

How Does the Eye Breathe?

EVIDENCE FOR NEUROGLOBIN-MEDIATED OXYGEN SUPPLY IN THE MAMMALIAN RETINA*

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Visual performance of the vertebrate eye requires large amounts of oxygen, and thus the retina is one of the highest oxygen-consuming tissues of the body. Here we show that neuroglobin, a neuron-specific respiratory protein distantly related to hemoglobin and myoglobin, is present at high amounts in the mouse retina (~100 μM). The estimated concentration of neuroglobin in the retina is thus about 100-fold higher than in the brain and is in the same range as that of myoglobin in the muscle. Neuroglobin is expressed in all neurons of the retina but not in the retinal pigment epithelium. Neuroglobin mRNA was detected in the perikarya of the nuclear and ganglion layers of the neuronal retina, whereas the protein was present mainly in the plexiform layers and in the ellipsoid region of photoreceptor inner segment. The distribution of neuroglobin correlates with the subcellular localization of mitochondria and with the relative oxygen demands, as the plexiform layers and the inner segment consume most of the retinal oxygen. These findings suggest that neuroglobin supplies oxygen to the retina, similar to myoglobin in the myocardium and the skeletal muscle.

Neuroglobin (Ngb)¹ has been identified as an intracellular respiratory protein expressed mainly in the brain (1) but also in the peripheral nervous system and endocrine tissues (2). Ngb displays only limited sequence similarities with vertebrate hemoglobin and myoglobin (less than 25% amino acid sequence identity) but resembles the nerve-specific globins known from some invertebrates, e.g. that of the annelid *Aphrodite aculeata* (3). Ngb binds oxygen reversibly via an iron (Fe^{2+}) ion of the heme group (1, 4, 5). The oxygen affinity (P_{50}) of Ngb was determined to be in the range of a typical vertebrate myoglobin (~1–2 torr (1, 4)), although the hexacoordinate structure of the Fe^{2+} in deoxy-Ngb renders the oxygen-binding kinetics more complex than in other globins (4–6). Ngb was suggested to play an important role in oxygen homeostasis of neuronal tissues (1, 7). In fact, Ngb expression is unregulated under hypoxic conditions *in vitro*, and the presence of Ngb enhances the survival of cultured neuronal cells at low oxygen levels (8).

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¹ The abbreviations used are: Ngb, neuroglobin; PBS, phosphate-buffered saline.

The visual process in the vertebrate eye requires large amounts of metabolic energy, which is provided mainly as ATP that derives from glycolysis and oxidative phosphorylation (9, 10). High ATP amounts are consumed, for example, for the functioning of photoreceptor cells (e.g. for the maintenance of the dark current, for the fast turnover of photosensitive sensitive membranes, or the phototransduction process itself (11)) and the transmission at the synapses of all neurons in the retina. Thus, it is not surprising that the relative oxygen consumption of the mammalian retina is higher than that of the brain and other tissues (12, 13). It is assumed that, on a per gram basis, the retina is one of the highest oxygen demanding tissues of the human body (10, 14). Lack of oxygen (hypoxia) has immediate and severe effects to the visual performance of man and other vertebrates (15, 16).

Oxygen is supplied to the retina by the hemoglobin of the blood. The degree of vascularization of the retina differs among mammals (10). In mouse and most other species, the inner parts are nourished by the central retinal vessels, whereas the outer parts are supplied via the pigment epithelium by capillaries of the choroidal circulation. Blood capillaries do not reach the retinal layers of photoreceptor cells directly. Therefore, oxygen delivered by the blood has to diffuse over large distances to reach e.g. the photoreceptor compartments. Nevertheless, the presence of an intracellular respiratory protein, which could enhance the uptake of oxygen by the vertebrate retina, has been hitherto unknown. Here we show that Ngb is highly expressed in the murine retina and that the intra-retinal distribution of Ngb correlates with the relative oxygen consumption.

EXPERIMENTAL PROCEDURES

Animals—The procedures concerning animals complied with German legislation for the protection of animals and were approved by the county government office (Bezirksregierung Rheinhessen-Pfalz). Balb/C and C57BL/6J mice were maintained under constant conditions (light:dark 12:12 h, room temperature $21 \pm 1^\circ\text{C}$) with food and water *ad libitum*. Bovine eyes were obtained from the local slaughterhouse. Bovine photoreceptor outer segments were purified from dark-adapted isolated bovine retinas as described previously (17).

Antibody Preparation—Two different polyclonal antibodies against neuroglobin were produced using a commercial service (Eurogentec). The first antibody was raised in chicken against recombinant mouse Ngb. The IgY fraction was purified from the yolk by the aid of the EGGstract kit (Promega). Specific anti-Ngb antibodies were affinity-purified using recombinant mouse Ngb coupled to a SulfoLink column (Pierce) according to the instructions of the manufacturer and stored in 50 mM Tris, 100 mM glycine, pH ~7.4) supplemented with 0.1% NaN_3 . The second antibody was raised in rabbits against a synthetic Ngb peptide, which covers the conserved amino acid positions 55–70 of mouse and human Ngb ($\text{H}_2\text{N-CLSSPEFLDHIRKVML-CONH}_2$). Affinity purification using the synthetic peptide coupled to a SulfoLink column was carried out as described above.

Gel Electrophoresis and Western Blotting—Tissue samples were ho-

mogenized in 1% SDS, 5% β -mercaptoethanol, 10% glycerol, 65 mM Tris, pH 6.8. The samples were heated at 95 °C for 5 min and loaded to a 14% SDS-polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue R-250. For Western blot detection, the heat-denatured protein samples were transferred to nitrocellulose for 2 h at 0.8 mA/cm². The membranes were blocked for several hours with TBST (0.3% Tween 20 in 10 mM Tris-HCl, pH 7.4, 140 mM NaCl) and incubated for 1–2 h with anti-Ngb antibodies diluted (1:1000 to 1:2000). The membranes were washed four times for 10 min in TBST and incubated with the appropriate secondary antibodies (goat anti-rabbit or goat anti-chicken; Promega) coupled with alkaline phosphatase. The filters were washed in TBST as described above, and detection was carried out with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Signals were quantified after scanning of the blots with the help of the Scion Image program (version Beta4.02).

Immunostaining—Immunofluorescence studies were essentially performed as previously described (17). Briefly, eyes from adult mice were prefixed in 4% paraformaldehyde in PBS for 1 h at room temperature, washed, soaked with 30% sucrose in PBS overnight, and cryofixed in melting iso-pentane. Cryosections were placed on poly-L-lysine-precoated coverslips (18). Specimens were incubated with 50 mM NH₄Cl and 0.1% Tween 20 in PBS and blocked with blocking solution (0.5% cold-water fish gelatin (Sigma) plus 0.1% ovalbumin (Sigma) in PBS). The sections were incubated with anti-Ngb antibodies or, in the case of double labeling, with a mixture of anti-Ngb and monoclonal anti-arrestin antibodies (clone 3D1.2, described previously in Ref. 19) in blocking solution overnight at 4 °C. The specimens were washed and subsequently incubated with secondary antibodies conjugated to Alexa[®] 488 or Alexa[®] 546 (Molecular Probes) in blocking solution for 1 h at room temperature in the dark. On some sections, instead of Alexa[®] probes, secondary anti-rabbit antibodies coupled with horseradish peroxidase (Sigma) were placed and incubated in the dark for 1 h. Washed sections were mounted in Mowiol 4.88 (Hoechst, Frankfurt, Germany), containing 2% *n*-propyl gallate and, in the case of triple staining, 1 μ g/ml 4',6-diamidino-2-phenylindole. Mounted retinal sections were examined with a Leica DMRP microscope. Images were obtained with a Hamamatsu Orca ER CCD camera (Hamamatsu, Japan) and processed with Adobe Photoshop (Adobe Systems).

In Situ Hybridization—Digoxigenin-labeled antisense RNA probes were created by *in vitro* transcription with T7 RNA polymerase (Roche Molecular Biochemicals), using PCR-generated templates covering the 453-base pair mouse *Ngb* coding region (EMBL/GenBank[™] accession no. AJ245945). A T7 RNA polymerase promoter sequence was attached to the 5' end of the sense or antisense PCR primers. *In situ* hybridization was as described previously (2). Briefly, the RNA probes were diluted in 2 \times SSC, 50% formamide and incubated on the sections at 42 °C overnight. Slides were washed in 2 \times SSC and subsequently in 0.1 \times SSC at 55 °C. Tissue sections were then treated with a mixture of RNase A (25 μ g/ml) and RNase T1 (25 units/ml) for 30 min at 37 °C in a wet chamber and then washed in PBS/Tween 20. After blocking for 15 min in Roche buffer 2, label was detected by alkaline phosphatase-coupled anti-digoxigenin antibodies (diluted 1:100 in Roche buffer 2; a 30-min incubation at room temperature), washing in PBS/Tween 20 and Roche buffer 3, and subsequent incubation with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (1:50 diluted in Roche buffer 3; 15–30 min at room temperature in the dark). The reaction was stopped by washing in PBS. The sections were covered by PBS/glycerol (1:1) and processed as described above.

RESULTS

High *Ngb* Expression in the Mammalian Retina—Specific anti-Ngb antibodies were raised in chicken (1) and rabbit and purified by affinity chromatography. Both types of antibodies detect *Ngb* in the protein extracts from the murine brain and the retina at ~17 kDa (Fig. 1A). The second band of the double molecular mass (about 34 kDa) likely results from the dimerization of *Ngb*, as frequently observed for many globins in SDS-PAGE. After preadsorption of the antibodies with the antigen, both bands disappeared in Western blots of retinal and brain tissue (data not shown), indicating the specificity of anti-Ngb antibodies. The apparent molecular mass of native *Ngb* in brain and retina is ~17 kDa and thus is about 1 kDa larger than that of the recombinant protein. The reason for this discrepancy is unknown, although posttranslational modification may be assumed. Further studies are required to address

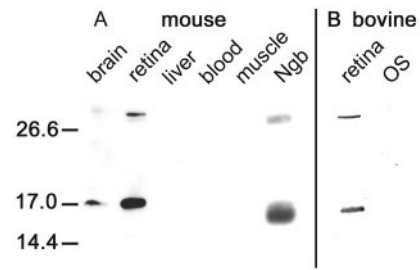


FIG. 1. Western blot analysis of *Ngb* protein tissue expression. A, ~30 μ g of total protein from selected mouse tissue extracts were applied per lane. *Ngb* was detected in the brain and the retina with an antibody raised against a synthetic *Ngb* peptide. 2 μ g of recombinant *Ngb* were applied as a positive control (lane "*Ngb*"). B, proteins from the total bovine retina and isolated outer segments (OS). *Ngb* is not present in the outer segment fraction, whereas the antibody stains *Ngb* in the bovine retina. On the left, the positions of the molecular mass standards are indicated.

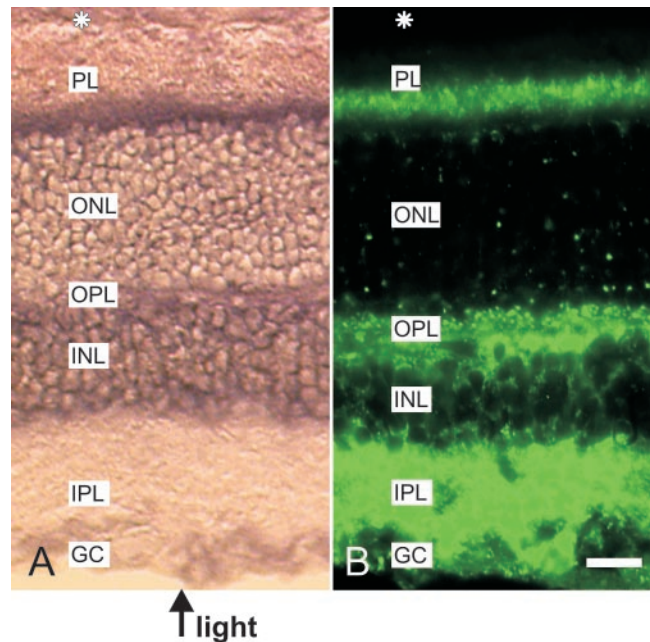


FIG. 2. Localization of *Ngb* mRNA and protein in the mouse retina. A, *in situ* hybridization of a longitudinal cryosection through the mouse retina. The mammalian retina is composed of well defined cellular layers: PL, layer of outer and inner segments of photoreceptor cells; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GC, layer of ganglion cells. The asterisk denotes the retinal pigment epithelium. The *Ngb in situ* hybridization signal (dark staining) is found mainly in the inner segments of the photoreceptor cells, the nuclear layers (ONL, INL) and the ganglion cells (GC) of the mouse retina but not in the pigment epithelium (asterisk). B, indirect anti-Ngb immunofluorescence. Bright indirect immunofluorescence anti-Ngb is visible in the inner segments of photoreceptors (PL), the plexiform layers (OPL, IPL), and the ganglion cells (GC) of the mouse retina but not in the pigment epithelium (asterisk). Bar: 10 μ m. The arrow at the bottom indicates the direction of the incoming light.

this question. No *Ngb* was detected in protein samples from murine liver, blood, or skeletal muscle. *Ngb* is also absent in the fraction of purified outer segments of bovine photoreceptor cells, whereas the antibody detects the protein in the retina of this species (Fig. 1B).

The intensity of the Western blot signals was quantified after scanning with the Scion Image program. The amount of *Ngb* in the retinal samples was estimated to be ~50–100-fold higher than in the total brain extracts. From the comparison with the known amounts of total protein and recombinant *Ngb* applied to the lanes, we estimate that *Ngb* makes up ~2–4% of the total retinal protein. The total concentration of soluble

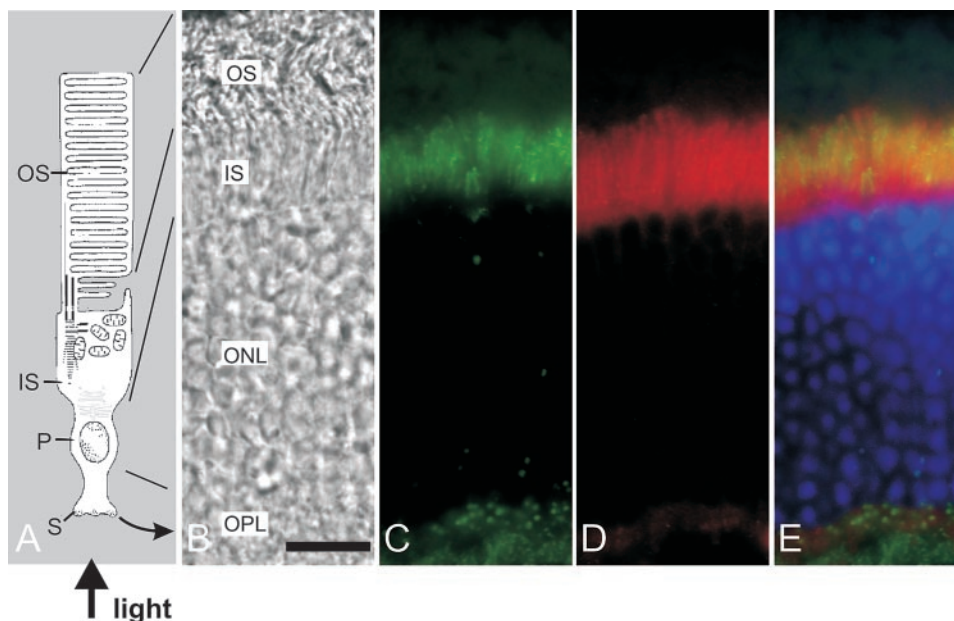


FIG. 3. Subcellular localization of Ngb in the mouse photoreceptor cells. *A*, schematic representation of a rod photoreceptor cell. Vertebrate photoreceptors are divided into distinct compartments: the photosensitive outer segment (*OS*), which is the place of visual transduction; the inner segment (*IS*), which contains the biosynthetic machinery of the cell, including numerous mitochondria in the ellipsoid region; the perikaryon or cell body (*P*), which is localized in the outer nuclear layer (*ONL* in *panel B*) of the retina and contains the nucleus; and the synaptic terminal (*S*) in the outer plexiform layer (*OPL* in *panel B*), which electrically connects the cell to downstream neurons of the retina. *B*, differential interference contrast image of a longitudinal cryosection through dark-adapted mouse retina. *C*, indirect anti-Ngb immunofluorescence in the section shown in *B*. Labeling is found predominantly in the inner segment of photoreceptor cells and the outer plexiform layer. *D*, indirect anti-arrestin immunofluorescence is present in the entire inner segment of the plexiform layer, in the outer nuclear layer, and in the synaptic terminal (*S*) of dark-adapted photoreceptor cells. *E*, merged images of *panels C* and *D* show partial localization of Ngb and arrestin in the ellipsoid region of the apical inner segment. The nuclei in the outer nuclear layer are blue-stained by 4',6-diamidino-2-phenylindole. Bar: 10 μm . The arrow at the bottom indicates the incoming light.

proteins in the retina was determined to be about 100 mg/ml (data not shown). Thus, the concentration of Ngb in the total retina is in the range of 100 to 200 μM .

Localization of Ngb in the Mouse Retina—The mammalian retina is composed of distinct layers that are easily distinguishable in retinal sections by light microscopy. To examine sites of mRNA expression of Ngb in the mammalian retina, cryosections through mouse retinæ and eyes were analyzed by *in situ* hybridization using an *in vitro* transcribed antisense probe. The specificity and selectivity of this method were demonstrated previously (2). Strong hybridization signals were observed in the inner segments of the photoreceptor cells, in the outer and inner nuclear layer, and in the ganglion cell layer of the neuronal retina (Fig. 2*A*). The perinuclear signal in the neuronal retina is typical for mRNA hybridization in neurons. Less intense signals were found in the plexiform layers, whereas in the layer of photoreceptor outer segments and in the cells of the pigment epithelium, no labeling was detected.

To determine the distribution of the Ngb protein, immunostaining experiments were carried out with rabbit antibody raised against the synthetic peptide. Both indirect immunofluorescence and indirect immunoperoxidase staining methods were applied and provided identical staining patterns in both fixed and unfixed samples (data not shown). No staining was observed in the appropriate control experiments. As shown in Fig. 2*B*, no anti-Ngb signal was detectable in the cells of the retinal pigment epithelium, whereas prominent indirect anti-Ngb immunofluorescence was visible in the photoreceptor layer, in the outer and inner plexiform layer, and at the ganglion cells. In both nuclear layers a less intense, but still detectable, fluorescence was present in the perinuclear cytoplasm of the perikarya. In addition, bright anti-Ngb-labeled foci were regularly detected in the outer nuclear layer, and

these may represent stained domains in the Müller glia cells that project through the outer nuclear layer to the "external limiting membrane." The localization of Ngb in the retina does not depend on light; a comparison of anti-Ngb-labeled dark-adapted (Fig. 3*C*) with light-adapted (Fig. 2*B*) mouse retinæ did not show any detectable differences in the staining patterns of Ngb protein.

Localization of Ngb in the Photoreceptor Cell—Vertebrate photoreceptor cells are highly polarized neurons with defined compartments; their outer segments, inner segments, perikarya, and synapses are organized into distinct layers in retinal sections (Fig. 3*A*). This feature makes it relatively simple to determine the subcellular localization of a polypeptide in retinal cryosections stained by indirect immunofluorescence. To analyze the subcellular localization of Ngb in photoreceptor cells, we performed double immunofluorescence labeling experiments with anti-Ngb and anti-arrestin antibodies in cryosections through dark-adapted mouse retinæ. As in other G protein-coupled receptor-mediated signal transduction mechanisms, arrestin is a major component in the desensitization of the receptor (rhodopsin) and in the process of molecular adaptation (20). It has been reported previously that arrestin shows a light-dependent movement from the inner to the outer segments. Under dark conditions, it is localized in the entire inner segment and also is distributed throughout the perinuclear cytoplasm down to the synapses (21, 22) (Fig. 3*D*). High magnification analysis of the photoreceptor compartments in these retinal cryosections revealed that Ngb is localized at the synapses and in the ellipsoid of the inner segments (Fig. 3*C*). Although anti-arrestin weakly stained photoreceptor synapse region, anti-Ngb immunofluorescence was found to be more intense in foci representing the presynaptic terminals of photoreceptors but was also present in the non-photoreceptor domains of the outer plexiform layer (extensions of bipolar, hor-

zontal, and amacrine cells). In the inner segment of the photoreceptor cells, in contrast to anti-arrestin, the anti-Ngb antibody did not stain the entire inner segment but prominently labeled the ellipsoid region of the apical inner segment. In photoreceptor outer segments, no Ngb labeling was detectable, which was confirmed with the Western blot analysis of retina fractions (Fig. 1B).

DISCUSSION

Because of the high demand for oxygen in the visual process, an adequate oxygen environment is crucial for the function of the retinal cells (10). Here we show that Ngb, an oxygen-binding respiratory protein (1), is synthesized at high levels in the mouse retina. Ngb has been identified previously mainly in the brain, where it may occur at a concentration in the range of $\sim 1 \mu\text{M}$ (1). Such a low amount was difficult to reconcile with the important role of Ngb in oxygen supply. For example, myoglobin typically occurs in the cells of the striated muscle and the myocardium at concentrations of several hundred μM (23–25). However, the estimated concentration of Ngb in the mouse retina is $> 100 \mu\text{M}$ and thus is within the range of myoglobin in the muscle (Fig. 1), indicating the important role of Ngb in retinal function. Our results are in agreement with the observations by Wittenberg and Wittenberg (26), who identified a heme protein with an absorption spectrum similar to Ngb that occurs at apparently high concentrations in the fish eye. Ngb is also present in fish, although its localization remains to be determined (27).

Ngb Expression Correlates with the Sites of Oxygen Consumption—Recent studies in rat have shown that oxygen consumption by the retina takes place mainly in the inner segments, the outer plexiform layer, and the inner plexiform layer (10, 28, 29). This is not surprising, because the energy demand is particularly high in both types of mammalian photoreceptor cells and the synaptic contact regions, which are located mainly in the plexiform layers (10, 30). As demonstrated by immunofluorescence, most of the Ngb protein is present in these three layers of high oxygen demand (Fig. 3). Subcellular localization within the photoreceptor cells shows Ngb to be mainly in the ellipsoid region of the apical inner segment, where mitochondria are known to be concentrated (9, 31). The same is true for the synaptic regions of the photoreceptors and the plexiform layers (32), which also showed strong Ngb signals in our immunocytochemical experiments. These observations further support the assumption that Ngb is involved in retinal oxygen consumption.

Intracellular Ngb Transport—The mammalian retina is a highly specialized structure that is divided into morphological and functional distinct layers (Fig. 3A). Immunostaining and *in situ* hybridization experiments reveal striking differences in intracellular distribution of Ngb protein and mRNA within the retinal layers (Figs. 2 and 3). Whereas the mRNA is present mainly in the large cell bodies that make up the nuclear and ganglion layers, as well as in the inner segments of the photoreceptors, the protein is restricted almost exclusively to the plexiform layers and the inner segments of the photoreceptors. This redistribution suggests a massive transport of Ngb protein after translation, at least in the plexiform layers.

Ngb Is the Respiratory Protein of the Mammalian Retina—Although the exact mechanism of myoglobin function is still a matter of debate, its important role in intracellular oxygen

supply is well established (33–35). The observed intracellular oxygen partial pressures in the retina and muscle are within the same range (10, 28, 33, 36). In addition, both the intracellular concentration and the oxygen binding affinities of Ngb and myoglobin are comparable (1, 4). Thus, a similar physiological function in intracellular oxygen supply can be expected. Therefore, it can be assumed that Ngb either facilitates the transport of oxygen to the respiratory chain of the mitochondria or may provide a short-term storage of oxygen that is required for peaks of retinal activity.

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