

2008

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Jelena Ostojic  
*Iowa State University*

Sinsia D. Grozdanic  
*Iowa State University*

Nasreen S. Syed  
*University of Iowa*

Mark S. Hargrove  
*Iowa State University, msh@iastate.edu*

James T. Trent III  
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**Authors**

Jelena Ostojic, Sinsia D. Grozdanic, Nasreen S. Syed, Mark S. Hargrove, James T. Trent III, Markus H. Kuehn, Young H. Kwon, Randy H. Kardon, and Donald S. Sakaguchi

# Patterns of Distribution of Oxygen-Binding Globins, Neuroglobin and Cytoglobin in Human Retina

Jelena Ostojić, DVM, PhD; Siniša D. Grozdanić, DVM, PhD; Nasreen A. Syed, MD; Mark S. Hargrove, PhD; James T. Trent III, PhD; Markus H. Kuehn, PhD; Young H. Kwon, MD, PhD; Randy H. Kardon, MD, PhD; Donald S. Sakaguchi, PhD

**Objective:** To determine the distribution of 2 intracellular oxygen-carrying molecules, neuroglobin (NGB) and cytoglobin (CYGB), in specific retinal cell types of human retinas.

**Methods:** Specific antibodies against NGB and CYGB were used in immunohistochemical studies to examine their distribution patterns in human retinal sections. Double-labeling studies were performed with the anti-NGB and anti-CYGB antibodies along with antibodies against neuronal (microtubule-associated protein 2, class III  $\beta$ -tubulin [TUJ1], protein kinase C alpha, calretinin) and glial (vimentin, glial fibrillary acid protein) markers. Confocal microscopy was used to examine the retinal sections.

**Results:** Immunohistochemical analysis of human retinal tissue showed NGB and CYGB immunoreactivity in the ganglion cell layer, inner nuclear layer, inner and outer

plexiform layers, and retinal pigment epithelium. Neuroglobin immunoreactivity was also present in the outer nuclear layer and photoreceptor inner segments. Neuroglobin and CYGB were coexpressed in the neurons in the ganglion cell layer and inner nuclear layer but not within glial cells.

**Conclusion:** Neuroglobin and CYGB are colocalized within human retinal neurons and retinal pigment epithelium but not within glial cells.

**Clinical Relevance:** Our results suggest that NGB and CYGB may serve a neuroprotective role as scavengers of reactive oxygen species and therefore should be considered when developing therapeutic strategies for treatment of hypoxia-related ocular diseases.

*Arch Ophthalmol.* 2008;126(11):1530-1536

**G**LOBINS ARE AN IMPORTANT family of small globular proteins containing a heme prosthetic group (iron protoporphyrin IX), by which they can reversibly bind gaseous ligands, such as oxygen, nitric oxide, and carbon monoxide.<sup>1</sup> Thus far, 4 mammalian globins have been described, hemoglobin, myoglobin, neuroglobin (NGB), and cytoglobin (CYGB), NGB and CYGB having been discovered most recently.<sup>2-4</sup>

Neuroglobin and CYGB may have significant roles in neuroprotection. For example, NGB was found to be neuroprotective during acute neuronal hypoxia<sup>5,6</sup>; increased levels of NGB were detected in the cerebral cortex and serum after ischemia/reperfusion insults; and NGB was detected in the cerebrospinal fluid of patients with chronic pain.<sup>7</sup> Cytoglobin, also known as liver fibrogenesis-related marker,<sup>8,9</sup> is a novel candidate tumor suppressor gene in upper aerodigestive tract squamous cancer.<sup>10</sup> In addition, studies of overexpressed NGB and CYGB have demonstrated increased cytoprotection and

survival of islets of Langerhans after transplantation<sup>11,12</sup> and protection of human neuroblastoma cells against oxidative stress-induced cell death.<sup>13</sup>

Recent reports have demonstrated the presence of NGB in human, dog, mouse, rat, guinea pig, chicken, and zebra fish eye<sup>14-21</sup> and the presence of CYGB in dog, mouse, and rat retinas.<sup>15,17,18</sup> To our knowledge, this is the first detailed report to describe NGB and CYGB distribution in human retina using immunohistochemical methods. Characterization of their expression patterns is essential for a better understanding of oxygen homeostasis in healthy and disease-affected eyes and may be useful in developing strategies to prevent neuronal death due to retinal ischemic diseases.

## METHODS

### HUMAN TISSUE

Archived eyes from 7 subjects (aged 1.5, 6, and 9 months and 6, 47, 58, and 60 years) were retrieved from the archives of the F. C. Blodi Eye Pathology Laboratory at the University of Iowa.

**Author Affiliations:** Departments of Genetics, Development, and Cell Biology (Drs Ostojić and Sakaguchi) and Biochemistry, Biophysics, and Molecular Biology (Drs Hargrove and Trent), Interdepartmental Neuroscience Program (Drs Ostojić and Sakaguchi), and Veterinary Clinical Sciences, College of Veterinary Medicine (Dr Grozdanić), Iowa State University, Ames; and Departments of Ophthalmology and Visual Sciences (Drs Syed, Kuehn, Kwon, and Kardon) and Pathology (Dr Syed), University of Iowa, and Veterans Administration (Dr Kardon), Iowa City.

Specimens were obtained either at the time of autopsy or as part of an exenteration procedure for an orbital neoplasm and were noted to be histologically normal for age. Globes were fixed in 10% formalin for a minimum of 48 hours immediately after tissue procurement and then transferred to an ethanol-formaldehyde mixture (Pen-Fix; Richard Allan Scientific, Kalamazoo, Michigan), paraffin embedded, and sectioned at 4  $\mu$ m. To investigate if NGB and CYGB localization patterns differed in early postnatal vs adult retinas, the experimenter was masked from the age of the subjects when analyzing the retinal tissue.

Two adult human donor eyes (ages 47 and 88 years) were obtained from the Iowa Lions Eye Bank (Iowa City) within 4 hours post mortem. Subjects had no visual problems and cause of death was reported to be cardiopulmonary arrest and gastrointestinal bleeding, respectively. Eyes were fixed for 3 hours in freshly prepared 4% paraformaldehyde in phosphate-buffered saline. Tissue was infiltrated with increasing concentrations of sucrose, frozen in optimal cutting temperature medium (Sakura Finetek USA, Inc, Torrance, California), and sectioned at 7  $\mu$ m.

Tissue collection adhered to the tenets of the Declaration of Helsinki and was also approved by the University of Iowa and Veterans Administration human subjects review committees.

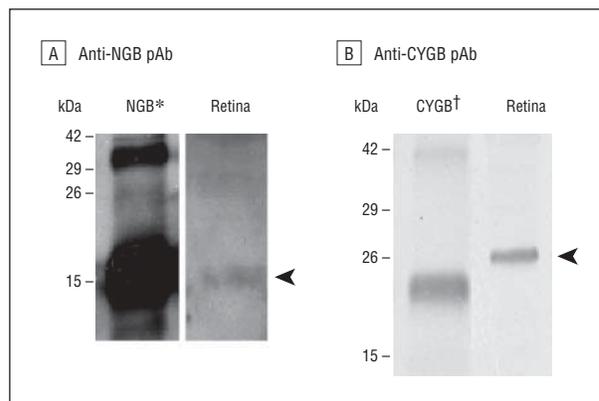
## FLUORESCENT IMMUNOHISTOCHEMISTRY

Human recombinant NGB and CYGB proteins were synthesized and polyclonal antisera against the full-length NGB or CYGB were generated and affinity purified as described previously.<sup>4,22</sup> Immunohistochemical procedures were carried out as previously described.<sup>15</sup> Briefly, sections were incubated for 2 hours in blocking solution that contained 2% blocking serum corresponding to the species in which the secondary antibody (Ab) was produced (normal donkey serum; Jackson ImmunoResearch, West Grove, Pennsylvania, or normal goat serum; Sigma, St Louis, Missouri), 1% bovine serum albumin (Sigma), and 0.4% Triton X-100 (Fisher Scientific Inc, Fairlawn, New Jersey). Sections were double labeled with a primary Ab cocktail during overnight incubation at room temperature in a sealed humid chamber. Primary Abs used in this study are summarized in eTable 1 (<http://archophthalmol.com>). Secondary Ab cocktails applied to slides were donkey antimouse biotinylated Ab (Jackson ImmunoResearch) and goat antirabbit Alexa 488 Ab (Molecular Probes, Eugene, Oregon); goat antirabbit biotinylated Ab (Vector Laboratories, Burlingame, California) and goat antimouse Alexa 488 Ab (Molecular Probes); goat antimouse Cy5 Ab (Jackson ImmunoResearch) and goat antirabbit Alexa 488 Ab (Molecular Probes); and goat antirabbit Cy5 Ab (Jackson ImmunoResearch) and goat antimouse Alexa 488 Ab (Molecular Probes). All primary and secondary Abs were diluted in potassium phosphate–buffered saline with 1% bovine serum albumin, 2% blocking serum, and 0.4% Triton X-100. All secondary Abs directly conjugated to a fluorochrome were used at a 1:200 dilution, and all biotinylated secondary Abs were used at a 1:500 dilution. Streptavidin Cy3 was diluted in potassium phosphate–buffered saline to 1:10 000. Negative controls were carried out in parallel during all processing by the omission of the primary or secondary Abs. Antibody specificity was verified by preadsorbing each with their respective recombinant proteins before incubation on tissue sections, in which case no specific staining was observed (eFigure 1).

## ANALYSIS OF TISSUE SECTIONS

Antibody-labeled tissue sections were visualized and images were captured using a Leica confocal scanning laser microscope (TCS-NT; Leica Microsystems Inc, Exton, Pennsylvania).

To discriminate Ab labeling from autofluorescence in the human retinal pigment epithelium,<sup>23,24</sup> confocal images of tis-

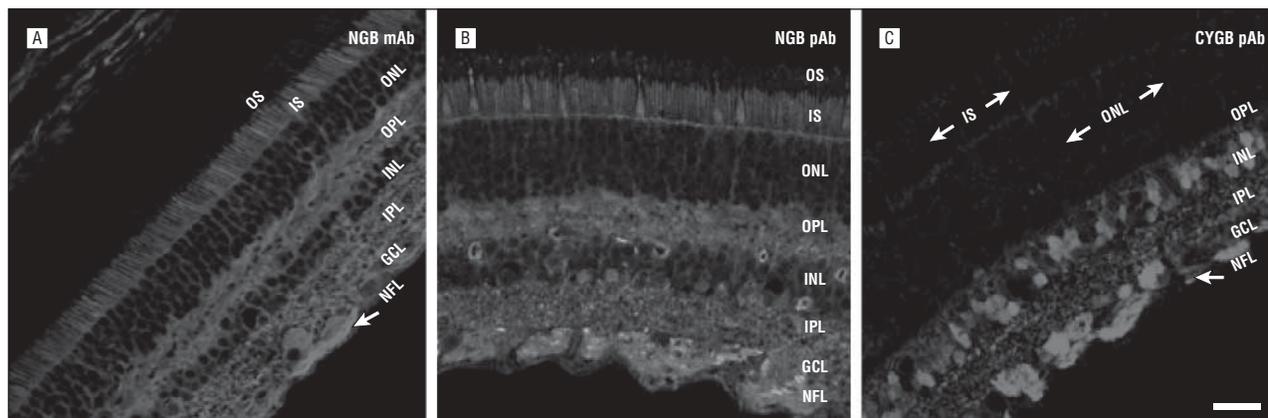


**Figure 1.** Western blot analysis of neuroglobin (NGB) and cytoglobin (CYGB) expression in human retina. A, Rabbit anti-NGB polyclonal antibody (pAb) detected recombinant NGB protein at approximately 17 kDa (left lane) and a similar band (right lane) in the protein extract from human retina. B, Rabbit anti-CYGB pAb detected specific recombinant CYGB protein at approximately 21 kDa (left lane) and CYGB in human retina (right lane) at approximately 26 kDa. The molecular weight markers are illustrated on the left side. Source of the protein: \*<sup>22</sup>; †.<sup>4</sup>

sue single labeled with primary Ab and appropriate Cy5 conjugated secondary Ab were captured at both the 488/525 nm and 633/645 nm excitation/emission wavelength channels. Cy5 is excited at 633 nm, and thus, the signal from the 488/525 nm channel was considered to be autofluorescence (background). Figures were made by subtracting images captured at the 488/525 nm channel (background) from images captured at the 633/645 nm channel (signal and background). As an additional control, autofluorescence was examined in nonlabeled tissue sections and images were also captured at both channels. Subtracted images prepared from these control images confirmed that intensity of autofluorescence was similar at both channels. All figures were prepared using Photoshop (version 7.0; Adobe, San Jose, California) and Freehand (version 10.0; Macromedia, San Francisco, California).

## WESTERN BLOT ANALYSIS

Characterization of antigens was performed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting procedures that have been described previously.<sup>15</sup> Briefly, for detection of NGB in human retinal samples, approximately 0.09  $\mu$ g of human recombinant NGB and 300  $\mu$ g of total human retinal protein were separated in 14% gels. After electrophoresis, proteins were renatured for 1 hour by incubating the gel in 50mM TRIS buffer, pH 7.4, containing 20% glycerol.<sup>25</sup> Proteins were then transferred to a polyvinylidene difluoride membrane in native transfer buffer and incubated in blocking buffer for 1 hour at room temperature and overnight in rabbit polyclonal anti-NGB Ab (1:1000). Subsequently, an alkaline phosphatase–conjugated goat antirabbit Ab (Promega, Madison, Wisconsin) was used at 1:7500 dilution. Lumi-Phos WB Chemiluminescent Substrate (Pierce Biotechnology, Inc, Rockford, Illinois) was used to visualize immunolabeled bands. For detection of CYGB in human retinal samples, approximately 0.06  $\mu$ g of human recombinant CYGB and 50  $\mu$ g of total human retinal protein were separated in 14% gels. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane in transfer buffer and incubated in blocking buffer for 1 hour at room temperature. The membrane was then incubated in rabbit polyclonal anti-CYGB Ab (1:1000), followed by incubation in alkaline phosphatase–conjugated goat antirabbit Ab (Promega). Immunoreactive (IR) bands were visualized with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium alkaline phosphatase color development reagents (Pro-



**Figure 2.** Antineuroglobin (anti-NGB) and anticytoglobin (anti-CYGB) immunoreactivity in human retina. A and B, Mouse anti-NGB monoclonal antibody (mAb) (A) and rabbit anti-NGB polyclonal antibody (pAb) (B) showed identical patterns of labeling in the retina. C, Pattern of immunoreactivity with rabbit anti-CYGB pAb. Scale bar=25  $\mu$ m. NFL indicates nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, photoreceptor inner segments, OS, photoreceptor outer segments.

mega). Molecular weights were estimated by comparison with prestained molecular weight standards (Sigma).

## RESULTS

### IMMUNOBLOT ANALYSIS OF NGB AND CYGB IN HUMAN RETINA

Rabbit anti-NGB Ab detected recombinant NGB protein at approximately 17 kDa (left lane) and a similar band (right lane) in the protein extract from human retina (**Figure 1A**). Rabbit anti-CYGB Ab detected recombinant CYGB protein at approximately 21 kDa (left lane) and an approximately 26-kDa protein (right lane) from the human retinal extract (**Figure 1B**).

### LOCALIZATION OF NGB AND CYGB IN HUMAN RETINA AND RETINAL PIGMENT EPITHELIUM

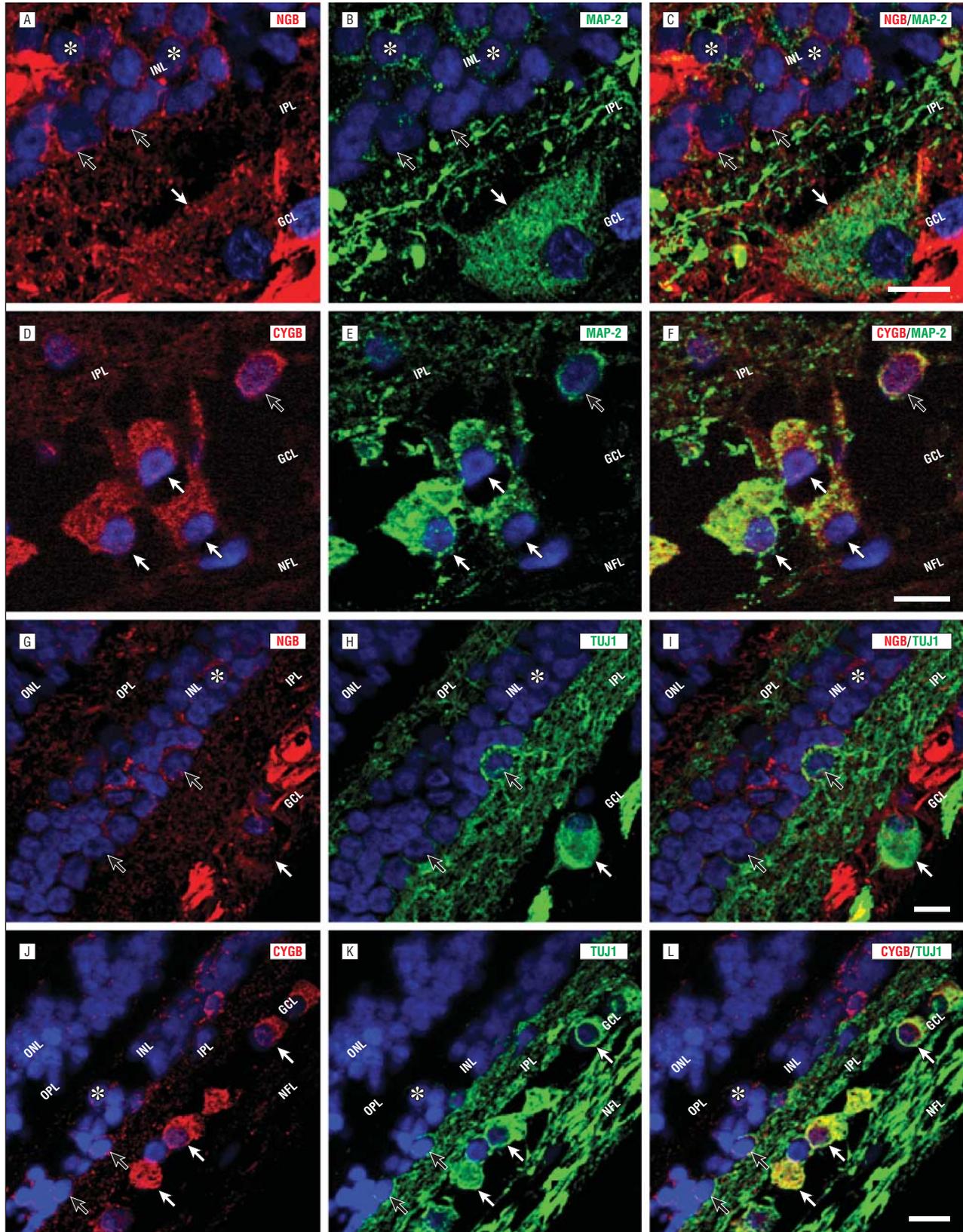
To examine the localization of NGB in human retinal sections, we used mouse monoclonal and rabbit polyclonal Abs generated against full-length human recombinant NGB protein and applied them on the whole retina. Both Abs showed identical patterns of labeling and were used for double-labeling studies. To illustrate patterns of IR of NGB and CYGB, we imaged similar parts of the retina. We did not observe any differences in patterns of IR of both NGB and CYGB in central vs peripheral retina. As illustrated in **Figure 2A** and B, NGB-IR in the retina was localized to the nerve fiber layer, ganglion cell layer, inner and outer nuclear layers, inner and outer plexiform layers, and photoreceptor inner segments. Cytoglobin IR was determined using a rabbit Ab generated against human recombinant CYGB protein. Cytoglobin IR was localized to the nerve fiber layer, ganglion cell layer, inner nuclear layer, and inner and outer plexiform layers (**Figure 2C**). Immunoreactivity for both NGB and CYGB was also detected in the retinal pigment epithelium, as shown in eFigure 2. In addition, no differences were observed in the labeling patterns of NGB or CYGB in the retinas that could be attributed to the age of the subjects. However, some variability in the labeling patterns of NGB or CYGB in the retinas

may be attributed to the quality and slight differences in timing of preparation of the human retinal specimens. eTable 2 summarizes the regional distribution of NGB and CYGB in human retina.

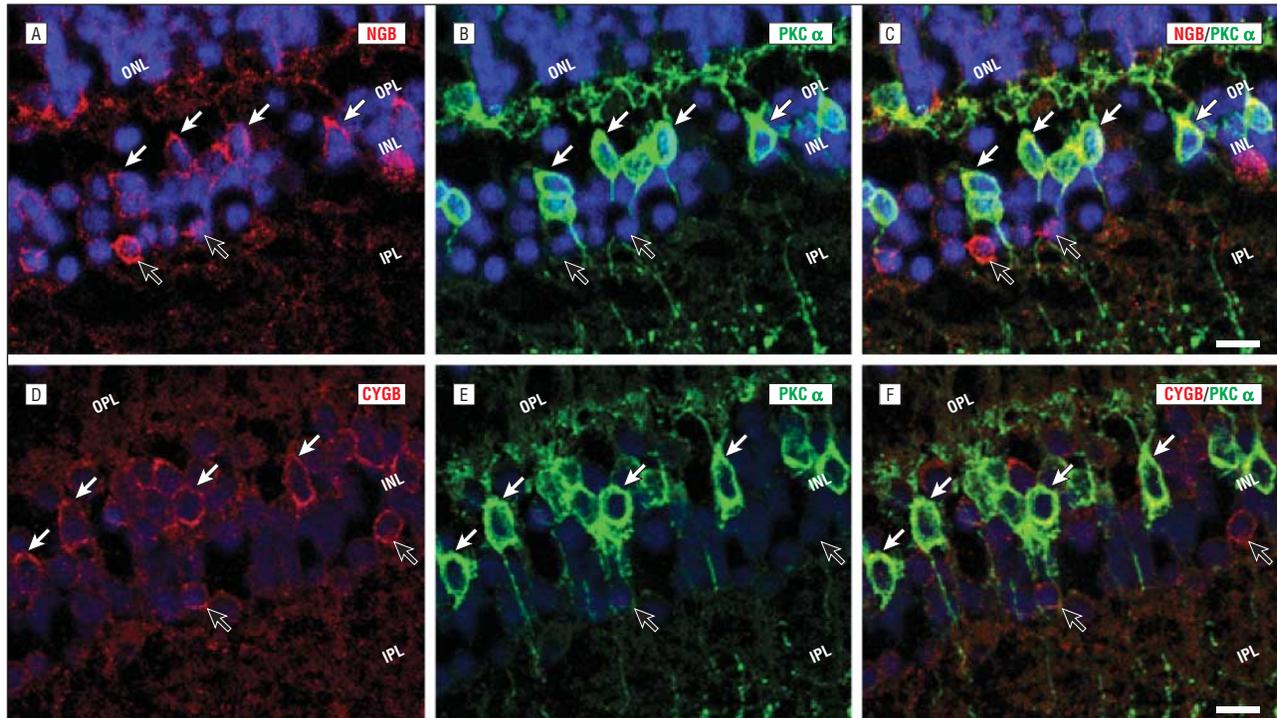
To determine if the cells' IR with the anti-NGB and anti-CYGB Abs were neurons, double labeling was performed with anti-microtubule-associated protein 2 (MAP-2) and TUJ1 antibodies. All cells in the ganglion cell layer and inner nuclear layer that were MAP-2 or class III  $\beta$ -tubulin (TUJ1) IR were also NGB- or CYGB-IR (**Figure 3**), thus confirming their neuronal identities. However, MAP-2 and TUJ1 are not ubiquitously expressed in mammalian retinal neurons. Therefore, we were not able to explicitly demonstrate that all NGB- and CYGB-IR cells are neurons.

To further investigate which cell types express NGB and CYGB within the inner nuclear layer, double labeling was performed with anti-protein kinase C alpha Ab. As illustrated in **Figure 4**, ON bipolar cell bodies containing protein kinase C alpha<sup>26</sup> were found to colocalize with NGB- and CYGB-IR. Furthermore, in addition to ON bipolar cells expressing NGB and CYGB, we observed NGB- and CYGB-IR OFF bipolar cells based on their laminar position in the inner nuclear layer. In addition, double labeling was performed with anti-NGB and anticalretinin, an amacrine cell marker in adult human retina.<sup>27</sup> Neuroglobin IR was observed to colocalize with calretinin-IR amacrine cells (eFigure 3). Moreover, we found that IR cells in the ganglion cell layer and the inner nuclear layer were double labeled for NGB (monoclonal Ab) and CYGB (polyclonal Ab) (**Figure 5**). Thus, NGB and CYGB are colocalized in retinal neurons but not photoreceptors. It is possible that some subpopulations of neurons express only 1 of the 2 proteins, rather than having ubiquitous coexpression.

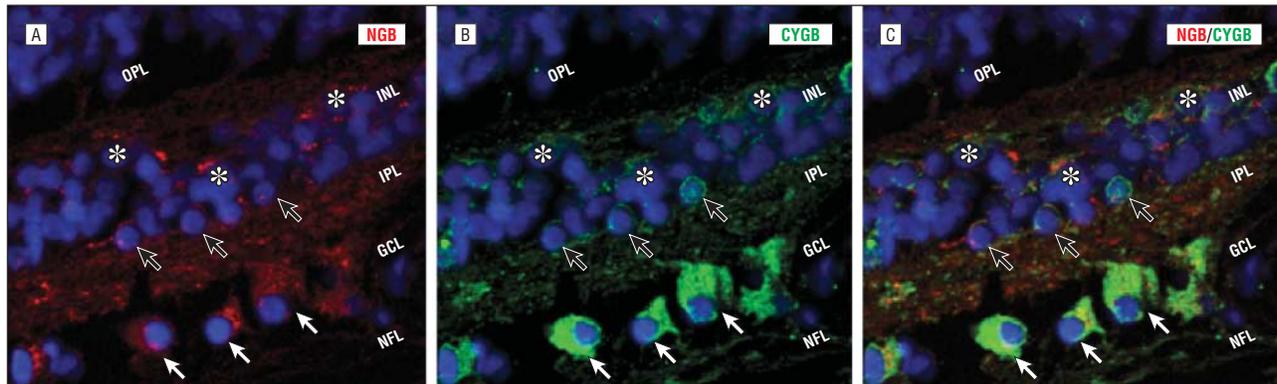
We investigated the possibility that NGB and CYGB are expressed in retinal glial cells in double-labeling studies using anti-glial fibrillary acid protein (labels astrocytes and reactive Müller glia) and antivimentin (labels astrocytes and Müller glia) Abs. We detected no colocalization of NGB or CYGB with glial fibrillary acid protein (data not shown) or vimentin Abs (**Figure 6**), thus confirming previous studies.<sup>28</sup>



**Figure 3.** Neuroglobin (NGB) and cytoglobin (CYGB) colocalize with microtubule-associated protein 2 (MAP-2)- and class III  $\beta$ -tubulin (TUJ1)-immunoreactive (IR) cells in the ganglion cell layer (GCL) and inner nuclear layer (INL). Images were captured using confocal microscopy. A-L, Neuroglobin IR (A) and CYGB-IR (D) (red) observed in ganglion cells and inner retinal neurons and MAP-2 labeling (green) (B and E). Merged images (C and F) confirmed neuronal localization of NGB and CYGB. Neuroglobin IR (G) and CYGB-IR (J) in the GCL and INL and TUJ1-IR (H and K) were found to colocalize in the merged images (I and L). White arrows point to examples of putative retinal ganglion cells, asterisks indicate examples of INL neurons, and black arrows point to examples of amacrine cells. Cell nuclei (blue) were labeled with DAPI (4',6-diamidino-2-phenylindole). Scale bars = 10  $\mu$ m. IPL indicates inner plexiform layer; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer.



**Figure 4.** Neuroglobin (NGB) and cytoglobin (CYGB) colocalized with protein kinase C alpha (PKC  $\alpha$ ) immunoreactive (IR) cells in the inner nuclear layer (INL). A-C, Neuroglobin IR (red) (A) and PKC  $\alpha$ -IR (green) (B) colocalized in bipolar cells as shown in the merged image (C). D-F, Cytoglobin IR (red) (D) and PKC  $\alpha$ -IR (green) (E) colocalized in bipolar cells as shown in the merged image (F). White arrows point to examples of bipolar cells, and black arrows point to examples of NGB- and CYGB-IR amacrine cells. Cell nuclei (blue) were labeled with DAPI (4',6-diamidino-2-phenylindole). Scale bars = 10  $\mu$ m. IPL indicates inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer.

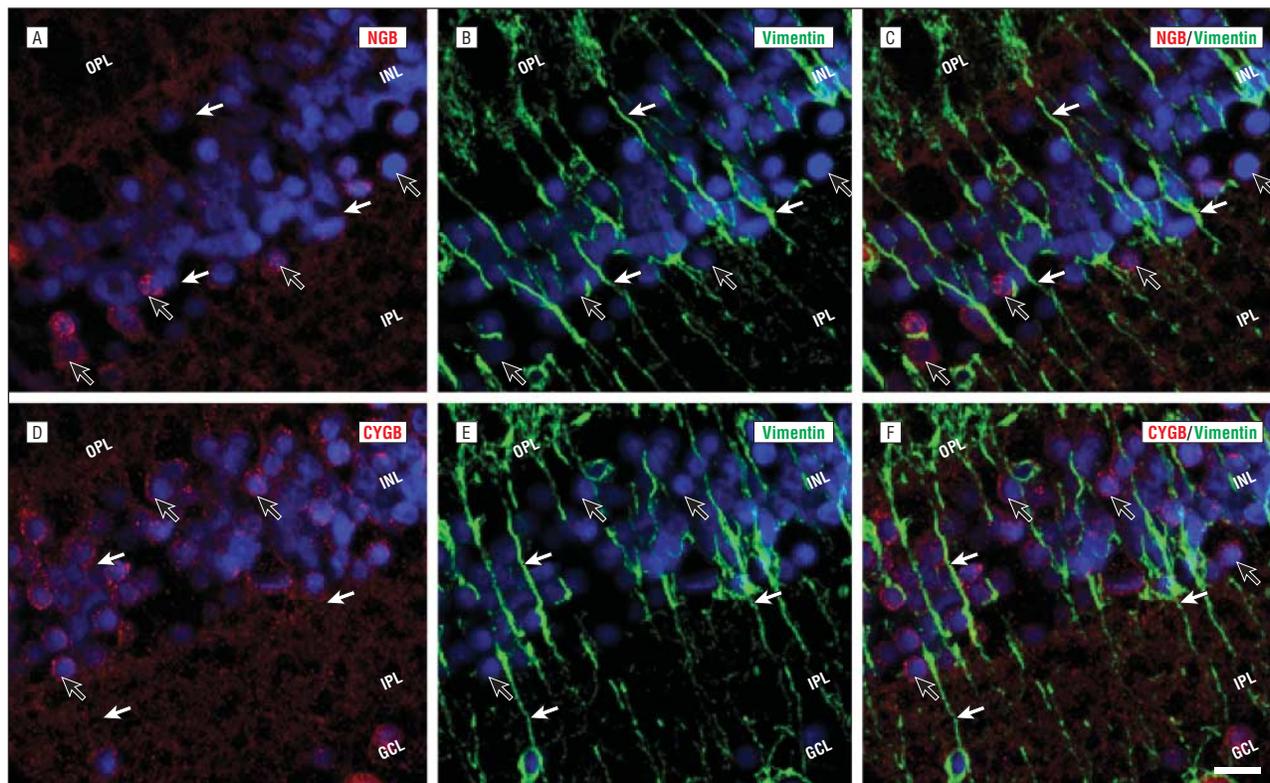


**Figure 5.** Neuroglobin (NGB) and cytoglobin (CYGB) immunoreactivity (IR) colocalized within neurons. A-C, Retina double labeled with NGB (red) (A) and CYGB (green) (B) displays colocalization of the proteins in the same neurons in the merged image (C). White arrows point to examples of presumptive retinal ganglion cells, asterisks indicate examples of inner nuclear layer (INL) neurons, and black arrows point to examples of amacrine cells. Cell nuclei (blue) were labeled with DAPI (4',6-diamidino-2-phenylindole). Scale bar = 10  $\mu$ m. IPL indicates inner plexiform layer; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer.

#### COMMENT

Oxygen is essential for retinal function,<sup>29</sup> but mechanisms of oxygen homeostasis in this highly metabolically active tissue remain poorly understood. Thus, this study aimed at investigating the presence and distribution patterns for NGB and CYGB, 2 oxygen-binding proteins, in human retina. Detection of recombinant NGB protein and NGB in the protein extract from human retina at approximately 17 kDa molecular weight is consistent with the molecular weight of human NGB. Also, detection of recombinant CYGB protein at approximately

21 kDa molecular weight is consistent with the molecular weight of human CYGB. The difference in the molecular weight between human recombinant CYGB (approximately 21 kDa) and CYGB detected in the protein extract from human retina (approximately 26 kDa) may be the result of posttranslational modification of the protein. To our knowledge, this study is the first to carefully examine and demonstrate colocalization of NGB and CYGB in human ganglion, amacrine, and bipolar cells by using double-labeling immunohistochemistry. The distribution of NGB and CYGB expression in human retina is consistent with the distribution pattern already de-



**Figure 6.** Neuroglobin (NGB) and cytoglobin (CYGB) did not colocalize with vimentin-immunoreactive (IR) cells. Images were captured using confocal microscopy. A-F, No colocalization of NGB (A) or CYGB (D) (red) with vimentin (green) (B and E) was detected on the merged images (C and F). Note also the absence of the radial pattern of IR characteristic for Müller glia in images A and D. White arrows point to examples of Müller glial radial processes, and black arrows point to examples of inner retinal neurons. Cell nuclei (blue) were labeled with DAPI (4',6-diamidino-2-phenylindole). Scale bar = 10  $\mu$ m. IPL indicates inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; GCL, ganglion cell layer.

scribed in other species (mice, rat, and dog).<sup>14,15,17,18</sup> Unfortunately, little is known about NGB and CYGB distribution patterns in disease conditions of the eye. The only study published so far that addressed that issue reported increased NGB-IR in advanced human glaucoma in photoreceptor inner segments and the plexiform layers as well as in the nuclear layers.<sup>16</sup> The absence of NGB- and CYGB-IR in glial cells, as well as the absence of CYGB-IR in the photoreceptor inner segments, has potentially important implications regarding the ability of these cell types to handle reactive oxygen species in neurodegenerative diseases of the retina.

Studies by Sun et al<sup>5,6,30</sup> provided important insight into the possible role of NGB in the pathology of the central nervous system. Neuroglobin expression was increased by neuronal hypoxia *in vitro* and focal cerebral ischemia *in vivo*,<sup>5</sup> and induction of NGB and its protective effect showed specificity for hypoxia over other stressors.<sup>5</sup> In addition, changes in NGB expression resulted in corresponding changes in severity of histological and functional deficits after focal cerebral ischemia.<sup>6</sup> Furthermore, in NGB-overexpressing transgenic mice, compared with wild-type littermates, the volume of cerebral infarcts after occlusion of the middle cerebral artery was reduced and the volume of myocardial infarcts produced by occlusion of the left anterior descending coronary artery was reduced.<sup>31</sup> Finally, NGB was prominently expressed in several areas of the brain that showed preferential vulnerability to neurodegenerative diseases, and NGB messenger RNA and protein levels in these areas de-

creased with aging.<sup>30</sup> Few studies have addressed CYGB function *in vivo*. A recent study demonstrated that CYGB is up-regulated in mouse brain, heart, liver, and muscle on hypoxia<sup>32</sup> and proposed that the mechanism of CYGB induction is regulated by hypoxia-inducible factor 1  $\alpha$ . Another study reported similar results: under a chronic hypoxic condition, the CYGB messenger RNA level was upregulated in rat heart and liver.<sup>33</sup> Furthermore, recent data have demonstrated a significant increase in hypoxia-inducible factor 1  $\alpha$  expression in glaucomatous human eyes.<sup>34</sup>

It has been proposed that NGB and CYGB might have roles in oxygen sensing and signal transduction<sup>35</sup> and/or free radical scavenging.<sup>36-38</sup> Possible mechanisms of NGB neuroprotection associated with oxygen-hypoxia sensing and signal transduction have been reviewed.<sup>39</sup> Furthermore, disparities between NGB and CYGB responses to hypoxia also suggest that these 2 globins may play divergent roles in the brain.<sup>40</sup> The existence of oxygen-binding molecules in retinal neurons may change the current dogma about oxygenation and hypoxic injury of the retina and optic nerve and is likely to facilitate the development of novel strategies for the treatment of hypoxia-related ocular diseases. Furthermore, identification of the functional properties of NGB and CYGB may significantly advance our understanding of retinal free radical scavenging in health and disease.

**Submitted for Publication:** September 15, 2007; final revision received February 26, 2008; accepted April 8, 2008.

**Correspondence:** Donald S. Sakaguchi, PhD, Department of Genetics, Development, and Cell Biology, 503 Science II, Iowa State University, Ames, IA 50011 (dssakagu@iastate.edu).

**Author Contributions:** Drs Grozdanić and Sakaguchi contributed equally to this article.

**Financial Disclosure:** None reported.

**Funding/Support:** This work was supported in part by The Glaucoma Foundation, National Institute of Neurological Disorders and Stroke grant NS44007, Carver Trust, Veterans Administration merit review grants (Rehabilitation Division), Iowa State University Biotechnology Foundation, and Research to Prevent Blindness.

**Additional Information:** The eTables and eFigures are available at <http://www.archophthalmol.com>.

**Additional Contributions:** Y. de Lathouder, MS, J. Orasky, MS, M. Harper, PhD, and M. Joksimović, PhD, provided technical assistance and D. Pospisilova, P. Kasperek, and J. Havlasek from BioVendor Laboratory Medicine, Inc provided the mouse monoclonal anti-NGB Ab and recombinant protein.

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