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Neuroglobin Expression in the Brain: a Story of Tissue Homeostasis Preservation

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Abstract

After its discovery in 2000, the notion grew that neuroglobin, a neuronal specific heme protein, is involved in cytoprotection. To date, neuroglobin levels have been positively correlated with a beneficial outcome in a plethora of neurotoxic insults, e.g., ischemic and traumatic brain injuries and Alzheimer's disease. The first part of this review goes further into these changes of neuroglobin expression upon different neuronal insults as well as the underlying regulation. In the second part, we shed light on the mechanisms by which neuroglobin contributes to neuroprotection, being (i) the scavenging and detoxification of reactive oxygen/nitrogen species, (ii) the augmentation of the threshold for apoptosis initiation, (iii) its contribution to an anti-inflammatory milieu, and (iv) tissue regeneration. We also consider different neuroglobin models to address as yet unanswered questions. Based on the recent findings and progress in the field, we invigorate the avenues of neuroglobin in neurological ailments to increase in the coming years.

Keywords Neuroglobin · Expression · Neuroprotection · Apoptosis · Inflammation · Neurogenesis

Introduction

Sustainment of homeostasis in the central nervous system is essential to support vital physiological functions. In multicellular organisms, the maintenance of tissue homeostasis is based on a balance between cell death on the one hand and cell survival and proliferation on the other [1]. In case of a toxic insult or pathology, tissue integrity is preserved through an interacting network of signaling pathways, often including the activation of hypoxia-inducible factor 1 (HIF1) [2]. HIF1 modulates processes as erythropoiesis, angiogenesis, glucose transport, and glycolysis, allowing adaptations to oxygen delivery and metabolism [2]. Interestingly, globins are a class of hypoxia-activated, gaseous ligand-binding, heme proteins of which the ascribed functions surpass transport and supply of oxygen, the function for which they are best known [3].

One of these globins is neuroglobin (Ngb). It shows a widespread expression in the central and peripheral nervous system [4, 5]. Highest expression levels are detected in the mammalian hypothalamus, which shows 100-fold higher transcription rates than other key *Ngb* expression regions as the cerebral cortex and hippocampus ([6] Fig. 1). Pronounced levels are also detected in endocrine active regions of, e.g., the pituitary and adrenal glands [5]. Like neurons, these cells sustain high metabolic rates. Being confined to high oxygen-consuming cell types, Ngb was first proposed to play a central role in the maintenance of cellular oxygen supply. This hypothesis, however, was hardly tenable in view of Ngb's relatively low concentration (up to a μM range, or below 0.01% of the cerebral protein content [4]) and its oxygen affinity (P50) value of 7.5 Torr. The latter is below the oxygen tension existing inside neurons under physiological conditions [8]. Further elucidation of Ngb's structure and subcellular distribution entailed the premise of a better understanding of the vital biological functions performed by Ngb, knowing it to exhibit a 3-fold slower evolutionary rate as in comparison to other globins as hemoglobin and myoglobin [9–11].

Though Ngb exhibits the classical three-over-three alpha-helical globin fold, generating a hydrophobic pocket for the central heme iron atom, its protein sequence only reveals an up to 25% identity with sequences of other globins as hemoglobin and myoglobin [12]. Ngb's unique sequence supports the heme iron atom to be both hexa- and pentacoordinated, with the HisE7 residue and

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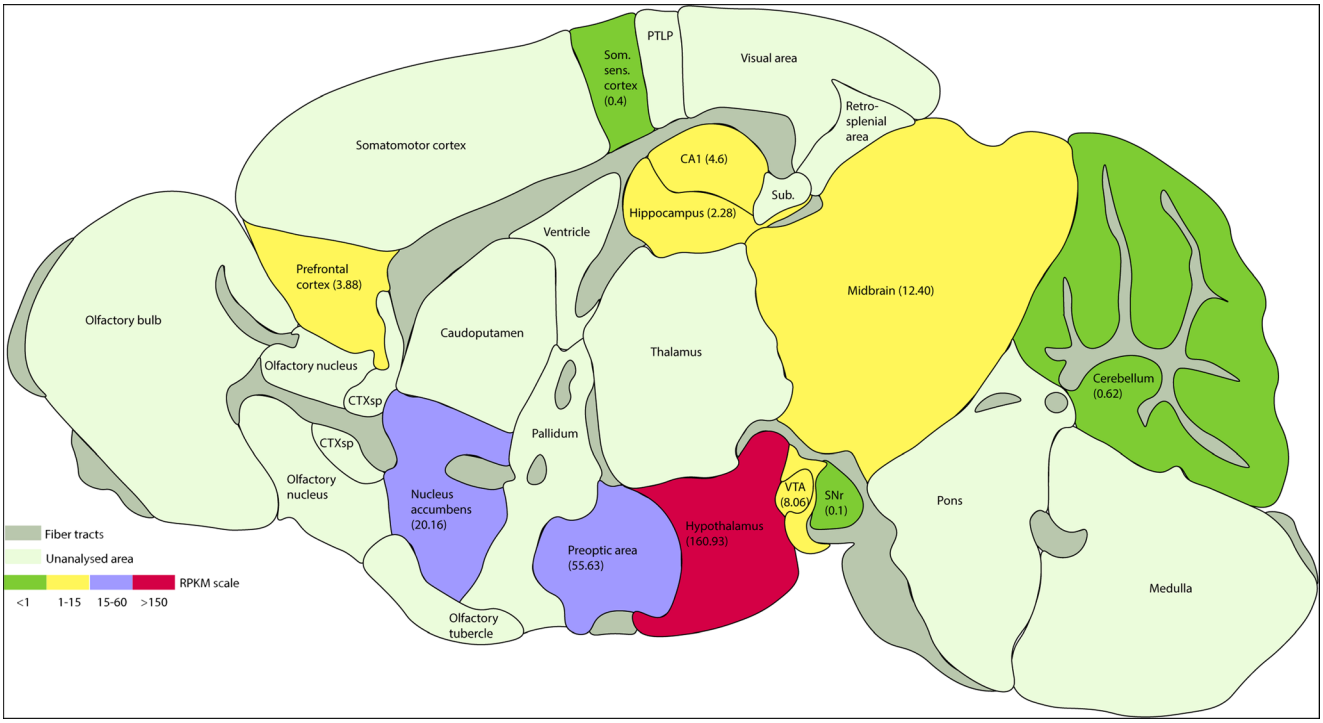

Fig. 1 Cerebral expression pattern of *Ngb*. Mean "reads per kilobase exon model per million mapped reads" (RPKM) values are indicated for the different areas. Values were obtained from a meta-analysis study on transcriptome data (RNA-Seq) [7]. PTLP posterior parietal association areas, Sub. subiculum, CTXsp cortical subplate, VTA ventral tegmental area, SNr substantia nigra

the ε-amino group of LysE10 to function as endogenous ligands in the hexacoordinated state [10, 12]. Beside the sixth coordination position of Ngb's iron atom being involved in the regulation of exogenous ligand binding [13], its valence state (ferrous or ferric) has been found implicated in Ngb's ability to interact with nitric (di)oxide [7, 14, 15] and reactive oxygen species (ROS) [16]. Moreover, ferrous (Fe^{2+}) Ngb buffers limited stress signals and maintains homeostasis by swiftly reducing leaked ferric (Fe^{3+}) cytochrome c to its inactive form [17], averting cytochrome c-induced apoptosis. In view of the neuroprotective nature of the latter interactions and the cellular redox state playing part in Ngb's ability to engage in cytoprotection, it is hypothesized that Ngb's functions are modest within physiological conditions and only come strongly into effect under cellular stress. In support of this idea, Ngb is a particularly stable protein. It retains most of its structural features under acidosis, a common side effect of cerebral pathological conditions as hemorrhages and pneumococcal meningitis [18]. *Ngb* expression is also upregulated in a plethora of neurotoxic insults; both studied in vitro (Table 1) as in vivo (Table 2). Given the wide variety of neurological injuries upon which *Ngb* expression is induced, this suggests the involvement of generalized or downstream pathways of tissue integrity preservation to which *Ngb* expression responses.

Ngb Expression Regulation

Hypoxia-Linked Elements—HIF1, SP1, mTOR

Despite the unlikelihood of Ngb having a primary role in maintaining oxygen homeostasis, its link with oxygen is strongly present. Many of the neurotoxic insults that increase *Ngb* expression are characterized by a direct (e.g., hypoxia) or indirect (e.g., ROS) dysregulated oxygen metabolism. Hence, it may be of no surprise that HIF1 levels are positively correlated with Ngb levels [11, 53, 75]. Though it is already noted that this is not a universal finding in all studies investigating *Ngb* expression under hypoxia (vide infra [50, 65]). The latter may be grounded in the regulatory elements upstream of *Ngb*'s promotor lacking a binding site for HIF1 [9]. As two GC boxes in the region around the transcription start site are bound by transcription factors specificity protein (Sp) 1 and Sp3 [9, 76], it is hypothesized that HIF1 works through Sp1 to upregulate *Ngb* expression. Such a HIF1-SP1-Ngb axis is proposed, knowing Sp1 to participate in sequential gene activation downstream of HIF1 upon cerebral ischemia ([77] Fig. 2).

Increases in Ngb levels originating from other cytological events could be promoted by HIF1 activity as well. The thyroid hormone 3,5,3′-triiodo-L-thyronine is involved in neuronal migration and differentiation and regulates transcriptional responses related to mitochondria, neurotrophic factors, and the myelination of axons.

Table 1 In vitro alterations and neuroprotective actions of Ngb

Neurotoxic insult	Model	Ngb mRNA	Ngb protein	Neuroprotection	
AD					
Extracellular Aβ(25–35) 20 μM	Primary cortical cultures of Ngb-overexpression mice	n.d.	n.d.	◦ Cell death about halved after 24 h ◦ Further ↘ when pre-incubated with 4 μM Ngb	[19]
Extracellular Aβ(1–42) 100 μM	PC12 cells, Ngb-transfected (~4-, 8-, and 16-fold ↗ Ngb mRNA)	n.d.	n.d.	◦ ROS/RNS ↘ is Ngb dose-dependent ◦ Lipid peroxidation, caspase-3/7 activity and cell death ↘	[20]
APP Swedish double mutation: K670N/M671L	SHSY5Y cells, Ngb-transfected	n.d.	n.d.	◦ ↘ $A\beta_{42}$: 34.1 to 21.3 pg/ml medium ◦ ↘ Late apoptotis: 4.43 to 2.53%	[21]
NMDA toxicity 300 μM and 2 mM	Primary cortical cultures of Ngb-overexpression mice	n.d.	n.d.	◦ At 300 μM: ↘ cell death ◦ At 2 mM: Ngb overexpression no effect	[19]
Tau pathology	HEK 293/tau cells, EGFP-Ngb-transfected	n.d.	n.d.	Counters tau-P at pS214, pT231, pS396, and pS404	[22]
Anoxia					
8, 10, 12, 16, 24, or 32 h of 95% N_2, 5% CO_2	SH-SY5Y cells	No significant increase	n.d.	n.d.	[23]
48 h of 95% N_2, 5% CO_2	Glioblastoma: M059J, M010b, M059K, M006x, M006xLo, U87T, and U87R	Between 2- and 10-fold ↗	Intensities ↗ up to 5.4-fold	n.d.	[24]
10 h of 90% N_2, 5% CO_2, 5% H_2	Primary cortical cultures of Ngb-overexpression mice (2.6-fold ↗)	n.d.	n.d.	◦ 14 h: ↘lactate dehydrogenase, not at 6 h ◦ Amelioration in oxi-HE formation, ATP level decline, and loss of mitochondrial ΔΨ	[25]
99.99% positive pressure flow-through of N_2	Primary neurons of *Trachemys scripta elegans*, siNgb-transfected	n.d.	72 ± 6% ↘	◦ 2.5-fold ↗ H_2O_2 release ◦ Knockdown no effect on cell death ◦ ↗ Levels of activated ERK (p-ERK)	[26]
Axon disruption					
Axonal damage seeing culturing	Primary retinal ganglion cells of male Long Evans rats, Ngb-siRNA-transfected	n.d.	n.d.	Cell survival at day seven decreased to 16.0% (control 46.6%)	[27]
Hypoxia-reoxygenation injury					
◦ 1 h Argon-degassing ◦ 2, 5, or 24 h recovery	ND15 cells	◦ 2 h: marginal↗ ◦ 5 h: 5–6-fold ↗ ◦ 24 h: baseline	n.d.	n.d.	[28]
◦ 1 h Argon-degassing ◦ 2, 5, or 24 h recovery	SH-SY5Y	◦ 2 h: 1.9-fold ↗ ◦ 5 h: 7.0-fold ↗ ◦ 24 h: 5.9-fold↗	n.d.	Pre-incubation with the phenolic antioxidant 3,3′,5,5′-tetra-t-butyl-biphenyl-4,4′-diol reduces *Ngb* expression	[29]
◦ 1 h Argon-degassing ◦ 1, 2, 5, or 24 h recovery	SH-SY5Y, Ngb-transfected	n.d.	~ 95% stably transfected	◦ Unchanged average cell area (Wt:1.6-fold ↘) ◦ Intracellular ATP preserved in transfected cells ◦ Less evident actin condensation	[30]
Infection					
500 ng/ml LPS for 5 h	Primary mouse cortical astrocytes	n.d.	~ 2.3-fold ↗	n.d.	[31]
500 ng/ml LPS for 5 h	Primary mouse cortical astrocytes, siNgb-transfected	n.d.	Reduced with ~ 70%	◦ ~ 2-fold higher IL-6 mRNA levels ◦ ~ 1.5-fold higher CXCL10 mRNA levels	[31]
500 ng/ml LPS for 5 h	Primary mouse cortical astrocytes, 10 pM E_2	n.d.	4.5-fold ↗	Prevents LPS-linked elevation of IL-6 and CXCL10 mRNA levels	[31]
Parkinson's disease					
C-terminally modified αSyn aggregation	Human neuroglioma cells (H4), Ngb-transfected	n.d.	n.d.	◦ ~ 2-fold lower number of aggregates per cell ◦ No impact on lactate dehydrogenase release	[32]
OGD					
◦ 8–32 h 95% N_2, 5% CO_2 ◦ No recovery	SH-SY5Y cells	◦ 24 h: ~ 2.5× ↗ ◦ 32 h: 4.2× ↗	n.d.	n.d.	[23]
◦ 16–24 h 95% N_2, 5% CO_2		n.d.	Ngb/actin ↗ from 100 to 25.000%	◦ More cell survival at both 16 and 24 h	[23]

Table 1 (continued)

Neurotoxic insult	Model	Ngb mRNA	Ngb protein	Neuroprotection	
◦ No recovery	SH-SY5Y cells, Ngb-EGFP-transfected			◦ H_2O_2 levels are about halved	
◦ 4 h 90% N_2, 5% CO_2 ◦ 20 h recovery	Primary mouse cortical neurons	n.d.	1.8-fold ↗	n.d.	[33]
◦ 4 h 90% N_2, 5% CO_2 ◦ 4 h recovery	Primary mouse cortical neurons	n.d.	1.8-fold ↗ in mitochondria	n.d.	[34]
◦ 4 h 90% N_2, 5% CO_2 ◦ 20 h recovery	Primary Ngb-overexpressing transgenic cortical neurons (~ 2.7-fold ↗ Ngb protein level)	n.d.	2.5-fold ↗	◦ ~ 50% less neuronal death ◦ Counteracts downregulation of *Camk2g*, *Hif-1a*, *Il6st*, and *Khsrp*	[33]
◦ 4 h 90% N_2, 5% CO_2 ◦ 4 or 20 h recovery	Primary mouse cortical neurons, AAV-Ngb-transduced	n.d.	4.6-fold ↗	◦ 16.4% less neuronal death ◦ 0.26-fold less NAD^+ level decline	[35]
◦ 4 h 90% N_2, 5% CO_2 ◦ 20 h recovery	Neuronal mitochondria of mice, rNgb (90 μg/ml) co-incubated	n.d.	n.d.	◦ 0.82-fold less NAD^+ release ◦ 0.47-fold less Cyt c release	[35]
◦ 4 h 90% N_2, 5% CO_2 ◦ 4 or 20 h recovery	Primary mouse cortical neurons, Ngb-shRNA-transfected	Transfection efficiency ~ 40%	n.d.	◦ 23.5% more neuronal death ◦ 1.61-fold more Cyt c release	[35]
◦ 36 h 95% N_2, 5% CO_2 ◦ 12 h recovery	SHSY5Y cells, Ngb-CPP-transduced (1 μM or 600 nM)	n.d.	n.d.	No significant improvement of viability	[36]
Oxidative stress 24 h 300 μM H_2O_2	SH-SY5Y cells, Ngb-EGFP-transfected	n.d.	Optimal 12 h after transfection	23.6% cell survival (only 11% in control cells)	[37]

AAV adeno-associated virus, *APP* amyloid precursor protein, *AD* Alzheimer's disease, *CPP* cell-penetrating peptide, E_2 17-oestradiol, *EGFP* enhanced green fluorescent protein, *LPS* lipopolysaccharide, *Ngb* neuroglobin, *NMDA* N-methyl-D-aspartate, *RNS* reactive nitrogen species, *ROS* reactive oxygen species, *OGD* oxygen-glucose deprivation, *Wt* wild-type

In addition to its classical activation of thyroid hormone response elements, a nonclassical mechanism was detected in which PI3K is activated by liganded thyroid receptor-β [78]. PI3K, in turn, upsurges transcription of *HIF1* [78]. Hence, when intravenously administered to thyrodectomized rats, 3,5,3′-triiodo-L-thyronine was found to upsurge both Ngb mRNA and protein levels to control levels in cortices, hippocampi, and cerebella 24 h after administration [79]. Higher mRNA levels were also detected at earlier time points, which did not correlate with still unaffected protein levels [79]. Such differences could be indicative for posttranscriptional processing of Ngb or for Ngb catabolism playing an important role in the regulation of its protein levels. By way of example, protein levels of HIF1 itself are mainly regulated by oxygen-sensitive prolyl hydroxylases, targeting HIF1 for ubiquitination and proteosomal degradation under normoxic conditions [80].

In addition, Ngb is reported to be upregulated by and protective against Alzheimer's disease (AD) (Tables 1 and 2). AD brains exhibit a constitutive upregulation of Akt activation (p-Akt/total Akt ratio) [81], which could lead to *Ngb* expression through the TSC1/2-Rheb-mTOR-HIF1 signaling pathway. This pathway could also contribute in Ngb levels being transiently affected during AD progression, i.e. being only elevated in up to moderately affected AD brains [39, 82]. Where prolyl hydroxylases are keys to fine-tune protein levels of HIF1, microRNAs form an alternative mechanism for its posttranscriptional regulation. In particular, microRNA-155 expression is induced upon prolonged hypoxia, silencing HIF1 translation [83]. Interestingly, we recently showed *Ngb* transcription to be downregulated in cortices and hippocampi of young APP23 mice subjected to whole body hypoxia [84], providing evidence for a key role in the downregulation of *Ngb* transcription to be played by AD angiopathogenesis.

Mitochondrial functioning and autophagy in relation to cellular senescence and AD are other downstream processes of mTOR signaling [85]. Not only do Ngb protein levels undergo an age-related decline under physiological aging [86], mitochondrial functioning and autophagy are cellular mechanisms to which Ngb has been linked (Fig. 2). Moreover, Ngb binds gamma-aminobutyric acid, which plays a key role in autophagosome formation [87]. Ngb silencing also downregulates expression of *Entpd4*. The associated Entpd4 protein participates in nucleotide turnover in the lysosomal-autophagolysosomal membrane [88]. Although indirect evidence suggests a link between the mTOR pathway and Ngb, such a pathway of events remains to be studied. Regardless of the detection of a direct link between Ngb and mTOR, reductions in Ngb, with its cytoprotective properties, could contribute to the

Table 2 In vivo alterations and neuroprotective actions of Ngb

Neurotoxic insult	Model	Ngb mRNA	Ngb protein	Neuroprotection	
AD					
28 days ICV $A\beta_{(1–40)}$ solution (4.6 nmol/rat)	◦ Male Wistar rats ◦ Cortical coronal sections	n.d.	Ngb^{+} area 0.32% (1.85% in control)	n.d.	[38]
APP Swedish, Indiana mutation: K670N, M671L, V717F	◦ 12-month-old $Ngb^{++}/APP_{Sw,Ind}$ double transgenics ◦ Brain sections	n.d.	n.d.	◦ 12 m: 1.8 ng/g $A\beta_{(1–40)}$ (32 ng/g in Wt) ◦ Normal somal microdomain distribution ◦ Restored performance in Y-maze	[19]
APP Swedish double mutation: K670N/M671L	◦ 13-month-old Tg2576 mice ◦ Brain sections and lysates	n.d.	~3-fold ↘ as age-matched controls	Negative correlation between Ngb and tau-P at pT231 and pS396 sites in neurons	[22]
APP Swedish + presenilin 1 (PS1-dE9) mutation	◦ 13-month-old APP/PS1 mice, 1 mg/ml Ngb-pCDNA3.1 ICV ◦ Brain lysates	n.d.	n.d.	◦ ↘ $A\beta_{42}$: 105.9 to 79.5 pg/mg protein ◦ Better Morris water maze performance	[21]
Pathologically confirmed AD	◦ Mixed gender patients ◦ Superior temporal lobe	23% higher in AD cases as in controls	n.d.	n.d.	[39]
Neuropathologies: AD, AGD and PD	◦ Mixed gender patients ◦ HC, subiculum and dorsal nucleus of the vagus	n.d.	≈ in neurons with and without tau or synuclein	n.d.	[40]
Combustion smoke inhalation					
◦ Wood shavings ◦ 1 h: 5 min smoke, 10 s venting	◦ Male Ngb^{++}-Tg mice ◦ Cerebrum	Overexpression in cerebrum-cerebellum	Broadly distributed in mid-brain region	% inhibition of complex I ◦ 0 h: 92.5 ± 7.2 (78.2 ± 4.4 in Wt) ◦ 2 h: 76.7 ± 7.4 (55.2 ± 6.3 in Wt)	[41]
◦ Wood shavings ◦ 1 h: 10 min smoke, 10–20 s venting	◦ Male Ngb^{++}-Tg mice ◦ Cortical mitochondria	n.d.	Detected on blots, not in those of Wt controls	◦ 1 h: 25% less OCR reduction as Wt ◦ 24 h: lactate to baseline (↗60% in Wt) ◦ 24 h: 120% ↗c-Fos (250% in Wt)	[42]
◦ Cigarette smoke ◦ 15 min/d for 5 d/w	◦ Male Wistar rats (8 w) ◦ Cortex and HC	◦ Cortex 1.1–1.4× ↗ ◦ HC 1.3–1.5× ↗	◦ Cortex 1.2–1.4× ↗ ◦ HC 1–1.4× ↗	n.d.	[43]
◦ 25 ppm CO in 22% O_2 ◦ 12 h/d up to age of 20 d	◦ Sprague-Dawley rats ◦ Cerebellum	No significant change	No significant change	n.d.	[44]
Eye pathology					
Predisposition to eye abnormalities, progressive glaucoma	◦ DBA/2J mice (2 and 8 m), AAV2/2-Ngb delivery ◦ Retina	3.4-/3.6-fold ↗ at 2/8 months as compared to untreated	Untreated: ↘ by age Treated: RGC 79% Ngb^{+} (2-fold ↗)	◦ Decreased glial cell activation ◦ Protection against RGC loss ◦ Retinal morphology better preserved ◦ Neuronal activity in the visual cortex ↗	[45]
Optic nerve injury	◦ C57BL/6 mice (8/9 w), 5 μM human/zebrafish chimeric Ngb ◦ Retina	n.d.	Day 5: untreated (50% ↘), treated (50% ↗)	◦ 3× more of surviving RGC (day 14) ◦ More than double # of regenerating axons as in control group	[46]
Huntington's disease					
Huntingtin gene, carries approximately 120 ±5 CAG-repeat expansions	◦ Male and female R6/2 mice (7 and 13 w) ◦ Striatum	n.d.	◦ ♂: ~2-fold ↗ as compared to Wt ◦ ♀: higher in Wt	Ngb protein mostly detected in neurons with large and medium perikarya	[47]
Huntingtin gene, carries approximately 125 CAG-repeat expansions	◦ Male and female R6/2 mice (4–13 w) ◦ Hippocampus	n.d.	↘ by ~half in both female and male R6/2 mice	Mutant Huntingtin impairs 17β-estradiol- and BDNF-induced *Ngb* expression and mitochondrial localization	[48]
Hypoxia					
1.5 h 7% O_2, 93% N_2	◦ Female $Ngb^{-/-}$ mice ◦ Brain lysates	n.d.	n.d.	◦ ↗ Hif1A, Cspp1, Adi1, and Prpf4b mRNA ◦ ↘ Ubc, Rplp0, and Kidins220 mRNA	[49]
2 h 7.6% O_2, 92.4% N_2	◦ Female BALB/c mice ◦ Cerebrum, cerebellum	◦ Cerebrum: 1.1× ↗ ◦ Cerebellum: 0.7× ↘	No upregulation across brain	Expression only detected in neurons, e.g., Purkinje cells	[50]
5 h, 6% O_2	◦ Sprague-Dawley rats ◦ Whole brains	↘to half the normoxic value	Slight downregulation	n.d.	[51]
48 h 7% or 12% O_2	◦ Swiss CD1 mice ◦ Total brains	◦ 7 h: 1.95 ± 1.11 ◦ 12 h: 3.23 ± 1.16	n.d.	n.d.	[52]
≤ 48 h 7% O_2, 93% N_2	◦ Swiss CD1 mice	◦ 2,4,6, 12 h: =	n.d.		[11]

Table 2 (continued)

Neurotoxic insult	Model	Ngb mRNA	Ngb protein	Neuroprotection	
	◦ Whole brains	◦ 12, 48 h: ↗		Negative correlation between Ngb and H_2O_2 concentration	
6 h 8% or 7 days 10% O_2	◦ Neonatal C57BL/6 mice ◦ Whole brains	7 days 10%: 2.01-fold ↗	P0: Ngb^+ ↗ in cortex P7: unchanged	n.d.	[53]
14 days 10% O_2, 90% N_2	◦ Male C57BL/6 mice ◦ Whole brains	Unchanged	Unchanged	n.d.	[54]
≤ 14 days 10% O2	◦ Male Sprague–Dawley rats ◦ Cortex	Sustained ↗ of ~ 2-fold the baseline	Sustained ↗ of ~ 1.8-fold the baseline	n.d.	[55]
≤ 14 days alternating 10%–21% O_2 every 90 s	◦ Male Sprague–Dawley rats ◦ Cortex	Only slight ↗ at day 1	Only slight ↗ at days 1 and 3	n.d.	[55]
Deep subcortical WML	◦ Mixed gender cohort ◦ Hypoxic milieu within WML	n.d.	# Ngb^+ cells ↗ by ~ 10%	*Ngb* expression in activated microglia	[56]
Ischemia					
◦ 10 min MCAO ◦ 3 days reperfusion	◦ C57/B6, Ngb transgene under ubiquitin C promoter (10 w) ◦ Hippocampus	Transgene: 4-fold ↗	Transgene: 2.5-fold ↗	◦ Unaltered SOD, GPX, catalase activity ◦ Ischemia injury ↘: i.e. lipid peroxidation, nitrotyrosine and ROS levels, cell death	[57]
◦ 30 min MCAO ◦ 24 h reperfusion	◦ BDF × CD1 mice: beta-actin promoter for Ngb overexpression ◦ Whole brains	n.d.	Increased intensity on western blot	◦ Overexpression: 30% ↘ infarct volume ◦ Cerebral blood flow is ≈ in Wt and transgenic mice	[58]
◦ 1.5 h MCAO ◦ 4–24 h reperfusion	◦ Rats ◦ Cortex, penumbra, and core	n.d.	↗ in cytoplasm of normal-appearing penumbric neurons	n.d.	[59]
◦ 1.5 h MCAO ◦ 24 h reperfusion	◦ Male Sprague–Dawley rats, 0.72 nM Ngb oligodeoxynucleotide ◦ Cortex	n.d.	Lower intensity on western blot	◦ Knockdown: 56–60%↗ infarct volume ◦ Worsened neurological severity score	[60]
◦ 1.5 h MCAO ◦ 24 h reperfusion	◦ Male Sprague–Dawley rats, intracerebral AAV-Ngb	n.d.	↗ IHC intensity in cortical neurons	◦ 49–52% ↘ in the size of cerebral infarcts, primary in the cortex ◦ Improved neurological function	[60]
◦ 2 h MCAO ◦ 24 h reperfusion	◦ Male C57BL/6 J mice, 10 mg/kg i.v. TAT–Ngb ◦ Brain hemispheres	n.d.	n.d.	◦ Decreased infarct volume and cell death ◦ Improved neurological deficit scores 72 h after 30-min MCAO	[61]
◦ 1,5 h MCAO ◦ 24 h–1 w reperfusion	◦ Spontaneously hypertensive rats ◦ Brain hemispheres	24 h: lowered by ~ 40%	1 w: no difference with sham animals	◦ No difference in Ngb/NeuN ratio ◦ Inverse correlation: infarct volume and # Ngb^+ neurons	[62]
◦ 45 min MCAO ◦ Up to 14 days reperfusion	◦ Male hypertensive rats, CAV-2-Ngb transduced ◦ Cortex and striatum	n.d.	19 days: 25% ↗(9.9% in Wt)	◦ No effect on SBP or survival ◦ ↘ infarct size in motor cortex and striatum ◦ 14 d: less footfalls in tapered beam walk	[63]
◦ 12.5 min two-vessel occlusion	◦ Male Wistar rats ◦ HC, cortex; dentate gyrus	No significant increases	n.d.	n.d.	[64]
◦ 1–2 h deep hypothermic circulatory arrest ◦ 6 h reperfusion	◦ Neonatal piglet model	◦ 1 h: unchanged ◦ 2 h:↘ by ~ half	n.d.	Neuronal cell necrosis correlates with neuroglobin *Ngb* expression ↘	[65]
Normal aging					
Neurologically normal subjects	◦ Mixed gender cohort, 62 ± 12.8 years ◦ Superior temporal lobe	◦180× ↘ as *ACTB* ◦ ↘1% per year	n.d.	Higher expression in rs8014408 carriers, but faster decrease upon aging	[66]
TBI					
Cryo-lesion in fronto-parietal cortex	◦ Mixed gender, C57BL/6j;129/Sv ◦ Sagittal and coronal sections	n.d.	Astroglia: TBI edge (3 days) and within (10 days)	n.d.	[67]
Subarachnoid autologous arterial blood injection	◦ Male Sprague–Dawley rats ◦ Temporal cortex	Peaks at 6 h post-TBI: 3-fold ↗	↗ up to 24 h after TBI and ↘ from 48 to 72 h after TBI	Ngb positivity co-localizes for 98.9% with NeuN, otherwise Iba1 but not GFAP	[68]
450-g weight drop from 2-m height	◦ Male Wistar rats ◦ Whole brain lysates	6/12/24/48 h: + 929/490/171/133%	6/12/24/48 h: + 447/259/195/135 pg/mg	n.d.	[69]
Needlestick injury		n.d.	◦ 1 day: +++ staining		[70]

Table 2 (continued)

Neurotoxic insult	Model	Ngb mRNA	Ngb protein	Neuroprotection	
	◦ Mixed gender Sprague-Dawley rats ◦ Neocortex		◦ 3 days: ++ staining ◦ 5–30 days: + staining	Decrease in Ngb level after injury is statistically significant	
◦ 3-mm injury tip ◦ 6 m/s, 0.6 mm depth	◦ Ngb^{++}-Tg mice	n.d.	6 h: 196% of Wt shams	◦ ↘ 3NT levels and brain lesion volume	[71]
◦ 3-mm injury tip ◦ 1.5 m/s, 1 mm depth	◦ Cerebral cortex ◦ Male C57/BL6 mice	◦ 1/3/14 days: =	7 days: glia/neurons of HC/cortex	◦ Unvaried sensorimotor/memory recovery Ngb reactivity is primarily	[72]
◦ 3-mm injury tip ◦ 1.5 m/s, 1 mm depth	◦ Cortex and coronal sections ◦ Male B6.Cg-Tg (CAG-Ngb,-EGFP)1Dgrn/J mice ◦ Cerebral cortex	◦ 7 days: ~2.2-fold ↗ n.d.	7 days: glia/neurons throughout the brain	found near the injury site Improved sensorimotor recovery ◦ 2 days: ~1.9 ft faults/min (~2.9 in Wt) ◦ 7 days: ~0.5 ft faults/min (~1.4 in Wt)	[72]
Severe TBI: hematoma-and hemorrhage-related	Caucasian patients	n.d.	n.d.	↘ outcome in rs3783988 alleles (Wt: TT): ◦ 3 months: TT (30.3% good), CC/CT (14.0%) ◦ 1 year: TT (51.5% good), CC/CT (29.4%)	[73]
Intracerebral hemorrhage	Patients with cerebral arteriovenous malformations	n.d.	↗ in parenchyma adjacent to lesions	Co-localization of Ngb with NeuN, but not GFAP	[74]

AD Alzheimer's disease, *AGD* argyrophilic grain disease, *APP* amyloid precursor protein, *BDNF* brain-derived neurotrophic factor, *HC* hippocampus, *ICV* intracerebroventricular, *MCAO* middle cerebral artery occlusion, *Ngb* neuroglobin, *OCR* oxygen consumption rate, *PD* Parkinson's disease, *RGC* retinal ganglion cells, *ROS* reactive oxygen species, *SBP* systolic blood pressure, *TAT* trans-activator of transcription, *TBI* traumatic brain injury, *WML* white matter lesions, *Wt* wild-type

age-related susceptibility to ailments of the neuronal system.

Neuronal Survival Linked Elements—REST, CREB

The *Ngb* promotor contains two RE1-silencing transcription factor (REST) sites −359 and − 127 bp relative to the transcription start site [76]. REST activation is known to work neuroprotective, repressing proapoptotic genes [89]. Expression of *REST* is upregulated along physiological aging. This is, at least partly, attributed to canonical Wnt-β-catenin signaling, a pathway known for controlling cell proliferation and fate throughout development and adult life [89]. In cases of aggregation pathology, e.g. AD and frontotemporal lobar degeneration, REST appears trapped in cytoplasmic inclusions. Subsequent depletion of its nuclear levels entails the loss of downstream oxidative stress resistance and apoptosis counteraction ([89] Fig. 2). The latter may also contribute in the *Ngb* transcription upregulation, seen in early AD stage brains, to be lost when the AD pathology reaches end-stages [39, 82]. *REST* transcription is indeed detected to correlate with the one of *Ngb* in cortices of mouse models of β-amyloid pathology and hypoxia [84]. REST-mediated *Ngb* expression regulation could be extended to other etiologies in which cellular damage reaches a certain threshold as well, e.g. *Ngb* expression is upregulated after subacute and chronic traumatic brain injuries but not after an acute injury [90]. Of note, the 5′-flanking region of the *Ngb* gene comprises two CpG methylation islands from − 874 to − 600 and from − 157 to + 885 relative to the transcription start site [76]. As REST activation also influences histone acetylation modifications [89], it remains to be elucidated to which extend (de)acetylation and (de)methylation patterns influence *Ngb* expression upon different cellular conditions.

Incubation with 10 nM 17β-estradiol (1 h) enriches the H3K4me3 epigenetic marker at the *Ngb* promoter site in SK-N-BE and NT2 differentiated cells [91]. The expression-enhancing, noncoding RNA transcript (chr14:77,735,963-77,736,462; hg19) in *Ngb*'s first exon gets marked by H3K4me1 and H3K27Ac as well [91]. Hence, *Ngb* could be one of the neuronal genes that are influenced by steroid hormones, i.e. 17β-estradiol, which are known for their mitogenic, anti-apoptotic, and anti-inflammatory effects. A 24-h incubation with 1 nM 17β-estradiol was detected to raise Ngb protein levels by 300% in SK-N-BE neuroblastoma cells and mouse hippocampal neurons [92]. Another study detected a 2- and 2.5-fold increase in SK-N-BE and NT2 cells at a higher concentration of 10 nM [91]. However, no (or almost no) Ngb reads could be identified in a recent RNA-seq study on the SK-N-BE cell line [6], putting the previous findings to the question. Other papers already pinpointed the need to consider the different detection methods and tissue preparations which are being used for Ngb quantification, as well as the

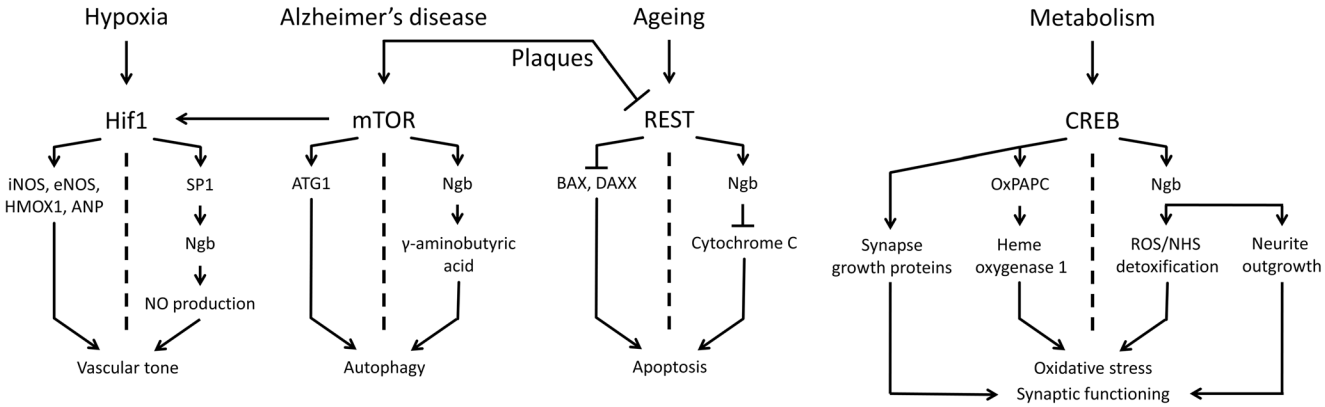

Fig. 2 *Ngb* expression regulation and downstream neuroprotective actions. Ngb is upregulated under different conditions of cellular stress, containing binding sequences for regulatory elements as AP1, CREB, Ergr1, NF1, NF-κB, REST, and SP1/3. Once expressed, downstream functions of Ngb can be paralleled to known effects of the respective pathways

necessity to validate antibodies [93]. Ngb is indeed characterized by a low antigenicity and, as a result, the occurrence of antibody cross reactivity has been described before [94]. Discrepancies between Ngb transcript and protein levels have been detected as well [68, 79]. Given the above, it could be of interest to investigate the endogenous epigenetic status of *Ngb* in the different experimental setups, in addition to displaying extra vigilance on the used Ngb detection system.

The cAMP response element binding (CREB) protein is another transcription factor with a binding site in *Ngb*'s regulatory region [95]. CREB-mediated transcription is linked to ROS detoxification, vertebrate synaptic plasticity, and neuronal survival (reviewed in [96]). Of note, once transcribed, Ngb can induce a phosphorylation cascade, starting from Akt, which leads to CREB activation [38]. Hence, an elevated Ngb protein level could be sustained through such a positive feedback loop. The latter still has to be demonstrated experimentally.

Pharmacological Induction of Ngb—Hemin, Short-Chain Fatty Acids, Deferoxamine, Tibolone, Ibuprofen, (R)-α-Lipoic, Natural Plant Compounds

In light of the neuroprotective effects of elevated Ngb levels, different pathologies and injuries of the nervous system might benefit from therapeutically increasing intracellular Ngb levels. The most self-evident way to address Ngb levels would be a recombinant protein replenishment therapy, which is incompatible with Ngb being membrane impermeable. Hence, studies have been elucidating the potential of pharmacologically increasing Ngb levels, in which the use of pharmaceuticals with a blood-brain barrier (BBB)-crossing capacity would be most preferable.

Administration of hemin, an iron-containing porphyrin, forms part of the clinical management of porphyria attacks, which are characterized by gastrointestinal and neuropathic symptoms, and etiologically grounded in a dysfunctional heme biosynthesis (OMIM #176000). When added to the culture medium of hippocampal HN33 cells, 50 μM hemin increases both Ngb transcript and protein levels up to 4-fold. The expression is regulated by sGC-PKG signaling [97], an upstream effector of vesicle cycling, actin remodeling, and neurite outgrowth.

HN33 cells were detected to have increased their Ngb protein level 5 to 14-fold above control levels when grown in valproic acid and cinnamic acid containing medium, respectively. Though the mechanism of action is still unclear, the *Ngb* expression inducing effect of both short-chain fatty acids is unlikely caused by histone deacetylase inhibition [98]. Of note, valproic acid is the active compound in a variety of anticonvulsant drugs, primarily used in the treatment of generalized and partial epilepsy. Cinnamic acids (derivates), on the contrary, are being investigated as histone deacetylase inhibitors for the treatment of solid tumors and hematological malignancies (e.g., registered at www.clinicaltrials.gov as NCT01496118, NCT01451268). Hence, these therapeutic indications are less in accordance with those in which Ngb has been detected to have a beneficial effect (Tables 1 and 2).

Ngb transcription in primary mouse cortical neurons is elevated by 100 μM deferoxamine [59]. This chelating BBB-crossing agent has therapeutic indications that include chronic iron overload (genetic or acquired hemochromatosis, OMIM #235200), acute iron intoxication, and chronic aluminum overload in end-stage renal failure. The chemical works through the activation of hypoxia-inducible genes, such as HIF1 [59]. Another molecule is Tibolone. This synthetic BBB-crossing steroid works through complex formation with SP1 downstream of HIF1 [99]. It has a clinical efficacy in the prevention of osteoporosis and the relief of the symptoms related to estrogen deficiency. Interestingly, it has important neuroprotective effects on the central nervous system as well [100]. This raises the question as to whether Ngb upregulation could be in part attributing to these effects. When administered at 10 nM to primary cortical cell cultures of mice, *Ngb*

expression levels increase ~1.8-fold [101]. The latter is increased to ~2.5-fold when combined with oxygen-glucose deprivation (OGD), an increase that is also observed on the protein level. Of interest, Ngb silencing prevents tibolone's support of cell survival under OGD, with cells losing their mitochondrial membrane potential [101].

CREB-linked *Ngb* transcription can be targeted by natural compounds as formononetin, providing protection against OGD-induced neurotoxicity in primary neuronal cultures [102]. In addition, given the cell survival characteristics of CREB and Ngb, an ibuprofen and (R)-α-lipoic acid conjugate was developed as to combine the known AD-ameliorating effects of both components and as to study the molecular events attributed to Ngb activation. Subcutaneous administration of the conjugate to AD rats (250 μL/kg) results in their brains maintaining Ngb levels to healthy control levels, even at vast amounts of intracerebroventricular Aβ(1–40). Moreover, the sustained Ngb levels correlate with less apoptosome formation and cell death [38].

Another fusion molecule, Tat-Ngb, was created, making recombinant Ngb cell- and possibly BBB-permeable. The protein, produced by *Pichia pastoris*, was found to increase Ngb levels in pheochromocytoma PC12 cells and, as such, to avert hypoxia-linked cellular damage [103]. Nonetheless, Ngb might have some cytoprotective effects in the periphery as well. Azarov et al. engineered human Ngb-H64Q-CCC, which is 1200 faster in displacing CO from carboxyhemoglobin in comparison to when only atmospheric oxygen is present [104]. Intravenous infusion of CO-poisoned mice with the Ngb ligand trap antidote increases their survival rate to 87.5% as compared to less than 10% in control mice. With CO-bound Ngb-H64Q-CCC to be detected in the mice's urine, this approach also appears promising with regard to safety of therapy [104].

Given the different transcription factors with a known link to neuroprotection regulating the expression of *Ngb*, the notion is underscored of Ngb being involved in the preservation of nervous system homeostasis.

Ngb Modes of Action

Constraint and Use of Oxidative Molecules

An essential element in the understanding of Ngb's functions and mechanisms of action lies in its unique structure. The eight alpha helices (3′-A-B-C-D-E-F-G-H-5′) of Ngb's secondary protein structure are stacked in the classical three-over-three alpha-helical fold to generate a three-dimensional hydrophobic pocket for the central heme iron atom [12, 105]. A structural deviation from other globins, such as hemoglobin and myoglobin, lies in Ngb's wide protein core cavity and its structural flexibility encompassing the C and E helix regions [105]. The latter enables the sixth position of the Ngb heme iron to reversibly coordinate with either an external ligand (hexacoordinated), the HisE7 residue (hexacoordinated) or being unbound (pentacoordinated) [10, 12]. Hence, an external ligand as O_2 or nitric oxide (NO) can only bind the heme atom after disruption of the iron-HisE7 bond, a process of which the kinetics are strongly influenced by the redox state of the internal Ngb CysCD7-CysD5 disulfide bond [106–109]. By way of example, the k_{on} of CO and O_2 shifts from 40 and 140 $\mu m^{-1}\ s^{-1}$ to 50 and 170 $\mu m^{-1}\ s^{-1}$ after disulfide bridge disruption, respectively [106].

Both cysteines lie at the internal cavity in the CD loop region, linking the C and D helices of the globin fold [9, 110]. The importance of the intramolecular disulfide bond is represented by the position of both cysteine residues being remarkably conserved [110]. Such conservation is grounded in the cysteine bridge being able to translate the redox state of the cell into differential ligand affinities (binding and release) by controlling the heme iron coordination (hexa <-> penta) (Fig. 3). Electron transfers induce conformational changes of the imidazole planes of the heme pocket, completing the transitioning between the reduced (Ngb_{SH}) and oxidized (Ngb_{SS}) states [106, 111]. Hence, it may be of no surprise that Ngb is also detected in cellular compartments which set the redox environment of the cell. Where generally the vast majority of the cellular Ngb content is detected within the cytosol, different studies also describe a Ngb mitochondrial pool to be present under different cellular conditions [27, 34, 112, 113]. The mitochondrial Ngb fraction ranges from near 8% in primary mouse cortical neuron cultures [34] to 70% in retinal neurons of the rat [27], even though Ngb lacks a mitochondrial signaling sequence.

Ngb exerts both a nitrite reductase function and a NO dioxygenase activity (Fig. 3). Hence, Ngb plays an important role in adapting NO levels to the cellular state. NO has beneficial effects under oxygen depletion. It evokes hypoxic vasodilation and reversibly inhibits cytochrome oxidase, limiting mitochondrial respiration and ROS production [114]. Interestingly, in case of oxidative stress, the cellular environment will favor the oxidized state of Ngb. Ngb_{SS} generates NO at a rate (k of $Ngb_{SS} = 0.12 \pm 0.02\ m^{-1}\ s^{-1}$) that is close to 2-fold higher as to when the disulfide bond is reduced (k of $Ngb_{SH} = 0.062 \pm 0.005\ m^{-1}\ s^{-1}$) [115]. The production of NO is further promoted by ERK1/2 and PKA activation upon hypoxic and ischemic insults. Both kinases have docking sites on Ngb (amino acids 17/19/50/51) and subsequent phosphorylation increases Ngb's nitrite reductase activity up to 4-fold the nonphosphorylated level [116]. Phosphorylation also facilitates the binding of 14-3-3 scaffold proteins, which counteract dephosphorylation by phosphatase activity [116].

However, excess of NO needs to be detoxified. NO interacts with superoxide to form peroxynitrite, which generates strong nitrating agents together with CO_2. Being located in the

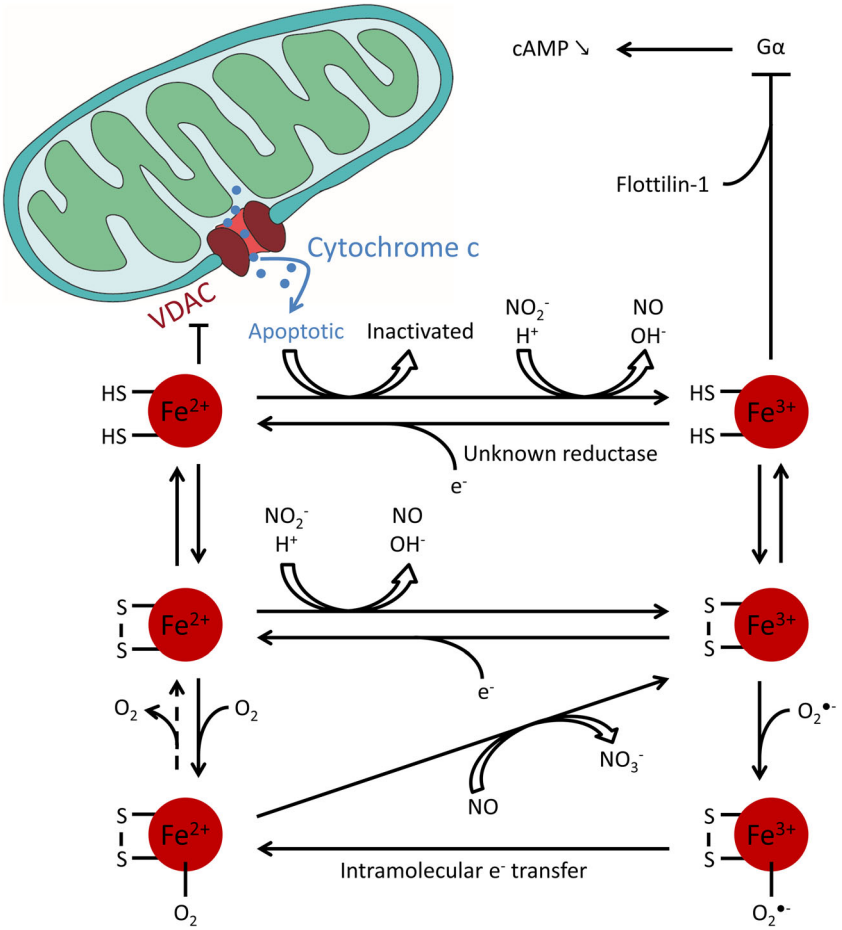

Fig. 3 Impact of the cellular redox state on (neuro)protective functions of Ngb. The CysCD7-CysD5 disulfide bond is indicated as present (S-S) or absent (depicted with reduced sulfhydryl –SH groups). Hexacoordinated Ngb interacts with mitochondrial voltage-dependent anion channels (VDAC), counteracting the release of apoptotic cytochrome c. Ferric (Fe^{3+}) Ngb, e.g. formed after the reduction of ferric cytochrome c, is recruited to lipid rafts by binding to flottillin-1. The interaction opposes the activation of α-subunits of heterotrimeric G proteins (Gα) by acting as a guanine nucleotide dissociation inhibitor. Preventing cellular cAMP levels to drop adds to neuroprotection. The oxidation state of Ngb further contributes in the scavenging and detoxification of reactive oxygen and nitrogen species

vicinity of mitochondria and within, Ngb appears to be an ideal candidate scavenger. Ferric (Fe^{3+}) Ngb can bind NO following bi-exponential kinetics ($6.6 \pm 0.5 \times 10^{-3}$ and $2.0 \pm 0.1 \times 10^{-3}$ s^{-1}). This generates a thermodynamically stable ferrous (Fe^{2+}) Ngb-NO species by reductive nitrosylation [14]. Once bound, NO initially shields the ferrous iron from (auto)oxidation [108]. For Ngb's NO dioxygenase activity, the globin contains multiple docking sites in which gaseous ligands can bind for up to several hundred microseconds before sequential reactions take place with the central heme atom [7]. The reaction could exist in a synergism with the scavenger activity of ferric Ngb against superoxide, which was detected to have a IC_{50} value of 7.4 ± 0.7 μM ([16] Fig. 3).

Cell Life and Death Decisions

Mitochondria are not only key players in cellular ATP production, providing in the high energy demand of neurons, they also initiate or contribute in the apoptosis process when cellular damage accumulates. The notion of Ngb playing an important role in the counteraction of cell death is consistent with Ngb's significant redistribution to the mitochondria upon OGD [34, 117]. Although the ratio of mitochondrial Ngb to the total intracellular Ngb level only slightly increases from 7.8% in healthy neurons to 9.9% upon OGD, this increase represents a significant 2.1-fold increase of mitochondrial Ngb [34]. Of interest, Ngb-interacting partners have been studied before, some of which are mitochondrial proteins involved in apoptosis (Table 3). One of these proteins is the voltage-dependent anion channel (VDAC) ([87] Fig. 3). VDAC is an important regulator of ATP- and Ca^{2+} transport, which spans the mitochondrial outer membrane. In cases of oxidative stress, superoxide initiates the fast release of cytochrome c through VDAC-dependent permeabilization [124]. Hence, Ngb is proposed to oppose neuronal apoptosis by its binding to VDAC, blocking the discharge of cytochrome c into the cytosol. Furthermore, VDAC blockers (DIDS and dextran sulfate) inhibit OGD-induced Ngb translocation to the mitochondria. Inhibition of the mitochondria permeability transition pore (mPTP) was detected to have a similar effect on mitochondrial Ngb transport [34]. Knowing mPTP activation to be linked to events of late apoptosis and necrosis, including matrix swelling

Table 3 In literature-described Ngb-protein interactions

Ngb binding partner	Cellular location	Model(s)	Identification	Validation	Ref.
14-3-3 protein	Cytosol	SH-SY5Y cells, brainstem and thalamus of sheep	Co-IP - WB	FRET analysis	[116]
Casein kinase 2, alpha prime polypeptide	Cytosol, nucleus, plasma membrane	HEK293	Co-IP - MS	Cross-laboratory comparison	[118]
Cystatin C	Extracellular, cytosol, ER	Yeast, recombinant proteins	Y2H screen	SPR analysis	[119]
Cytochrome C1	Mitochondrion, nucleus	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
			IHC co-localization	Co-IP - WB	[117]
		Primary mouse cortical neurons			
Cytochrome B5, C	ER, extracellular, mitochondrion, cytosol	Recombinant proteins	Absorbance spectrum	Kinetic traces	[17]
Disheveled homolog 1	Cytosol, nucleus, plasma membrane	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
Electron transferring flavoprotein α subunit	Extracellular, mitochondrion	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
Flotillin-1	Cytosol, plasma membrane	Yeast, rat brain extract	Y2H screen	GST pull-down - WB	[120]
			Co-IP - WB		[121]
		Primary mouse cortical neurons			
$GABA_A$ receptor-associated protein-like 1	Cytosol, ER, Golgi	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
Heterotrimeric G protein α subunit	Plasma membrane, lysosome, cytoskeleton	Recombinant human Ngb	SPR analysis	Functional assays	[122]
Huntingtin	Cytosol, nucleus	SK-N-BE cells	Co-IP - WB	Proximity ligation assay; Duolink	[48]
Microtubule-associated protein 1	Cytosol, cytoskeleton	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
Na/K ATPase β1,2,3	Plasma membrane	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
P21 (RAC1) activated kinase 1	Cytosol, Golgi, membranes	Primary mouse cortical neurons	Co-IP - WB	Functional assay	[121]
Synaptotagmin I	Plasma membrane	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
Thioredoxin reductase	Cytosol, extracellular, nucleus	Recombinant proteins	Absorbance spectrum	MS	[123]
Ubiquitin C	Cytosol, extracellular, nucleus	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]
Voltage-dependent anion channel 1	Mitochondrion, nucleus, plasma membrane, cytosol	Yeast, primary mouse cortical neurons	Y2H screen	Co-IP - WB	[87]

ER endoplasmic reticulum, *FRET* fluorescence resonance energy transfer, *GFP* green fluorescent protein, *GST* glutathione S-transferase, *IHC* immunocytochemistry, *IP* immunoprecipitation, *MS* mass spectrometry, *Ngb* neuroglobin, *SPR* surface plasmon resonance, *WB* western blot, *Y2H* yeast two-hybrid

[125], it is questioned whether Ngb might be involved in those processes in some or other way as well. In support of this idea, Ngb overexpressing SH-SY5Y cells are protected against Ca^{2+} influxes and increases of cellular uptake of Fe, Cu, and Zn, associated with hypoxia-reoxygenation injury [30].

Of the cytosolic Ngb fraction, a chief part has been detected in close proximity with mitochondria [126]. During hypoxic events, Ngb protects against the formation of a lipid raft-mitochondrial-actin cytoskeletal lattice death signal by opposing Pak1 kinase activity, reducing Rac1-GDP dissociation, and the inhibition of actin assembly [121]. When localized in such mitochondrial vicinity, Ngb is also thought to prevent leaked ferric cytochrome c to interact with apoptotic protease-activating factor-1 (Apaf-1) and, hence, its oligomerization into an Apaf-1 apoptosome. Such impediment is achieved by ferrous Ngb being able to reduce ferric cytochrome c very fast ($k = 2 \times 10^7\ M^{-1}\ s^{-1}$) [17]. A cytochrome reducing role for Ngb is further supported by the redox potential of cytochrome c, which is more positive as the one of Ngb [127]. Of note, in order to inactivate more than one cytochrome c, ferric Ngb is to be reduced back to its ferrous form. Such a reductase has not yet been identified (Fig. 3).

Ferric Ngb also exerts a pro-survival function, evinced by its recruitment to lipid rafts by binding to flottillin-1 [120, 128]. The interaction is not carried out by ferrous ligand-bound Ngb [120, 128]. At the membrane, it is proposed that ferric Ngb counteracts ROS-induced activation of α-subunits of heterotrimeric G proteins, acting as a guanine nucleotide dissociation inhibitor. The subsequent maintenance of cAMP levels contributes to cell survival [128]. Glu53, Glu60, Arg97, Glu118, and Glu151 residues of Ngb are crucial for this interaction [129]. It is also reported that Ngb interacts with Na/K-ATPase subunits, of which the function reaches further then the establishment of concentration gradients. Na/K-ATPase can form a functional complex with Src family kinases, leading to downstream effects on the Ras/Raf/ERK1/2 cascade and mitochondrial ROS production [130, 131]. However, a direct link between Ngb and this pathway remains to be shown.

Consideration should be given to the fact that the chronic inhibition of apoptosis paves the way for malignancy. To circumvent chronic promotion of tumor formation, only ~ 30% of the neuronal Ngb fraction is present in the anti-apoptotic ferrous state [132]. In case of lowered oxygen concentrations (e.g., as seen in stroke), oxygen quickly dissociates from the previously oxygenated form (dissociation rate constant of $4\ s^{-1}$) [132]. Reduction equivalents ($NADH + H^+$) further accumulate within the cell, making Ngb to potentially function as an oxidator of NADH to increase ATP generation by glycolysis [133]. The latter could contribute to the observation of a readjusted redox cycle towards the active hexacoordinated Ngb_{SS} form of the protein (to ~ 80% of the cellular Ngb content), and this in a seconds time scale ([106, 132] Fig. 3). Moreover, Ngb levels need to outweigh those of Apaf-1, having both proteins binding to the same docking site on the cytochrome c surface [134]. Quantitative modeling even revealed the requirement of a high stoichiometric ratio of Ngb/cytochrome c to fully constrain apoptosis initiation [134]. The latter is in accordance with the significant increases of Ngb expression under neuronal stress (Tables 1 and 2). Hence, Ngb should be regarded as a protein that augments the threshold for apoptosis initiation rather than one that completely averts cell death.

Cancer cells, however, are known to circumvent cell cycle control [135] and to evade immune surveillance [136]. Hence, it is not unthinkable that, if of physiological relevance, malignant cells also dysregulate Ngb's biology in their advantage, i.e., as part of their triad of defense mechanisms against cell death and the hypoxic tumor environment. *Ngb* expression is indeed found upregulated in some squamous cell carcinomas, adenocarcinomas [137], and glioblastomas [24, 138]. The ectopic activation of *Ngb* expression, however, does not appear to be a classic constituent of malignancy. No Ngb upregulations were detected in a wide screening on samples of Burkit's lymphoma, lymphoblastic leukemia, melanoma, glioblastoma, and astrocytoma [139]. Hence, increased *Ngb* transcription could be rather attributed to a specific tumor environment as to tumor types. In support of this idea, Ngb levels were found to be upregulated in MCF-7 breast cancer cells upon addition of ROS inducers as hydrogen peroxide and lead(IV), but to be unaltered in a PO_2 of 2%, representing the medium hypoxic tumor microenvironment [140]. Moreover, a better comprehension is needed on whether and/or how Ngb upregulation is contributing to the impediment of malignant cell eradication. In line with this indistinctness, Ngb levels have been detected to be upregulated in SK-N-LP neuroblastoma cells upon treatment with anti-cancer agents of the di-2-pyridylketone thiosemicarbazone series [141]. These compounds are highly potent copper chelators, inhibiting neuroblastoma growth [141]. In this case, elevated Ngb levels do not impact cell death. On the other hand, increased levels of Ngb have been detected to negatively influence the sensitivity of MCF-7 breast cancer cells to the active chemotherapeutic agent paclitaxel (Taxol) [142]. The latter acts through inhibition of microtubule depolymerization, opposing the dynamic reorganization of the microtubule network during mitosis.

Implication of Ngb on Astrocytic Inflammatory and Pro-Healing Pathways

Consensus has not yet been reached on astroglial expression of *Ngb*. Though Ngb was detected in primary cultures of murine cortical astrocytes and in human T98G astroglial cells [101, 143], positivity was not detected in glioblastoma cell

lines (DKMG, GAMG) [144], in astroglia across the adult subventricular zone of rats [145] or in astrocytes of whole mouse brain sections under control conditions [86]. Given the fact that culturing of astrocytes can endorse cellular stress due to the culture conditions or the disruption of process extensions [27], it was hypothesized that resting astroglia express negligible levels of Ngb. As for neurons, transcription could be increased in regions with or in the proximity of cellular damage. In addition to cell culturing conditions, the latter includes traumatic brain injury [31, 67, 72], metabolic stress [146], cerebral malaria, and autoimmune encephalitis [67]. *Ngb*-expressing astroglia are also linked to tolerance towards chronic environmental hypoxia as found in brains of aquatic mammals and the subterranean mole rat *Spalax* [51, 147]. Moreover, where neuronal transcription of *Ngb* returns to baseline after the insult [69, 70], Ngb immunoreactive astrocytes persist up to 12 months after brain trauma in patients [90]. The latter is in accordance with astrocytes playing an important role in the maintenance of homeostasis in neural tissue, regulating glutamate transport and neuroinflammation, and releasing neuroprotective molecules [148]. One of these restorative and protective molecules is the platelet-derived growth factor subtype BB. The latter was shown to also upregulate *Ngb* expression in T98G astrocytic cells [149]. Even in cases of cellular stress, not every study so far has observed a link between Ngb and astroglia. Astroglial Ngb immunoreactivity was not detected in white matter lesions of a postmortem cohort [56], hypoxic murine brains [50, 68], and in ischemic stroke tissue of patients [150]. Dissimilarities could be the result of experimental differences, including the antibodies used, as mentioned earlier. The induction of astroglial *Ngb* expression could also be constraint to specific tissue regions and/or insults. A recent meta-analysis study of Fabrizius et al. indicated conserved patterns of *Ngb* expression, i.e., high- and low-expressing cells and tissues [6].

Irrespective of whether or not astroglia express *Ngb*, Ngb could indirectly effectuate changes in astroglial functions. When looking at neuron-specific AAV2-mediated overexpression of *Ngb*, mRNA levels of the glial fibrillary acidic protein (GFAP) are reduced by 19% in a murine model of Wallerian degeneration [151]. Knowing increased *GFAP* expression to be a hallmark of reactive astrocytes, it can be presumed that a Ngb-associated anti-inflammatory environment contributes to the observed preservation of axons [151]. Ngb silencing also impairs the reduction of the lipopolysaccharide-induced expression of the universal astroglial marker vimentin [31]. To boost neuronal regeneration and anti-apoptotic mechanisms, injury-induced astroglia characteristically produce transforming growth factor beta 1 (TGF-β1) [152]. Given the link with functions associated with Ngb, the synergism between TGF-β1 and Ngb has been investigated in postmortem cortical tissue of depressed and nondepressed individuals. A significant change was found for neither of the molecules [153]. Hence, the white matter lesions and ischemia associated with depression might be of a too low severity for Ngb's neuroprotection to act.

Finally, an anti-inflammatory effect associated with Ngb increases could not be limited to astroglia. Glial Müller cells increase their Ngb content above the one of resting cells as a response to retinal injury [154]. Moreover, postmortem tissue with white matter ischemia shows Ngb to be colocalized with microglial CD68 [56]. Ngb was also found in Iba1-positive cells in a rat model of subarachnoid hemorrhage [68]. The anti-inflammatory, regenerative effect could be a conjunction between actions of different cells as well. Culture medium of human adipose tissue-derived mesenchymal stem cells (2%) upsurges the transcription of *Ngb* by 251% in human astrocyte T98G cells upon mechanical injury and glucose deprivation [155]. The subsequent protective effect is abrogated when *Ngb* expression is silenced: reducing the mitochondrial membrane potential, lowering mitochondrial mass, and upsurging both superoxide and hydrogen peroxide levels [155]. In addition, transgenic overexpression of *Ngb* in a mouse model of acute myocardial infarction attenuates the expression of inflammatory markers in infarct border tissue, i.e. of *HIF1*, *ICAM1*, and *VCAM1* [156].

Neurogenesis, Improving Brain Integrity

Like most tissues, the nervous system contains stem cells which, through amplification and differentiation, are capable of replacing damaged and/or aged cells [1]. Hence, the observed ameliorations in tissue integrity upon *Ngb* expression induction (Table 2) could be attributed to not only an enhanced cell survival of existing cells but also to a boosted cell proliferation and differentiation as well. Of interest, neuronal stem cells in the subventricular zone of adult rats show a clear Ngb immunoreactivity [145]. Transcription factors SOX-3 and SOX-4, both associated with the preservation of the progenitor neuronal cell pool, are also cotranscribed with Ngb in the neurogenic subventricular zone [157]. Moreover, the expression appears correlated to the differentiation state of the cells. Where the Ngb levels are barely noticeable in embryonic stem cells, the Ngb/actin density ratio increases to 0.10 in neuronal stem cells and is still further elevated by about 40% in neuronally differentiated neuronal stem cells [145]. Mouse Ngb transcript levels further increase steadily from embryonic brains over newborns to a yield which is the highest in young adult brain [6]. The inverse relationship is observed when looking at nonneuronal cells as peripheral blood hematopoietic stem/progenitor cells. Ngb protein levels are 2-fold higher in uncommitted hematopoietic stem/progenitor cells ($CD34^{high/bright}$) as in more mature ones ($CD34^{low}$) [158]. These results are in accordance with a role for Ngb in the maintenance of the stem cell pool and with Ngb playing an even more pronounced role in neuronal

differentiation, but not in the differentiation of other cell types. Accordingly, GATA2 binding to its transcription factor binding site upstream of the human *Ngb* gene was identified to increase *Ngb* transcription [159]. Hence, Ngb might take part in known effects of GATA2 activation, being migration and differentiation of immature neuronal precursors [160, 161]. The gene neighborhood of *Ngb* also contains other conserved vertebrate transcription factor binding sites linked to neuronal cell fate and development (AP2α, Churchill, INSM1, Klf4, REST) [162]. Developmental regulation was already described for two other globins, i.e. hemoglobin [163] and myoglobin [164].

Basal ganglia and parietal lobes of patients with intracerebral hemorrhage exhibit increased levels of proliferation and neuronal stem cell markers involved in neurogenesis [165]. A role may be reserved for Ngb in this tissue repair process. Perihematomal neurons and those adjacent to unruptured arteriovenous malformations show an enhanced immunohistochemical staining of Ngb [74]. In line with a role for Ngb in neurogenesis, a main part of the cellular Ngb fraction is present in axons and dendrites during neuronal development [166]. This localization is associated with neurite outgrowth. Ngb silencing in N2a cells reduces the length of neurites to 0–20 μm in over 70% of the cells [166]. On the other hand, overexpression of mouse *Ngb* significantly enhances neurite outgrowth in RGC-5 cells [167]. An underlying mechanism is found in Ngb levels positively correlating with elevated Akt (Ser473) and downscaled PTEN (Ser380/Thr382/Thr383) phosphorylation levels [166].

Considerations of Cell Lineage and Phylogeny

It is very important to choose the correct model for analysis of Ngb levels and functions. Some variation has been detected in *Ngb* transcription as a response to stress signals, e.g., on whether *Ngb* expression is increased [11, 53, 75], unaltered or decreased [50, 65] upon hypoxia. These results could be attributed to the expression of *Ngb* being strongly influenced by (I) the kind and degree of cellular stress, (II) the maturational stage of the cell or organism, and (III) the type of cell and organism.

Ngb transcription is indeed upregulated under moderate traumatic brain injury [90] and early-stage AD pathology [39, 82] but returns to baseline when cellular damage increases in both models. An important factor in such downregulation could be the loss of REST as a *Ngb* expression regulatory element. REST can get trapped within the cytosol through interaction with pathological misfolded proteins, including Aβ [89], and is downregulated by hypoxia-induced miRNAs [168]. The total degree of cellular dysregulation could function as some threshold for Ngb involvement as well. In support of this idea, *Ngb* expression correlates with the level of cell death (necrosis and apoptosis) in a neonatal piglet model of deep hypothermic circulatory arrest [65]. Cortices with less than 10% necrosis stably express *Ngb*. In cases of high-grade (> 50%) neuronal cell necrosis, *Ngb* transcription is reduced by approximately half [65]. Moreover, while Ngb is ~ 6 times upregulated in P0 whole mouse brains after 6 h of 8% oxygen, the expression stays unaltered after a chronic exposure to 10% oxygen of 7 days [53]. The window of Ngb involvement appears to have a lower limit as well. Adult Swiss CD1 mice show a significantly increased Ngb transcript level after 24 and 48 h of 7% O_2, but not yet after 2 up to 12 h of hypoxia [11]. A similar *Ngb* expression profile is seen in HN33 cells that are exposed to 1% O_2, i.e. only a significant upregulation is detected as from 24 h hypoxia [64]. Additionally, where *Ngb* expression responds to sustained hypoxia (10% O_2), no effect is seen after an insult of intermittent hypoxia of 10% O_2 every 90 s [55]. Such stress type-dependent upregulation of Ngb is not limited to terrestrial animals, with the freshwater turtle *Trachemys scripta elegans* showing increased Ngb protein levels to 416 ± 14% of the control level after 4 h of hypoxia at 5% O_2 [26]. In case of a 4 h anoxic insult, Ngb protein levels are only elevated by 246 ± 10% over normoxic controls [26]. The zebrafish (*Danio rerio*) does not increase its *Ngb* transcription when subjected to mild hypoxia (PO_2 = ~ 8.6 kPa) for 48 h. Under sever hypoxia of 4.1 kPa PO_2, *Ngb* expression is four times elevated after 24 h but is almost returned back to baseline level after 48 h [169].

An age-dependent *Ngb* expression is detected across different studies as well. Mouse cerebral *Ngb* expression increases along the aging process up till its highest level is reached in young adults [6]. A similar trend is seen in the human carotid body in which the Ngb^+ area on histology increases from 1 ± 1.8% in children of 2 years to 4.4 ± 2.8% in pensioners with a mean age of 73.5 years [170]. Age does also impact *Ngb* transcription under stress conditions. Cerebral Ngb mRNA concentrations are increased 6-fold in newborn mice subjected to an acute model of systemic hypoxia (8% O_2, 6 h). At P6, the Ngb transcript levels do not significantly differ from those of the normoxic controls [53]. A study on Wistar rats detected a *Ngb* expression induction in carotid bodies at the age of 3 months after 10–12% oxygen for 12 days. However, Wistar rats of 24 months did not show a significant upsurge in *Ngb* transcription after the hypoxic insult [171]. It is noted, however, that the different studies cannot be compared between one another as different kinds or levels of insults are studied, in addition to the use of diverse model systems and tissues.

Some organisms and cells are indeed better evolutionary adapted to certain types of stressors as others, being a third reason as to why differences in *Ngb* expression and functions are observed. By way of example, the human brain will develop irreversible brain injury after 5 to 10 minutes of oxygen

depletion while aquatic mammals as Weddell seals do not encounter neurological deficits after dives of over 1.5 h [172]. Ngb, as one of the resident neural globins, could be one part of the adaptation mechanisms. Goldfish (*Carassius auratus*), which live in isolated ponds with low oxygen partial pressures, have Ngb protein levels that are five times the level of zebrafish [173]. Ngb protein levels of coastal marine animals (e.g., the sea lion, sea otter, and bottlenose dolphin) are 1.7–1.9-fold higher as those of terrestrial animals [172]. Here too is a limit observed with regard to the neuroprotective effect of Ngb, with an inverse relationship existing between resident neural globin levels and maximum dive duration [172]. A basal elevated *Ngb* expression due to adaptations to extremely hypoxic/hypercapnic conditions could also explain the different *Ngb* expression patters observed in such animals under hypoxic conditions. The subterranean mole rat *Spalax* has up to 2.8- and 3.5-fold higher transcript and protein levels as the rat under 21% O_2, respectively [51]. Given such a condition to represent an abnormal environment for the *Spalax* mole, the latter can be regarded as a stress condition with an excessive ROS production as a result of the higher as normal oxygen level. This is in line with the observed downregulation of Ngb mRNA levels to 40–75% of the normoxic condition after 22 and 44 h of 10% O_2, which is similar as the Ngb transcript levels of the rat under 21% O_2 [51]. A similar trend is seen in total fish preparations of the Japanese ricefish medaka (*Oryzias latipes*), showing a decrease in Ngb transcript levels after 24 and 48 h of $PO_2 = 4$ kPa [174]. However, brain samples alone showed an increase in Ngb transcripts, though significance was not reached [174].

It can be deduced from the foregoing that Ngb ligand affinities and functions differ between organisms throughout the phylogenetic tree; in particular, Ngb's structure and functions can be adapted to the specific circumstances and environmental requirements of the organism in which it resides. It is not only necessary to reflect on this when choosing a model system, but one can as well directly derive information from it regarding sequence-structure-function correlations. By way of example, gene orthologs of vertebrate Ngb are detected in organisms without a nervous system (e.g., placozoans) [162]. Hence, Ngb will exert more basic cellular functions within these organisms as compared to in higher order animals, in which its transcription is restricted to specific (neural) cell types. Such functional diversification was enabled through whole-genome duplication events in the stem lineage of vertebrates [175]. Indeed, with the increasing size of animals and the emergence of blood circulatory systems, vertebrate hemoglobin arose from co-option of duplicated *Ngb* genes [176]. Functional diversification can also be retrieved from phylogenetic footprinting. As such, neuron-specific expression of *Ngb* might be promoted by Klf4 and REST of which the binding sites around *Ngb* are conserved in vertebrates, but are not detected in or around other globin genes [162]. Many other excellent additions on phylogenetic analyses of Ngb were excluded here as they went beyond the scope of the present review. The reader is directed to Ascenzi et al. [177] and Burmester et al. [3] for comprehensive reviews of the topic.

Discussion and Future Perspective

Our understanding of Ngb's genetics, structure, evolution, and biological functions has greatly increased over the last decade. Given the positive correlation between Ngb levels and beneficial outcomes in numerous ailments (Table 2), the notion is underscored of Ngb having cytoprotective traits. While the focus was first on intrinsic neuroprotective contributions of Ngb (i.e., the reduction of proapoptotic cytochrome c [17], Ngb's nitrite reductase activity [115], and its scavenger activity against superoxide [16]), the research field is now starting to move to studying the involvement of Ngb in signaling pathways in neuronal and nonneuronal cell populations [69, 70, 166].

To obtain such biological insights, it might be required to study $Ngb^{-/-}$ mice or other model organisms. So far, most studies on Ngb have been on *Ngb*-overexpressing transgenic mice [19, 41, 58, 178]. While *Ngb* silencing has been shown to augment neuronal vulnerability to cytotoxic events in vitro [27, 35, 179], only a limited number of studies has thus far been published that used a $Ngb^{-/-}$ mouse strain. Most recently, a $Ngb^{-/-}$ mouse was generated, using the knockout-first allele Ngbtm1a(EUCOMM)Wtsi, to study a possible role for Ngb in auditory functioning [180]. Ngb depletion resulted in minimal defects in hearing ability and had no impact on noise trauma outcomes. Conversely, auditory brainstem peak-to-peak amplitudes were reduced in $Ngb^{-/-}$ mice 4 weeks after noise trauma. The latter may be grounded in a continuing loss of central neurons, which leads to fewer neurons in the inferior colliculus to transmit signals [180]. $Ngb^{-/-}$ mice have also been used to elucidate the light-induced retinal gene expression response in the mouse retina [88]. Ngb deficiency only caused a small effect on the light-induced retinal gene expression response. However, some other systemic differences were observed with regard to the wild-type condition, e.g. different expression levels of *Akap6*, *Entpd4*, and *Atp8a2* [88]. The first study investigated the transcriptional response to hypoxia in whole $Ngb^{-/-}$ mouse brains [49]. Neuronal viability in these $Ngb^{-/-}$ mice was not altered as compared to wild-type controls, though changes were observed in transcription patterns of the glycolytic pathway as well as increases in HIF1 and c-FOS expression [49]. The latter could be indicative of cells being depleted of Ngb to be more susceptible to hypoxia-induced transcription [49]. However, some genetic buffering might be expected as well, knowing it was already seen in myoglobin knockout mice. Only finding

the correct trigger to induce myoglobin's physiological response and studying its regulatory networks in more detail revealed myoglobin's vital functions [181]. Hence, studying Ngb-linked signaling pathways, using *Ngb* knockout models, may also shed light on cytotoxic pathways in which advantageous participation of Ngb can be expected.

In conclusion, (non)neuronal tissue benefits from an increase in Ngb cellular levels upon cytotoxic events, which contributes to the preservation of tissue integrity. Through an increased attention for Ngb's neuroprotective traits, our understanding has grown on the underlying mechanisms, though most have been intrinsic to Ngb itself. With current research still broadening on the topic of Ngb, we have a good eye on the expansion of our knowledge on Ngb's physiological functions and modes of action.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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