*Neuroscience* 163 (2009) 552–560

# WHEN THE BRAIN GOES DIVING: GLIAL OXIDATIVE METABOLISM MAY CONFER HYPOXIA TOLERANCE TO THE SEAL BRAIN

**S. A. MITZ,[a,b] S. REUSS,[b] L. P. FOLKOW,[c] A. S. BLIX,[c] J.-M. RAMIREZ,[d] T. HANKELN[e] AND T. BURMESTER[a]***

[a]*Institute of Zoology and Zoological Museum, University of Hamburg, Germany*

[b]*Department of Anatomy and Cell Biology, University Medical Center, Mainz, Germany*

[c]*Department of Arctic Biology, University of Tromsø, Tromsø, Norway*

[d]*Center for Neuroscience, Seattle Children's Research Institute, Seattle, WA, USA*

[e]*Institute of Molecular Genetics, University of Mainz, Germany*

**Abstract—Deep diving mammals have developed strategies to cope with limited oxygen availability when submerged. These adaptations are associated with an increased neuronal hypoxia tolerance. Brain neurons of the hooded seal *Cystophora cristata* remain much longer active in hypoxic conditions than those of mice. To understand the cellular basis of neuronal hypoxia tolerance, we studied neuroglobin and cytochrome c in *C. cristata* brain. Neuroglobin, a respiratory protein typically found in vertebrate neurons, displays three unique amino acid substitutions in hooded seal. However, these substitutions unlikely contribute to a modulation of $O_2$ affinity. Moreover, there is no significant difference in total neuroglobin protein levels in mouse, rat and seal brains. However, in terrestrial mammals neuroglobin resided exclusively in neurons, whereas in seals neuroglobin is mainly located in astrocytes. This unusual localization of neuroglobin is accompanied by a shift in the distribution of cytochrome c. In seals, this marker for oxidative metabolism is mainly localized in astrocytes, whereas in terrestrial mammals it is essentially found in neurons. Our results indicate that in seals aerobic ATP production depends significantly on astrocytes, while neurons rely less on aerobic energy metabolism. This adaptation may imbue seal neurons with an increased tolerance to hypoxia and potentially also to reactive oxygen species, and may explain in part the ability of deep diving mammals to sustain neuronal activity during prolonged dives.** 

**Key words: astrocytes, cytochrome c, deep diving, lactate shuttle, neuroglobin, seal.**

Energy metabolism of mammalian brains is fueled by carbohydrates and ketone bodies. For many years, it was thought that glucose constitutes the sole energy substrate for neurons. Glucose was assumed to be directly provided to neurons via the extracellular space by the cerebral circulation. Recently, it has been proposed that—in addition to glucose—neurons largely rely on lactate to sustain their activity (for review, see Pellerin, 2005, 2008). According to this "lactate shuttle" hypothesis, glycolysis occurs predominantly in glial cells (astrocytes), which produce substantial amounts of lactate (Peng et al., 1998). Lactate is taken up by neurons, which appear to have a preference to oxidize imported lactate instead of producing lactate/pyruvate by their own glycolysis (Peng et al., 1998; Itoh et al., 2003). Thus most oxygen is consumed by the respiratory chain in the mitochondria of brain neurons. As mammals rely on oxidative metabolism to meet their energy demands, an acute lack of oxygen (hypoxia) usually leads to severe consequences for the animal, especially in hypoxia-sensitive tissues. Most terrestrial mammals show massive brain dysfunctions already after seconds of oxygen shortage, resulting from decreasing ATP levels, which affects ion homeostasis and therefore the excitation of neurons (Katsura et al., 1994; Lutz and Nilsson, 2004).

Interestingly, diving mammals have developed various physiological adaptations to cope with severe hypoxia (for review, see: Blix and Folkow, 1983; Butler and Jones, 1997; Butler, 2004; Ramirez et al., 2007). High concentrations of hemoglobin (Hb) in blood and myoglobin (Mb) in skeletal and heart muscle increase the capacity to store oxygen (Scholander, 1940; Lenfant et al., 1970; Snyder, 1983; Polasek and Davis, 2001). Selective vasoconstriction assures the blood circulation in $O_2$-sensitive organs such as the brain and reduces the $O_2$-consumption in other organs (e.g. kidney) to a minimum. Some species may further reduce their metabolic rate by regional hypothermia (Butler and Jones, 1997). Moreover, seals may also actively cool their brains to reduce oxygen demand (Odden et al., 1999). On the cellular level, almost nothing is known concerning how diving mammals maintain neuronal activity during severe hypoxia (Ramirez et al., 2007).

The hooded seal (*Cystophora cristata*) is known to dive as deep as 1000 m for up to 1 h (Folkow and Blix, 1999). Recently, Folkow et al. (2008) showed that cortical neurons from the hooded seal are able to cope with severe oxygen deprivation and remain much longer active under hypoxic stress than mouse neurons. Folkow et al. (2008) speculated that neuroglobin (Ngb) (Burmester et al., 2000) contributes to the remarkable hypoxia tolerance of the seal's brain. Ngb, a globin related to Mb and Hb, is expressed in neurons of the CNS and peripheral nervous system (Burmester et al., 2000). Ngb binds oxygen reversibly via an iron-ion with an oxygen affinity that is roughly similar to

*Correspondence to: T. Burmester, Biozentrum Grindel und Zoologisches Museum, Universität Hamburg, Martin-Luther-King-Platz 3, D-20146, Hamburg, Germany. Tel: +49-40-42838-3913; fax: +49-40-42838-3937.
E-mail address: thorsten.burmester@uni-hamburg.de (T. Burmester).

*Abbreviations:* CNS, central nervous system; Cyt c, cytochrome c; GFAP, glial fibrillary acidic protein; Hb, hemoglobin; Mb, myoglobin; Ngb, neuroglobin; nHb, nerve hemoglobin; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline.

0306-4522/09 $ - see front matter 
doi:10.1016/j.neuroscience.2009.06.058

that of Mb (Burmester et al., 2000; Dewilde et al., 2001). Several studies have shown that Ngb has neuroprotective properties (Sun et al., 2001, 2003; Khan et al., 2006). On the cellular level, Ngb might have a similar role as Mb in muscle cells, thus acting as respiratory protein that supplies $O_2$ to the metabolically highly active neurons. Alternatively, Ngb may scavenge reactive oxygen or nitrogen species (ROS/RNS) (e.g. Herold et al., 2004; Wang et al., 2008). Various other or additional functions of Ngb have been proposed (for review, see: Hankeln et al., 2005; Burmester and Hankeln, 2009), but regardless of its actual physiological role, there is convincing evidence that Ngb is tightly linked to the oxidative metabolism (Schmidt et al., 2003; Bentmann et al., 2005).

Here we have investigated the presence and distribution of Ngb in the hooded seal (*C. cristata*). Ngb levels did not differ in brains of seals, rats and mice, but we found an unprecedented localization of Ngb and cytochrome c (Cyt c) in astrocytes of the seal neocortex. We hypothesize that in seals, the cerebral energy metabolism fundamentally differs from that of terrestrial mammals in that astrocytes assume at least partly oxidative metabolism, while neurons largely function anaerobically.

## EXPERIMENTAL PROCEDURES

### Animals

Adult rats (Sprague–Dawley, $n=2$) and mice (Balb/C; C57BL/6, $n=6$) were maintained under constant conditions (12-h light/dark cycle, room temperature 21±1 °C; food and water *ad libitum*). Rats and mice were killed with an overdose of isoflurane at the middle of the light period. Adult female hooded seals (*C. cristata*, $n=5$) were collected off the coast of East Greenland under permits of the national authorities of Denmark and Norway. The seals were decapitated under full anesthesia (i.m. injection of zolazepam/tiletamine, 1.5–3.0 mg per kg of body mass), as approved by the National Animal Research Authority of Norway. The procedures concerning rats, mice and seals reported in this study were conducted to minimize the number of animals used and their suffering. They complied with German, Norwegian and European laws for the protection of animals.

### Cloning and sequencing of hooded seal Ngb-cDNA

Seal brain tissue was cut into small pieces and stored frozen. Total RNA was extracted using the RNeasy Mini-Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Oligonucleotide primers were designed according to the conserved segments of the aligned mammalian Ngb-cDNA sequences (Burmester et al., 2004): 5′-GAGCTGATCCGGCAGAGCTGGCG-3′ and 5′-GCCATGAGTCGAGGCTGGGA-3′. Ngb-cDNA fragments were amplified by reverse transcription–PCR experiments employing the Qiagen OneStep-kit according to the manufacturer's instructions. Missing 5′- and 3′-ends of the cDNA were obtained using the GeneRacer™ Kit (Invitrogen, Karlsruhe, Germany). The PCR products were cloned into the pCR4-TOPO TA vector (Invitrogen, Karlsruhe, Germany). Sequences were obtained from both strands using a commercial sequencing service (GENterprise, Mainz, Germany). The seal cDNA sequence was deposited at the GenBankTM/EMBL database under the accession number FM999827.

A homology model of *C. cristata* Ngb was built applying the online facility SwissModel (GlaxoWellcome Experimental Research, Geneva, Switzerland) at the following address: http://www.expasy.ch/spdbv/ using the known human Ngb crystal structures (Pesce et al., 2003) as template. The structure was visualized by the aid of POLYVIEW-3D (Porollo and Meller, 2007).

### Immunohistochemistry

For immunohistochemistry, rat and mouse brain tissues were fixed by perfusion with 4% paraformaldehyde (Reuss et al., 2002). Fresh seal brain tissue (neocortex, cerebellum) was cut into appropriate pieces ($15\times15\times15$ mm$^3$) and fixed overnight in 4% paraformaldehyde in PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$) and stored in cold PBS until use. Brain samples from mouse, rat and seal were immersed overnight in 30% sucrose/PBS at 4 °C and cryo-sectioned at 40 μm thickness. Brain sections were incubated free-floating with the first antibody diluted in PBS/0.1%–0.4% Triton X-100/1% bovine serum albumin overnight at room temperature. A commercial polyclonal rabbit antibody against Ngb (1:500, Sigma Aldrich, Deisenhofen, Germany) was used. In addition, we employed a monoclonal mouse antibody against glial fibrillary acidic protein (GFAP; 1:200, Abcam, Cambridge, UK) and a polyclonal anti–Cyt c antibody produced in sheep (1:8, Sigma Aldrich). The sections were washed three times 10 min in PBS and incubated for 90 min at room temperature in the dark with the corresponding secondary antibodies, which had been diluted in PBS: donkey anti-rabbit $F(ab)_2$-fragment coupled to Cy3 (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), donkey anti-mouse $F(ab)_2$-fragment coupled to Cy2 (1:200 in PBS; Jackson ImmunoResearch) or donkey anti-sheep $F(ab)_2$-fragment coupled to Cy2 (1:200 in PBS; Jackson ImmunoResearch). Sections were washed three times 10 min in PBS, mounted on glass slides and embedded in Mowiol (Calbiochem, Darmstadt, Germany). The Hoechst dye 33258 (0.3 μg/ml) was added to the Mowiol to stain the nuclei. Sections were analyzed using an Olympus BX51 research microscope equipped with a digital camera. Images were combined using Adobe Photoshop 7.0.

### Western blotting

For Western blot analyses, frozen mouse and seal brains were microdissected to obtain samples enriched in tissues of cerebrum, cerebellum, and medulla spinalis. If adequate, we separated grey (cortical) from underlying white (medullary) matter. Proteins were extracted by homogenizing the tissues in PBS with 0.1% SDS. Protein concentrations were estimated according to Bradford (1976). Tissue extracts (about 80 μg protein per lane) were diluted with sample buffer (65 mM Tris–HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol) and denatured at 95 °C for 5 min. Recombinant mouse Ngb was used as positive control. After gel electrophoresis on a 15% SDS–polyacrylamide gel, the proteins were transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked by incubation for 1 h with pure soy milk. The membranes were incubated with a custom antibody raised against a conserved Ngb peptide (amino acid positions 55–70: $H_2N$-CLSSPEFLDHIRKVML-CONH2; Eurogentec, Seraing, Belgium) diluted 1:100 in soy milk at 4 °C overnight. The membranes were then washed four times 10 min in TBS (10 mM Tris–HCl, 150 mM NaCl, pH 7.5) and incubated with a goat anti-rabbit antibody coupled with alkaline phosphatase (Dianova, Hamburg, Germany; 1:10,000 in TBS) for 45 min at room temperature. The membranes were washed in TBS as above and detection was carried out with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

## RESULTS

### Sequencing of hooded seal Ngb cDNA

Degenerated primers were deduced from a multiple sequence alignment of known mammalian Ngbs (Burmester et al., 2004). A 323 bp fragment of *C. cristata* Ngb cDNA

was amplified by RT-PCR from total RNA extracted from cerebral cortex (neocortex) tissue. The missing 5 and 3′ ends were completed and verified by the GeneRacer method. The full sequence was confirmed by RT-PCR with specific primers complementary to start and stop codon and subsequent sequencing (Supplemental Fig. 1). Like in all mammalian Ngbs studied so far, the coding region of the hooded seal Ngb covers 465 base pairs. The amino acid sequence of 151 residues shows a high identity to the other known mammalian Ngb proteins, with all key determinants required for reversible oxygen binding being conserved. The highest identity score was observed with Ngb from the dog, *Canis familiaris* (96.7% identity, 98.7% similarity on the amino acid level). However, hooded seal Ngb harbors three unique substitutions, which have not been observed so far in any other mammalian Ngb (Fig. 1). These substitutions are located in positions D2 (i.e. second amino acid of globin helix D: E→K), H13 (G→A) and H17 (Q→E) (cf. Supplemental Figs. 2 and 3).

### Quantification of Ngb protein content in mouse and seal brains

Total proteins were extracted from dissected brain areas of mouse and seal. To analyze Ngb protein levels of mouse and seal brains, a semi-quantitative Western blotting method was applied. A constant amount of total protein extract per lane was applied and detected by specific anti-Ngb antibodies (Fig. 2). In all samples, Ngb protein

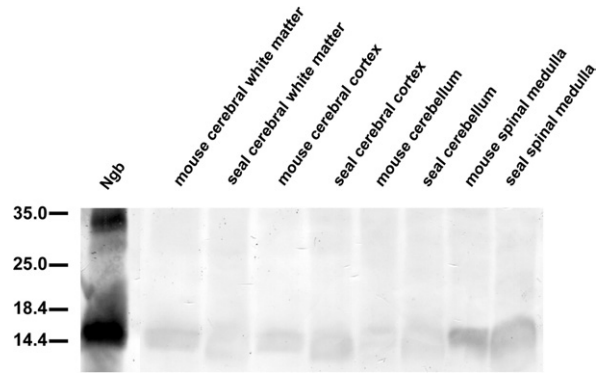


**Fig. 2.** Western blot analysis of Ngb protein tissue expression. Eighty micrograms of total protein from selected mouse and seal tissue extracts was applied per lane. Recombinant mouse Ngb was applied as a positive control (lane "Ngb"). Ngb was detected in all brain regions of mouse and seal.

was detected with the expected mass of about 15 kDa (Burmester et al., 2000). We observed no significant differences in Ngb protein levels throughout all samples. Similar results were obtained on RNA level by using semi-quantitative RT-PCR (data not shown).

### Immunohistochemical detection of Ngb and Cyt c

The distribution of Ngb protein in the seal, rat and mouse brains (neocortex and cerebellum) was further characterized by immunostaining using a commercial antibody

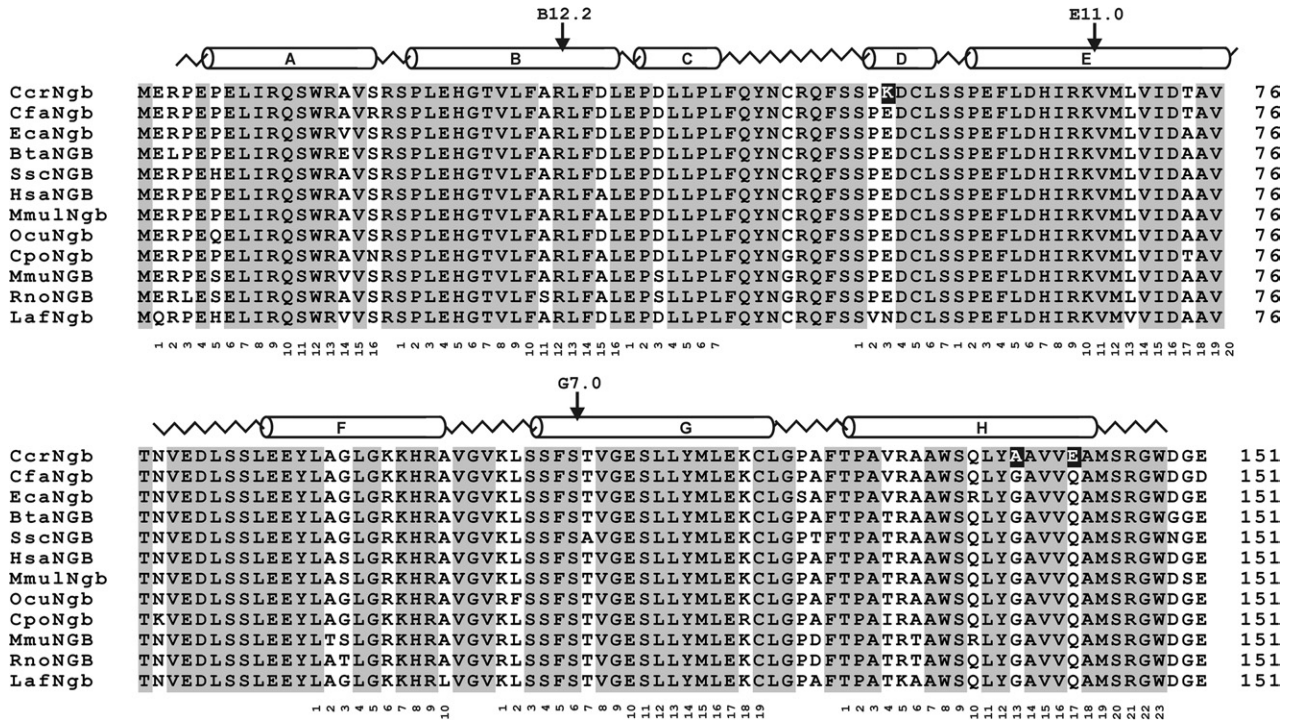


**Fig. 1.** Alignment of Ngb amino acid sequences from various mammals. Shaded positions indicate conserved residues. The unique substitutions of hooded seal Ngb are marked in black. Intron positions in the Ngb gene are indicated by arrows. The species abbreviations are: Ccr, *Cystophora cristata* (hooded seal); Cfa, *Canis familiaris* (dog); Eca, *Equus caballus* (horse); Bta, *Bos taurus* (cattle); Ssc, *Sus scrofa* (pig); Hsa, *Homo sapiens* (man); Mmul, *Macaca mulatta* (rhesus monkeys); Ocu, *Oryctolagus cuniculus* (rabbit); Cpo, *Cavia porcellus* (guinea pig); Mmus, *Mus musculus* (mouse); Rno, *Rattus norvegicus* (rat); Laf, *Loxodonta africana* (elephant).

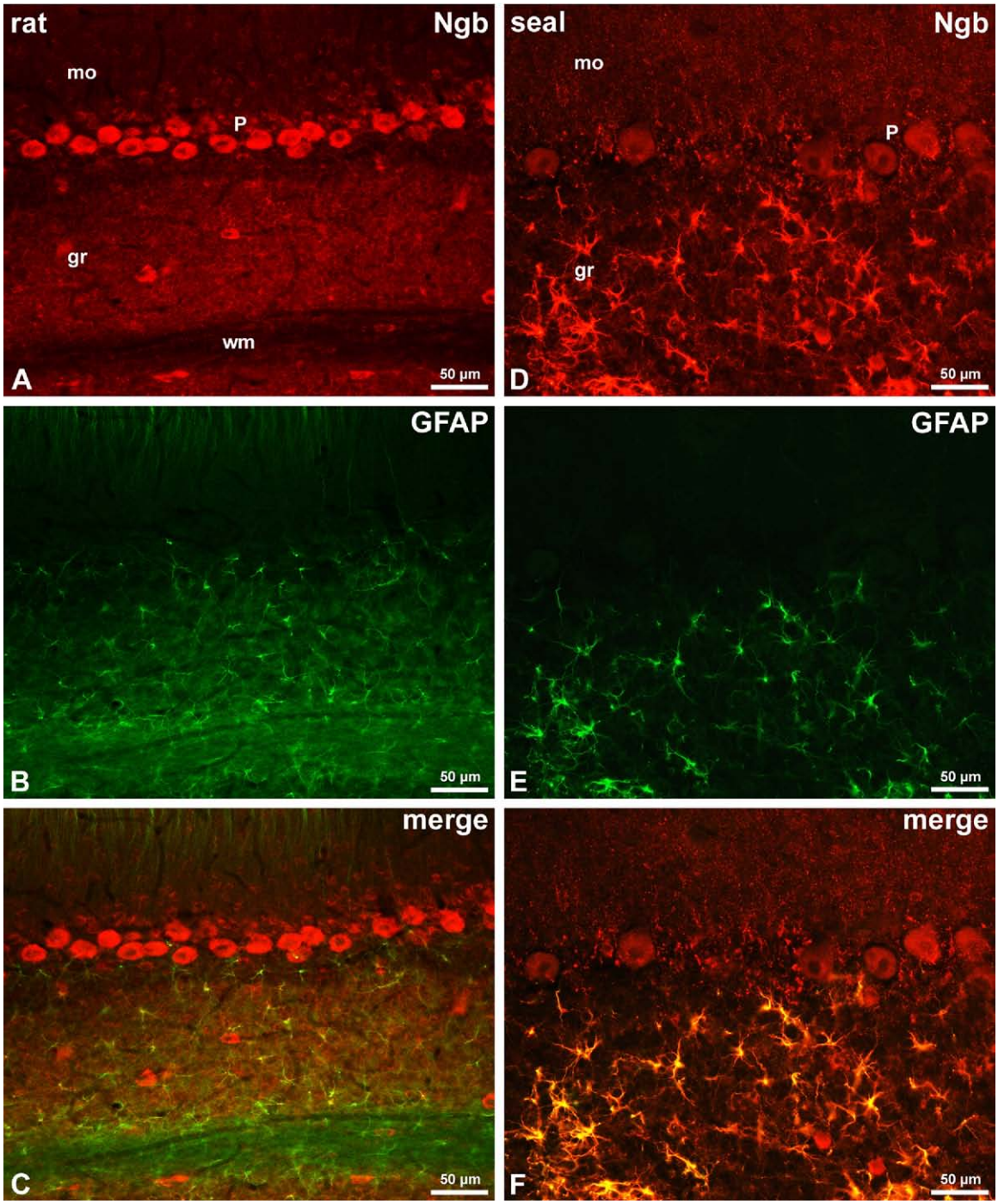


**Fig. 3.** Immunofluorescence of Ngb (red) and GFAP (green) in the cerebellum of rat (A–C) and seal (D–F). (A) Ngb-immunoreactivity was detected in neurons of the rat molecular layer (mo), the granular layer (gr) and in Purkinje cells (P), but not in the white matter (wm) (B) GFAP-positive astrocytes were found in gr and in the wm of rat. (D) In seals, weak Ngb staining was observed in neurons of the mo, in P; astrocytes of the gr show Ngb labeling. (E) GFAP staining in astrocytes was found of gr and wm of seal cerebellum. Merged figures show co-localization of Ngb and GFAP (yellow) in seal (F) but not in rat (C) cerebellum.

raised against Ngb (Figs. 3 to 5; Supplemental Fig. 4). Rat and mouse brains showed identical neuronal anti-Ngb staining patterns (Figs. 3 and 4; Supplemental Fig. 4), which correspond to those described in previous studies (Reuss et al., 2002; Wystub et al., 2003). The specificity of the Ngb antibody in all three species was verified by pre-absorption tests employing recombinant Ngb protein. Negative controls, in which either primary or secondary antibodies were omitted, did not show any signal (data not shown). Astrocytes were identified using the abovementioned antibody directed against a specific glial protein, GFAP.

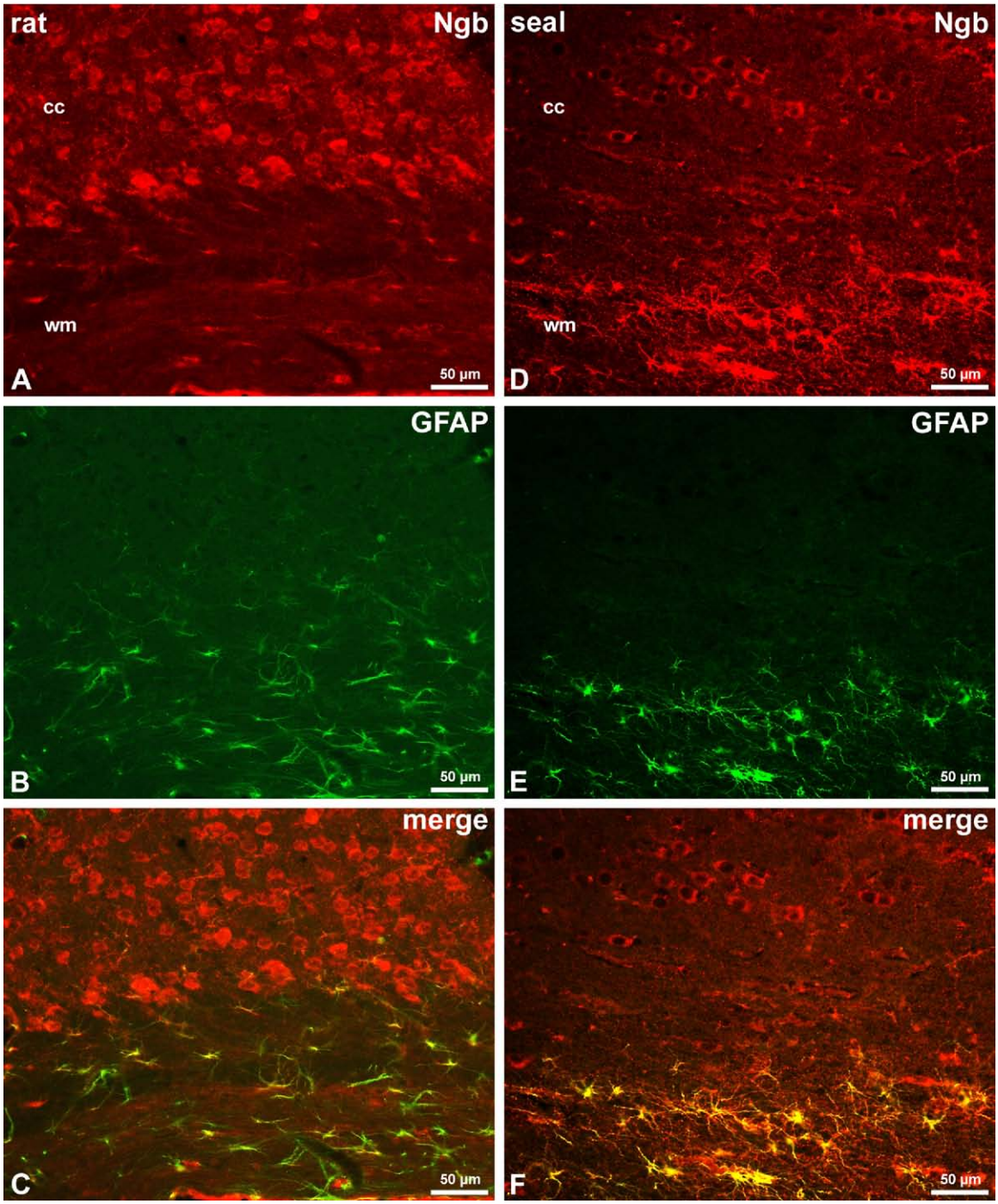


**Fig. 4.** Immunofluorescence of Ngb (red) and GFAP (green) in the cerebrum of rat (A–C) and seal (D–F). (A) In rat, strong Ngb staining was detected in neurons of the cerebral cortex (cc). (B) GFAP staining was found in the white matter (wm) of rat. (D) In seals, Ngb-immunoreactivity was detected in neurons of the cerebral cortex (cc) and in astrocytes of the wm. (E) GFAP staining of astrocytes in the wm of seal cerebrum. Merged figures show co-localization of Ngb and GFAP (yellow) in seal (F) but not in rat (C) cerebrum.

In all three species, Ngb immunoreactivity was observed in most Purkinje cells and in several neurons of the molecular and granular layers of the cerebellum (Fig. 3A, D). Neuronal staining in rodent was stronger than in seal, when sections were incubated in the same test tube under identical conditions. In the rat cerebrum, the majority of neurons in the cortex was stained (Fig. 4A, D). In the seal brain, Ngb was additionally present in astrocytes of the granular layer and of the white matter of the cerebellum (Fig. 3D). In the cerebrum, Ngb-labeled astrocytes were seen predominantly in the white matter but some were also observed between cortical neurons (Fig. 4D). The GFAP

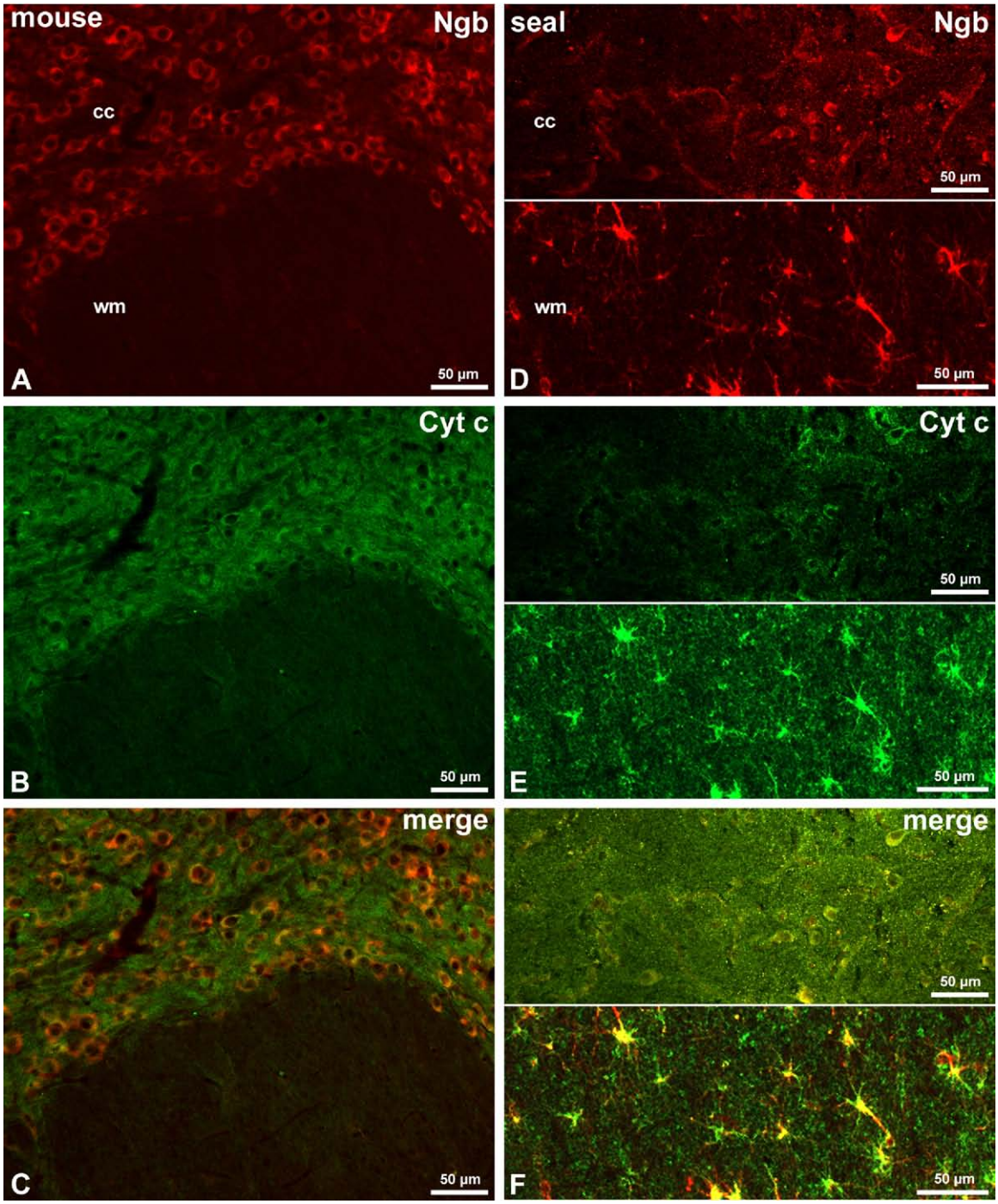


**Fig. 5.** Immunofluorescence of Ngb (red) and Cyt c (green) in the cerebrum of mouse (A–C) and seal (D–F). (A, B) Ngb- and Cyt c-immunoreactivity was restricted to neurons in the cerebral cortex (cc) of mouse cerebrum. (D, E) In seal cerebrum, Ngb- and Cyt c-immunoreactivity was detected in neurons of the cc, and stronger in astrocytes in the white matter (wm). Merged figures show co-localization of Ngb and Cyt c. (yellow) in both the (C) mouse and hooded (F) seal brain.

staining pattern (Fig. 3B, E and Fig. 4B, E) demonstrated that these Ngb-expressing cells in the seal brain were astrocytes. Double-staining experiments confirmed the co-localization of Ngb and GFAP in seal brain regions (Fig. 3F and Fig. 4F) but very little overlapping in rat (Fig. 3C and Fig. 4C).

To identify the main sites for oxidative energy metabolism, we stained brain sections of mice (Fig. 5A–C), rats (Supplemental Fig. 5) and seals (Fig. 5D–F) for Cyt c, a component of the respiratory chain in the mitochondria that transfers electrons between complexes III and IV. In rat and mouse brains, this enzyme was located predominantly

in neurons (Fig. 5B; Supplemental Fig. 5), whereas in seals, Cyt c staining was much stronger in astrocytes than in neurons (Fig. 5E). In all three species (mice, rats and seals), Cyt c essentially co-localized with Ngb (Fig. 5C, F).

## DISCUSSION

In humans, apnea leads to the loss of consciousness within minutes. Diving mammals, however, remain submerged for extended periods of time and usually have high activity levels during their dive. A large body of literature deals with the various physiological adaptations of diving animals, which allow them to endure apnea and thus tolerate hypoxia better than their terrestrial relatives (for review, see: Blix and Folkow, 1983; Butler and Jones, 1997; Butler, 2004; Ramirez et al., 2007). Most recently, is has been demonstrated that *C. cristata* neurons remain longer active under hypoxia than mice neurons (Folkow et al., 2008). However, little is known about the molecular mechanisms that sustain neuronal integrity and activity while the seal brain experiences oxygen deficiency (Ramirez et al., 2007).

### Ngb in diving mammals

Respiratory proteins reversibly bind $O_2$ for the purpose of transport or storage. They enhance the $O_2$ carrying capacity of the body fluid, facilitate intracellular $O_2$ diffusion or enable $O_2$ storage. It is well known that diving mammals have higher concentrations of Hb in blood and more Mb in striated muscles than non-diving mammals (Scholander, 1940; Lenfant et al., 1970; Snyder, 1983; Polasek and Davis, 2001). Therefore, Ngb, which was found to be expressed predominantly in neurons of various mammalian species (Burmester et al., 2000; Reuss et al., 2002; Wystub et al., 2004), is a candidate that could contribute to an enhanced $O_2$ supply and thus neural hypoxia tolerance, also in seals.

In fact, recently Williams et al. (2008) suggested that higher levels of intracellular globins may convey enhanced cerebral hypoxia tolerance to aquatic mammals by facilitating $O_2$ diffusion. However, in that study, neuro- and cytoglobin were lumped together, although a respiratory role is unlikely for cytoglobin (Schmidt et al., 2004; Hankeln et al., 2005). In addition, the similarity of absorption spectra of hemoglobin that may remain in cerebral blood vessels, of neuro- and cytoglobin, and those of other intracellular heme proteins, makes it questionable whether the spectrophotometric quantification employed by Williams et al. (2008) yielded reliable results. Indeed, our Western blot studies showed no significant differences in Ngb protein levels between hooded seal and mouse brains (Fig. 2). Therefore, it is rather unlikely that a higher Ngb protein concentration per se contributes to enhanced hypoxia tolerance of the seal brain.

An alternative hypothesis is that an enhanced $O_2$-affinity of Ngb from diving mammals may improve $O_2$ supply. The sequence of Ngb from the hooded seal contains three unique substitutions. We did not measure $O_2$ affinity of seal Ngb, but homology-modeling approaches show that these mutations are located on the surface of the protein (Supplemental Figs. 2 and 3), suggesting that it is rather unlikely that there are functional differences e.g. in $O_2$ affinity between Ngb from the hooded seal and non-diving mammals. Rather the unique distribution of Ngb in this diving species may either be the consequence of or contribute to a divergent oxidative metabolism on the cellular level of the brain (see below).

### Ngb in the seal astrocytes

Hitherto, Ngb was demonstrated to be restricted to neurons in brain sections (Reuss et al., 2002; Wystub et al., 2003; Hankeln et al., 2004) and in cultured cells (Laufs et al., 2004) of terrestrial mammals. In the hooded seal, however, Ngb was primarily found in astrocytes. It is tempting to assume that this different distribution of Ngb in the seal brain is related to its unusual hypoxia tolerance. The tight correlation of Ngb and Cyt c location in neurons of both rodents and seals (cf. Fig. 5), which holds despite fundamental differences in cellular distribution of Ngb between the species, also corroborates the linkage of Ngb to oxidative metabolism (Schmidt et al., 2003; Bentmann et al., 2005; Hankeln et al., 2005).

Interestingly, observations of a distribution of nerve hemoglobins (nHbs) similar to that of the hooded seal have been made in some invertebrates. In mollusks and annelids that do not regularly face hypoxic conditions, nHb typically resides in neurons; in hypoxia-tolerant species nHb is present at high concentrations in glial cells (Wittenberg, 1992; Burmester and Hankeln, 2008). Accordingly, neurons of the mollusk *Tellina alternate*, which has glial cells that are rich in nHb, maintain their functional integrity for up to 30 min of anoxia, whereas neurons of the closely related *Tagellus plebeius*, which does not have glial nHb, cease functioning already after 5 min of anoxia (Kraus and Colacino, 1986). It is tempting to assume that, analogous to the situation in hypoxia-tolerant invertebrates, Ngb in astrocytes of the seal brain may secure adequate supply of oxygen to the cell, to enable them to sustain neuronal function during dives and/or support the recovery from dives. However, we cannot rule out that Ngb also protects the metabolically active neurons from ROS generated e.g. in the respiratory chain (Herold et al., 2004; Hankeln et al., 2005; Wang et al., 2008; Burmester and Hankeln, 2009).

### A unique oxidative metabolism in the seal brain

Oxidative metabolism requires the electron transport chain in mitochondria. As early as in the 1970s it was demonstrated that the activity of the mitochondrial marker-enzyme cytochrome oxidase correlates positively with neural activity (for review, see: Wong-Riley, 1989). Ngb and Cyt c are two independent markers for oxidative metabolism, which are tightly correlated within the nervous system (Figs. 3 to 5; see also: Schmidt et al., 2003; Bentmann et al., 2005; Hankeln et al., 2005). In mouse and rat brains, Ngb and Cyt c almost exclusively reside in neurons, while astrocytes are essentially devoid of these proteins. This distribution has also been found in other terrestrial animals and indicates that brain neurons generally depend on ATP

generated by the respiratory chain in mitochondria (Wong-Riley, 1989). Our data thus agree with recent findings on the energy metabolism of the brain: according to the lactate shuttle hypothesis, oxidative metabolism in brains of terrestrial mammals essentially takes place in neurons and is fueled by lactate, which is continuously produced by astrocytes by anaerobic glycolysis (Pellerin, 2005, 2008).

The distribution of Ngb and Cyt c in hooded seal (Figs. 3 to 5), however, suggests that there are fundamental differences in the localization of aerobic and anaerobic metabolism between hooded seals and non-diving rodents. The principal presence of Cyt c in the seal's astrocytes (Fig. 5) indicates that these cells consume most of the available oxygen. Ngb may support oxidative metabolism in these cells, either by supplying oxygen or by other means (Hankeln et al., 2005; Burmester and Hankeln, 2009). By contrast, astrocytes in the brains of rat and mouse are largely anaerobic (see above). In the hooded seal's brain, however, neurons appear to have much less Cyt c and Ngb compared to astrocytes of the seal, and compared to neurons in mouse and rat brains. This observation indicates that in the brain of *C. cristata* neurons depend less on oxidative metabolism than the astrocytes and than the neurons of mouse and rat brains. We thus hypothesize that at the end of long dives when arterial $PO_2$ drops to tensions as low as 15 to 20 Torr (Blix and Folkow, 1983; Qvist et al., 1986) neuronal activity in seal brains may not be fueled by oxidative metabolism. Therefore, an alternative ATP production process in seal brain neurons must be considered. Given the low concentration of Cyt c, it is likely that at least some brain neurons of seals employ anaerobic glycolysis. Anaerobic energy production is also required in neurons of avascular retinae, which are present in some mammalian species (Bentmann et al., 2005). The reduction of the reliance on oxygen-consuming energy production may explain the unusual hypoxia tolerance of seal brain neurons observed *in vitro* (Folkow et al., 2008). It is well established that mitochondria are the main source of ROS (Halliwell, 2006) and that mitochondrial ROS production strongly increases during hypoxia and reoxygenation. A lower oxidative metabolism in seal neurons is advantageous to avoid damage by ROS. Shifting the oxidative metabolism from neurons to astrocytes may therefore protect the particularly sensitive neurons from ROS-induced damage.

However, what is the advantage for seal astrocytes relying on aerobic metabolism? The end product of anaerobic energy production in seal neurons is most likely lactate. When the brain becomes hypoxic during long dives, lactate from the neurons may be transported by the blood into the liver and converted to glucose or glycogen. In fact, Kerem and Elsner (1973) observed an increase of lactate concentration in the cerebral venous effluent at the end of long dives in harbor seals (*Phoca vitulina*). Under normoxia during the recovery period between dives, a "reverse" lactate-shuttle mechanism may be functional, in which astrocytes convert lactate that is released by the essentially anaerobic neurons, into pyruvate, which is then consumed in aerobic metabolism by the astrocyte.

There is increasing awareness that glial cells are crucial in regulating neuronal oxidative metabolism and baseline activity in mammals (Pellerin, 2005; Magistretti, 2006; Pellerin, 2008). It is conceivable that astrocytes play a particularly prominent role in maintaining neocortical activity under extreme hypoxia in diving seals. Therefore, insights gained from studies of diving animals could potentially be useful for identifying novel neuroprotective strategies that enable cortical networks to function also under pathological conditions (Choi, 1990; Ramirez et al., 2007).

*Acknowledgments—We wish to thank Simone Zehm for her help in the laboratory and Jürgen Markl for excellent working facilities. This work was supported by the Deutsche Forschungsgemeinschaft (Bu956/10, Bu956/12; Ha2103/3), the Norwegian Research Council (164791/V40) and the Roald Amundsen Centre for Arctic Research, University of Tromsø, Norway.*

## REFERENCES

Bentmann A, Schmidt M, Reuss S, Wolfrum U, Hankeln T, Burmester T (2005) Divergent distribution in vascular and avascular mammalian retinae links neuroglobin to cellular respiration. J Biol Chem 280:20660–20665.

Blix AS, Folkow B (1983) Cardiovascular adjustments to diving in mammals and birds. In: Handbook of physiology—the cardiovascular system III (Shepherd JT, Abboud FM, eds), pp 917–945. Bethesda: American Physiological Society.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Burmester T, Haberkamp M, Mitz S, Roesner A, Schmidt M, Ebner B, Gerlach F, Fuchs C, Hankeln T (2004) Neuroglobin and cytoglobin: genes, proteins and evolution. IUBMB Life 56:703–707.

Burmester T, Hankeln T (2008) Neuroglobin and other nerve globins. In: Protein reviews, Vol. 9 (Bolognesi M, et al., eds), pp 211–222. Milan: Springer Verlag Italia.

Burmester T, Hankeln T (2009) What is the function of neuroglobin? J Exp Biol 212:1423–1428.

Burmester T, Weich B, Reinhardt S, Hankeln T (2000) A vertebrate globin expressed in the brain. Nature 407:520–523.

Butler PJ (2004) Metabolic regulation in diving birds and mammals. Respir Physiol Neurobiol 141:297–315.

Butler PJ, Jones DR (1997) Physiology of diving of birds and mammals. Physiol Rev 77:837–899.

Choi DW (1990) Cerebral hypoxia: some new approaches and unanswered questions. J Neurosci 10:2493–2501.

Dewilde S, Kiger L, Burmester T, Hankeln T, Baudin-Creuza V, Aerts T, Marden MC, Caubergs R, Moens L (2001) Biochemical characterization and ligand binding properties of neuroglobin, a novel member of the globin family. J Biol Chem 276:38949–38955.

Folkow LP, Blix AS (1999) Diving behaviour of hooded seals (*Cystophora cristata*) in the Greenland and Norwegian Seas. Polar Biol 22:61–74.

Folkow LP, Ramirez JM, Ludvigsen S, Ramirez N, Blix AS (2008) Remarkable neuronal hypoxia tolerance in the deep-diving adult hooded seal (*Cystophora cristata*). Neurosci Lett 446:147–150.

Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? J Neurochem 97:1634–1658.

Hankeln T, Ebner B, Fuchs C, Gerlach F, Haberkamp M, Laufs TL, Roesner A, Schmidt M, Weich B, Wystub S, Saaler-Reinhardt S, Reuss S, Bolognesi M, De Sanctis D, Marden MC, Kiger L, Moens L, Dewilde S, Nevo E, Avivi A, Weber RE, Fago A, Burmester T (2005) Neuroglobin and cytoglobin in search of their role in the vertebrate globin family. J Inorg Biochem 99:110–119.

Hankeln T, Wystub S, Laufs T, Schmidt M, Gerlach F, Saaler-Reinhardt S, Reuss S, Burmester T (2004) The cellular and subcellular localization of neuroglobin and cytoglobin—a clue to their function? IUBMB Life 56:671–679.

Herold S, Fago A, Weber RE, Dewilde S, 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.

Itoh Y, Esaki T, Shimoji K, Cook M, Law MJ, Kaufman E, Sokoloff L (2003) Dichloroacetate effects on glucose and lactate oxidation by neurons and astroglia in vitro and on glucose utilization by brain in vivo. Proc Natl Acad Sci U S A 100:4879–4884.

Katsura K, Kristian T, Siesjo BK (1994) Energy metabolism, ion homeostasis, and cell damage in the brain. Biochem Soc Trans 22: 991–996.

Kerem D, Elsner R (1973) Cerebral tolerance to asphyxial hypoxia in the harbor seal. Respir Physiol 19:188–200.

Khan AA, Wang Y, Sun Y, Mao XO, Xie L, Miles E, Graboski J, Chen S, Ellerby LM, Jin K, Greenberg DA (2006) Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. Proc Natl Acad Sci U S A 103:17944–17948.

Kraus DW, Colacino JM (1986) Extended oxygen delivery from the nerve hemoglobin of *Tellina alternata* (Bivalvia). Science 232: 90–92.

Laufs TL, Wystub S, Reuss S, Burmester T, Saaler-Reinhardt S, Hankeln T (2004) Neuron-specific expression of neuroglobin in mammals. Neurosci Lett 362:83–86.

Lenfant C, Johansen K, Torrance JD (1970) Gas transport and oxygen storage capacity in some pinnipeds and the sea otter. Respir Physiol 9:277–286.

Lutz PL, Nilsson GE (2004) Vertebrate brains at the pilot light. Respir Physiol Neurobiol 141:285–296.

Magistretti PJ (2006) Neuron-glia metabolic coupling and plasticity. J Exp Biol 209:2304–2311.

Odden A, Folkow LP, Caputa M, Hotvedt R, Blix AS (1999) Brain cooling in diving seals. Acta Physiol Scand 166:77–78.

Pellerin L (2005) How astrocytes feed hungry neurons. Mol Neurobiol 32:59–72.

Pellerin L (2008) Brain energetics (thought needs food). Curr Opin Clin Nutr Metab Care 11:701–705.

Peng L, Arystarkhova E, Sweadner KJ (1998) Plasticity of Na,K-ATPase isoform expression in cultures of flat astrocytes: species differences in gene expression. Glia 24:257–271.

Pesce A, Dewilde S, Nardini M, Moens L, Ascenzi P, Hankeln T, Burmester T, Bolognesi M (2003) Human brain neuroglobin structure reveals a distinct mode of controlling oxygen affinity. Structure 11:1087–1095.

Polasek LK, Davis RW (2001) Heterogeneity of myoglobin distribution in the locomotory muscles of five cetacean species. J Exp Biol 204:209–215.

Porollo A, Meller J (2007) Versatile annotation and publication quality visualization of protein complexes using POLYVIEW-3D. BMC Bioinformatics 8:316.

Qvist J, Hill R, Schneider R, Falke K, Liggins G, Guppy M, Elliot R, Hochachka P, Zapol W (1986) Hemoglobin concentrations and blood gas tensions of free-diving Weddell seals. J Appl Physiol 61:1560–1569.

Ramirez JM, Folkow LP, Blix AS (2007) Hypoxia tolerance in mammals and birds: from the wilderness to the clinic. Annu Rev Physiol 69:113–143.

Reuss S, Saaler-Reinhardt S, Weich B, Wystub S, Reuss MH, Burmester T, Hankeln T (2002) Expression analysis of neuroglobin mRNA in rodent tissues. Neuroscience 115:645–656.

Schmidt M, Gerlach F, Avivi A, Laufs T, Wystub S, Simpson JC, Nevo E, Saaler-Reinhardt S, Reuss S, Hankeln T, Burmester T (2004) Cytoglobin is a respiratory protein in connective tissue and neurons, which is up-regulated by hypoxia. J Biol Chem 279:8063–8069.

Schmidt M, Giessl A, Laufs T, Hankeln T, Wolfrum U, Burmester T (2003) How does the eye breathe? Evidence for neuroglobin-mediated oxygen supply in the mammalian retina. J Biol Chem 278:1932–1935.

Scholander PF (1940) Experimental investigations on the respiratory function in diving mammals and birds. Hvalradets Skrifter 22:1–131.

Snyder GK (1983) Respiratory adaptations in diving mammals. Respir Physiol 54:269–294.

Sun Y, Jin K, Mao XO, Zhu Y, Greenberg DA (2001) Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. Proc Natl Acad Sci U S A 98:15306–15311.

Sun Y, Jin K, Peel A, Mao XO, Xie L, Greenberg DA (2003) Neuroglobin protects the brain from experimental stroke in vivo. Proc Natl Acad Sci U S A 100:3497–3500.

Wang X, Liu J, Zhu H, Tejima E, Tsuji K, Murata Y, Atochin DN, Huang PL, Zhang C, Lo EH (2008) Effects of neuroglobin overexpression on acute brain injury and long-term outcomes after focal cerebral ischemia. Stroke 39:1869–1874.

Williams TM, Zavanelli M, Miller MA, Goldbeck RA, Morledge M, Casper D, Pabst DA, McLellan W, Cantin LP, Kliger DS (2008) Running, swimming and diving modifies neuroprotecting globins in the mammalian brain. Proc Biol Sci 275:751–758.

Wittenberg JB (1992) Functions of cytoplasmatic hemoglobins and myohemerythrin. Adv Comp Environ Physiol 13:60–85.

Wong-Riley MT (1989) Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. Trends Neurosci 12:94–101.

Wystub S, Ebner B, Fuchs C, Weich B, Burmester T, Hankeln T (2004) Interspecies comparison of neuroglobin, cytoglobin and myoglobin: sequence evolution and candidate regulatory elements. Cytogenet Genome Res 105:65–78.

Wystub S, Laufs T, Schmidt M, Burmester T, Maas U, Saaler-Reinhardt S, Hankeln T, Reuss S (2003) Localization of neuroglobin protein in the mouse brain. Neurosci Lett 346:114–116.

## APPENDIX

### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2009.06.058.

*(Accepted 27 June 2009)*
*(Available online 2 July 2009)*