

## Commentary

### What is the function of neuroglobin?

Thorsten Burmester<sup>1,\*</sup> and Thomas Hankeln<sup>2</sup>

<sup>1</sup>Institute of Zoology and Zoological Museum, University of Hamburg, Martin-Luther-King-Platz 3, D-20146 Hamburg, Germany and

<sup>2</sup>Institute of Molecular Genetics, Johannes Gutenberg University of Mainz, J. J. Becherweg 30a, D-55099 Mainz, Germany

\*Author for correspondence (e-mail: thorsten.burmester@uni-hamburg.de)

Accepted 3 March 2009

#### Summary

For a long time, haemoglobin and myoglobin had been assumed to represent the only globin types of vertebrates. In 2000, however, we discovered a third globin type by mining the genome sequence data. Based on a preferential expression in the nervous system, this globin is referred to as neuroglobin. Despite nine years of research, its function is still uncertain and a number of hypotheses have been put forward. Neuroglobin enhances cell viability under hypoxia and under various types of oxidative stress in transgenic systems, but does not appear to be strongly upregulated in response to stress. A close phylogenetic relationship with invertebrate nerve globins and its positive correlation with the oxidative metabolism and mitochondria suggest a role in O<sub>2</sub> supply. *In vitro* studies and cell culture experiments imply that neuroglobin may detoxify reactive oxygen or nitric oxide. Still other studies propose neuroglobin as being part of a signalling chain that transmits the redox state of the cell or that inhibits apoptosis. Although some functions are more probable than others, we conclude that it is still too early to definitively decide what may be the physiological role(s) of neuroglobin in vertebrates. Nevertheless, there is no doubt that neuroglobin has an essential, conserved function and is beneficial to neurons.

Key words: globin, mitochondria, ischemia, gene regulation, reactive oxygen species.

#### Introduction

Haemoglobin (Hb) and myoglobin (Mb) are without doubt the best-known proteins, and most intensively studied in biology. They have the ability to bind O<sub>2</sub> for the purpose of transport or storage, thereby enhancing the O<sub>2</sub> carrying capacity of the blood (Hb) or the availability of O<sub>2</sub> inside muscle cells (Mb). For a long time, Hb and Mb were considered as the only globins found in vertebrates. In 2000, however, we discovered a third globin that was named neuroglobin (Ngb) because of its preferential localisation in the nervous system (Burmester et al., 2000). Since then, Ngb orthologues have been identified in various mammalian, avian, reptilian, amphibian and fish species (Burmester et al., 2004). The structure of Ngb largely resembles that of a typical Mb: Ngb is a monomer of about 150 amino acids with a molecular mass of 16 kDa (Burmester et al., 2000; Dewilde et al., 2001) that features a typical globin fold (Pesce et al., 2003). Ngb binds to various gaseous ligands such as O<sub>2</sub>, CO or NO (Dewilde et al., 2001). In the absence of an external ligand there are significant structural differences between Hb or Mb on the one hand, and Ngb on the other. Hb and Mb are so-called pentacoordinate globins, in which the iron (Fe<sup>2+</sup>) is bound by four nitrogen atoms of the porphyrin ring and the proximal histidine in the F helix (HisF8). Upon oxygenation, O<sub>2</sub> binds to the free sixth coordination site of the Fe<sup>2+</sup>. Ngb is a hexacoordinate globin: in the deoxy state, the distal histidine in the E helix (HisE7) is bound to the sixth coordination position of the Fe<sup>2+</sup>. Any external ligand has therefore to compete with HisE7 for the binding of Fe<sup>2+</sup>. Hexacoordination is a feature of various plant, bacterial, invertebrate and vertebrate globins, but its functional significance is not yet understood (Hankeln et al., 2005; Trent and Hargrove, 2002).

Ngb is mainly expressed in neurons of the central and peripheral nervous systems (CNS, PNS), as well as some endocrine tissues (Burmester et al., 2000; Reuss et al., 2002). In the CNS all or at least most neurons express Ngb, although there are quantitative differences among distinct neuron populations. Whereas the total concentration of Ngb protein in the mouse brain is low [probably <1 μmol l<sup>-1</sup> (Burmester et al., 2000)], the level in the neuronal retina, which requires large amounts of O<sub>2</sub>, is about 50- to 100-fold higher (Schmidt et al., 2003).

#### The family of 'novel' vertebrate globins

In addition to Ngb, other 'novel' vertebrate globins have been found, which differ in expression patterns and evolutionary history (Fig. 1): cytoglobin [Cygb (Burmester et al., 2002; Trent and Hargrove, 2002)], globin E [GbE (Kugelstadt et al., 2004)], globin X [GbX (Roesner et al., 2005)] and globin Y [GbY (Fuchs et al., 2006)]. Cygb resides in fibroblast-like cells and in distinct populations of neurons. Cygb is related to Mb from which it diverged early in the evolution of vertebrates (Burmester et al., 2002) (Fig. 1). It is unlikely that Cygb functions in O<sub>2</sub> supply to the respiratory chain, but it may be involved in detoxification of reactive oxygen or nitrogen species (ROS/RNS), or in oxygen supply to particular enzymatic reactions (Hankeln et al., 2005). While Ngb and Cygb are presumably present in all vertebrates, three other globin types only occur in some taxa. GbX is weakly expressed in amphibia and fish and its function is not yet known (Fuchs et al., 2006; Roesner et al., 2005). In the eye of birds, another globin type (GbE) has been detected (Kugelstadt et al., 2004). GbY has only been identified so far in *Xenopus* (Fuchs et al., 2006). Both, GbE and GbY are distant relatives of Cygb or Mb, but little is known about ligand binding characteristics or functions.

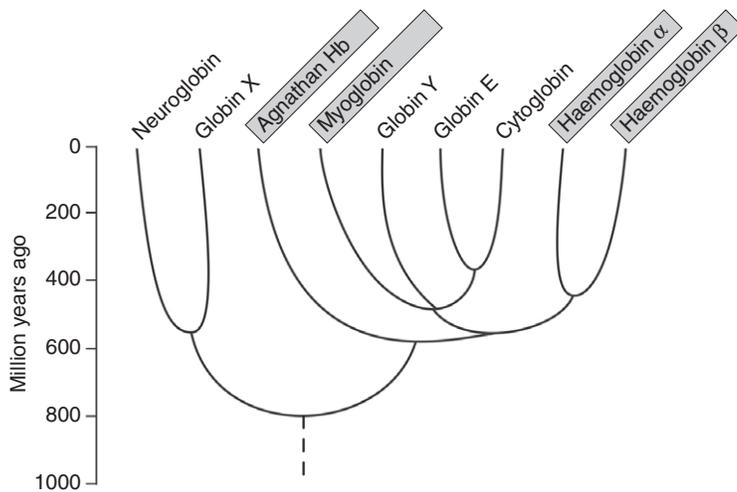


Fig. 1. Phylogenetic history of vertebrate globins. The simplified tree has been combined from different sources (Burmester et al., 2002; Burmester et al., 2000; Kugelstadt et al., 2004; Roesner et al., 2005). Globins with clear respiratory function (i.e. Mb and Hb) are shaded in grey. Note that haemoglobins from Agnatha and Gnathostomata (haemoglobin  $\alpha$  and  $\beta$ ) are not monophyletic.

### Putative neuroglobin functions

Until recently, it has generally been accepted that any intracellular globin has a similar function to that of Mb, either storing  $O_2$  for hypoxic phases (e.g. in diving mammals) or facilitating  $O_2$  diffusion from the capillaries to the respiratory chain in the mitochondria. However, within the past ten years it has become evident that certain globins may carry out various other functions. For example, maize globin regenerates  $NAD^+$ , which is used to promote glycolysis in low oxygen environments (Sowa et al., 1998). Some prokaryotic globin and globin domains are involved in  $O_2$  sensing (Hou et al., 2001). *Escherichia coli* flavohaemoglobin decomposes NO, conferring resistance to NO poisoning and aconitase inactivation (Gardner et al., 1998). A similar function has been demonstrated for mammalian Mb, which scavenges NO and thus protects cellular respiration (Flögel et al., 2001). Mb may also be involved in decomposition of harmful reactive oxygen species (ROS) (Flögel et al., 2004).

Although Ngb is the best-investigated 'novel' globin type, its exact physiological role is still uncertain. Ngb is a highly conserved protein, with an evolutionary rate that is about threefold slower than that of Mb and Hb (Burmester et al., 2004). Thus Ngb has remained largely unchanged during evolution, pointing to an important role of this protein. Several functions of Ngb have been proposed (Fig. 2).

1. Ngb may exert a Mb-like role, enhancing  $O_2$  supply to the mitochondria of the metabolically active neurons (Fig. 2A).
2. Ngb may scavenge damaging reactive oxygen or nitrogen species (ROS/RNS), which are generated for example by the respiratory chain (Fig. 2B).
3. Ngb may detoxify harmful excess of nitric oxide (NO) to nitrate ( $NO_3^-$ ) at normoxia or produce NO for signalling functions from nitrite ( $NO_2^-$ ) at hypoxia for the control of blood pressure (Fig. 2C).
3. Ngb may be involved in a signal transduction pathway, e.g. by inhibiting the dissociation of GDP from G protein  $\alpha$  (Fig. 2D).
4. Ngb may be part of a redox process that is for example instrumental in preventing apoptosis via reduction of cytochrome c (Fig. 2E).

All these functions have been supported by some experimental data or based on analogy with other globins. However, we consider it unlikely that Ngb has so many distinct roles and careful evaluation of the evidence, which is outlined below, is required.

### A role of neuroglobin in $O_2$ supply?

Globins sporadically occur in glial cells or neurons of various invertebrates and there is little doubt that these nerve globins are involved in  $O_2$  supply (Burmester and Hankeln, 2008; Wittenberg, 1992). In fact, the lack of nerve globins reduces the viability of invertebrate neurons under hypoxia. Sequence comparisons and phylogenetic analyses showed that Ngb is related to invertebrate nerve globins (Burmester and Hankeln, 2008; Burmester et al., 2000). From the evolutionary perspective, an  $O_2$  supply function of vertebrate Ngb is the most parsimonious hypothesis. Analyses of the phylogenetic history of the vertebrate globins (Fig. 1) show that globins with clear respiratory functions (Mb, Hb, agnathan Hb) are not monophyletic, i.e. they do not derive from a single most recent common ancestor. One must either assume a principal role of all vertebrate globins in  $O_2$  supply or must propose a frequent switch in globin functions. A specific function of Ngb in  $O_2$  delivery basically requires (1) an  $O_2$  affinity that is sufficient to deliver oxygen to the tissue, (2) an expression in cells with special needs for  $O_2$ , (3) an expression level roughly equivalent to that of muscle Mb and (4) a system that counterbalances the tendency of Ngb to autoxidise.

Ngb binds reversibly to  $O_2$  with an affinity that is within the range of a typical Mb [half saturation pressure  $P_{50}=0.12\text{--}0.29\text{ kPa}$  (0.9–2.2 Torr)] at 20–37°C (Burmester et al., 2000; Dewilde et al., 2001; Hundahl et al., 2006a). Ngb combines with  $O_2$  at high  $P_{O_2}$  and releases it at low  $P_{O_2}$ , fulfilling the basic requirements of a respiratory protein employed in  $O_2$  supply (Fig. 2A).

The hypothesis that Ngb functions as an  $O_2$  supply protein is supported by the observation that Ngb preferentially resides in metabolically active cells and subcellular compartments (Schmidt et al., 2003). Regions with low  $O_2$  partial pressures ( $P_{O_2}$ ) usually have no or much less Ngb, as exemplified by the striking differences between mammalian retinae with different  $O_2$  supply modes. For example, oxygen is supplied to the vascular retinae of most mammals by a network of capillaries such that high  $P_{O_2}$  are found in the inner segments of the photoreceptor cells, in the outer and inner plexiform layers, and in the ganglion cell layer (Bentmann et al., 2005; Schmidt et al., 2003). However, in the avascular retina of the Guinea pig,  $O_2$  supply by capillaries is restricted to the inner segments, whereas the other parts of the retinae rely on an anaerobic metabolism (Bentmann et al., 2005). In both types of retinae, Ngb is restricted to regions that are supplied with, and consume, oxygen. In addition, the concentration of Ngb is also tightly correlated with the distribution of

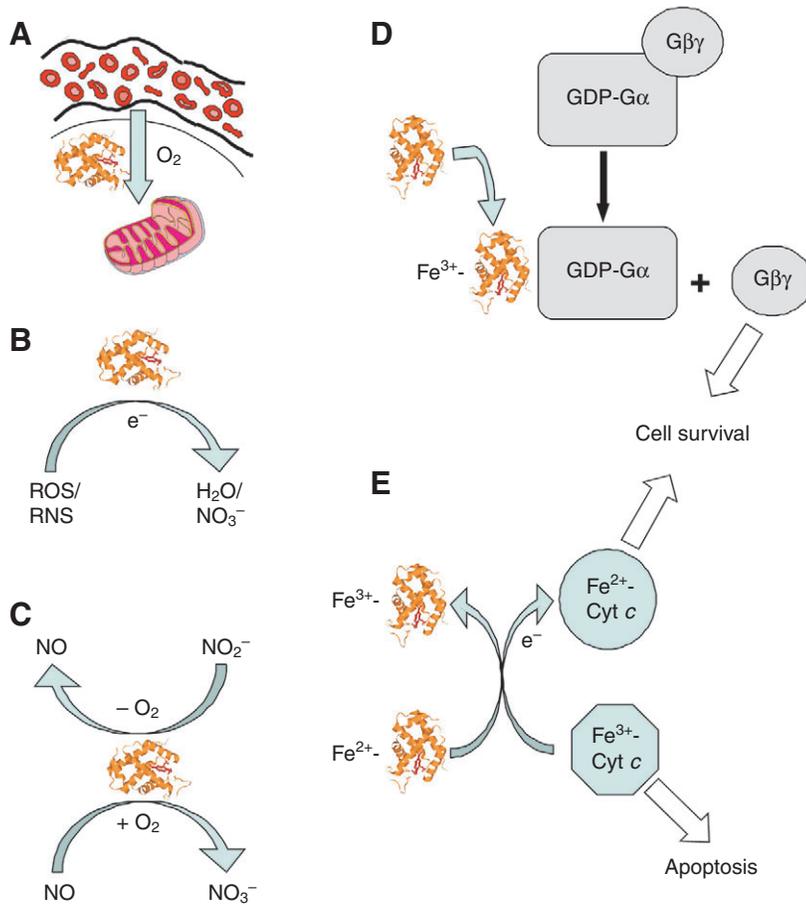


Fig. 2. Some postulated functions of neuroglobin. Neuroglobin may support the supply of  $O_2$  to the electron transport chain in mitochondria (A), may detoxify reactive oxygen or nitrogen species (B), may convert NO to  $NO_3^-$  at high  $P_{O_2}$  and  $NO_2^-$  to NO at low  $P_{O_2}$  (C), act as a signal protein by inhibiting the dissociation of GDP from  $G\alpha$  (D) or prevent hypoxia-induced apoptosis *via* reduction of cytochrome *c* (E).

mitochondria, although Ngb is not localised within this organelle. This observation provides strong evidence for a connection between Ngb and oxidative metabolism and is an additional argument in favour of a prominent role of Ngb in  $O_2$  storage or delivery. This holds in particular for the retina, given its rather high levels of Ngb expression, which approach the same level as that of Mb in muscle (Wittenberg, 1992). As pointed out by Fago et al. (Fago et al., 2004), an argument against a prominent role of Ngb in  $O_2$  supply in the brain has been its low concentration of only  $1 \mu\text{mol l}^{-1}$  in total brain extracts (Burmester et al., 2000), which is considered to be much too low to sustain  $O_2$  flow to mitochondria. However, there are distinctly different Ngb expression levels in different brain regions. In addition, Ngb is restricted to the thin cytoplasmic volume of neurons, so that local concentrations will be much higher than estimated for the total brain, which largely consists of glial cells. Most recently we found that in zebrafish total Ngb and Mb mRNA levels are in the same range, with Ngb levels that are tenfold higher than Mb levels during some developmental stages (J. Tiedke, F. Gerlach, T.H. and T.B., unpublished results). If one accepts a respiratory function for Mb in fish, such a role cannot be excluded for Ngb on the basis of total concentrations. However, the fast autoxidation of ferrous ( $Fe^{2+}$ ) Ngb to ferric ( $Fe^{3+}$ ) Ngb observed *in vitro* (Dewilde et al., 2001) would rather preclude an efficient binding of  $O_2$  to Ngb, but favours an involvement of Ngb in some type of redox reaction (see below). Any hypothesis that suggests a function of Ngb in  $O_2$  supply must therefore propose the existence of an efficient Ngb reductase or a reducing cellular environment, which permits the conversion of ferric Ngb to the ferrous form.

But if the hypothesis that Ngb functions as an  $O_2$  supply protein is true, why then are neurons and myocytes the only cell types with respiratory proteins? One possible explanation relates to the specific functions and morphologies of these tissues, which result in relatively long  $O_2$  diffusion distances from blood vessels to mitochondria. Myocytes of skeletal muscles are composed of syncytia, large multinucleate cells that had been formed by fusion of myoblasts. In the brain, the  $O_2$  must pass the blood brain barrier consisting of astrocytes to reach the neurons. This is not the case in most other tissues. A respiratory protein may therefore be required in brain and muscles to enhance the flow of  $O_2$  from the capillaries to the respiratory chain.

#### Hypoxia regulation of Ngb

If Ngb is associated with  $O_2$  consumption, it may be expected that alterations of external  $O_2$  supply may also affect Ngb expression (for review, see Burmester et al., 2007). In cell culture systems, Ngb expression may actually be moderately induced by hypoxia (Schmidt-Kastner et al., 2006; Sun et al., 2001). *In vivo*, however, Mammen et al. (Mammen et al., 2002) and Hundahl et al. (Hundahl et al., 2005) found no difference in Ngb levels in hypoxic and normoxic brains of rodents. Our own experiments employing various hypoxia regimes never showed increased Ngb mRNA or protein levels in rat brain (A. Avivi, F. Gerlach, S. Reuss, T.B., E. Nevo, T.H., unpublished data). Likewise, *in situ* hybridisation, micro-array and quantitative real-time PCR studies showed that ischemia did not induce significant changes of Ngb (Hundahl et al., 2006b; Schmidt-Kastner et al., 2006). It should be therefore considered unlikely that Ngb expression levels are increased by

exposure to hypoxia in rodents. However, it must be considered that under non-pathological conditions most terrestrial mammals will never experience hypoxic phases during their adult life. Therefore, it is unlikely that their nervous systems are well adapted to O<sub>2</sub> shortage and that Ngb expression responds to hypoxia (Burmester et al., 2007). By contrast, certain fish species and aquatic turtles live in environments with low O<sub>2</sub> availability. Experimental hypoxia induces significant upregulation of Ngb in the zebrafish *Danio rerio* (Roesner et al., 2006) and in the anoxia-tolerant turtle *Trachemys scripta* (Milton et al., 2006). In goldfish (*Carassius auratus*), a species that survives extended periods of reduced O<sub>2</sub> availability, hypoxia does not cause increased Ngb levels (Roesner et al., 2008). However, the level of Ngb protein in goldfish brain is approximately fivefold higher than in the brain of the less hypoxia-tolerant zebrafish. These observations are in line with the hypothesis that Ngb is involved in hypoxia tolerance, at least in some species.

#### Reaction of Ngb with reactive oxygen and nitrogen species

Soon after the discovery of Ngb there was speculation that – in analogy to Mb – it protects neurons from noxious ROS or RNS (Fig. 2B,C). Like any globin, Ngb associates with gaseous ligands other than O<sub>2</sub>. Under an excess of NO applied *in vitro*, an Ngb-Fe<sup>2+</sup>-NO form is established by reductive nitrosylation, which then decomposes the very toxic ROS component peroxynitrite, resulting in Ngb-Fe<sup>3+</sup>-NO (Herold et al., 2004). *In vitro*, oxygenated Ngb (Ngb-Fe<sup>2+</sup>-O<sub>2</sub>) may also react with NO, yielding Ngb-Fe<sup>3+</sup> and NO<sub>3</sub><sup>-</sup> (Brunori et al., 2005). Thus Ngb was proposed to have a similar role to that of Mb, acting as a NO-dioxygenase when P<sub>O<sub>2</sub></sub> is low (e.g. after an ischemic insult) and NO levels are increased (Fig. 2C). Under low-oxygen conditions deoxygenated Ngb may react with NO<sub>2</sub><sup>-</sup>, resulting in the formation of NO (Petersen et al., 2008). Thus, depending on the oxygen partial pressures, Ngb may either decompose or produce NO, which may be instrumental in the control of vasoconstriction or -relaxation, and the level of mitochondrial respiration. Jin et al. (Jin et al., 2008) showed that Ngb-transfected cells are protected from NO-induced stress. However, almost any globin will react with NO and similar compounds *in vitro*, and the *in vivo* importance of this reaction for Ngb remains to be demonstrated. In fact, Smagghe et al. (Smagghe et al., 2008) questioned the relevance of Ngb (and several other globins) in NO metabolism because this reaction lacks specificity.

Owing to the O<sub>2</sub> consumption process, mitochondria may generate various harmful ROS. This effect is most pronounced under hypoxia, when the flow of O<sub>2</sub> is reduced. Globins may protect the cells from ROS stress (Flögel et al., 2004). Several studies have suggested that Ngb may have a similar protective function in neuronal cells (Milton et al., 2006; Wang et al., 2008; Weber and Fago, 2004). The hypothesis that Ngb has a role in protection from ROS is in line with the observed neuroprotective effect of Ngb after ischemia and reperfusion (Khan et al., 2006; Liu et al., 2009; Sun et al., 2001; Sun et al., 2003; Wang et al., 2008), when ROS are known to form. In addition, ROS are released by the mitochondria and Ngb concentrations tightly correlate with this organelle (Bentmann et al., 2005). Moreover, Fordel et al. (Fordel et al., 2007) observed in the eye a negative correlation of Ngb and levels of H<sub>2</sub>O<sub>2</sub> production in hypoxia/reoxygenation studies, which they interpreted in terms of a ROS-scavenging function of Ngb. Cell culture experiments showed that overexpression of Ngb may confer a higher tolerance towards H<sub>2</sub>O<sub>2</sub> stress to the neuronal cell lines SH-SY5Y or PC12 (Fordel et al., 2006; Li et al., 2008). However, we did not observe any correlation of ROS formation or

ROS-decomposing enzymes with Ngb expression *in vivo* (T. Laufs, S. Reinhardt, S. Reuss, T.B., T.H., unpublished data). For example, Ngb expression levels in the mouse brain do not increase around time of birth when ROS and known ROS defence proteins are known to peak. In addition, exposure of cultured neurons to ROS stress does not trigger an upregulation of Ngb. The specificity of the ROS protective effect of Ngb remains to be demonstrated, as it does also for NO.

#### Neuroprotective properties of neuroglobin

Neurons are particularly sensitive to hypoxia-related stress. A protein that either enhances O<sub>2</sub> supply or that depresses the ROS-induced injuries should therefore enhance cell viability under ischemic stress. In fact, it has been demonstrated that experimentally decreased Ngb levels reduced, whereas overexpression of Ngb improved survival of cultured neurons (Liu et al., 2009; Sun et al., 2001) or isolated islet of Langerhans cells (Mendoza et al., 2005). Ngb may also protect neurons from β-amyloid-induced neurotoxicity and thus from Alzheimer's disease (Khan et al., 2007). Mice over-expressing Ngb in the brain showed reduced tissue infarction and improved pathology after ischemia (Khan et al., 2006; Sun et al., 2003; Wang et al., 2008). Surprisingly, in transgenic mice, Ngb has a similar ischemia-protective effect for the heart (Khan et al., 2006), even in the presence of a vast excess of Mb. However, in our hands, transfection of HN33, NS20SY or 3T3 cell lines with Ngb does not significantly improve the viability, either under normoxia or under hypoxia (S. Mitz, T.H. and T.B., unpublished data). Thus both the significance *in vivo* as well as the exact mechanism of neuroprotection by Ngb is still uncertain.

#### Neuroglobin interactions

Proteins that interact with Ngb may provide important clues for its function. For example, an alternative role of Ngb may be in intracellular signalling, which may be similar to gas-sensing bacterial globins (Hou et al., 2001). In several papers, Wakasugi et al. suggested that ferric (Fe<sup>3+</sup>) Ngb binds to the α subunit of heterotrimeric G proteins (e.g. Wakasugi and Morishima, 2005; Wakasugi et al., 2003; Watanabe and Wakasugi, 2008). The authors proposed that this interaction inhibits the dissociation of GDP from Gα and triggers the release of Gβγ, thereby protecting the cell from apoptosis (Fig. 2D). Most recently, Khan et al. (Khan et al., 2008) suggested that Ngb inhibits Rac1-GDP-dissociation inhibitor disassociation, thereby controlling actin assembly and cell death. The association of Ngb with G proteins was postulated on the basis of an alleged sequence similarity between Ngb and regulators of G protein signalling (RGS) and RGS domains (Wakasugi et al., 2003). However, our own sequence comparisons did not reveal any similarities between Ngb and RGS that are better than random (Burmester et al., 2004; Hankeln et al., 2005). In addition, a GDP dissociation-inhibiting function was not found for zebrafish Ngb (Wakasugi and Morishima, 2005). Given the conservation of Ngb sequences and expression patterns, these findings cast some doubts on the relevance of the interaction between Ngb and G proteins *in vivo*. Other studies identified various putative interacting partners of Ngb, such as the β<sub>2</sub> subunit of the Na<sup>+</sup>,K<sup>+</sup>-ATPase ion pump, the lipid raft protein flotillin 1, and the cysteine proteinase inhibitor cystatin C (for a review, see Hankeln et al., 2005). Although the specificity of these interactions and the exact role of Ngb herein remain to be established, some cellular and intracellular distributions of Ngb and the alleged interacting proteins are very distinct, making the *in vivo* relevance of these interactions highly questionable.

Fago et al. (Fago et al., 2006) suggested that the neuroprotective effect of Ngb is the result of the reduction of ferric ( $\text{Fe}^{3+}$ ) cytochrome *c* by ferrous ( $\text{Fe}^{2+}$ ) Ngb, thereby preventing cytochrome *c*-induced apoptosis (Fig. 2E). This hypothesis, which is based on *in vitro* experiments, is in line with many expression data, such as the colocalization of Ngb and mitochondria (Bentmann et al., 2005), which actually release cytochrome *c* to trigger apoptosis. Future studies must show whether electron transfer from Ngb to cytochrome *c* actually occurs *in vivo* and whether a neuroprotective effect can be related to this reaction.

### Conclusions

Ten years ago nobody would have questioned a respiratory function of any globin. Today, it is obvious that various globins may have other or additional physiological roles. Unfortunately, most of these functions are still poorly understood. Ngb is certainly one of the best-studied examples of the 'novel globins', but the current evidence neither convincingly supports nor excludes an involvement of Ngb in  $\text{O}_2$  supply. Various alternative hypotheses have been put forward (Fig. 2). We consider it unlikely that Ngb actually fulfils all of them, and the specificity of these functions *in vivo* remains to be demonstrated. For example, any heme globin will possibly react with ROS or NO, or to some extent transfer electrons to cytochrome *c* *in vitro*. Likewise, many proteins will combine with G proteins. Therefore, future experiments should more rigorously compare Ngb with other globins such as Mb, for which functions are comparatively well established.

### List of abbreviations

Cygb	cytoglobin
Gb	globin
Hb	haemoglobin
Mb	myoglobin
Ngb	neuroglobin
NO	nitric oxide
RGS	regulators of G protein signalling
RNS	reactive nitrogen species
ROS	reactive oxygen species

We wish to thank Frank Gerlach, Stephanie Mitz and Jessica Tiedke (Hamburg), Bettina Ebner, Christine Fuchs, Mark Haberkamp, Tilmann L. Laufs, Anja Roesner, Marc Schmidt, Bettina Weich, Sylvia Wystub, Sigrid Saaler-Reinhardt and Stefan Reuss (Mainz), Alessandra Pesce and Martino Bolognesi (Milano), Michael C. Marden and Laurent Kiger (Paris), Luc Moens and Sylvia Dewilde (Antwerp), Eviatar Nevo and Aaron Avivi (Haifa) and Roy E. Weber and Angela Fago (Aarhus) for discussions and sharing data. Our work has been supported by grants from the DFG (Bu956/5 and /11; Ha2103/3), the European Union (QLG3-CT2002-01548), the Stiftung für Innovation Rheinland-Pfalz (695), and the Fonds der Chemischen Industrie.

### References

- Bentmann, A., Schmidt, M., Reuss, S., Wolfrum, U., Hankeln, T. and Burmester, T. (2005). Divergent distribution in vascular and avascular mammalian retinae links neuroglobin to cellular respiration. *J. Biol. Chem.* **280**, 20660-20665.
- Brunori, M., Giuffrè, A., Nienhaus, K., Nienhaus, G. U., Scandurra, F. M. and Vallone, B. (2005). Neuroglobin, nitric oxide, and oxygen: functional pathways and conformational changes. *Proc. Natl. Acad. Sci. USA* **102**, 8483-8488.
- Burmester, T. and Hankeln, T. (2008). Neuroglobin and other nerve globins. In *Protein Reviews: Dioxygen Binding and Sensing Proteins*, vol. 9 (ed. M. Bolognesi, G. di Prisco and C. Verde), pp. 211-222. Milan: Springer.
- Burmester, T., Weich, B., Reinhardt, S. and Hankeln, T. (2000). A vertebrate globin expressed in the brain. *Nature* **407**, 520-523.
- Burmester, T., Ebner, B., Weich, B. and Hankeln, T. (2002). Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. *Mol. Biol. Evol.* **19**, 416-421.
- Burmester, T., Haberkamp, M., Mitz, S., Roesner, A., Schmidt, M., Ebner, B., Gerlach, F., Fuchs, C. and Hankeln, T. (2004). Neuroglobin and cytoglobin: genes, proteins and evolution. *IUBMB Life* **56**, 703-707.
- Burmester, T., Gerlach, F. and Hankeln, T. (2007). Regulation and role of neuroglobin and cytoglobin under hypoxia. *Adv. Exp. Med. Biol.* **618**, 169-180.
- Dewilde, S., Kiger, L., Burmester, T., Hankeln, T., Baudin-Creuz, V., Aerts, T., Marden, M. C., Caubergs, R. and Moens, L. (2001). Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. *J. Biol. Chem.* **276**, 38949-38955.
- Fago, A., Hundahl, C., Malte, H. and Weber, R. E. (2004). Functional properties of neuroglobin and cytoglobin: insights into the ancestral physiological roles of globins. *IUBMB Life* **56**, 689-696.
- Fago, A., Mathews, A. J., Moens, L., Dewilde, S. and Brittain, T. (2006). The reaction of neuroglobin with potential redox protein partners cytochrome b5 and cytochrome *c*. *FEBS Lett.* **580**, 4884-4888.
- Flögel, U., Merx, M. W., Gödecke, A., Decking, U. K. and Schrader, J. (2001). Myoglobin: a scavenger of bioactive NO. *Proc. Natl. Acad. Sci. USA* **98**, 735-740.
- Flögel, U., Gödecke, A., Klotz, L. O. and Schrader, J. (2004). Role of myoglobin in the antioxidant defense of the heart. *FASEB J.* **18**, 1156-1158.
- Fordel, E., Thijs, L., Martinet, W., Lenjou, M., Laufs, T., Van Bockstaele, D., Moens, L. and Dewilde, S. (2006). Neuroglobin and cytoglobin overexpression protects human SH-SY5Y neuroblastoma cells against oxidative stress-induced cell death. *Neurosci. Lett.* **410**, 146-151.
- Fordel, E., Thijs, L., Moens, L. and Dewilde, S. (2007). Neuroglobin and cytoglobin expression in mice: evidence for a correlation with reactive oxygen species scavenging. *FEBS J.* **274**, 1312-1317.
- Fuchs, C., Burmester, T. and Hankeln, T. (2006). The amphibian globin gene repertoire as revealed by the *Xenopus* genome. *Cytogenet. Genome Res.* **112**, 296-306.
- Gardner, P. R., Gardner, A. M., Martin, L. A. and Salzman, A. L. (1998). Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc. Natl. Acad. Sci. USA* **95**, 10378-10383.
- Hankeln, T., Ebner, B., Fuchs, C., Gerlach, F., Haberkamp, M., Laufs, T. L., Roesner, A., Schmidt, M., Weich, B., Wystub, S. et al. (2005). Neuroglobin and cytoglobin in search of their role in the vertebrate globin family. *J. Inorg. Biochem.* **99**, 110-119.
- Herold, S., Fago, A., Weber, R. E., Dewilde, S. and Moens, L. (2004). Reactivity studies of the Fe(III) and Fe(II)NO forms of human neuroglobin reveal a potential role against oxidative stress. *J. Biol. Chem.* **279**, 22841-22847.
- Hou, S., Freitas, T., Larsen, R. W., Piatibratov, M., Sivozhelozov, V., Yamamoto, A., Meleshkevitch, E. A., Zimmer, M., Ordal, G. W. and Alam, M. (2001). Globin-coupled sensors: a class of heme-containing sensors in Archaea and Bacteria. *Proc. Natl. Acad. Sci. USA* **98**, 9353-9358.
- Hundahl, C., Stoltenberg, M., Fago, A., Weber, R. E., Dewilde, S., Fordel, E. and Danscher, G. (2005). Effects of short-term hypoxia on neuroglobin levels and localization in mouse brain tissues. *Neuropathol. Appl. Neurobiol.* **31**, 610-617.
- Hundahl, C., Fago, A., Dewilde, S., Moens, L., Hankeln, T., Burmester, T. and Weber, R. E. (2006a). Oxygen binding properties of non-mammalian nerve globins. *FEBS J.* **273**, 1323-1329.
- Hundahl, C., Kelsen, J., Kjaer, K., Ronn, L. C., Weber, R. E., Geuens, E., Hay-Schmidt, A. and Nyengaard, J. R. (2006b). Does neuroglobin protect neurons from ischemic insult? A quantitative investigation of neuroglobin expression following transient MCAo in spontaneously hypertensive rats. *Brain Res.* **1085**, 19-27.
- Jin, K., Mao, X. O., Xie, L., Khan, A. A. and Greenberg, D. A. (2008). Neuroglobin protects against nitric oxide toxicity. *Neurosci. Lett.* **430**, 135-137.
- Khan, A. A., Wang, Y., Sun, Y., Mao, X. O., Xie, L., Miles, E., Graboski, J., Chen, S., Ellerby, L. M., Jin, K. et al. (2006). Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. *Proc. Natl. Acad. Sci. USA* **103**, 17944-17948.
- Khan, A. A., Mao, X. O., Banwait, S., Jin, K. and Greenberg, D. A. (2007). Neuroglobin attenuates beta-amyloid neurotoxicity *in vitro* and transgenic Alzheimer phenotype *in vivo*. *Proc. Natl. Acad. Sci. USA* **104**, 19114-19119.
- Khan, A. A., Mao, X. O., Banwait, S., DerMardirossian, C. M., Bokoch, G. M., Jin, K. and Greenberg, D. A. (2008). Regulation of hypoxic neuronal death signaling by neuroglobin. *FASEB J.* **22**, 1737-1747.
- Kugelstadt, D., Haberkamp, M., Hankeln, T. and Burmester, T. (2004). Neuroglobin, cytoglobin, and a novel, eye-specific globin from chicken. *Biochem. Biophys. Res. Commun.* **325**, 719-725.
- Li, R. C., Morris, M. W., Lee, S. K., Pournafar, F., Wang, Y. and Gozal, D. (2008). Neuroglobin protects PC12 cells against oxidative stress. *Brain Res.* **1190**, 159-166.
- Liu, J., Yu, Z., Guo, S., Lee, S. R., Xing, C., Zhang, C., Gao, Y., Nicholls, D. G., Lo, E. H. and Wang, X. (2009). Effects of neuroglobin overexpression on mitochondrial function and oxidative stress following hypoxia/reoxygenation in cultured neurons. *J. Neurosci. Res.* **87**, 164-170.
- Mammen, P. P., Shelton, J. M., Goetsch, S. C., Williams, S. C., Richardson, J. A., Garry, M. G. and Garry, D. J. (2002). Neuroglobin, a novel member of the globin family, is expressed in focal regions of the brain. *J. Histochem. Cytochem.* **50**, 1591-1598.
- Mendoza, V., Klein, D., Ichii, H., Ribeiro, M. M., Ricordi, C., Hankeln, T., Burmester, T. and Pastori, R. L. (2005). Protection of islets in culture by delivery of oxygen binding neuroglobin via protein transduction. *Transplant. Proc.* **37**, 237-240.
- Milton, S. L., Nayak, G., Lutz, P. L. and Prentice, H. M. (2006). Gene transcription of neuroglobin is upregulated by hypoxia and anoxia in the brain of the anoxia-tolerant turtle *Trachemys scripta*. *J. Biomed. Sci.* **13**, 509-514.
- Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T. and Bolognesi, M. (2003). Human brain neuroglobin structure reveals a distinct mode of controlling oxygen affinity. *Structure* **11**, 1087-1095.
- Petersen, M. G., Dewilde, S. and Fago, A. (2008). Reactions of ferrous neuroglobin and cytoglobin with nitrite under anaerobic conditions. *J. Inorg. Biochem.* **102**, 1777-1782.
- Reuss, S., Saaler-Reinhardt, S., Weich, B., Wystub, S., Reuss, M. H., Burmester, T. and Hankeln, T. (2002). Expression analysis of neuroglobin mRNA in rodent tissues. *Neuroscience* **115**, 645-656.
- Roesner, A., Fuchs, C., Hankeln, T. and Burmester, T. (2005). A globin gene of ancient evolutionary origin in lower vertebrates: evidence for two distinct globin families in animals. *Mol. Biol. Evol.* **22**, 12-20.

- Roesner, A., Hankeln, T. and Burmester, T. (2006). Hypoxia induces a complex response of globin expression in zebrafish (*Danio rerio*). *J. Exp. Biol.* **209**, 2129-2137.
- Roesner, A., Mitz, S. A., Hankeln, T. and Burmester, T. (2008). Globins and hypoxia adaptation in the goldfish, *Carassius auratus*. *FEBS J.* **275**, 3633-3643.
- Schmidt, M., Giessi, A., Laufs, T., Hankeln, T., Wolfrum, U. and Burmester, T. (2003). How does the eye breathe? Evidence for neuroglobin-mediated oxygen supply in the mammalian retina. *J. Biol. Chem.* **278**, 1932-1935.
- Schmidt-Kastner, R., Haberkamp, M., Schmitz, C., Hankeln, T. and Burmester, T. (2006). Neuroglobin mRNA expression after transient global brain ischemia and prolonged hypoxia in cell culture. *Brain Res.* **1103**, 173-180.
- Smagghe, B. J., Trent, J. T., 3rd and Hargrove, M. S. (2008). NO dioxygenase activity in hemoglobins is ubiquitous *in vitro*, but limited by reduction *in vivo*. *PLoS ONE* **3**, e2039.
- Sowa, A. W., Duff, S. M., Guy, P. A. and Hill, R. D. (1998). Altering hemoglobin levels changes energy status in maize cells under hypoxia. *Proc. Natl. Acad. Sci. USA* **95**, 10317-10321.
- Sun, Y., Jin, K., Mao, X. O., Zhu, Y. and Greenberg, D. A. (2001). Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. *Proc. Natl. Acad. Sci. USA* **98**, 15306-15311.
- Sun, Y., Jin, K., Peel, A., Mao, X. O., Xie, L. and Greenberg, D. A. (2003). Neuroglobin protects the brain from experimental stroke *in vivo*. *Proc. Natl. Acad. Sci. USA* **100**, 3497-3500.
- Trent, J. T., 3rd and Hargrove, M. S. (2002). A ubiquitously expressed human hexacoordinate hemoglobin. *J. Biol. Chem.* **277**, 19538-19545.
- Wakasugi, K. and Morishima, I. (2005). Preparation and characterization of a chimeric zebrafish-human neuroglobin engineered by module substitution. *Biochem. Biophys. Res. Commun.* **330**, 591-597.
- Wakasugi, K., Nakano, T. and Morishima, I. (2003). Oxidized human neuroglobin acts as a heterotrimeric G alpha protein guanine nucleotide dissociation inhibitor. *J. Biol. Chem.* **278**, 36505-36512.
- Wang, X., Liu, J., Zhu, H., Tejima, E., Tsuji, K., Murata, Y., Atochin, D. N., Huang, P. L., Zhang, C. and Lo, E. H. (2008). Effects of neuroglobin overexpression on acute brain injury and long-term outcomes after focal cerebral ischemia. *Stroke* **39**, 1869-1874.
- Watanabe, S. and Wakasugi, K. (2008). Neuroprotective function of human neuroglobin is correlated with its guanine nucleotide dissociation inhibitor activity. *Biochem. Biophys. Res. Commun.* **369**, 695-700.
- Weber, R. E. and Fago, A. (2004). Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Respir. Physiol. Neurobiol.* **144**, 141-159.
- Wittenberg, J. B. (1992). Functions of cytoplasmic hemoglobins and myohemerythrin. *Adv. Comp. Environ. Physiol.* **13**, 60-85.