu et al., 2014). Ngb was envisioned also as a biomarker of stroke severity and poor outcomes in aneurysmal subarachnoid hemorrhage owing to its large presence in human serum (Cai et al., 2018; Ding et al., 2019). Notably, amongst the proportion of aneurysmal subarachnoid hemorrhage patients, those who experienced delayed cerebral ischemia had significantly enhanced levels of Ngb, indicating that Ngb could also be used as a predictor of delayed cerebral ischemia. Although discrepancies may arise from the variety of models utilized as in the case of work carried out in cellular models, studies are again in favor of a neuroprotective effect carried out by Ngb (Luyckx et al., 2019).

7. Final remarks

The effort by different and complementary approaches to unravel the function and mechanism of neuroglobin started in 2000 has led to a remarkable collection of experimental findings, witnessing a great advancement from the oxygen delivery/reservoir initial hypothesis.

The analysis of Ngb structure and structural dynamics highlighted specific features that are consistent with enzymatic and sensing/signalling roles. The heme relocation upon ligand binding is linked to variations in structure and mobility of the CD loop and EF loop. The presence of an internal cavity and tunnel network might sustain sequential redox activity aimed at scavenging, generation or trapping of physiologically relevant radicals.

These potential activities are suggested by biochemical and biophysical characterization carried out on the isolated protein however, the complex metabolism of the involved species in the cellular environment does not allow to assign physiologically relevant activity(es) to Ngb based on the sole biochemical characterization.

The reducing activity against cytochrome *c* and the identification of Ngb interactors that participate to (anti)apoptotic pathways is based on multiple experimental evidence *in vitro* and *in cellulo* and it is compatible with a parallel action in the homeostasis of radical species due to enzymatic or trapping activity. Several interactors of Ngb have been identified, beyond cytochrome c and they point to the involvement in several cell biology pathways.

Pathologies in which Ngb up-regulation correlates with less adverse outcomes include Alzheimer's disease, Huntington disease, brain ischemia, glaucoma (Wei et al., 2011) and traumatic brain injury (Shang et al., 2012), this is consistent with an underlining protective function that comes into play in pathologies that challenge nervous cells survival, but not necessarily imply a distinct mode of action for each condition.

The possible implication of Ngb in different, yet mostly cell survival related, pathways is certainly fascinating however, a reductionist approach might be sensible, aiming at a conclusive and comprehensive picture that might include only some of the interactions reported in the scientific literature.

Of remarkable interest, given the concurring evidence of Ngb induction upon several pathologies or cellular insults, is the possibility of utilizing Ngb upregulation or even delivery for the therapy of neurological pathologies or its downregulation as tool to reduce malignant cell survival. These

developments deserve a clear definition of Ngb mode of action in physiological and pathological conditions, that is a requisite for their approval in modern pharmacology.

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Declarations of interest

The authors declare no conflict of interest.

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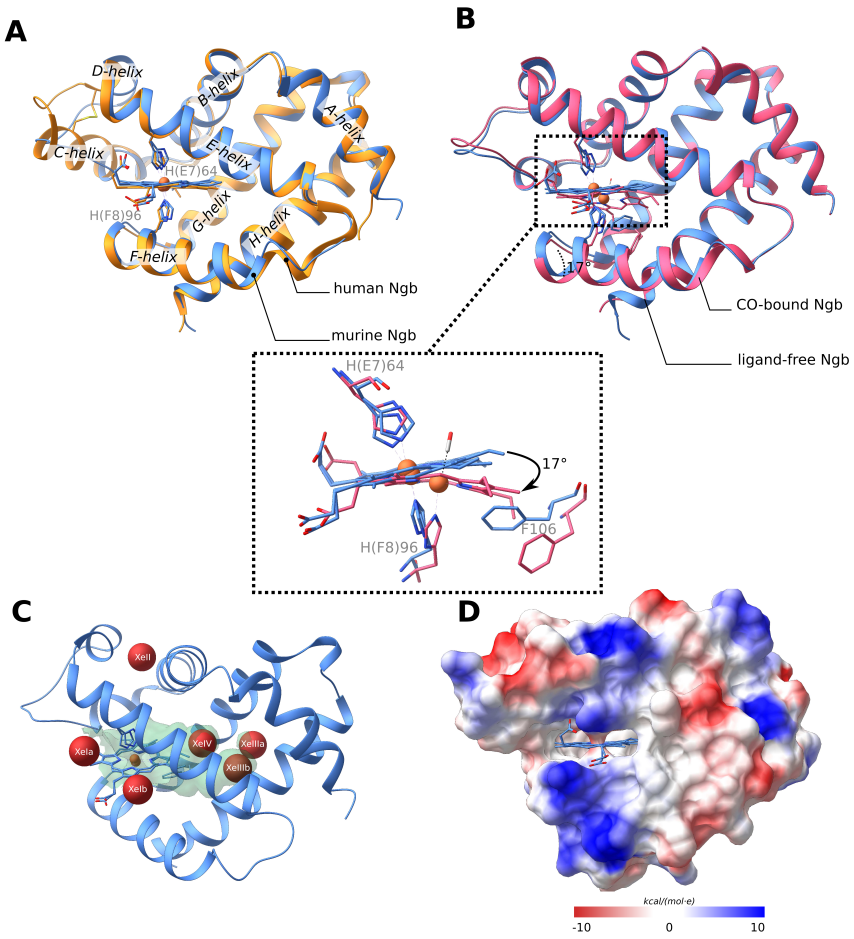
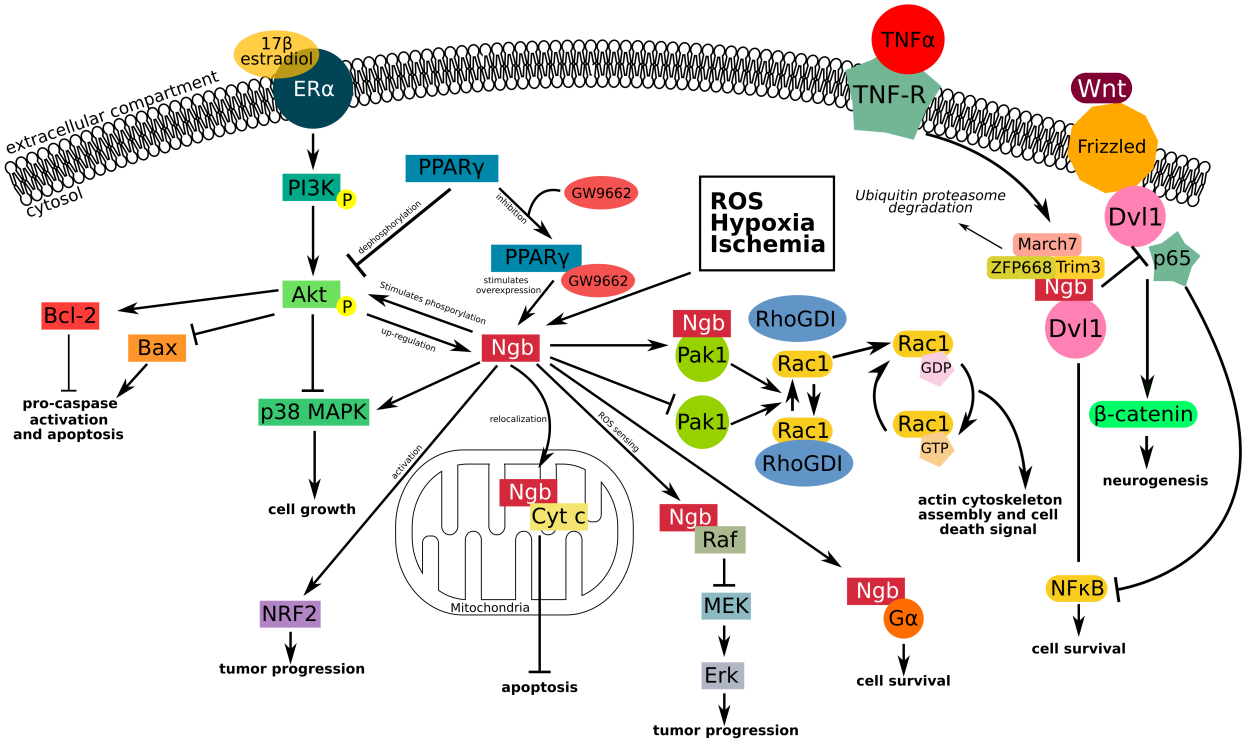


1086 ***Figure 1. Structural features of neuroglobin.*** A, The subtle structural differences between ferric
1087 human (4MPM PDB accession code, in orange (Guimarães et al., 2014)) and murine Ngb (1Q1F
1088 PDB accession code, in blue (Vallone et al., 2004a)) is most likely due to crystal packing
1089 constraints. B, The superposition of murine ligand-free (1Q1F PDB accession code, in blue
1090 (Vallone et al., 2004a)) and CO-bound (1W92 PDB accession code, in pink (Vallone et al., 2004b))
1091 structures of Ngb highlights the conformational rearrangements occuring upon CO binding, notably
1092 the heme sliding (inset). C, Ngb is endowed with a large internal cavity (green, calculated using
1093 CASp), and x-ray crystallography in the presence of noble gases allowed the identification of
1094 diatomic gas docking sites (XeI to XeIV as red spheres (Moschetti et al., 2009)) which regulate the
1095 internal ligand dynamics. D, Surface charges are represented according to Chimera Coulombic
1096 surface coloring function.



1097 ***Figure 2. Ngb seems to intervene in several signaling pathways.*** This figure recapitulates the

1098 Ngb interactors reported in the literature and the relative pathways involved.