

2006

Neuroglobin and cytoglobin distribution patterns in human and canine eye

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Neuroglobin and cytoglobin distribution patterns in human and canine eye

by

Jelena (Marka) Ostojić

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-majors: Neuroscience; Veterinary Anatomy

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2006

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LIST OF ABBREVIATIONS

AB - anterior border layer

Abs – antibodies

AC - anterior chamber

BCIP/ NBT - 5,bromo-4-chloro-3 indolylphosphate/nitroblue tetrazolium

BM - Bowman's membrane

CEn - corneal endothelium

CEp - corneal epithelium

Ch – choroid

CM - ciliary muscle

CNS - central nervous system

CO - carbon monoxide

CRALBP - cellular retinaldehyde binding protein

CS - canal of Schlemm

Cygb – cytoglobin

DAPI - 4',6-diamino-2-phenylindole

DIC - differential interference contrast

DM - Descemet's membrane

GbE - eye-specific globin from chicken

GbX - globin X

GCL - ganglion cell layer

GDI - guanine nucleotide dissociation inhibitor

GFAP - glial fibrillary acidic protein

G $\alpha_{i/o}$ - α -subunit of heterotrimeric G protein

H₂O₂ - hydrogen peroxide

Hb – hemoglobin

HIF-1 α - hypoxia-inducible factor 1 alpha

HRE - hypoxia responsive element

ILM - inner limiting membrane

INL - inner nuclear layer

IPL - inner plexiform layer

IR – immunoreactivity

IR - iris root

LC - lens capsule

LE -lens epithelium

mAb - monoclonal antibody

MAP-2 - microtubule-associated protein 2

Mb – myoglobin

NE - nonpigmented epithelium

NFL - nerve fiber layer

Ngb – neuroglobin

nNOS - neuronal nitric oxide synthase

NO - nitric oxide

O₂ - oxygen

OLM - outer limiting membrane

ONL - outer nuclear layer

OPL - outer plexiform layer

OS - photoreceptor outer segments

P - pupillary border

pAb - polyclonal antibody

PC - posterior chamber

PE -pigmented epithelium

PKC α - protein kinase C alpha

PVDF - polyvinylidene difluoride

ROS - reactive oxygen species

RPE - retinal pigment epithelium

S - stroma

S – stroma

SDS-PAGE - SDS-polyacrylamide gel electrophoresis

SM - sphincter muscle

STAP - stellate cell activation-associated protein

T – tapetum

TM - trabecular meshwork

TUJ1 - class III β - tubulin

VEGF - vascular endothelial growth factor

ABSTRACT

Globins are a family of heme-containing proteins that reversibly bind gaseous ligands such as oxygen, nitric oxide and carbon monoxide. Neuroglobin (Ngb) and cytoglobin (Cygb) have been recently added to mammalian globin family. The general hypothesis of this dissertation was: The presence of Ngb and Cygb can be detected by biochemical and immunohistochemical methods in different structures of the eye.

The research was divided into three parts: 1) Determining Ngb and Cygb presence and distribution in different retinal cell types in healthy canine retina; 2) Determining Ngb and Cygb presence and distribution in different retinal cell types in healthy human retina; 3) Determining Ngb and Cygb presence and distribution in structures of the anterior eye segment of human and dog.

Ngb immunoreactivity (IR) in the canine retina was located in the ganglion cell layer (GCL), inner (INL) and outer nuclear layers (ONL), inner (IPL) and outer plexiform layers (OPL), photoreceptor inner segments (IS) and retinal pigment epithelium (RPE). Cygb-IR in the canine retina was found in the GCL, INL and ONL, IPL and OPL and RPE. Ngb and Cygb were expressed in the same cells in the GCL and INL. Distribution pattern of Ngb and Cygb in human retina was similar to distribution found in the canine retina.

Ngb and Cygb-IR in the human and canine anterior segment structures was detected in the corneal epithelium and endothelium. Furthermore, in the iris, Ngb and Cygb-IR was localized to the anterior border layer and the stroma, iridal sphincter and dilator muscle. In the iridocorneal angle, both Ngb and Cygb were detected in endothelial cells of the human and canine trabecular meshwork and canal of Schlemm in human. In the ciliary body, Ngb- and Cygb-IR was localized to the nonpigmented ciliary epithelium of the pars plana and pars

plicata, as well as in ciliary body musculature. Weak Ngb and Cygb-IR was detected in the lens epithelium. Ngb and Cygb distribution was consistent between human and canine anterior segments and was co-localized within the same cells in all structures.

Our studies are the first detailed description of Ngb and Cygb presence detected by immunohistochemical methods in different structures of the canine and human eye. Based on Ngb and Cygb localization and their previously reported biochemical features, we hypothesize that Ngb and Cygb may have important roles in scavenging reactive oxygen species and/or facilitating oxygen diffusion in the eye.

CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction

Globins are a family of small globular proteins containing a heme prosthetic group (Fe-protoporphyrin IX), by which they can reversibly bind gaseous ligands such as oxygen (O₂), nitric oxide (NO) and carbon monoxide (CO). Four mammalian globins have been described so far: hemoglobin (Hb), myoglobin (Mb), neuroglobin (Ngb) and cytoglobin (Cygb), Ngb and Cygb being recently discovered.

The importance of Ngb and Cygb has been suggested from studies spanning a wide array of research fields: Ngb was found to be neuroprotective during acute neuronal hypoxia^{1, 2}; increased levels of Ngb were detected in the cerebral cortex and serum after ischemia-reperfusion insults, and Ngb was detected in cerebrospinal fluid of patients with chronic pain³. Cygb was found to be liver fibrogenesis-related marker^{4, 5}, and is a novel candidate tumor suppressor gene in upper aero-digestive tract squamous cancer⁶. In addition, recent reports have demonstrated increased cytoprotection and survival of islets of Langerhans after transplantation when Ngb or Cygb were overexpressed^{7, 8}.

Recent studies have demonstrated the presence of Ngb and Cygb in mouse and rat retinas^{9, 10}, but no information about Ngb and Cygb presence in the retina of other species has thus far been published, nor their presence in other eye structures except retina. Since dogs are emerging as a viable large-animal model for study of ocular disorders^{11, 12}, especially due to the recent completion of their genome sequence (<http://www.ncbi.nih.gov/Genomes/>), this dissertation addressed the presence of Ngb and Cygb in canine and human eye. We hope that

this knowledge will advance our current insight into key features of Ngb and Cygb function in human ocular physiology and disease.

The research conducted was divided into three parts: **The first part** aimed to determine Ngb and Cygb presence and distribution in different retinal cell types in healthy canine retina. **The second part** aimed to determine Ngb and Cygb presence and distribution in different retinal cell types in healthy human retina. **The third part** aimed to determine Ngb and Cygb presence and distribution in structures of the anterior eye segment of human and dog.

The **general hypothesis** of the dissertation was: **The presence of neuroglobin and cytoglobin can be detected by biochemical and immunohistochemical methods in different structures of the eye; moreover, the distribution pattern of the two proteins in human and canine eye will be similar.**

A set of **focused hypotheses** was developed to address our general hypothesis:

1. Ngb and Cygb are present in different retinal cell types in healthy canine retina.
2. Ngb and Cygb are present in different retinal cell types in healthy human retina, and their distribution pattern is similar to the pattern described in dog.
3. Ngb and Cygb are present in structures of the anterior segment of the eye, and their distribution pattern is similar in human and dog.

A list of **specific aims** was defined in order to address the above hypotheses:

1. Characterize mouse monoclonal and rabbit polyclonal anti-Ngb antibody and rabbit polyclonal anti-Cygb antibody, which are to be employed in subsequent immunohistochemical studies, by using Western blot technique.

2. Determine Ngb and Cygb distribution in different retinal cell types in healthy canine retina by performing double-labeling immunohistochemistry with various cell markers.
3. Determine Ngb and Cygb distribution in different retinal cell types in healthy human retina by performing double-labeling immunohistochemistry with various cell markers.
4. Compare Ngb and Cygb distribution in different retinal cell types in healthy human and canine retina.
5. Determine Ngb and Cygb distribution in human and canine anterior eye structures by performing double-labeling immunohistochemistry.

1.2. Dissertation organization

The dissertation is organized in the alternative format, including three manuscripts that have been published or submitted for review. Each of the manuscripts is represented as a separate thesis chapter in the form prepared for publication:

1. Ostojić, J, Sakaguchi DS, de Lathouder Y, Hargrove MS, Trent III JT, Kwon YH, Kardon RH, Kuehn MH, Betts DM, Grozdanić S. Neuroglobin and Cytoglobin: Oxygen-Binding Proteins in Retinal Neurons. *Investigative Ophthalmology and Visual Science* 2006;47:1016–1023.
2. Ostojić, J, Syed NA, Hargrove MS, Trent III JT, Kuehn MH, Kwon YH, Kardon RH, Betts DM, Grozdanić S, Sakaguchi DS. Neuroglobin and Cytoglobin Distribution in the Human Retina: A Detailed Immunohistochemical Study. (Prepared for submission)

3. Ostojić, J, Grozdanić S, Syed NA, Hargrove MS, Trent III JT, Kuehn MH, Kardon RH, Kwon YH, Betts DM, Sakaguchi DS. Neuroglobin and Cytoglobin – Novel Candidates for Oxygen Mediated Metabolism in Anterior Eye Segment (Submitted)

In addition to three manuscript chapters, a chapter with general introduction and literature review, as well as a closing chapter with conclusions and recommendations for future research are included in the dissertation. References are listed at the end of each chapter.

1.3. Literature Review

Globins, small globular heme-containing proteins, typically consist of about 150 amino acids and are comprised of eight α -helical segments (named A-H)¹³. The main helices within the globin fold are organized into a two-layer structure, termed ‘three-over-three’ α -helical sandwich¹⁴ (Figure 1A). Globins contain a heme prosthetic group (Fe-protoporphyrin IX), by which they can reversibly bind gaseous ligands such as O₂, NO and CO. The heme group is located in a deep protein pocket, defined sideways by the E- and F-helices (distal and proximal to the heme, respectively), and by the G- and H-helices at the dead end of the pocket (Figure 1B)¹⁵. In hemoglobin and myoglobin, the heme-Fe atom is pentacoordinated by four pyrrole nitrogen atoms (within the heme plane) and the nitrogen atom at proximal HisF8 (the fifth coordination position). Binding of O₂, in hemoglobin and myoglobin, occurs on the distal side of a pentacoordinated heme, where O₂ establishes a sixth coordination bond to the heme Fe, while the main O₂ stabilizing interaction is provided by a hydrogen bond donated by distal residue HisE7 (Figure 1B)¹⁵. In neuroglobin and cytoglobin, the heme-Fe atom is hexacoordinated, where residue HisE7 is the sixth heme-Fe ligand both in the ferrous deoxygenated (Fe²⁺) and ferric (Fe³⁺) forms. Heme hexacoordination implies that O₂ (and

other gaseous ligands) compete with the endogenous HisE7 ligand for the sixth coordination position of the heme-Fe atom¹⁵.

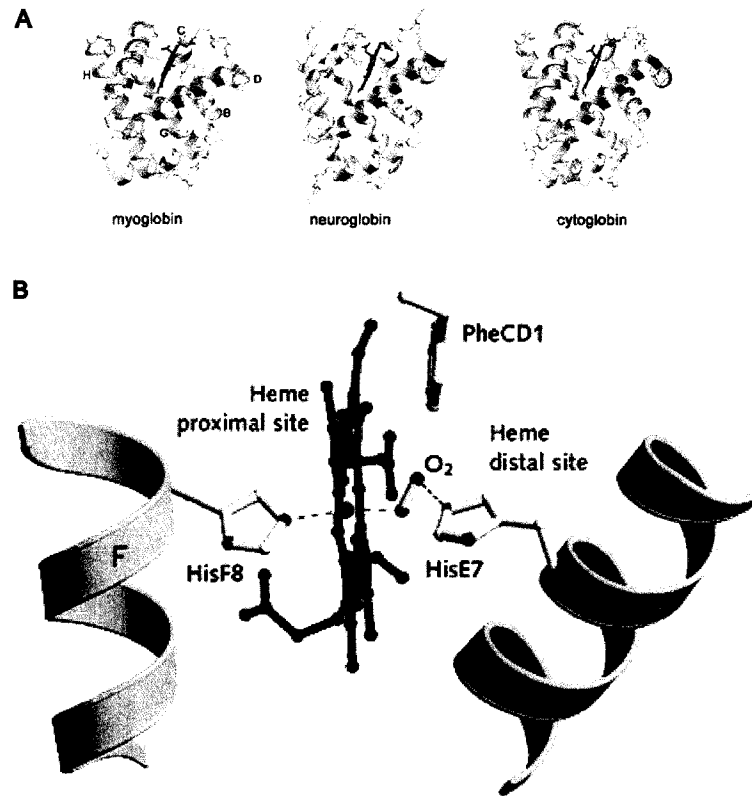


Figure 1. (A) 3-D protein structure of human myoglobin, neuroglobin and cytoglobin. In myoglobin, eight alpha helices are designated A-H. The globin fold is conserved in all three proteins (Adapted from Hankeln et al., 2005). (B) A view of the heme proximal and distal sites, defined by E- and F-helices, together with the key residues PheCD1, HisE7 and HisF8 and the heme O₂ ligand. The Fe coordination bonds with the proximal HisF8 residue and the O₂ ligand are indicated by red dashed lines, while the hydrogen bond between O₂ and the distal HisE7 residue is indicated in blue (Adapted from Pesce et al., 2002).

Globins are phylogenetically ancient molecules, found in virtually all kingdoms of organisms, including eubacteria, unicellular eukaryotes, plants, and animals¹⁶. As globins are being found in more and more distantly related species, the estimated existence of the last common ancestral globin gene before invertebrate/vertebrate divergence moves further back

to at least 670 million years ago¹⁷. Six vertebrate globins have been identified so far: hemoglobin (Hb), myoglobin (Mb), neuroglobin (Ngb), cytoglobin (Cygb), globin X (GbX) and eye-specific globin from chicken (GbE)^{15, 18-23}.

1.3.1. Hemoglobin

Normal adult Hb (MW 64 500), found in the blood, is a heterotetramer of two α -globin and two β -globin polypeptides, with a heme tightly bound to a pocket in each globin monomer¹⁵. Interactions between the α - and β -globin subunits lead to the cooperative binding of O₂ to this heterotetramer, thus binding of one O₂ molecule to deoxyHb increases the O₂ affinity of the remaining binding sites on the same Hb molecule. This cooperativity during oxygen transport allows Hb to pick up O₂ readily in the lungs and to unload it efficiently in the peripheral respiring tissues¹⁶. In addition to O₂ homeostasis, Hb also interacts with NO homeostasis by oxidative inactivation of NO, binding of NO to deoxygenated heme moieties and formation of S-nitrosothiols²⁴. The amino acid sequences of the α -globins and β -globins in Hb are about 50% identical, regardless of which vertebrate species is the source, and are about equally divergent from the monomeric myoglobin, indicating that these two genes are descended from a common ancestor about 450 million years ago²⁵.

1.3.2. Myoglobin

Myoglobin, an intracellular heme protein, found in the cytoplasm of vertebrate type I and IIa skeletal and cardiac muscle tissue, is a monomer consisting of a polypeptide chain of 153 amino acid residues (MW 16 700)²⁶. Mb lacks the cooperativity of the blood Hb, but has the

classical globin fold and a heme prosthetic group¹⁵. While in mammals Mb is a short-term O₂ reservoir in exercising skeletal muscle and in the beating heart, in diving mammals, whose Mb concentration is higher 10- to 30-fold, Mb serves for the extension of diving time when pulmonary ventilation ceases^{27, 28}. Also, it has been proposed that Mb facilitates intracellular delivery of O₂, through a process termed “facilitated O₂ diffusion”²⁹. This process, which makes a critical link between capillary O₂ supply and O₂-consuming cytochromes within mitochondria, starts when Mb adjacent to the sarcoplasm of the cardiac myocyte or of red skeletal muscle fibers picks up O₂, traverses the cytosol by translational diffusion to unload O₂ in the vicinity of mitochondria, and finally diffuses back to the cell membrane in the deoxygenated state²⁹. An important prerequisite for Mb to serve this function is its high concentration in tissues; Mb content in the heart is close to 200-300 μmol/kg wet mass tissue, and may reach 400-500 μmol/kg wet mass in skeletal muscles²⁹. Furthermore, it has been reported that oxyMb plays a pivotal role in controlling the level of NO within the cell; by converting endogenous NO to the innocuous nitrate with concomitant formation of ferric Mb, which is recycled through the action of intracellular metMb reductase³⁰. Since NO is a potent but reversible inhibitor of cytochrome oxidase³¹, as well as vasodilator, continuous removal of NO by oxyMb controls both the rate of capillary oxygen delivery to the cell and the rate of oxygen utilization, thus determining the dose-response curve of the NO effects on coronary blood flow and cardiac contractility³⁰. In addition, since lack of Mb leads to increased vulnerability of the heart when challenged by oxidative stress, it has been proposed recently that Mb may be regarded as a molecular radical scavenger protecting against transient rises of cytosolic reactive oxygen species (ROS) produced after short periods of ischemia, thereby complementing the known muscular antioxidant defense mechanisms³².

Interestingly, a very recent study reported that, in a hypoxia-tolerant fish model, Mb is also expressed in a range of other tissues, including liver, gill and brain³³. Moreover, a second, unique Mb isoform, distinct from Ngb and not upregulated by environmental hypoxia, was found to be expressed exclusively in neural tissue³³. This discovery suggested that Mb may play a much wider role than previously understood.

1.3.3. Globin X

The identification of globin X gene in fish and amphibians was recently reported²². Globin X sequences obtained from the zebrafish (*Danio rerio*), the goldfish (*Carassius auratus*), the pufferfish (*Tetraodon nigroviridis*), and the clawed frog (*Silurana tropicalis*), were reported to be distinct from vertebrate hemoglobins, myoglobins, neuroglobins, and cytoglobins. While globin X, whose function is presently unknown, displayed the highest identity with Ngb (~26% to 35%), RT-PCR experiments on goldfish RNA from various tissues demonstrated that it was not a neuronal protein²². The distal ligand-binding and the proximal heme-binding histidines (E7 and F8), as well as the conserved phenylalanine CD1 were found to be present in the globin X sequence, but because of extensions at the N-terminal and C-terminal, the globin X protein seemed to be longer than the typical eight α -helical globins, comprising of about 200 amino acids. Furthermore, phylogenetic analysis confirmed an ancient evolutionary relationship of globin X with Ngb and suggested that the gene for this protein has been lost in the evolution of higher vertebrates²².

1.3.4. An eye-specific chicken globin (globin E)

The gene encoding eye-specific globin from chicken (GbE) was discovered in the systematic database searches of the chicken genome sequence, with no apparent orthologous gene in the genomes of fish or mammals²³. The predicted coding sequence of this globin covered 456 base pairs, resulting in a protein of 151 amino acids with a MW of 17.2 kDa. Amino acid sequence comparison with the sequences of other vertebrate globins demonstrated the presence of a conserved globin fold and the key residues important for oxygen-binding (the proximal and distal histidines in position E7 and F8, as well as the phenylalanine at CD1). Although the function of GbE is currently unknown, computer predictions indicated that this globin does not contain any signal peptide and is most likely localized in the cytoplasm. This globin appeared to be distantly related to Cygb (35% identity at the amino acid level), and phylogenetic inference suggested that it had diverged from the vertebrate Cygb lineage some 420 million years ago³⁴. Taking into account the apparent early evolutionary origin of GbE, the absence of any orthologous gene in the genomes of fish or mammals could be caused either by independent gene loss events, or by Cygb gene duplication only within the avian lineage²³. An additional argument supporting the possibility that the common presence of Cygb and GbE in chicken eye tissue resulted from gene duplication was a similar finding in the case of duplicated fish Cygb genes³⁵.

1.3.5. Neuroglobin

Neuroglobin has been identified as a 151 amino acid (17 kDa) protein distantly related to vertebrate myoglobins (< 21% amino-acid identity) and hemoglobins (< 25% amino-acid identity)¹⁸. Intracellular nerve myoglobin of the annelid worm *Aphrodite aculeate* shares the

highest similarity with Ngb (30% amino-acid identity)¹⁸. Ngb presence has been detected in human, mouse, rat, chicken, zebrafish and pufferfish brain^{18, 23, 36-41}, as well as in mouse, chicken and zebrafish eyes^{9, 10, 23, 40}. Recent double-staining experiments of primary hippocampal cultures and mouse brain confirmed that neuroglobin is present exclusively in neurons, but not in astroglial cells⁴². Interestingly, Ngb has also been detected in non-neural cells, such as β cells of the islets of Langerhans in pancreas⁴³, spermatogonia and spermatocytes in testes, endocrine cells of the anterior pituitary, medulla and glomerular zone of the cortex of the adrenal gland³⁶, and it remains to be determined what role Ngb might have in these particular tissues. A series of *in vitro* and *in vivo* experiments^{1, 2, 44, 45} has provided very important information about Ngb-mediated neuroprotection:

a) Ngb expression was increased by neuronal hypoxia *in vitro* and focal cerebral ischemia *in vivo*¹.

b) Both induction of Ngb and its protective effect showed specificity for hypoxia over other stressors (staurosporine and the NO donor sodium nitroprusside), suggesting the specific involvement of hypoxia-signaling pathways in Ngb induction¹.

c) Neuronal survival of cultured cortical neurons after hypoxia was reduced by inhibiting Ngb expression and enhanced by Ngb overexpression. However, Ngb could not protect neurons from the NO-mediated neuronal death after sodium nitroprusside (potent NO donor) exposure, and increased Ngb expression did not cause increase in extracellular oxygen consumption¹.

d) Hemin (the ferric chloride salt of heme) and hypoxia regulated Ngb expression by different mechanisms (protein kinase G and MAPK pathway, respectively)⁴⁴.

e) Changes in Ngb expression resulted in corresponding changes in the severity of histological and functional deficits after focal cerebral ischemia in the rat (Ngb antisense oligodeoxynucleotide exacerbated focal cerebral ischemia and overexpression of Ngb reduced ischemic cerebral injury)².

f) Ngb was prominently expressed in several areas of the brain that showed preferential vulnerability to neurodegenerative diseases (frontal, parietal and temporal cortex, hippocampus, caudate-putamen and cerebellum). Ngb mRNA and protein levels in these areas decreased with aging⁴⁵.

While the functions of hemoglobin and myoglobin have been extensively studied, the roles that Ngb could play in the organism are still uncertain¹⁸⁻²¹, and are grouped around three possible functions:

1. Role in facilitated O₂ diffusion to the mitochondria. Ngb concentration in the mouse retina was reported to be in the range of 50-100 μM, and thus about 100 times higher than in total brain extracts¹⁰. Since such concentration might be considered to be in the range of Mb concentration in muscle, and Mb is facilitating O₂ diffusion in muscle tissue, the finding was taken as indirect evidence for Ngb role in facilitated O₂ diffusion. Furthermore, the same investigators reported divergent distribution of Ngb in vascular (mouse and rat) and avascular (guinea pig) retina⁴⁶. In avascular retina, where O₂ is delivered only by the choroidal vascular bed, both Ngb and high concentration of mitochondria were localized only in the inner segments of the photoreceptors, in close proximity to the blood supply. In vascular retina, where O₂ is delivered by a dual blood supply, both Ngb and high concentration of mitochondria were localized to inner segments of the photoreceptors, outer and inner plexiform layers and ganglion cell layer, which are all in close proximity to the blood supply.

This divergent distribution of Ngb in avascular and vascular retina was used as additional indirect evidence that Ngb is a respiratory protein that supplies O₂ to the respiratory chain⁴⁶.

However, it has been recently demonstrated⁴⁷ that the O₂ affinity of human Ngb was in the lower end of the range of values for O₂-binding heme proteins, with a P_{50} value of 7.5 torr at 37 °C and a neutral pH, while Mb for example had a P_{50} of 2-3 torr²⁹. This finding implied that only a small fraction of Ngb in nervous tissue would be saturated with O₂ under normal conditions, and was not supportive of a role for Ngb as a significant source of the O₂ reserve in the retina.

2. Role in scavenging reactive oxygen and nitrogen species. Following hypoxia, the levels of NO and ROS such as superoxide increases. A major fate of NO produced during hypoxia is that it reacts with superoxide, forming peroxynitrite, a potent oxidizing and nitrating agent, whose role in cellular damage during brain hypoxia has been described⁴⁸. It has been shown recently that in the Fe²⁺ - NO form Ngb reacts more rapidly with peroxynitrite than Hb does⁴⁹, and this scavenging property may protect the neurons and contribute to their survival following hypoxic episodes. In addition, the reaction of met(Fe³⁺) Ngb with peroxynitrite or hydrogen peroxide does not appear to generate cytotoxic ferryl (Fe⁴⁺) species, which is another feature that may contribute to cellular survival⁴⁹. A role of Ngb in the metabolism of NO is further supported by the reported cellular localization of neuronal nitric oxide synthase (nNOS) in the human and rat retina⁵⁰. Neuronal NOS has been localized to the inner segments of the photoreceptors, cells in the inner nuclear and ganglion cell layer, layers where Ngb has also been found. However, as recently described, NO scavenging by Ngb in the neurons (similar to Mb in the heart) could be of physiological value only if an efficient metNgb reductase system existed to restore reduced Ngb⁵¹.

3. Role in intracellular signaling during hypoxic conditions. Since the binding affinity of Ngb to O₂ and NO is similar, Ngb binding to a ligand will depend only on the relative concentrations of the two gasses in the tissue⁵¹. In an hypoxic environment containing both O₂ and NO at comparable concentrations, the formation of NgbO₂ is very dynamic, because NgbO₂ reacts very rapidly with free NO, and the half-life of this reaction is milliseconds even at NO concentrations in the micromolar range, yielding as products NO₃⁻ and metNgb⁵¹. It has been demonstrated that metNgb interacts specifically with a component of GDP/GTP signal transduction pathway⁵². MetNgb can bind to the GDP-bound form of the α -subunit of heterotrimeric G protein (G $\alpha_{i/o}$) and act as a guanine nucleotide dissociation inhibitor (GDI) by inhibiting the rate of exchange of GDP for GTP on G $\alpha_{i/o}$ ^{52, 53}. The interaction of metNgb with GDP-bound G $\alpha_{i/o}$ keeps G $\alpha_{i/o}$ in its inactive state and liberates G $\beta\gamma$, leading to protection against neuronal death⁵⁴. However, as noted earlier, in order to be physiologically relevant, this pathway demands the presence of an efficient metNgb reductase, which is yet to be discovered⁵¹. In addition, Ngb was found to interact with flotillin-1 and cystatin C^{55, 56}. Since flotillin-1, a lipid raft microdomain-associated protein, has been shown to recruit signaling proteins to lipid rafts, it has been hypothesized that it might recruit Ngb to lipid rafts as a means of preventing neuronal death⁵³. Cystatin C, a cysteine proteinase inhibitor, was found to prevent neuronal death after severe ischemic damage to the brain by regulating cysteine protease activities^{57, 58}. In the retina, cystatin C has been identified in cones, horizontal, bipolar, amacrine and ganglion cells⁵⁹. Under conditions of oxidative stress, it was hypothesized that interaction between Ngb and cystatin C might protect neurons against apoptosis⁵⁶.

1.3.6. Cytoglobin

Cytoglobin has been identified for the first time in a proteome study that showed upregulation of this protein in rat stellate cells of fibrotic liver and was thus termed a stellate cell activation-associated protein (STAP)⁶⁰. Subsequently, Cygb has been identified as a 190 amino acid (20.9 kDa) protein in virtually all human, mouse and zebrafish tissues^{20,21} and because of its ubiquitous expression was termed cytoglobin^{20,21}. Cygb shares less than 30% identity with Mb and Hb²⁰ and phylogenetic analysis suggested that Cygb had a common ancestor with vertebrate Mb²¹. Cygb has been found in the cytoplasm of hepatic stellate cells⁶⁰, connective tissue fibroblasts, chondroblasts and osteoblasts⁶¹ and splanchnic fibroblast-like cells⁴. and retinal neurons⁹. In addition, Cygb has been found in subpopulations of CNS and retinal neurons^{9,61}. Very few studies addressed Cygb function *in vivo*. A recent study demonstrated that Cygb is upregulated in mouse brain, heart, liver and muscle upon hypoxia⁶², and proposed that the mechanism of Cygb induction is regulated by hypoxia-inducible factor 1 α (HIF-1), since:

- a) Cygb upregulation is dependent on the severity of hypoxia.
- b) The regulation of Cygb expression in HIF-1 (+/-) knockout mice is affected.
- c) The variations of the expression regulation are in the same manner as seen in the expression of VEGF (vascular endothelial growth factor) that is proven to be regulated by HIF-1 α pathway.
- d) Cygb promoter region contains hypoxia responsive element (HRE) sites.

Another study by a different group of investigators reported similar results: under chronic hypoxic condition, Cygb mRNA level has been upregulated in rat heart and liver⁶¹. It has been proposed that Cygb role is linked to hydroxylation of proline residues during collagen

synthesis⁶¹ and organ fibrogenesis⁴, although some other possible functions such as facilitation of oxygen transport, NO detoxification and peroxidase activity could not be excluded^{20, 21, 60}. Cygb O₂ affinity and measured P₅₀ values lie within the range 0.7-1.8 torr calculated from kinetic experiments²⁰ which is similar to Mgb oxygen affinity. Also, O₂ binding to Cygb is cooperative, which could permit greater O₂ loading and unloading within a narrow range of low O₂ tensions⁴⁷, thus implying that the *in vivo* function of Cygb could relate to reversible O₂ binding.

1.3.7. Invertebrate Nerve Globins

The presence of globins in neuronal tissue has been reported in different invertebrates subjected to intermittent O₂ supply, especially in cyclic-ventilating animals and gut parasites⁶³⁻⁶⁵. The concentration of invertebrate nerve globins was often found to be very high (for example 5.9 mM in bivalve *Tellina alternata*), and these globins were located in glial cells, with the exception of globin from gastropod *Aplysia deplians*, which was found exclusively in neurons⁶⁶. Based on heme iron coordination, some of invertebrate nerve globins have been classified as pentacoordinated (such as the globins from gastropod *Aplysia spec*, polychaete annelid *A. aculeata* and nemertean *Cerebratulus lacteus*), and some as hexacoordinated (such as the globins of the bivalves *T. alternata* and *Spisula solidissima*)⁶⁶.

Many studies focused on the functional significance of heme bound O₂ as a vital O₂ store in these invertebrates. In the minute gastrotrichan *Neodasys* the nerve globin concentration sufficed for 17 min of O₂ consumption by an active animal under anaerobic conditions⁶⁷. A comparative electrophysiological study of several bivalve species with and without globins in their nerve tissues demonstrated that tissue with nerve globins consumed much less O₂

during the process of action potential conduction⁶⁸⁻⁷¹. In addition, O₂ bound to the nerve tissue globins in the clams *Tellina alternata* and *Spisula solidissima* and nemertean worm *Cerebratulus lacteus* were shown to support the O₂ requirements of the nerves for up to 30 min during anoxic periods^{68, 70, 72}. These reports suggested that the presence of invertebrate nerve globins, which play a role as an O₂ store, is a highly preserved evolutionary mechanism for enabling a continuous nerve activity during periods of prolonged anoxic conditions⁶⁶.

Figure 2 summarizes phylogenetic relationships between vertebrate globins discovered so far.

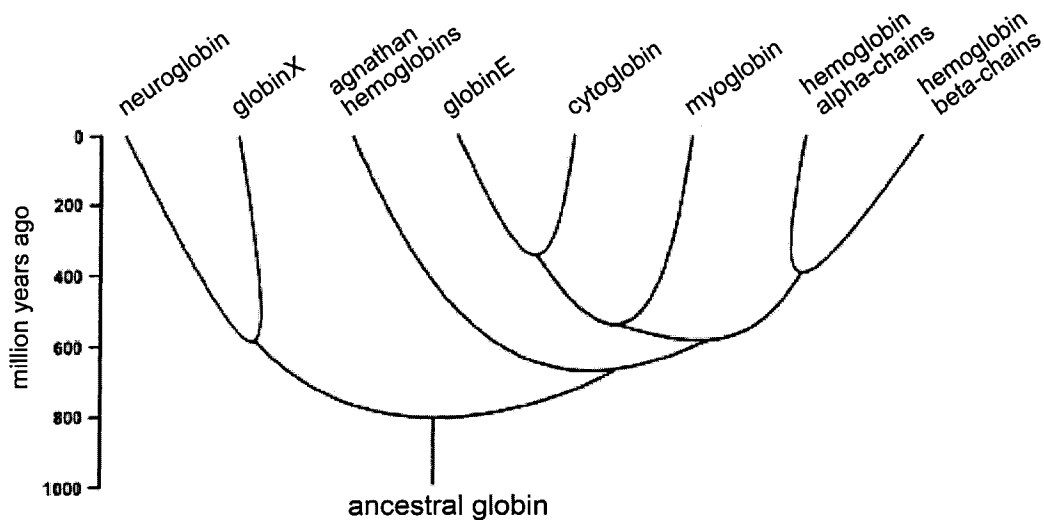


Figure 2. Simplified phylogenetic tree of vertebrate globins. (Adapted from Burmester et al., 2004).

1.3.8. Anatomy and histology of the mammalian eye

Mammalian eye is composed of three basic layers, the outermost fibrous tunic (cornea and sclera), middle uvea (choroid, ciliary body and iris), and innermost nervous coat (neural retina and optic nerve, ciliary body epithelium and iris epithelium)⁷³. Topographically, the

eye can also be divided into anterior segment, which is comprised of the cornea, lens, iris, ciliary body, and highly specialized tissue at the iridocorneal angle and posterior segment, comprised of retina, choroid and sclera. Figure 3 illustrates major anatomical structures of the human eye.

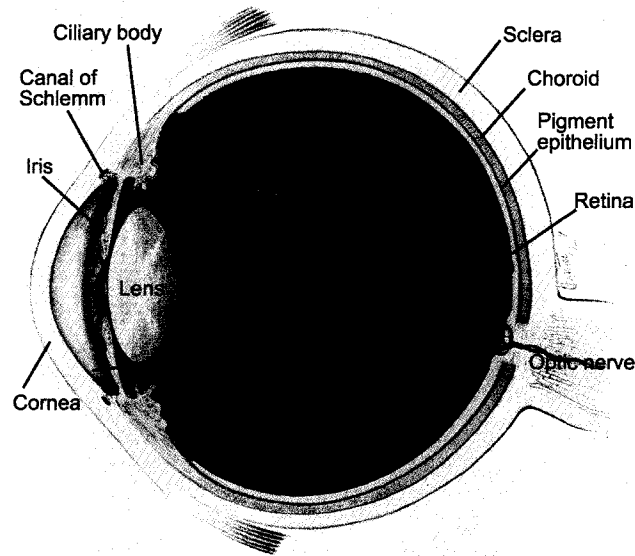


Figure 3. Diagram of the human eye. Adapted from: National Eye Institute, National Institutes of Health, Ref#: NEA04.

The fibrous tunic structures, the cornea and sclera, provide constant shape and form for the eye; in addition, incoming light passes through the transparent cornea before being focused onto the neural retina. The human cornea can be divided into six layers: tear film, epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium⁷⁴.

The main function of the uvea, which consists of choroid, ciliary body, and iris, is to provide nourishment for the eye and modify light reflection and scatter. The ciliary body is a structure that is subdivided into six layers: the suprachoroidal space, the ciliary muscle, the layer of vessels, the external basement membrane, epithelium (pigmented and non-

pigmented), and internal basement membrane⁷⁵. In all vertebrates, intraocular pressure, which keeps the eye's firm shape, is maintained by the balance between aqueous humor production and outflow. Aqueous humor is being produced by ciliary body epithelial cells, and its drainage has been regulated by the trabecular meshwork, situated at the iridocorneal angle overlying Schlemm's canal⁷³. Ciliary muscle is composed of smooth muscle fibers and in human is highly developed, having three components: radial, meridional and circular. Topographically, the ciliary body is divided into pars plicata (an anterior portion consisting of a ring of ciliary processes) and pars plana (posterior flat part). The iris, a circular diaphragm separating anterior and posterior chamber, can be divided into anterior uveal portion (consisting of anterior border layer and stroma) and posterior retinal portion (consisting of iris sphincter and dilator muscle and iris pigment epithelium)⁷⁵.

The neural retina is a laminar structure, whose function is to receive light stimuli from the external environment, transduce these stimuli into a nervous impulse and transmit them to the brain via the optic nerve. Histologically, the retina can be divided into: retinal pigment epithelium, photoreceptor layer, outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and inner limiting membrane (Figure 4). Since light must travel through all the layers of the neural retina before reaching the photoreceptors, the vertebrate retina is said to be "inverted"⁷⁶.

The retinal pigment epithelium, the outermost layer of the retina, is a monolayer of polygonal densely pigmented cells which reduce light scattering by absorbing light not captured by photoreceptors. In addition, the retinal pigment epithelium cells transport nutrients from the choriocapillars to the outer layers of the retina and phagocytize

photoreceptor outer segments. Some species, including dog, possess an adaptation of the choroid called a tapetum lucidum, which increases their sensitivity to light. The tapetum is a mirror-like layer behind the retinal pigment epithelium which reflects photons that were not captured by the photoreceptors back, thus giving the receptors a "second chance" to capture them. Retinal pigment epithelium overlying tapetum is devoid of melanin granules⁷⁶.

The photoreceptor layer contains the inner and outer segments of rod and cone photoreceptors. Outer segments of both the rod and cone photoreceptors arise from outpouching of the photoreceptor cell plasma membrane, and are connected to the inner segments by the cilium. Outer segments contain photopigment molecules, located in free-floating disks in rods or in folded membrane layers in cones, which absorb individual photons and thus initiate phototransduction cascade. Photoreceptor inner segments contain organelles, such as mitochondria, ribosomes, endoplasmic reticulum, Golgi apparatus, and are the major site of protein synthesis⁷⁶.

The outer limiting membrane is a thin membrane which coincides with the base of inner segments of photoreceptors, and is composed of the densities of the cell junction that attaches inner segments to Müller glia cells and the Müller cells to each other⁷⁶.

The outer nuclear layer contains the nuclei of photoreceptors; cone cell bodies situated in a single row right below the outer limiting membrane and rod cell bodies making up the remainder of the outer nuclear layer below the cone cell bodies⁷⁶.

The outer plexiform layer contains bipolar cell, horizontal cell and photoreceptor synapses. If the term outer plexiform layer is used for the entire zone between outer and inner nuclear layer, it has to be emphasized that it is truly plexiform only in its inner third; its outer

two-thirds, known as Henle fiber layer, are composed of axonal extensions of the photoreceptors enveloped by Müller glial cells⁷⁶.

The inner nuclear layer contains bipolar, amacrine, and horizontal cell bodies. In human retina eleven different bipolar cell types have been described based on their morphology; cones make synapses with ten types, and rods with one type of bipolar cells. Amacrine cells of the vertebrate retina are interneurons that integrate and modulate the signals presented to the ganglion cells; more than twenty different morphological subtypes of amacrine cell have been described in human retina. Horizontal cells are second order neurons interconnecting photoreceptors laterally across the plane of the outer plexiform layer; they can be both pre- and post-synaptic to photoreceptors. Two morphological types of horizontal cells have been described in the vertebrate retina: axonless and with axons⁷⁶.

The inner plexiform layer contains the synapses made between bipolar, amacrine and ganglion cells. Since inner plexiform layer can be subdivided into five sublayers into which amacrine, bipolar and ganglion cells synapse, all cell types can also be classified by the stratum or strata in which their dendrites or axons are located⁷⁶.

The ganglion cell layer comprises the cell bodies and axons of ganglion cells, as well as of some displaced amacrine cells. Ganglion cells collect the electrical messages concerning the visual signal from the two layers of nerve cells preceding it in the retinal wiring scheme. There are at least 18 different morphological types of ganglion cell in the human retina⁷⁶.

The nerve fiber layer is made up of axons of retinal ganglion cells which transmit information to the brain. Inner limiting membrane is the boundary between the vitreous humor in the posterior chamber and the retina itself. Glial cell types found in the human retina are Müller cells, astroglia and microglia. Müller cell bodies are located in the inner

nuclear layer and project to the outer and inner limiting membrane. Astrocytes, whose cell bodies and processes are almost entirely restricted to the nerve fiber layer, originate from the brain; they migrated into the developing retina along the optic stalk. Microglia, found in every layer of the retina, are of mesodermal origin; they entered the retina during development with the mesenchymal precursors of retinal blood vessels⁷⁶.

Figure 4 of the canine dorsal retina overlying tapetum lucidum illustrates laminar retinal organization present in all vertebrates.

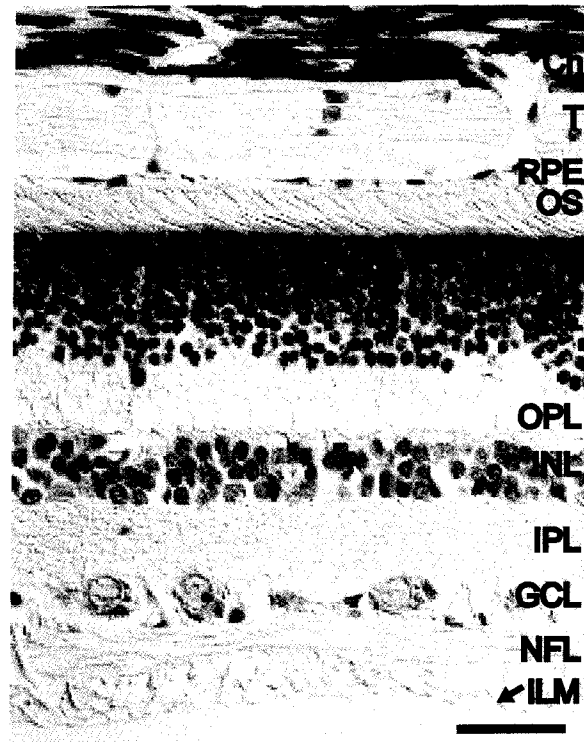


Figure 4. Hematoxylin stained canine retina. Abbreviations: Ch, choroid; T, tapetum; RPE, retinal pigment epithelium; OS, photoreceptor outer segments; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer; ILM, inner limiting membrane. Scale bar 20 μm .

1.3.9. O₂ and NO in the retina

Since Ngb and Cygb reversibly bind gaseous ligands such as O₂ and NO, and Cygb has been shown to have peroxidase activity, it is possible that the function of these two proteins in the eye can be related to O₂ transport and NO/free radical scavenging. Thus, understanding the current knowledge about O₂ and NO homeostasis in the eye is an important prerequisite in investigating the potential role of Ngb and Cygb.

Blood is supplied to the human retina via a dual circulatory system: the posterior ciliary arteries in the choroid that supply O₂ to the outer portion of the retina, and via retinal vessels (branches of the central retinal artery) that supply O₂ to the inner portion of the retina⁷⁷. The retina is one of the highest O₂ consuming tissues in our body⁷⁸⁻⁸⁰, and as such requires a continuous and sufficient supply of O₂. Inadequate O₂ supply to the retina, in conditions such as ischemic optic neuropathies, retinopathy of prematurity, diabetic retinopathy and numerous proliferative retinopathies, result in severe visual deficits secondary to retinal neuronal death^{81, 82}. Also, vascular and ischemic disorders of the retina and optic nerve head are a common cause of visual loss in the middle-aged and elderly population⁸³. Normal-tension glaucoma can often be preceded by decreased ocular blood perfusion and ischemia⁸⁴; in fact, decreased optic nerve blood flow in human patients was correlated with functional and morphologic measures of glaucomatous progression⁸⁵, and treatments designed to improve ocular blood flow have been shown to benefit glaucoma patients⁸⁶. It has been reported recently that hypoxia-inducible factor 1 α (HIF-1 α) increased in human glaucomatous retinas and optic nerve heads compared to age-matched controls⁸⁷, supporting arguments that tissue hypoxia is present in the retina and optic nerve head of glaucomatous eyes⁸⁸⁻⁹⁰.

In vivo measurements of O₂ consumption in rats have shown that under light-adapted conditions, O₂ uptake by the inner retina (primarily the inner plexiform layer) exceeds the O₂ uptake of the outer retina⁹¹. It has been proposed that this high O₂ demand of the inner retina presumably renders it more vulnerable to the hypoxic insult⁹¹. Under the dark-adapted conditions, the highest O₂ consumption in the retina occurs in the photoreceptor inner segments⁹². Thus, it has been proposed that high levels of O₂ consumption appear to be correlated with the distribution of mitochondria in the retina, the highest being in both plexiform layers and in photoreceptor inner segments⁴⁶.

Nitric oxide (NO) in the retina is formed by three isoforms of nitric oxide synthase: NOS-1 (brain NOS), NOS-2 (inducible NOS), and NOS-3 (endothelial NOS). While NOS-1 and NOS-3 are constitutive enzymes, NOS-2 makes excessive quantities of NO, which can become cytotoxic⁹³. In both human and rat retina, NOS-1 was detected in the inner segments of photoreceptors, cells in the inner nuclear layer, particularly amacrine cells, nerve fibers of the outer and inner plexiform layers and retinal ganglion cells^{94, 95}. In the rat optic nerve, NOS-1 was constitutively present in astrocytes, pericytes and nerve terminals in the walls of the central artery, and NOS-3 was detected in the vascular endothelia of large and small vessels. While NOS-2 was not present in eyes with normal intraocular pressure, it has been detected in reactive astrocytes and microglia from the eyes with chronic elevated intraocular pressure⁹⁵. It has been hypothesized that NOS-2 may contribute to the neurotoxicity of the retinal ganglion cells⁹⁵⁻⁹⁷. Similar findings confirming that retinal glial cells are the major source of NO production under hypoxic conditions were also reported for the *in vitro* conditions⁹⁸. Furthermore, iNOS mRNA was found to be upregulated in non-neuronal cells of the inner retina during reperfusion following transient retinal ischemia, and

it was suggested that NO produced by iNOS may mediate retinal ischemia-reperfusion injury⁹⁹.

1.3.10. O₂, NO and ROS in the anterior segment of the eye

The diverse and highly specialized structures of the anterior segment of the mammalian eye, comprised of the ciliary body, iris, iridocorneal angle, cornea and lens, serve two main functions: focusing incoming light to the retina and regulating intraocular pressure. The cornea and lens, both avascular tissues, are transparent and have the ability to refract light, the iris protects the retina from excess light, while the ciliary body secretes aqueous humor. In humans, aqueous humor leaves the eye by passing through the trabecular meshwork into the canal of Schlemm and from there into the venous system. The trabecular meshwork creates resistance to the flow of aqueous humor, and thus generates intraocular pressure¹⁰⁰. In order to function properly, the anterior segment of the eye requires a sufficient supply of O₂¹⁰¹⁻¹⁰⁴, the existence of mechanisms for ROS scavenging¹⁰⁵⁻¹⁰⁷, and the ability to regulate levels of NO¹⁰⁸⁻¹¹².

Blood supply to anterior eye segment is dual: via the major arterial circle of the iris formed by branches of the two long posterior ciliary arteries and via a second arterial circle derived from branches of the anterior ciliary arteries (termed intramuscular circle)¹¹³. The vascular system related to the main arterial circle can be further divided into iris vasculature, vasculature of the ciliary processes of the pars plicata and vasculature of the pars plana of the ciliary body, while intramuscular circle supplies mainly the ciliary muscle and the limbal region¹¹³. While O₂ homeostasis in the anterior segment has not been studied in great detail^{103, 104}, the existing reports of O₂ partial pressure measurements in the anterior chamber

point out that O_2 is supplied to the aqueous humor at the anterior iris surface¹⁰³. Oxygen supply to avascular tissues of the cornea and lens is thought to be accomplished from aqueous humor or diffusion from the corneal surface^{102, 103, 114, 115}.

Diverse nitric oxide synthase (NOS) activity was reported in the anterior eye segment structures under both physiological and pathological conditions¹⁰⁹. Under physiological conditions, NO was localized in the cornea, lens epithelium, ciliary body and trabecular meshwork¹⁰⁹. Moreover, NOS activity was detected in corneal epithelium, stromal fibroblasts and endothelium during experimental inflammation^{109, 111}, and in iris due to endotoxin-induced uveitis¹⁰⁹. In addition, it has been shown that apoptosis of corneal endothelium after corneal grafting is mediated through NO¹¹⁶. Furthermore, it has been reported that NO acts as a vasodilator to regulate aqueous production in the ciliary body^{117, 118}, and can influence aqueous outflow in the trabecular meshwork and ciliary muscle^{112, 117, 119}.

The oxidants in the cornea originate from two main sources: cornea absorbs most of the ultraviolet light that enters the eye (and thus is the site for the generation of the ROS caused by UVB rays), and is exposed to hydrogen peroxide (H_2O_2) from the corneal endothelial side¹²⁰. Hydrogen peroxide is present in tissues of the anterior uvea and in mammalian aqueous humor at concentrations between 30 and 70 μM ¹⁰⁵. In order to protect against oxidative stress, both corneal epithelium and endothelium are rich in antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase¹²⁰. In the lens, oxidative damage from ROS produced by UV radiation has been regarded as the main factor in the development of age-onset cataracts, and the lens is also rich in antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase¹²¹. In addition, it has been proposed that an accessory function of mitochondria in the lens is preserving the lens core

proteins by keeping them in a hypoxic state¹¹⁴. ROS and oxidative stress have also been associated with ocular inflammation of the anterior segment¹¹⁰, as well as with the morphologic and physiologic alterations in the aqueous humor outflow in aging and glaucoma¹²², and catalase has been found in the aqueous humor, trabecular meshwork, and ciliary body¹⁰⁵.

Current information about mechanisms of oxygen and nitric oxide metabolism in the eye will help to establish a framework from which to design rational future studies to investigate proposed roles of Ngb and Cygb as facilitators of O₂ diffusion and NO/free radical scavengers in the retina and anterior eye segment.

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CHAPTER 2: Neuroglobin and Cytoglobin: Oxygen-Binding Proteins in Retinal Neurons

Published in Investigative Ophthalmology and Visual Science, 2006;47:1016–1023

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2.1. Abstract

Purpose. The goal of this study was to describe the detailed localization of the novel oxygen-binding molecules, neuroglobin (Ngb) and cytoglobin (Cygb), in mammalian retinas and to determine whether Ngb and Cygb are neuronal or glial proteins in the retina.

Methods. Antibodies directed against Ngb and Cygb were used to examine their patterns of distribution in normal canine retinas. Immunoblot analysis was performed to verify antibody specificity and the presence of Ngb and Cygb in canine tissues. Double-labeling

immunohistochemistry was performed with the Ngb and Cygb antibodies along with antibodies against neuronal (MAP-2, class III β -tubulin (TUJ1), PKC α , and calretinin) and glial antigens (vimentin and CRALBP). Tissue sections were analyzed with light and confocal microscopy.

Results. Ngb and Cygb proteins were observed in different retinal cells. Cygb (but not Ngb) was also present in canine kidney, liver, lung, and heart tissue. Immunohistochemical analysis of canine retinas demonstrated Ngb immunoreactivity (IR) in the ganglion cell layer (GCL), inner (INL) and outer (ONL) nuclear layers, inner (IPL) and outer plexiform (OPL) layers, photoreceptor inner segments (IS), and retinal pigment epithelium (RPE). Ngb IR was localized within retinal neurons, but not in glia. Cygb IR was found in neurons and their processes in the GCL, IPL, INL, and OPL and within the RPE, but not in glia.

Conclusions. Ngb and Cygb are widely distributed in retinal neurons and RPE, but not in glial cells of the canine retina. Their structure and distribution is suggestive of a possible role in oxygen transport in the mammalian retina.

2.2. Introduction

Visual processing in the retina has considerable oxygen demands, which makes the retina one of the highest oxygen-consuming tissues in the human body.¹⁻³ Continuous supply of sufficient O₂ to the retina is a fundamental physiological need, since even transient O₂ deficits can produce irreversible cellular damage.⁴ Vascular and ischemic disorders of the retina and optic nerve head are a common cause of visual loss in the middle-aged and elderly population.⁵ Furthermore, retinal hypoxia is considered to be an important factor contributing

to many retinal diseases.⁶⁻⁸ However, the mechanisms of oxygen homeostasis in the retina remain poorly understood.

Globins are a family of heme-containing proteins that reversibly bind oxygen and they have been described in bacteria, fungi, protists, plants, and animals.⁹ Four mammalian globins have been identified so far (hemoglobin [Hb], myoglobin, neuroglobin [Ngb], and cytoglobin [Cygb]).¹⁰⁻¹⁴ Hb is localized in erythrocytes and has a major role in oxygen transport between the lungs and other tissues via the circulatory system.⁹ Myoglobin is localized in the cytoplasm of skeletal and cardiac muscle, acts in intracellular oxygen storage, and enhances oxygen diffusion to the mitochondria for use in oxidative phosphorylation.¹⁵ In addition, Hb and myoglobin can act as scavengers of bioactive nitric oxide.¹⁶ Ngb and Cygb are two recently described members of the globin family with functions that are still not completely understood.^{10,12,13}

Ngb has been identified as a 17-kDa protein distantly related to vertebrate myoglobins (<21% amino-acid identity) and Hbs (<25% amino acid identity).¹⁰ Ngb expression has been found in human, mouse, rat, chicken, zebrafish, and pufferfish brain,^{10,17-23} as well as in human (Grozđanić et al. *IOVS* 2004; 45:ARVO E-Abstract 2586), mouse, chicken, and zebrafish eyes.^{21,22,24} Some of the proposed functions for Ngb include enhancement of oxygen delivery to mitochondria,^{22,24-26} detoxification of NO,^{19,27-29} and hypoxia sensing.³⁰⁻³² A potentially neuroprotective role of Ngb was suggested from studies demonstrating increased Ngb expression in vivo and in vitro during acute neuronal hypoxia and enhanced survival of cortical neurons by Ngb overexpression in vivo and in vitro.^{33,34} Moreover, it has been shown in rats that Ngb is prominently expressed in several areas of the brain that show preferential vulnerability to neurodegenerative diseases and that Ngb mRNA

and protein levels in these areas decrease with aging.³⁵ Cygb has been identified as a 20.9-kDa protein in virtually all human, mouse, and zebrafish tissues.^{12,13} Cygb expression has been observed in the cytoplasm of splanchnic fibroblast-like cells³⁶⁻³⁸ and also in subpopulations of central nervous system (CNS) and retinal neurons.^{37,39} Because of their heme-based structure and oxygen-binding properties, Ngb and Cygb probably serve as oxygen transport molecules and/or mediators of intracellular signaling during hypoxic conditions.^{14,32,40,41}

In this study, Western blot analysis was used to verify the specificity of the anti-Ngb and -Cygb antibodies, and these antibodies were then used to investigate for the presence of Ngb and Cygb in mammalian (canine) tissues. Immunohistochemical procedures were used to describe cell-specific histologic localization of Ngb and Cygb in the canine retina. Both Ngb and Cygb were present in retinal ganglion cells and inner retinal neurons of the canine retina, which are particularly sensitive to ischemic damage. Furthermore, both Ngb and Cygb were present in the retinal pigment epithelium, and Ngb was detected in photoreceptors, which makes these proteins potential candidates for facilitating oxygen metabolism in the outer retina.

2.3 Materials and Methods

Canine Tissue

Eyes, liver, kidney, lung, and heart tissue from nine healthy adult beagles (2–4 years of age) was collected immediately after euthanasia. Before euthanasia, all eyes were examined for signs of ocular abnormalities (slit lamp examination, fundus examination) and the presence of elevated intraocular pressure (tonometry). Eyes with detectable abnormalities

of the anterior segment, lens, or the fundus and/or the presence of elevated intraocular pressure (>25 mm Hg) were not collected for use in this study. Globes were fixed for 12 hours at 4°C in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). After fixation, globes were embedded in paraffin, and sections were cut at 7- μ m thickness. All research conducted in this study was in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Iowa State University Committee on Animal Care regulations.

Antibodies

Full-length human Ngf and Cx36 recombinant proteins were synthesized and purified as described previously.^{11,12} Molecular weights of the recombinant proteins were determined by matrix-assisted desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Polyclonal antisera against synthesized Ngf or Cx36 proteins were raised in rabbits and antibodies (Abs) were affinity purified from the serum using the recombinant proteins coupled to a column (SulfoLink; Pierce Biotechnology, Rockford, IL). Mouse monoclonal anti-Ngf antibody and the human recombinant Ngf protein used to produce this antibody were kindly provided by BioVendor Laboratory Medicine, Inc. Primary Abs used in this study and their dilutions are summarized in Supplementary Table S1 online at <http://www.iovs.org/cgi/content/full/47/3/1016/DC1>.

Immunohistochemistry

Fluorescent immunohistochemistry was performed as a modification of a previously described procedure.⁴² Briefly, tissue sections were deparaffinized, rehydrated in a graded alcohol series, and incubated for 2 hours in blocking solution. Sections were double-labeled with primary Ab cocktail overnight and incubated in one of the following secondary Ab

cocktails: donkey anti-mouse biotinylated Ab (Jackson ImmunoResearch, West Grove, PA) and goat anti-rabbit Alexa 488 Ab (Molecular Probes, Eugene, OR); goat anti-rabbit biotinylated Ab (Vector Laboratories, Burlingame, CA) and goat anti-mouse Alexa 488 Ab (Molecular Probes); goat anti-mouse Cy5 (Jackson ImmunoResearch) and goat anti-rabbit Alexa 488 Ab (Molecular Probes); and goat antirabbit Cy5 (Jackson ImmunoResearch) and goat anti-mouse Alexa 488 Ab (Molecular Probes). After a 2-hour incubation, sections were washed in potassium phosphate-buffered saline (KPBS) with Triton X-100. If a biotinylated secondary Ab was used, sections were subsequently incubated with streptavidin Cy3 (Jackson ImmunoResearch) and washed in KPBS. Finally, sections were counterstained with 1 µg/mL of 4',6-diamino-2-phenylindole (DAPI; Molecular Probes), washed in KPBS and coverslipped.

For peroxidase immunohistochemistry, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide solution in KPBS. Sections were processed for antigen retrieval, incubated in blocking solution, and stored overnight at room temperature in primary Ab solution. Sections were incubated with biotinylated secondary Ab and then with horseradish peroxidase-avidin-biotin complex (Vector Elite ABC Kit; Vector Laboratories) according to the manufacturer's instructions. To visualize the antibody staining pattern, tissue was exposed to a substrate kit for peroxidase (NovaRed; Vector Laboratories). Sections were dehydrated through a graded ethanol series, cleared with xylene, and coverslipped.

Negative controls were run in parallel during all processing and included the omission of the primary Ab, secondary Ab or preadsorption of the primary Ab (mouse and rabbit anti-Ngb and rabbit anti-Cygb) with excess recombinant proteins.

Analysis of Tissue Sections

Canine tissue sections labeled with fluorescent antibodies were visualized and images captured using a confocal scanning laser microscope (TCS-NT; Leica Microsystems Inc., Exton, PA). A color digital camera (Sony DXC-S500; Labtek, Campbell, CA) was used for the bright-field images in Supplementary Figure S2. Sections stained with the red substrate (NovaRed; Vector Laboratories) were examined with an upright microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc, Thornwood, NY), and images were captured with a color camera (AxioCam MRc; Carl Zeiss Meditec, Inc.). All figures were prepared on computer (Photoshop ver. 7.0; Adobe, San Jose, CA, and Freehand ver. 10.0; Macromedia, San Francisco, CA).

Western Blot Analysis

Characterization of antigens was performed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot procedures. For SDS-PAGE, canine tissue samples and recombinant proteins were homogenized in SDS-reducing buffer. Approximately 25 µg of total protein from retina, kidney, liver, and heart tissue extracts, and approximately 50 µg of total protein from lung tissue extract were separated in 14% gels using a vertical system (Mini-Protean III; Bio-Rad, Hercules, CA). After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF; BioRad) membrane in transfer buffer and incubated in blocking buffer for 1 hour at room temperature. The membrane was incubated in rabbit polyclonal anti-Cygb Ab (1:1000), rabbit polyclonal anti-Ngb Ab (1:1000), or mouse monoclonal anti-Ngb Ab (1:1000), followed by incubation in alkaline phosphatase conjugated goat anti-rabbit Ab (Promega, Madison, WI) or goat anti-mouse Ab (1:7500), respectively. Immunoreactive (IR) bands were visualized with 5-bromo-

4-chloro-3 indolylphosphate (BCIP)/nitroblue tetrazolium (NBT) alkaline phosphatase color development reagents (Promega). Molecular weights were estimated by comparison with prestained molecular weight standards (Bio-Rad).

For detection of Ngb in canine tissue samples, samples were homogenized in SDS-reducing buffer and approximately 25 µg of total protein in the retina, kidney, liver, and heart tissue extracts and approximately 50 µg of total protein in the lung tissue extract was separated in 14% gels. After electrophoresis, proteins were renatured for 1 hour in SDS gel by incubating the gel in 50 mM Tris buffer (pH 7.4), containing 20% glycerol.⁴³ Proteins were then transferred to a PVDF membrane in native transfer buffer, 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 anti-rabbit Ab was used at 1:7500 dilution. Chemiluminescence (Lumi-Phos WB Chemiluminescent Substrate; Pierce Biotechnology, Inc., Rockford, IL) was used to visualize immunolabeled bands.

2.4. Results

Distribution of Ngb and Cygb in Canine Tissues

Mouse monoclonal and rabbit polyclonal anti-Ngb Abs detected recombinant Ngb protein at ~17 kDa (Fig. 1A) under reducing conditions. However, only the polyclonal anti-Ngb Ab was capable of detecting native Ngb in the protein extract from the canine retina (Fig. 1B). No specific bands were detected in protein extracts from canine kidney, liver, lung, and heart when examined under the same conditions (data not shown). The second band at ~34 kDa probably represents a stable dimer, as also reported by Schmidt et al.²⁴ Rabbit polyclonal anti-Cygb Ab detected specific Cygb protein at ~21 kDa (Fig. 1C). Anti-Cygb Ab

also detected Cygb in protein extracts from canine retina, kidney, liver, lung, and heart at ~29 kDa. The second bands at approximately double the molecular mass probably represent a dimer, as also reported by Schmidt et al.^{37,39}

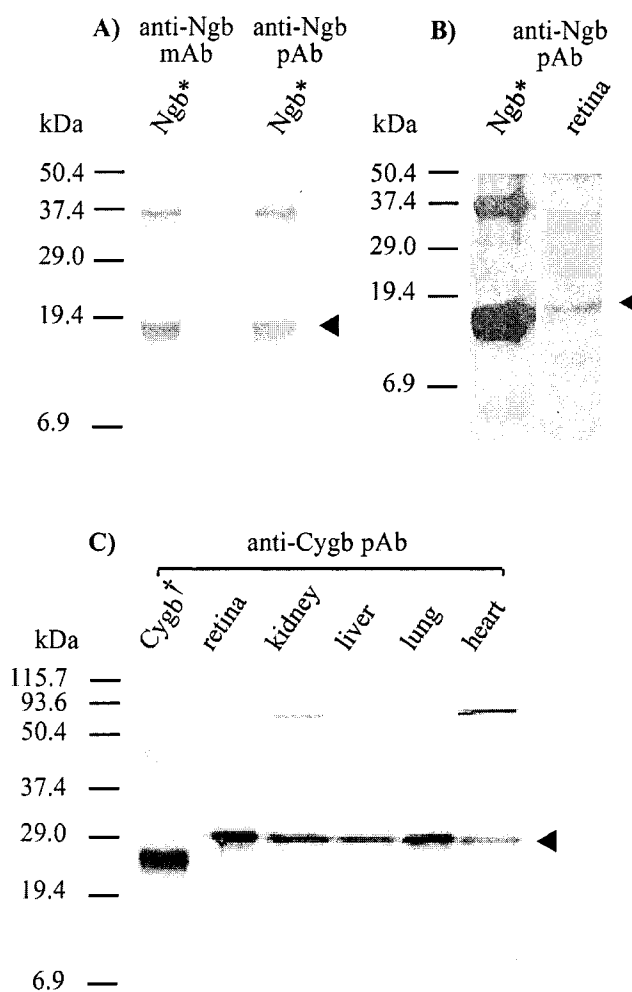


Figure 1. Western blot analysis of Ngb and Cygb expression in canine tissues. (A) Mouse monoclonal anti-Ngb Ab (mAb) and rabbit polyclonal anti-Ngb Ab (pAb) detected recombinant Ngb protein at ~17 kDa. Approximately 0.08 μ g of the recombinant protein was loaded per lane. (B) Rabbit polyclonal anti-Ngb Ab detected Ngb in the canine retina. Approximately 0.003 μ g of the recombinant Ngb was applied as the positive control. (C) Rabbit polyclonal anti-Cygb Ab detected specific recombinant Cygb protein at ~21 kDa and Cygb in canine retina, kidney, liver, lung, and heart at ~29 kDa. Approximately 0.02 μ g of the recombinant protein was applied per lane. *Left:* molecular mass markers.

Retinal Localization of Ngb and Cygb

To examine the expression pattern of Ngb in canine retina, we used the mouse monoclonal and rabbit polyclonal anti-Ngb Abs. Both Abs displayed similar labeling patterns, and were further used in double-labeling studies. As illustrated in Figures 2A and 2B, Ngb IR in the retina was localized to the ganglion cell layer, inner and outer nuclear layers, inner and outer plexiform layers, and photoreceptor inner segments (IS). Cygb expression was determined using a rabbit polyclonal antibody. As shown in Figure 2C, Cygb IR in the retina was localized to the ganglion cell layer, the inner nuclear layer (INL), and inner (IPL) and outer (OPL) plexiform layers. Ngb and Cygb IR was also observed in the retinal pigment epithelium (Figs. 2A–C). To verify the fluorescent immunolocalization patterns for Ngb and Cygb within the retina, we also performed peroxidase immunolabeling studies (Figs. 2D–F) with the same Ngb and Cygb primary antibodies. Both methods revealed similar patterns of labeling. To verify Ab specificity, each Ab was preadsorbed with their respective recombinant protein before incubation on tissue sections, and no specific staining was observed (Supplementary Fig. S1, <http://www.iovs.org/cgi/content/full/47/3/1016/DC1>). In addition, negative control studies were performed in parallel by omission of primary or secondary antibodies. No antibody labeling was observed in these control experiments (data not shown).

To discriminate antibody labeling from autofluorescence in the retinal pigment epithelium (RPE), which can be due to the presence of lipofuscin or oxidized melanin,^{44,45} confocal as well as bright-field images were taken from nonpigmented RPE in the tapetal retina (Supplementary Fig. S2, <http://www.iovs.org/cgi/content/full/47/3/1016/DC1>).

Confocal images of tissue sections single-labeled with primary antibody and appropriate Cy5 conjugated secondary antibody were captured at both 633/645-nm (red channel) and 488/525-nm (green channel) excitation/emission wavelengths, respectively. Cy5 is excited at 633 nm and thus, any signal from the 488/525-nm channel is considered background autofluorescence. This comparison would reveal lipofuscin autofluorescence if present (Supplementary Fig. S2). To investigate whether retinal neurons express both Ngf and Cygf, we performed double labeling with mouse monoclonal anti-Ngf Ab and rabbit polyclonal anti-Cygf Ab and found that the two proteins are expressed in all IR cells in the ganglion cell layer (GCL) and in the inner nuclear layer (INL). (Figs. 2G–I). In addition, extensive localization of both proteins was observed in the IPL and OPL.

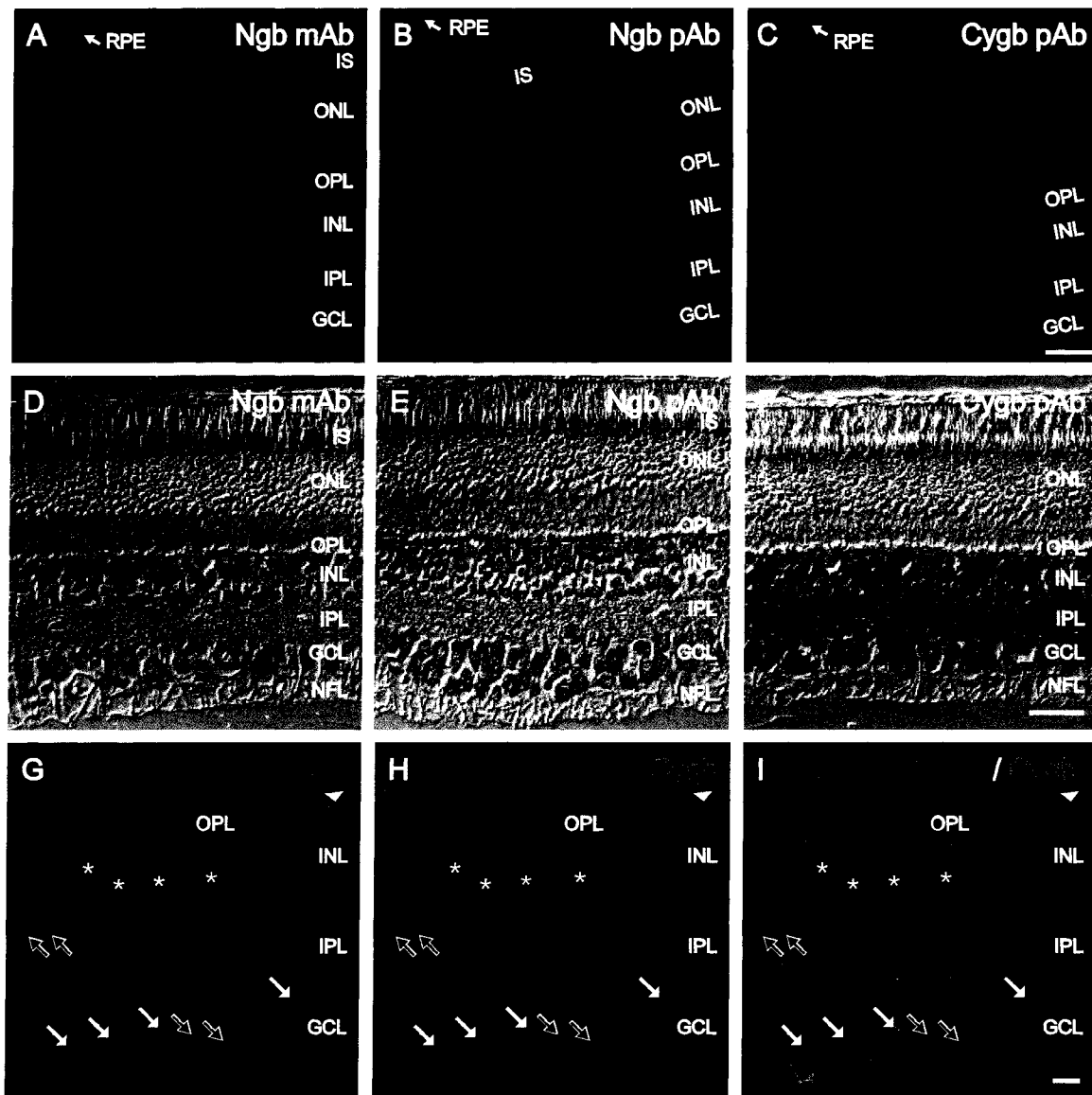


Figure 2. Ngb and Cygb immunolocalization in the canine retina. Confocal images illustrating patterns of IR for (A) anti-Ngb mouse monoclonal antibody (mAb), (B) anti-Ngb rabbit polyclonal antibody (pAb), and (C) anti-Cygb rabbit polyclonal antibody. Both anti-Ngb antibodies showed similar patterns of labeling in the retina (compare A and B). Differential interference contrast (DIC) images of Ngb and Cygb immunolocalization in canine central retina of (D) anti-Ngb mouse monoclonal antibody, (E) anti-Ngb rabbit polyclonal antibody, and (F) anti-Cygb rabbit polyclonal antibody. Confocal images of the retina double-labeled for (G) Ngb (red) and (H) Cygb (green) displays IR of both proteins in the same neurons, as illustrated in the merged image (I). *White arrows*: examples of retinal ganglion cells; *open arrows*: examples of cells that are presumably amacrine cells; *asterisks*: bipolar cells; *arrowhead*: example of a horizontal cell. Cell nuclei (*blue*) were labeled with DAPI. Scale bar: (A–C) 25 μ m; (D–F) 20 μ m; (G–I) 10 μ m. Abbreviations the same for all figures: RPE, retinal pigment epithelium; IS, inner segments; ONL, outer nuclear layer; OPL,

outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer.

Localization of Ngb and Cygb in Neurons

Double labeling was performed with anti-Ngb and anti-Cygb antibodies with anti-MAP-2 and TUJ1 antibodies to investigate whether the cells expressing Ngb and Cygb also express these neuronal markers. Cells in the GCL were identified as α , β , and γ ganglion cells or displaced amacrine cells, based on their morphology and relative size using parameters described by others.⁴⁶ α -cells are polygonal in shape and have the largest somata—21 to 44 μm in diameter. β -cells have a more globular cell body and medium-sized somata—14 to 30 μm , and γ cells have small somata, which makes them hardly distinguishable from displaced amacrine cells.⁴⁶ Cells in the INL were identified as amacrine, bipolar, or horizontal, based on their laminar position within the INL. All cells in the GCL and INL that expressed the neuronal proteins MAP-2 and class III β -tubulin (TUJ1 IR) were also Ngb- or Cygb IR (Fig. 3).

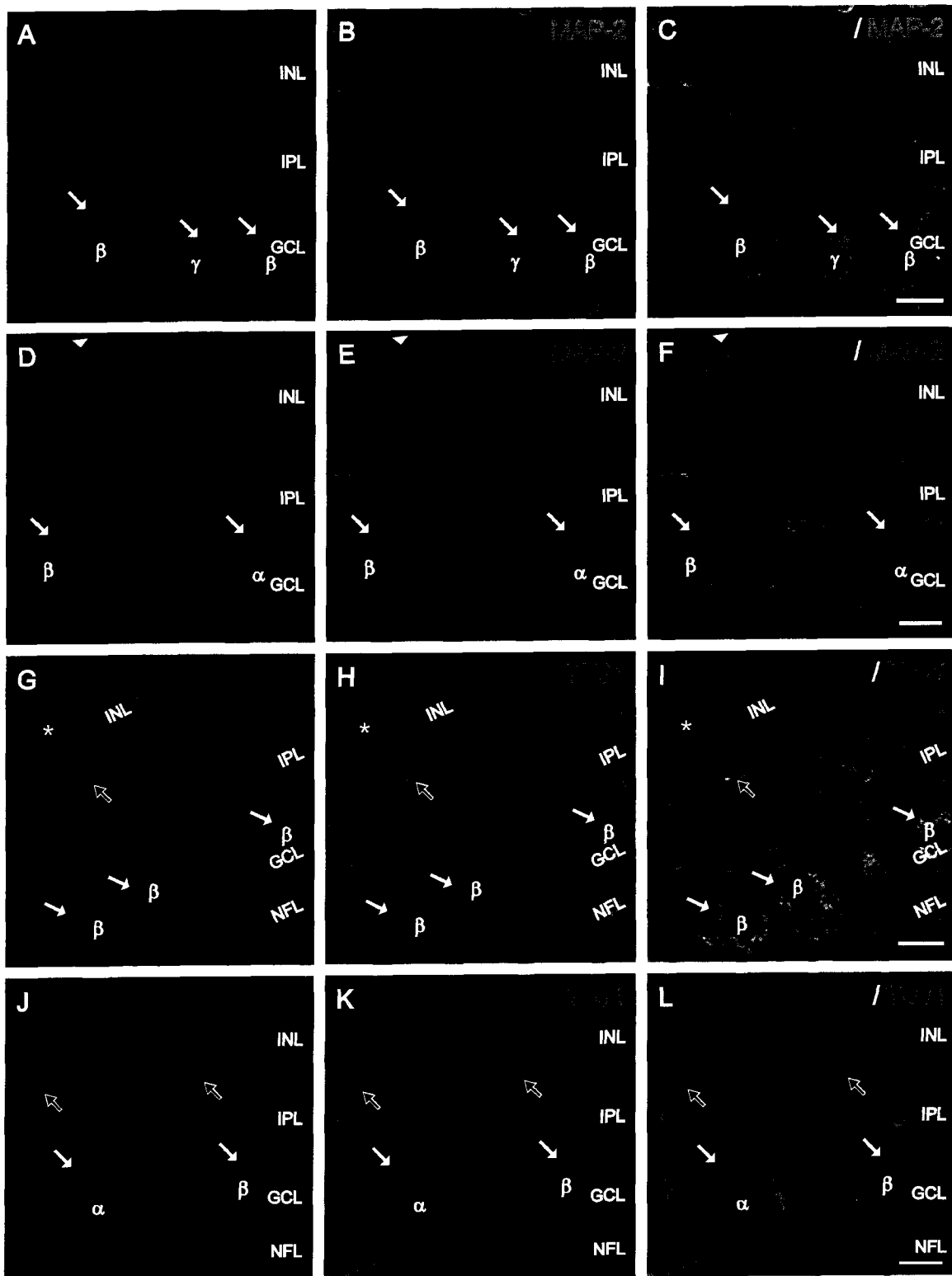


Figure 3. Ngb and Cygb were expressed in neurons. Images were captured with confocal microscopy. (A) Ngb IR (red) in two β -ganglion cells and one smaller cell between them

(either γ -ganglion cell or a displaced amacrine cell) and (B) MAP-2 IR (*green*) displayed positive double labeling in a merged image (C). (D) Cygb IR (*red*) in α - and β -ganglion cells and a horizontal cell and (E) MAP-2 IR (*green*) displayed double labeling in a merged image (F). (G) Ngb IR (*red*) in three β -ganglion cells, amacrine cell, and presumably a bipolar cell and (H) TUJ1 IR (*green*) showed double labeling in a merged image (I). (J) Cygb IR in α and β ganglion cells and two amacrine cells and (K) TUJ1 IR (*green*) showed double labeling in a merged image (L). *White arrows*: examples of retinal ganglion cells; *open arrows*: examples of amacrine cells; *arrowhead*: horizontal cell; *asterisk*: putative bipolar cell. Blue: cell nuclei labeled with DAPI. Scale bar, 10 μ m.

We investigated the possible immunolocalization of Ngb and Cygb in astrocytes and Müller glia with an anti-vimentin and anti-CRALBP antibodies. No double labeling of Ngb or Cygb with vimentin- or CRALBP IR was detected (Supplementary Fig. S3, <http://www.iovs.org/cgi/content/full/47/3/1016/DC1>). This finding suggests that Ngb and Cygb are present in neurons, but not in glial cells in the retina.

Localization of Ngb and Cygb in the Inner Retina

To further investigate expression of Ngb and Cygb within different cell types in the INL, double labeling was performed with rabbit polyclonal anti-Ngb or anti-Cygb and mouse monoclonal anti-PKC α Ab. Bipolar cells containing PKC α were found to be Ngb- and Cygb IR (Figs. 4A–F). In addition, double labeling was performed with mouse monoclonal anti-Ngb and rabbit polyclonal anti-calretinin Ab. Horizontal and amacrine cells containing calretinin⁴⁷ were found to be Ngb IR (Figs. 4G–I).

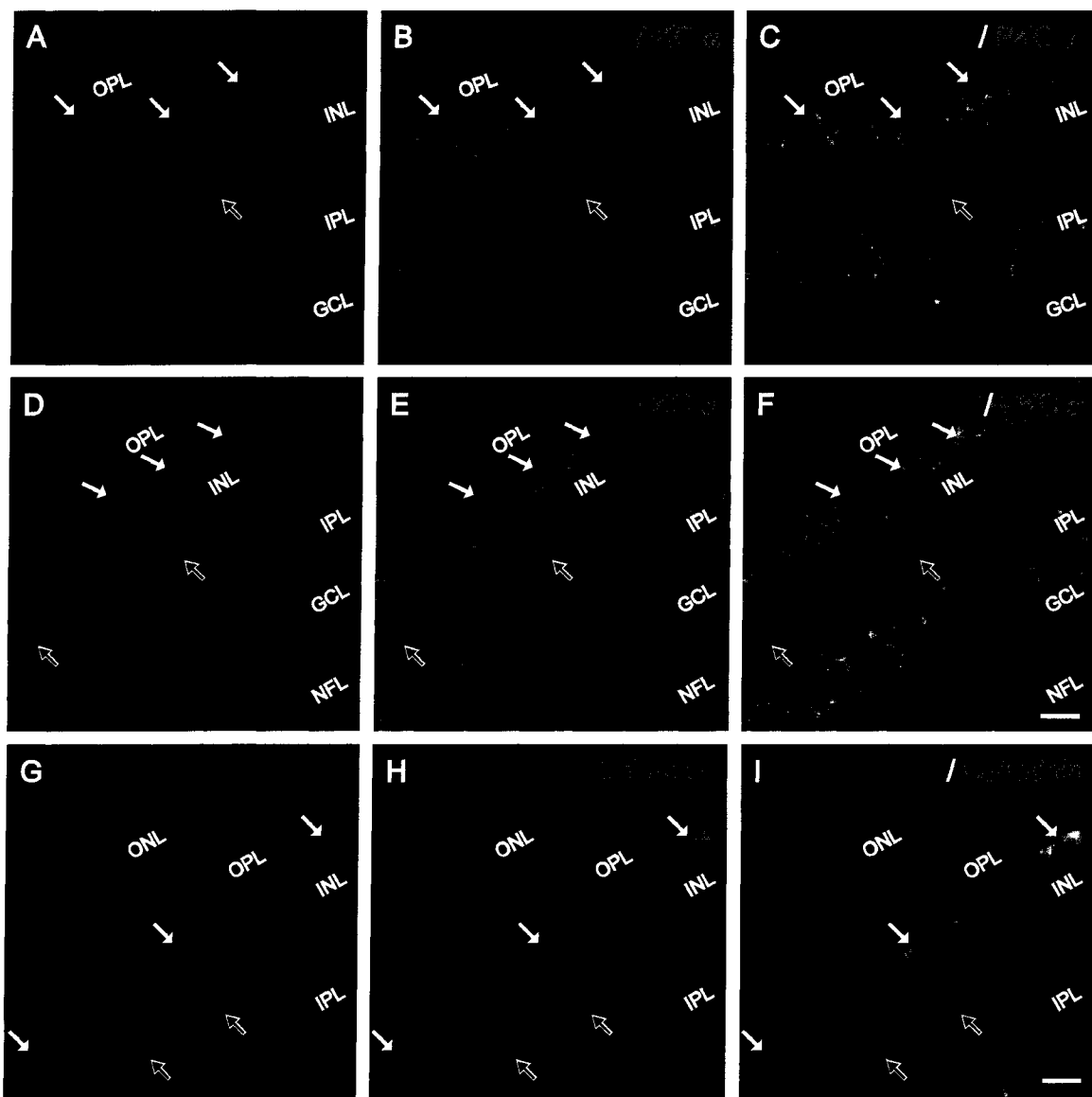


Figure 4. Ngb and Cygb localization in the INL and IPL. Images were captured with confocal microscopy. (A) Ngb IR (*red*) and (B) PKC α IR (*green*) double labeling in bipolar cells and their axonal terminals as shown in (C) a merged image. (D) Cygb IR (*red*) and (E) PKC α IR (*green*) double labeling in bipolar cells and their axonal terminals as shown in (F) a merged image. (G) Ngb IR (*red*) and (H) calretinin IR (*green*) double labeling in horizontal and amacrine cells as shown in (I) a merged image. (A–F) *White arrows*: examples of bipolar cells; (G–I) *white arrows*: examples of horizontal cells; *open arrows*: examples of amacrine cells. *Blue*: cell nuclei with DAPI. Scale bars, 10 μ m.

2.5. Discussion

The retina is one of the highest oxygen-consuming tissues in our body.¹⁻³ As such, a continuous and sufficient supply of O₂ is essential for the normal physiological function of the retina, and hypoxic conditions can lead to severe deficits in visual function.⁴

The presence of Hb in neural tissue has been extensively described in different nonvertebrate species. Hbs are essential oxygen stores in invertebrates subjected to intermittent O₂ supply, particularly in gut parasites, and in nerve and muscle tissues that exhibit sporadic high-level activities.⁴⁸⁻⁵⁰ The duration of the oxygenation from the globin stores increases with a reduction in metabolic rates under hypoxic conditions.⁵¹ A comparative electrophysiological study of several invertebrates, with and without Hb in their nervous tissues,⁵²⁻⁵⁵ demonstrated that neural tissue containing Hbs (neuroHb) consumes much less O₂ during the process of action potential conduction than does neural tissue without neuroHb. Furthermore, experiments showed that oxygen bound to the neural tissue Hb in clams and some worms can support the oxygen requirements of the nervous tissue for up to 30 minutes during anoxic periods.^{52,54,56} These results are suggestive of mechanisms in which the neuroHb-containing neural tissue may effectively use the neuroHb oxygen supplies to enable continued neuronal activity under hypoxic conditions as a highly preserved evolutionary mechanism in different species.

The detection of Ngb and Cygb in protein extracts of canine retina and detection of Cygb, but not Ngb, in protein extracts of canine liver, kidney, lung, and heart is consistent with previously published work.^{24,37,39} Detection of Ngb at 17 kDa molecular mass in the canine retinal tissue extract is consistent with the molecular mass of human Ngb, since the canine Ngb sequence shares 99% identity with that of human (source of the human and

canine Ngb sequence: <http://us.expasy.org/> provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland). Although the canine Cygb sequence has not yet been reported and assuming that it also shares high identity with the human protein, the difference in the molecular mass between human recombinant Cygb (21 kDa) and Cygb in the canine tissue samples (29 kDa) may be the result of posttranslational modification of the protein.

A study by Schmidt et al.²⁴ demonstrated the distribution of Ngb in ganglion cells and inner and outer plexiform layers, weak IR in both nuclear layers, and strong IR of the photoreceptor IS of the mouse retina. Although the pattern of IR was highly suggestive of neuronal localization, the lack of double-labeling experiments with neuronal and glial markers did not rule out the presence of Ngb in glial cells. Our double-labeling studies demonstrated colocalization of Ngb within retinal neurons, but not in glial cells. This finding is consistent with earlier double-labeling studies reporting neuronal localization of Ngb in different brain regions,^{35,57,58} but differs from the reported Ngb presence in cultured astrocytes.⁵⁹ Schmidt et al.³⁹ also recently reported Cygb distribution in the GCL, INL, and IPL and some minor IR in the OPL of the mouse retina, which is consistent with the Cygb IR pattern observed in our study. However, we detected both Ngb and Cygb IR in the RPE in the dog. The possible difference in the IR pattern of the RPE may be attributed to the different antibodies used. Whereas the antibodies used in our study were generated against whole recombinant proteins, Schmidt et al.³⁷ used antibodies generated against Ngb and Cygb peptide sequences. As in the case of Ngb, our double-labeling studies demonstrated localization of Cygb within retinal neurons, but not glial cells, in accordance with previous reports of the distribution of Cygb in the brain. Our findings of Ngb localization in the

cytoplasm of canine retinal neurons is in agreement with reports of Ngb expression in the cytoplasm of neurons.^{35,58,60} Several studies have described differing subcellular distribution of Cygb. Geuens et al.⁵⁷ reported strict localization to the nuclear region in mouse brain neurons, whereas Schmidt et al.³⁹ reported nuclear and cytoplasmic distribution in neurons within the caudate putamen, cerebral cortex, and colonic myenteric plexus³⁷ and the mouse retinal neurons,³⁹ with exclusive cytoplasmic location in fibroblasts.³⁷ However, our study demonstrated Cygb IR in the cytoplasm of canine retinal neurons. Although some of our low-magnification images may suggest that Cygb is present within cytoplasm and nuclei, high-magnification confocal images of single optical sections revealed that our anti-Cygb antibody detected Cygb only in the cytoplasm.

Our double-labeling experiments, together with the known morphologic characteristics of canine retinal cells^{46,61,62} and identification of retinal cell types, revealed Ngb and Cygb colocalization in ganglion, amacrine, bipolar, horizontal, and retinal pigment epithelial cells and Ngb localization in both rod and cone photoreceptor IS. Although our results suggest different roles of Ngb and Cygb in the mammalian retina, they do not permit discrimination between possible distinct Ngb and Cygb functions. Further *in vivo* and *in vitro* functional studies are needed before the exact function of these proteins in the retina can be established.

Although the neuroprotective function of Ngb in the brain has been reported in recent studies,^{33,34} the role of Cygb in the nervous system is still under active investigation.

Different theories exist for Ngb and Cygb function, such as a role in oxygen storage and facilitated oxygen transport. However, this particular hypothesis has been recently disputed

for Ngb by mathematical modeling of retinal oxygen consumption, which suggests that even a concentration of 100 μM is not sufficient to provide adequate oxygen supply.⁶³ Furthermore, Sun et al.³³ showed that increased Ngb expression did not cause an increase in oxygen consumption, and hypothesized that Ngb's neuroprotective activity was not related to increased neuronal oxygen transport. This finding was not unexpected, considering the small concentration of Ngb within the brain.¹⁰

Recent studies have suggested that Ngb may serve as an oxygen-hypoxia sensing and signaling molecule due to the ability of metNgb to bind to G proteins (G α i).³² In addition, studies using a yeast two-hybrid system demonstrated that cystatin C (a cysteine proteinase inhibitor) is an Ngb-binding protein,⁴⁰ as is flotillin 1, a lipid raft protein. These findings have been reviewed in light of the possible neuroprotective mechanism of Ngb by Wakasugi et al.⁶⁴

Furthermore, it has been hypothesized that Ngb and Cygb may act as enzymes for detoxification of free oxidative radicals, and it was suggested that scavenging of radical-derived organic peroxides by Cygb could be an adaptive reaction to normalize the cellular redox status during postischemic cell activation.³⁶ Also, it has been shown that an oxygenated derivative of Ngb reacts very rapidly with free NO, yielding as a product metNgb, which may interact specifically with components of GDP-GTP signal-transduction pathways.⁴¹

The present study has demonstrated the immunolocalization of Ngb and Cygb in retinal layers with high oxygen demand and particularly in the retinal ganglion cells, which are the primary cell population affected by glaucoma and retinal cell population that is the most sensitive to ischemic diseases of the retina and optic nerve. Hemodynamic alterations,

leading to decreased ocular blood perfusion and ischemia, can often precede normal-tension glaucoma.⁶ In addition, decreased optic nerve blood flow in humans correlates with functional and morphologic measures of the progression of glaucoma,⁷ and treatments designed to improve ocular blood flow have been shown to benefit patients with glaucoma.⁸ Furthermore, the presence of tissue hypoxia in the retina and optic nerve head of glaucomatous eyes⁶⁵⁻⁶⁷ is consistent with the finding of increased hypoxia-inducible factor (HIF)-1 α in human glaucomatous retinas and optic nerve heads.⁶⁸ Our recent preliminary results suggest an upregulation of *Cygb* mRNA in glaucomatous mouse eyes (Grozđanić et al. *IOVS* 2004;45:ARVO E-Abstract 2586). Also, it has been reported recently that *Ngb* expression is increased in the retina of human eyes with chronic glaucoma (Rajendram et al. *IOVS* 2005;46:ARVO E-Abstract 1313). These data are not surprising, considering previous reports which demonstrated *Cygb* upregulation by HIF-1 α ⁶⁹ and increased *Ngb* and *Cygb* protein expression in different tissues after exposure to hypoxia.^{33,37} It has been known for almost 40 years that Hb is the principal oxygen-transporting molecule that is significantly upregulated when an organism is exposed to hypoxia, to provide adequate oxygenation of all vital organs and maintain cell survival. Despite the enormous retinal oxygen and energy demands, it is not known why the retina is capable of tolerating an almost 10 times longer period of ischemia than is brain tissue.^{70,71} We hypothesize that *Ngb* and *Cygb* may have a vital role in retinal oxygen homeostasis and enable the retina to sustain longer periods of ischemia. Exact identification of the functional properties of *Ngb* and *Cygb* will significantly advance our understanding of retinal oxygenation in health and disease.

2.6. Acknowledgments

The authors thank Matt Harper, Jeffrey Orasky, Milan Joksimović, Daniel Zamzow, and Janice Buss for technical assistance during preparation of the manuscript; Daria Pospisilova, Petr Kasperek, and Jiri Havlasek from BioVendor Laboratory Medicine, Inc. for providing us with mouse monoclonal anti-Ngb Ab and recombinant protein; and John C. Saari (Department of Ophthalmology, University of Washington, Seattle, WA) for the CRALBP antibody.

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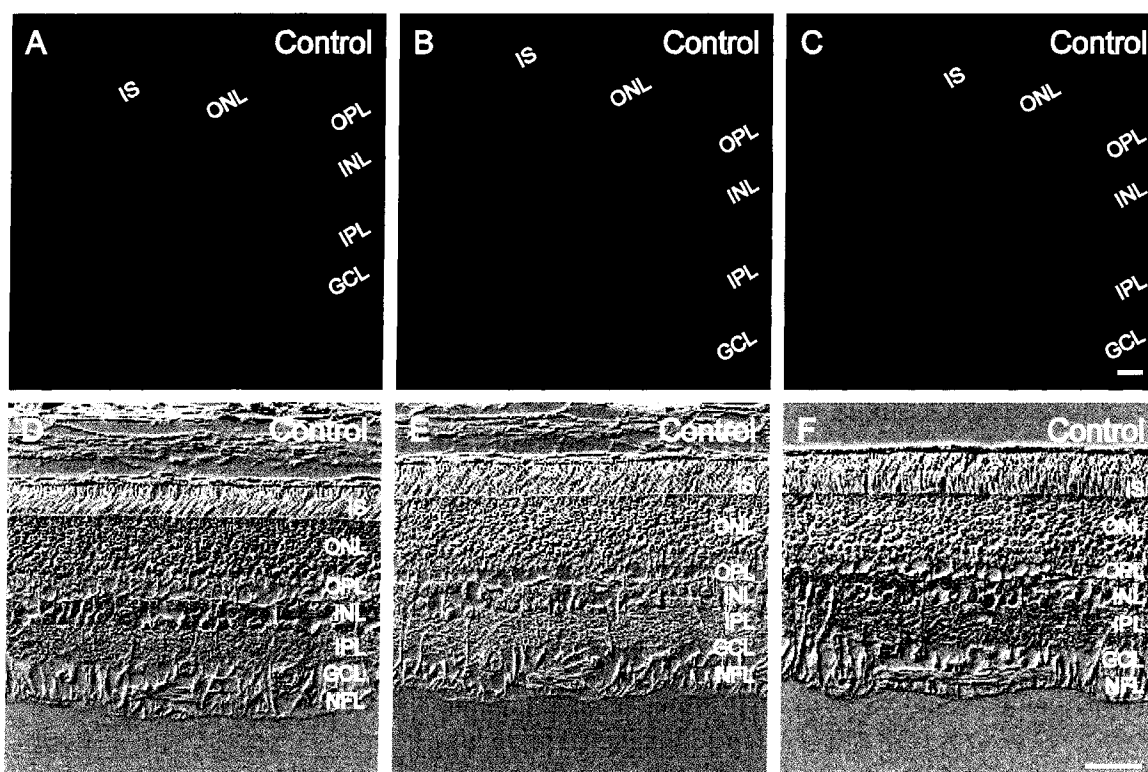
2.8. Supplemental Material (published online)

Supplemental Table 1. Primary Antibodies Used in the Study

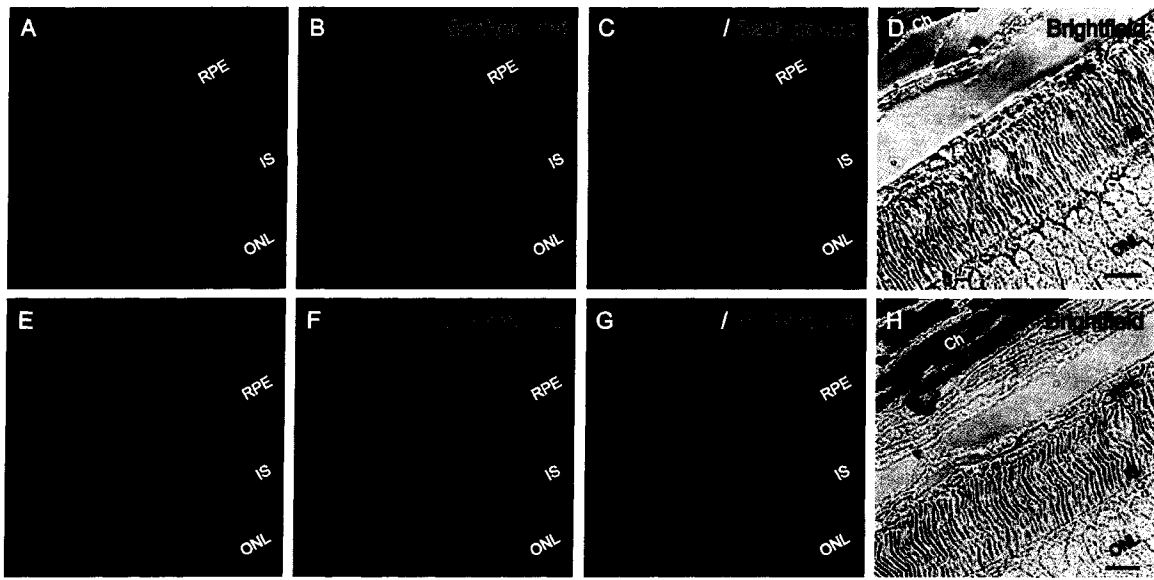
Antibody	Specificity	Used for detecting:	Species	Dilution	Source
Anti-neuroglobin	Human recombinant neuroglobin	Ngb	mouse	1:1000 1:100* 1:750 [†]	BioVendor Laboratory Medicine, Inc.
Anti-neuroglobin	Human recombinant neuroglobin	Ngb	rabbit	1:1000 1:100* 1:750 [†]	M. Hargrove, Iowa State University, IA
Anti-cytoglobin	Human recombinant cytoglobin	Cygb	rabbit	1:1000 1:100* 1:750 [†]	M. Hargrove, Iowa State University, IA
Anti-MAP-2	Microtubule-associated protein 2	Ganglion cells, amacrine cells	mouse	1:400	Chemicon International, Temecula, CA
Anti-TUJ1	Class III β -tubulin	Ganglion cells, amacrine cells	mouse	1:300	R&D Systems Inc., Minneapolis, MN
Anti-PKC α	Protein kinase C alpha	Rod bipolar cells	mouse	1:100	Santa Cruz Biotechnology, Inc., Santa Cruz, CA
Anti-Calretinin	Calretinin	Horizontal cells, amacrine cells	rabbit	1:150	Chemicon International
Anti-Vimentin	Vimentin	Müller glia and astrocytes	mouse	1:200	DakoCytomation, Carpinteria, CA
Anti-CRALBP	CRALBP (cellular retinaldehyde binding protein)	Müller glia and astrocytes	rabbit	1:200	Dr John Saari, Dept of Ophtalmology, University of Washington, WA

*when a fluorescent secondary antibody was used for indirect immunofluorescence

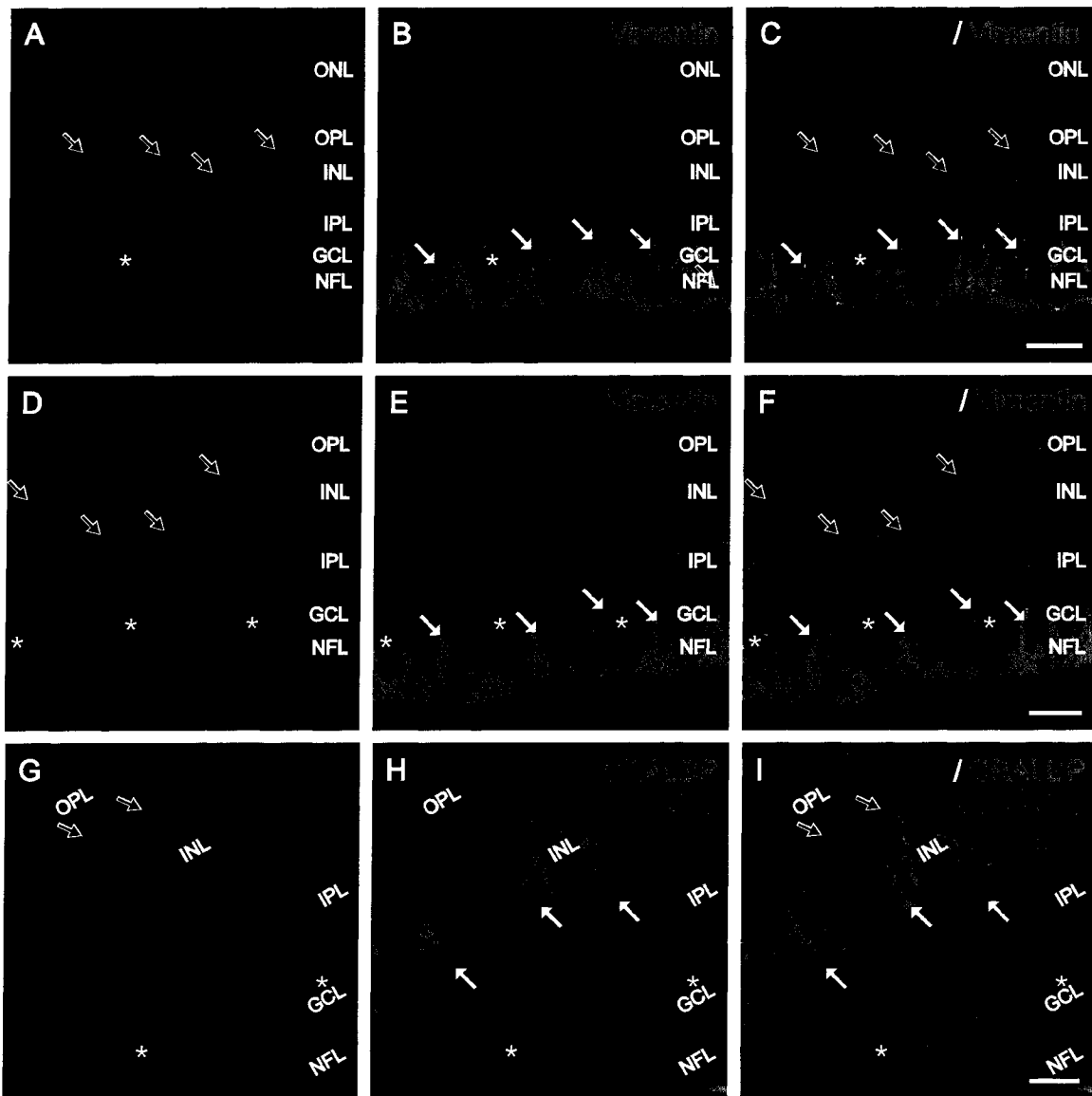
[†]for peroxidase immunohistochemistry



Supplementary Figure S1. Preadsorption controls. Confocal images of staining with (A) mouse monoclonal anti-Ngb, (B) rabbit polyclonal anti-Ngb, and (C) rabbit polyclonal anti-Cygb antibody (red) preadsorbed with their specific proteins before incubation on tissue sections. Cell nuclei (blue) were labeled with DAPI. Differential interference contrast (DIC) images of staining with (D) anti-Ngb mouse monoclonal antibody, (E) anti-Ngb rabbit polyclonal antibody and (F) anti-Cygb rabbit polyclonal antibody (red) preadsorbed with their specific proteins before incubation on tissue sections. Specific immunoreactivity was not observed in any of the cases (A-F). Scale bar: (A-C) 10 μm ; (D-F) 20 μm .



Supplementary Figure S2. Ngb and Cygb localization in the RPE. Confocal images of (A) RPE labeled with rabbit polyclonal anti-Ngb antibody, (B) background demonstrating absence of lipofuscin, (C) merged image of anti-Ngb labeling and background, and (D) corresponding brightfield image demonstrating absence of melanin granules in tapetal RPE. Confocal images of (E) RPE labeled with rabbit polyclonal anti-Cygb antibody, (F) background demonstrating absence of lipofuscin, (G) merged image of anti-Cygb labeling and background, and (H) corresponding brightfield image demonstrating absence of melanin granules in tapetal RPE. Scale bar, 10 μ m. Abbreviations: T (tapetum), Ch (choroid).



Supplementary Figure S3. Ngb and Cygb did not co-localize with Müller glia or astrocytes. Confocal micrographs of dog retinal sections double-labeled for (A) Ngb and (D) Cygb (red) and (B, E) vimentin (green). No co-localization of Ngb- or Cygb-IR with vimentin-IR was detected (C, F, merged images). In addition, no co-localization of (G) Ngb-IR (red) with (H) CRALBP IR (green) was detected in the merged image (I). Note the absence of the radial pattern of immunoreactivity characteristic for Müller glia in images A, D and G. *White arrows*: examples of Müller glia radial processes; *open arrows*: examples of inner retinal neurons; *asterisks*: examples of ganglion cells. Cell nuclei (blue) were labeled with DAPI. Scale bar: (A-F) 25 μm ; (G-I) 10 μm .

2.9. Appendix

In order to check for the total protein loading of the different tissue samples, approximately 25 µg of total protein from retina, kidney, liver and heart tissue extracts, and approximately 50 µg of total protein from lung tissue extract were separated in 14% gels and transferred to a PVDF membrane. As already described in Material and Methods section (Chapter 2), membrane was incubated in blocking buffer, followed by incubation in rabbit polyclonal anti-Cygb Ab (1:1000), and in alkaline phosphatase conjugated goat anti-rabbit secondary Ab (1:7500). Immunoreactive bands were visualized with Lumi-Phos WB Chemiluminescent Substrate (Pierce Biotechnology). After the chemiluminescent signal has been detected, PVDF membrane was incubated for 15 minutes in Restore Western Blot Stripping Buffer (Pierce Biotechnology), rinsed, than incubated for one hour in mouse monoclonal anti- α actin (Sigma), diluted to 1:1000. Finally, after rinsing, membrane was incubated for one hour in alkaline phosphatase conjugated goat anti-mouse secondary Ab (1:7500) and immunoreactive bands were visualized with Lumi-Phos WB Chemiluminescent Substrate (Pierce Biotechnology).

As shown in Figure 1, rabbit polyclonal anti-Cygb Ab detected specific Cygb protein at ~21 kDa, and Cygb in protein extracts from canine retina, kidney, liver, lung, and heart at ~29 kDa. The second bands at approximately double the molecular mass might represent a dimer, as also reported by Schmidt et al.^{37,39}, or non-specific binding of the antibody. It is also possible that the second bands at approximately double the molecular mass for Ng2 and Cygb (Figure 1A-C in the Results section) are a result of the non-specific binding of the protein rather than a dimer.

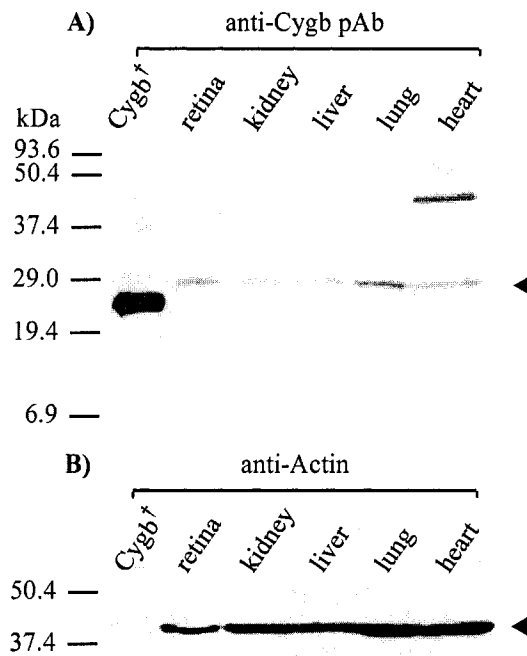


Figure 1. Western blot analysis of Cygb expression in dog tissues. **(A)** Rabbit polyclonal anti-Cygb Ab detected specific recombinant Cygb protein at ~ 21 kDa and Cygb in dog retina, kidney, liver, lung and heart at ~ 29 kDa. Approximately 0.02 μ g of the recombinant protein was applied per lane. **(B)** After the membrane was stripped and reprobed, mouse monoclonal anti- α actin detected actin in dog tissue samples. The molecular weight marks are given on the left side. Source of the protein: [†]Ref¹².

CHAPTER 3: Neuroglobin and Cytoglobin Distribution in the Human Retina: A Detailed Immunohistochemical Study

Prepared for submission

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3.1. Abstract

Purpose. Neuroglobin (Ngb) and cytoglobin (Cygb) are two novel oxygen-binding globins. In addition, Ngb has been implicated in neuronal protection from ischemic insults. Our goal was to determine the patterns of Ngb and Cygb localization in human retinas and their distribution in specific retinal cell types.

Methods. Specific antibodies directed 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 –Cygb antibodies along with antibodies against neuronal (MAP-2, class III β -tubulin (TUJ1), PKC α , calretinin) and glial

(vimentin, GFAP) markers. Tissue sections were visualized and images captured with a confocal scanning laser microscope.

Results. Immunohistochemical analysis of human retinal tissue showed Ngb immunoreactivity (-IR) in the retinal ganglion cell layer, inner and outer nuclear layers, inner and outer plexiform layers, photoreceptor inner segments and retinal pigment epithelium. Cygb-IR was found in the retinal ganglion cell layer, inner nuclear layer, inner and outer plexiform layers and retinal pigment epithelium. Ngb and Cygb were expressed in the same cells in the ganglion cell layer and inner nuclear layer. Ngb-IR and Cygb-IR was co-localized within neuronal, but not glial cells.

Conclusions. Ngb and Cygb, two recent additions to the globin family, are co-localized within human retinal neurons and retinal pigment epithelium. Based on Ngb and Cygb immunolocalization and their previously reported biochemical features, we hypothesize that Ngb and Cygb may function as facilitators of oxygen diffusion and/or scavengers of reactive oxygen species.

3.2. Introduction

Globins, a family of heme-containing proteins that reversibly bind oxygen, have been found in bacteria, fungi, protists, plants and animals¹. In vertebrates, heterotetrameric hemoglobin is localized exclusively in erythrocytes and transports oxygen between lungs and other tissues via the circulatory system¹. Monomeric myoglobin is found in muscle, where it acts as an intracellular oxygen store and facilitates oxygen diffusion to the mitochondria². Myoglobin also acts as a NO dioxygenase³. Both hemoglobin and myoglobin are pentacoordinate globins, in which the heme iron has five out of six binding sites occupied⁴.

Recently, two additional members of the vertebrate globin family have been described, neuroglobin (Ngb) and cytoglobin (Cygb)⁵⁻⁷. In contrast to hemoglobin and myoglobin, Ngb and Cygb are hexacoordinated; thus, ligand binding involves a competition between the exogenous ligand and intramolecular coordination by a local amino-acid chain⁴. Neuroglobin has been identified for the first time in the databases of anonymous mouse and human complementary DNAs (expressed sequence tags; ESTs)⁵. Subsequently, its presence has been confirmed in human, mouse, rat, chicken, zebrafish and pufferfish brain^{5, 8-14} as well as in dog¹⁵, mouse, rat, guinea pig, chicken and zebrafish eye^{12, 13, 16}. Cytoglobin (Cygb) was identified for the first time in a proteomic study that showed increased synthesis of this protein in rat stellate cells of fibrotic liver and was thus termed a stellate cell activation-associated protein (STAP)¹⁷. Subsequently, Cygb was identified as a 190 amino acid heme-containing protein in virtually all human, mouse and zebrafish tissues^{6, 7}. While phylogenetic analysis showed that Ngb, together with invertebrate nerve globins, diverged very early from an ancestral globin, Cygb had a common ancestor with vertebrate myoglobin¹⁸.

The retina is one of the highest oxygen-consuming tissues in the body, requiring more oxygen than the brain^{19, 20}. It has been hypothesized that inadequate oxygen supply to the retina, in conditions such as ischemic optic neuropathies, retinopathy of prematurity, diabetic retinopathy and numerous proliferative retinopathies, can result in severe visual deficits secondary to retinal neuronal death²¹. Furthermore, it has been hypothesized that decrease in optic nerve blood flow could lead to ischemia²², and may contribute to functional and morphologic deficits in human glaucomatous patients²³.

To our knowledge, this is the first report to describe Ngb and Cygb presence in the human retina using immunohistochemical methods. Their detailed localization demonstrated

here will be essential for our understanding of oxygen homeostasis in healthy and disease-affected eyes. Furthermore, this information may be useful in developing more effective methods to prevent neuronal death due to retinal ischemic diseases.

3.3. Materials and Methods

Human Tissue

Globes from seven donors (ages: 1.5, 6 and 9 months, 6, 47, 58 and 60 years) were fixed for 48 hours in 10% neutral buffered formalin immediately after tissue procurement. They were then transferred to an ethanol-formaldehyde mixture (Penfix -proprietary formula), embedded in paraffin and sectioned at 4 μm thickness. To investigate if Ngf and Cygf localization patterns differed in early postnatal versus adult retinas, the experimenter was masked from the age of the subjects when analyzing the retinal tissue.

In addition, two adult human donor eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA) within 4 hours postmortem. 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 OCT medium (Sakura Finetek USA, Inc, Torrance, CA), and sectioned to a thickness of 7 μm ²⁴.

Tissue collection adhered to the tenets of the Declaration of Helsinki and University of Iowa guidelines.

Fluorescent Immunohistochemistry

Human recombinant Ngf and Cygf proteins were synthesized as described previously^{4, 6}. Polyclonal antisera produced against Ngf or Cygf were raised in rabbits and antibodies were affinity purified from the serum using the respective synthetic proteins

coupled to a SulfoLink column (Pierce Biotechnology, Rockford, IL). All primary antibodies used in this study are summarized in Table 1.

Table 1. Primary antibodies used in this study

Antibody	Specificity	Used for detection of:	Species	Dilution	Source
Anti-neuroglobin	Human recombinant neuroglobin	Ngb	Mouse	1:1000* 1:100	BioVendor Laboratory Medicine, Inc.
Anti-neuroglobin	Human recombinant neuroglobin	Ngb	Rabbit	1:1000* 1:100	Dr Mark Hargrove, Iowa State University, IA
Anti-cytoglobin	Human recombinant cytoglobin	Cygb	Rabbit	1:1000* 1:100	Dr Mark Hargrove, Iowa State University, IA
Anti-MAP-2	Microtubule-associated protein-2	Ganglion, amacrine and horizontal cells	Mouse	1:400	Chemicon International, Temecula, CA
Anti- Class III β -tubulin (TUJ1)	Class III β -tubulin	Ganglion, amacrine and horizontal cells	Mouse	1:300	R&D Systems Inc., Minneapolis, MN
Anti-Vimentin	Vimentin	Müller glia and astrocytes	Mouse	1:200	DakoCytomation, Carpinteria, CA
Anti-GFAP	Glial fibrillary acidic protein	Astrocytes and reactive Müller glia	Mouse	1:250	ICN ImmunoBiologicals, Costa Mesa, CA
Anti-PKC α	Protein kinase C alpha	Rod bipolar cells	Mouse	1:100	Santa Cruz Biotechnology, Inc., Santa Cruz, CA
Anti-Calretinin	Calretinin	Amacrine cells	Rabbit	1:150	Chemicon International

*biotinylated secondary antibody was used for detection

Paraffin embedded tissue sections were kept for 30 min at 56°C, deparafinized in xylene, rehydrated in a graded alcohol series and treated for antigen recovery. This procedure was omitted for cryostat sectioned tissue. Sections were washed in distilled water and potassium PBS (KPBS; 0.15 M NaCl, 0.034 M K₂HPO₄, 0.017 M KH₂PO₄, pH=7.2). Sections were then incubated for 2 hours in blocking solution which contained: 2% blocking

serum corresponding to the species in which the secondary antibody was produced (normal donkey serum; Jackson ImmunoResearch, West Grove, PA or normal goat serum; Sigma, St Louis, MO), 1% bovine serum albumin (BSA; Sigma) and 0.4% Triton X-100 (Fisher Scientific Inc., Fairlawn, NJ). Sections were double-labeled with a primary Ab cocktail during overnight incubation at room temperature in a sealed humid chamber, washed in KPBS with Triton X-100, then incubated in one of the following secondary Ab cocktails: donkey anti-mouse biotinylated Ab (Jackson ImmunoResearch) and goat anti-rabbit Alexa 488 Ab (Molecular Probes, Eugene, OR); goat anti-rabbit biotinylated Ab (Vector Laboratories, Burlingame, CA) and goat anti-mouse Alexa 488 Ab (Molecular Probes); goat anti-mouse Cy5 Ab (Jackson ImmunoResearch) and goat anti-rabbit Alexa 488 Ab (Molecular Probes); goat anti-rabbit Cy5 Ab (Jackson ImmunoResearch) and goat anti-mouse Alexa 488 Ab (Molecular Probes). After incubating for 2 hours, sections were washed again in KPBS with Triton X-100. If a biotinylated secondary antibody was used, sections were subsequently incubated for 30 minutes with streptavidin Cy3 (Jackson ImmunoResearch) and washed in KPBS. Finally, sections were counterstained with 1 μ g/ml of 4', 6 -diamino-2-phenylindole (DAPI; Molecular Probes), rinsed and cover-slipped with Gel/Mount antifade mounting medium (Biomedex, Foster City, CA).

All primary and secondary Abs were diluted in KPBS with 1% BSA, 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 KPBS to 1:10,000. Negative controls were carried out in parallel during all processing by the omission of the primary or secondary Abs.

Additional negative controls were performed by preabsorbing primary Abs (mouse and rabbit anti-Ngb and rabbit anti-Cygb) with their respective recombinant proteins.

Analysis of Tissue Sections

Human tissue sections labeled with fluorescent Abs were visualized and images captured using a Leica confocal scanning laser microscope (TCS-NT; Leica Microsystems Inc., Exton, PA).

To discriminate antibody labeling from autofluorescence in the human retinal pigment epithelium^{25, 26}, confocal images of slides single-labeled with primary antibody and appropriate Cy-5 conjugated secondary antibody were captured at both 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 488/525 nm channel (background) from images captured at 633/645 nm channel (signal and background). As an additional control, autofluorescence was examined in non-labeled 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 (ver. 7.0, Adobe, San Jose, CA) and Freehand (ver.10.0, Macromedia, San Francisco, CA).

3.4. Results

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 antibodies generated against full length human

recombinant Ngb protein. Both antibodies showed identical patterns of labeling, and were subsequently used for double-labeling studies. As illustrated in Figure 1A and B, Ngb-immunoreactivity (-IR) in the retina was localized to the nerve fiber layer, the ganglion cell layer, inner and outer nuclear layers, inner and outer plexiform layers and photoreceptor inner segments (Table 2). Cygb localization was determined using a rabbit polyclonal antibody generated against human recombinant Cygb protein. Cygb- IR in the retina was localized to the nerve fiber layer, the ganglion cell layer, the inner nuclear layer and inner and outer plexiform layers (Fig. 1C, Table 2). No differences were observed in the labeling patterns of Ngb and Cygb in the retinas that could be attributed to the age of the subjects. In addition, no obvious difference in Ngb-IR and Cygb-IR in central vs. peripheral retina was observed. To verify antibody specificity, each antibody was pre-adsorbed with their respective recombinant protein before incubation on tissue sections, in which case no specific staining was observed (Supplemental Fig. 1). In addition, negative controls were performed in parallel by omission of primary or secondary antibodies. No antibody labeling was observed in these controls (data not shown).

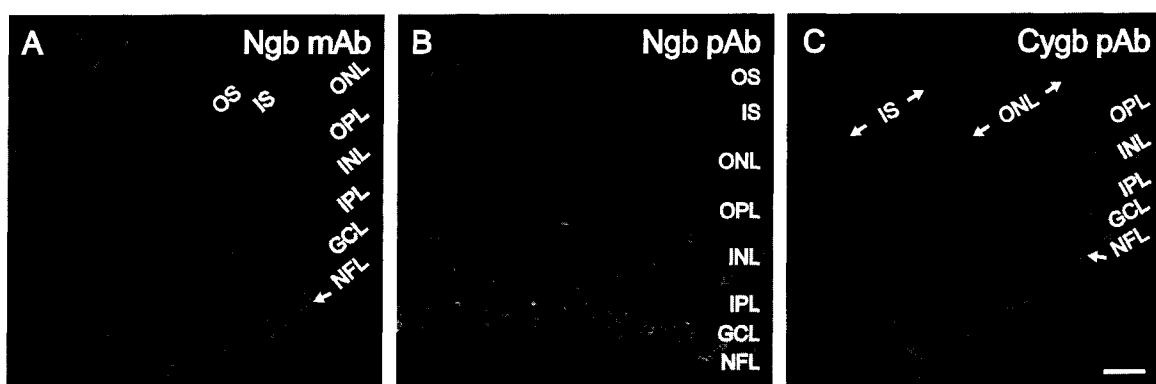


Figure 1. Anti-Ngb and anti-Cygb IR in the human retina. (A) Mouse monoclonal anti-Ngb antibody (mAb) and (B) rabbit polyclonal anti-Ngb antibody (pAb) showed identical patterns of labeling in the retina. (C) Pattern of IR with rabbit polyclonal anti-Cygb antibody. Scale bar, 25 μ m. Abbreviations for all figures: NFL- 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.

Immunoreactivity for both Ngb and Cygb was detected in the RPE, as shown in

Figure 2.

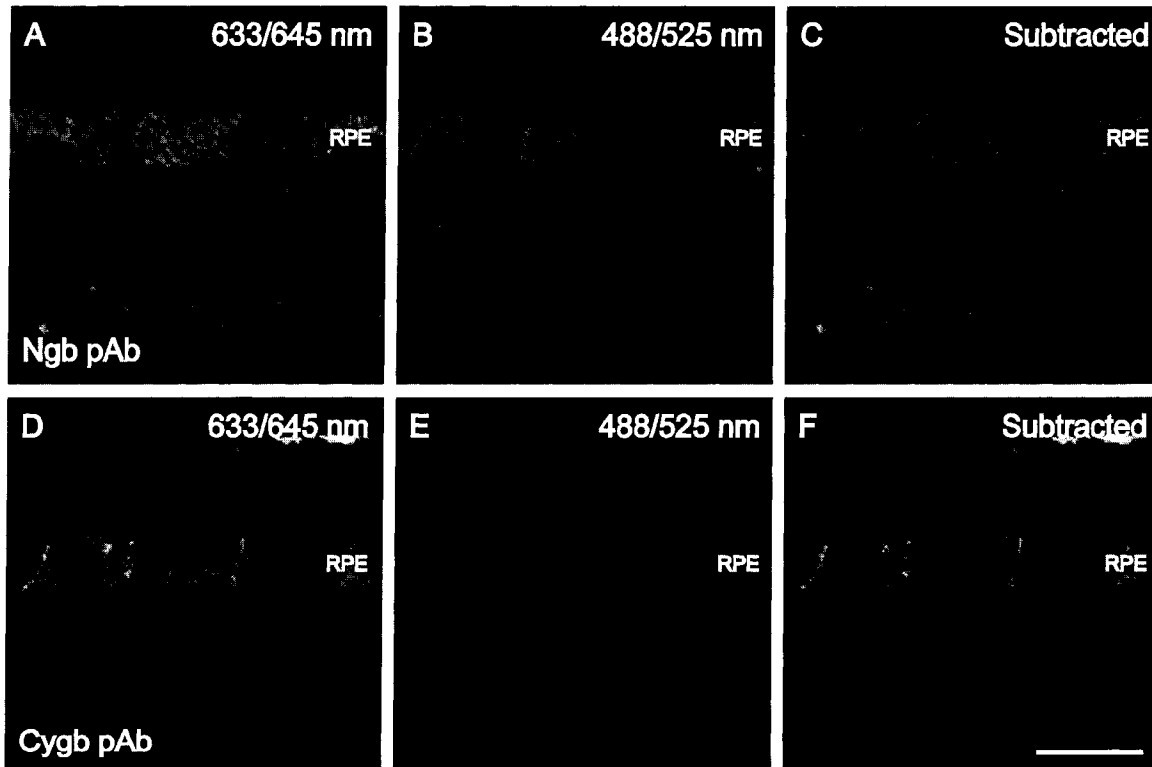


Figure 2. Anti-Ngb and anti-Cygb IR in the human RPE. (A) Confocal image of the retinal section labeled with Cy5 secondary antibody captured at 633/645 nm excitation/emission wavelength shows both Ngb-IR and lipofuscin autofluorescence (B) Lipofuscin autofluorescence captured at 488/525 excitation/emission wavelength (C) Image generated by subtraction of image B from A, shows Ngb-IR only. (D) Confocal image of the retinal section labeled with Cy5 secondary antibody captured at 633/645 nm excitation/emission wavelength shows both Cygb-IR and lipofuscin autofluorescence (E) Lipofuscin autofluorescence captured at 488/525 excitation/emission wavelength (F) Image generated by subtraction of image E from D, shows Cygb-IR only. Scale bar, 10 μ m.

Table 2 summarizes the regional distribution of Ngb and Cygb in human retina.

Table 2. Summary of the distribution of Ngb and Cygb in the human retina and pigment epithelium.

	NFL	GCL	IPL	INL	OPL	ONL	IS	OS	RPE
Ngb	+	+	+	+	+	+	+	-	+
Cygb	+	+	+	+	+	-	-	-	+

NFL: 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, RPE: retinal pigment epithelium, +: detectable immunoreactivity, -: no detectable immunoreactivity.

Localization of Ngb and Cygb in Retinal Neurons

To determine if the cells immunoreactive with the anti-Ngb and anti-Cygb antibodies were neurons, double-labeling was performed with anti-MAP-2 and TUJ1 antibodies. All cells in the GCL and INL that expressed the neuronal proteins MAP-2 and class III β - tubulin (TUJ1) were also Ngb or Cygb-IR (Fig. 3).

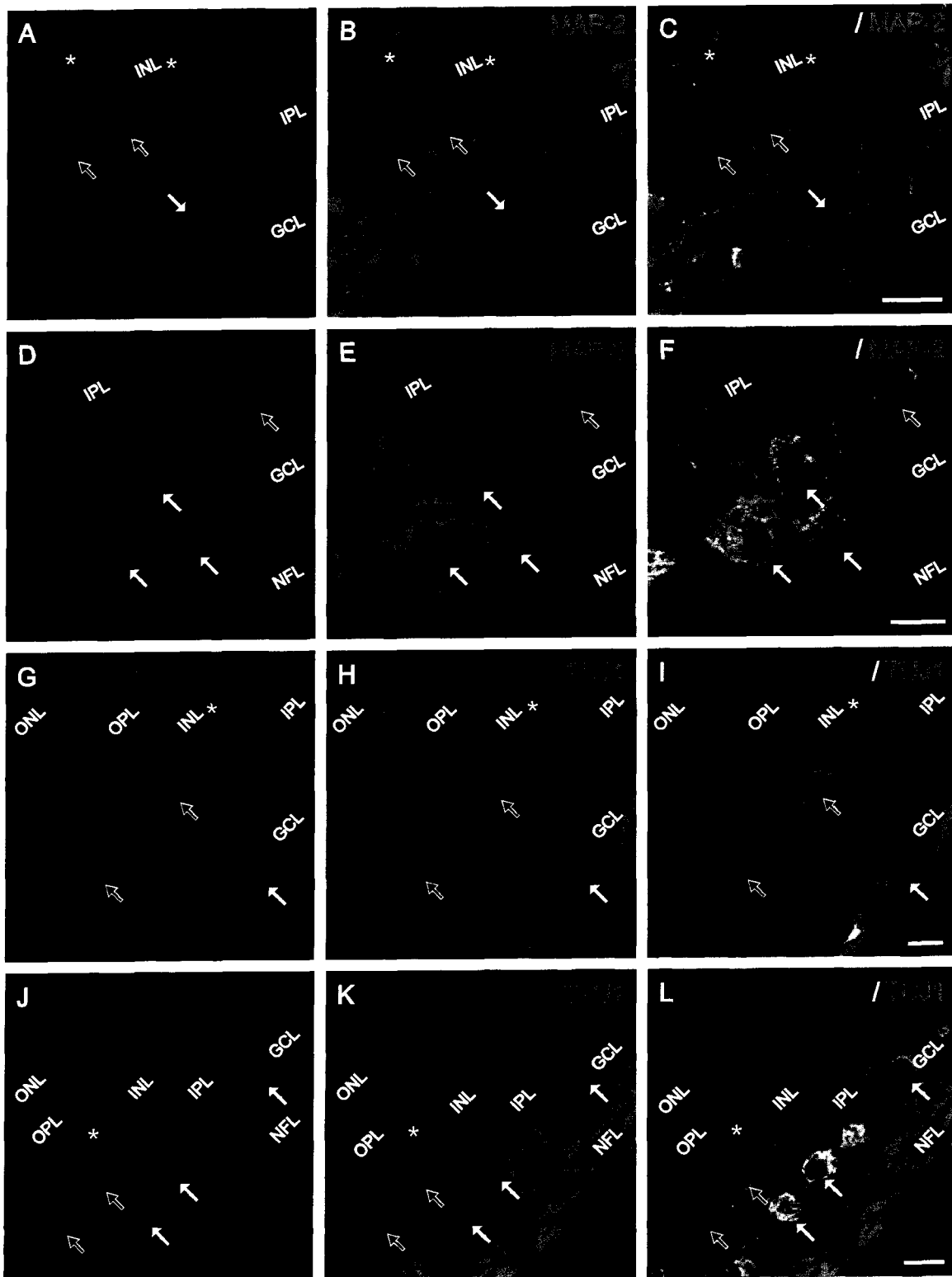


Figure 3. Ngb and Cygb co-localized with MAP-2 and TUJ1-IR cells in the retinal ganglion cell layer (GCL) and inner nuclear layer (INL). Images were captured using confocal

microscopy. (A) Ngb- and (D) Cygb-IR (red) observed in ganglion cells and inner retinal neurons and (B, E) MAP-2 labeling (green). Merged images (C, F) confirmed neuronal localization of Ngb and Cygb. (G) Ngb- and (J) Cygb-IR in ganglion cell layer and inner nuclear layer and class III β - tubulin-IR (H, K) was found to co-localize in the merged images (I, L). Arrows point to examples of putative retinal ganglion cells, asterisks indicate examples of inner nuclear layer neurons and open arrows point to examples of amacrine cells. Cell nuclei (blue) were labeled with DAPI. Scale bars, 10 μ m.

Localization of Ngb and Cygb in the Inner Retina

Ngb and Cygb immunoreactivity was prominent within the inner nuclear layer. In order to further investigate which cell types express Ngb and Cygb, double-labeling was performed with anti-PKC α antibody. As illustrated in Figure 4, ON bipolar cell bodies, containing PKC α^{27} , were found to be immunoreactive for Ngb and Cygb.

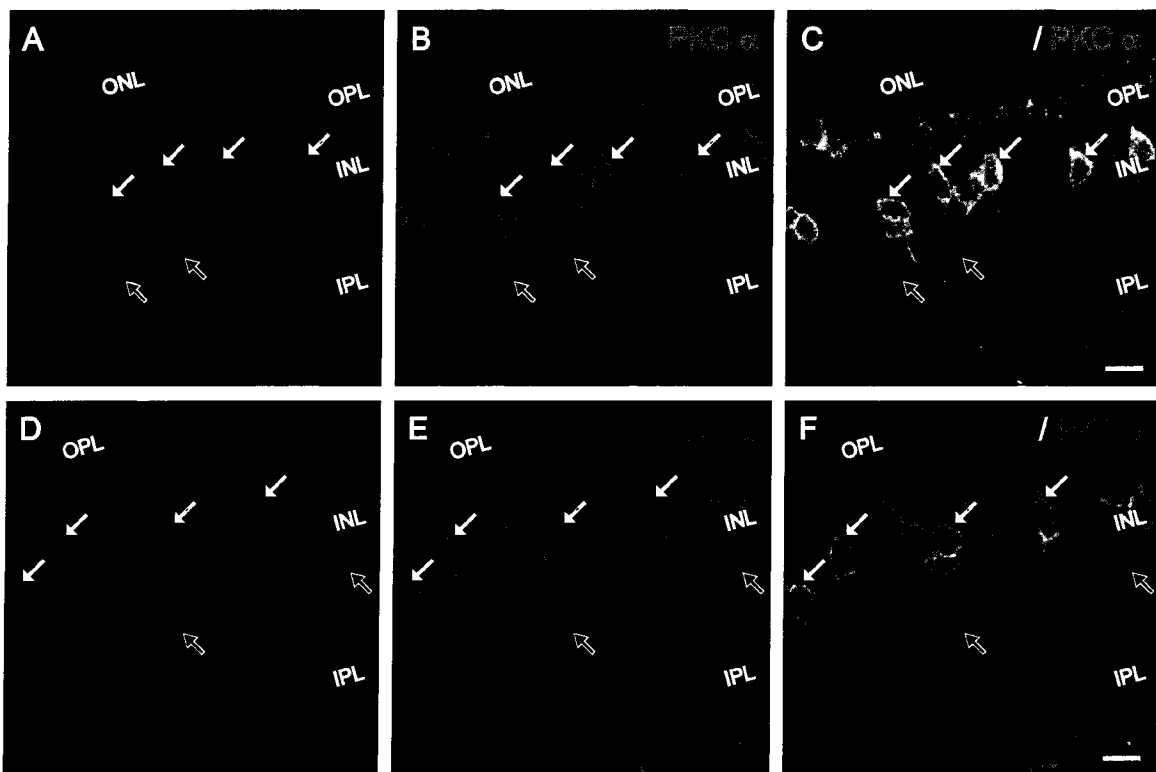


Figure 4. Ngb and Cygb co-localized with PKC α immunoreactive cells in the inner nuclear layer. (A) Ngb-IR (red) and (B) PKC α -IR (green) co-localized in bipolar cells as shown in the (C) merged image. (D) Cygb-IR (red) and (E) PKC α -IR (green) co-localized in bipolar cells as shown in the (F) merged image. Arrows point to examples of bipolar cells, and open

arrows point to examples of Ngb- and Cygb-IR amacrine cells. Cell nuclei (blue) were labeled with DAPI. Scale bars, 10 μ m.

In addition, double-labeling was performed with anti-Ngb and anti-calretinin, an amacrine cell marker in adult human retina²⁸. Ngb-IR was observed to co-localize with calretinin-IR amacrine cells (Supplemental Fig. 2).

Previous studies have reported that Ngb presence is restricted to neurons²⁹. We investigated the possible presence of Ngb and Cygb in retinal glial cells by performing double-labeling studies using anti-GFAP and anti-vimentin antibodies. Anti-GFAP labels astrocytes and reactive Müller glia while anti-vimentin labels astrocytes and Müller glia. We detected no co-localization of Ngb or Cygb with GFAP (data not shown) or vimentin antibodies (Supplemental Fig. 3).

Ngb and Cygb are Co-localized in Retinal Neurons but not Photoreceptors

As demonstrated in Fig. 5, we found that all immunoreactive cells in the ganglion cell layer and the inner nuclear layer were double-labeled for Ngb (mAb) and Cygb (pAb).

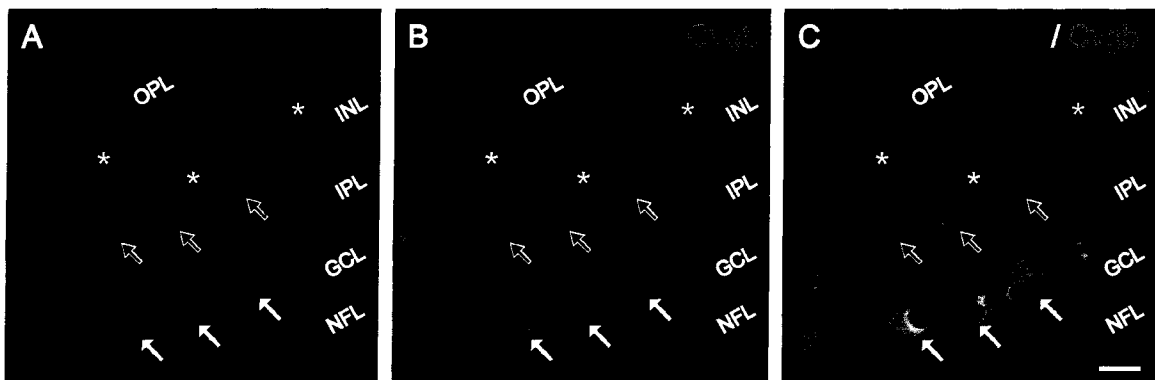


Figure 5. Ngb- and Cygb-IR co-localized within neurons. Retina double-labeled with (A) Ngb (red) and (B) Cygb (green) displays co-localization of the proteins in the same neurons (C). Arrows point to examples of presumptive retinal ganglion cells, asterisks indicate examples of inner nuclear layer neurons and open arrows point to examples of amacrine cells. Cell nuclei (blue) were labeled with DAPI. Scale bar, 10 μ m.

3.5. Discussion

Oxygen is essential for retinal function³⁰, 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, two recently discovered oxygen-binding proteins, in the human retina. The widespread distribution of Ngb in all layers of the human retina and Cygb expression in the ganglion cell layer, inner and outer plexiform layers, and inner nuclear layer of the human retina is consistent with the distribution pattern recently described in the mouse^{16,31} and dog retina¹⁵. This study is the first to examine and demonstrate co-localization of Ngb and Cygb in human ganglion, amacrine, bipolar and horizontal cells by using double-labeling immunohistochemistry and laminar position of cells within the retina.

The exact roles that Ngb and Cygb play are still under debate. Elegant studies by Greenberg's group^{32, 33} provided important insight into the possible role of these molecules in the pathology of the CNS. They demonstrated that Ngb is up-regulated *in vivo* and *in vitro* in neurons exposed to ischemia or to cobalt and deferoxamine (a potent inducers of hypoxia-inducible factor-1 α (HIF-1 α)). Furthermore, they showed that up-regulation of Ngb expression diminished hypoxia induced injury, while reduction of Ngb expression exacerbated hypoxic injury. On the other hand, a small number of studies have begun to address a possible role for Cygb. Under chronic hypoxic conditions, an upregulation of Cygb mRNA levels has been observed in rat heart and liver³⁴, as well as in mouse muscle, heart, liver and brain³⁵ It has been proposed that the mechanism of Cygb induction is regulated by HIF-1 α , since the Cygb promoter region contains hypoxia responsive element sites³⁵. In addition, Cygb and Ngb expression was decreased in HIF-1 (+/-) knockout mice upon

hypoxic stimuli, which further strengthens the hypothesis that these molecules are regulated by HIF-1 α ³⁵. Recent data³⁶ demonstrated significant increase in HIF-1 α expression in glaucomatous human eyes.

Currently, several hypotheses exist about Ngb and Cygb roles in the human retina:

a) Role in facilitating oxygen diffusion

Widespread distribution of both Ngb and Cygb in the retinal ganglion cells, inner and outer plexiform layers and retinal pigment epithelium, and the presence of Ngb in photoreceptor inner segments correlates with previous reports of intraretinal oxygen consumption. *In vivo* measurements of oxygen consumption in rats have shown that under light-adapted conditions the oxygen uptake by the inner retina, primarily the inner plexiform layer, exceeds that of the outer retina³⁷. This high oxygen demand of the inner retina presumably renders it more vulnerable to hypoxic insult³⁷. Moreover, high oxygen consumption in photoreceptor inner segments of the dark-adapted retina²⁰, and the presence of Ngb in the photoreceptor inner segments, as well as in retinal pigment epithelium, suggests that Ngb may play a role as a possible mediator of oxygen transport from the choroidal circulation to the photoreceptor layer.

Ngb and Cygb must abide to several physical requirements to be considered as candidates for oxygen transport and/or facilitated diffusion in normoxic conditions: First, the transporter (Ngb, Cygb) has to be present in hundreds of micromolar to millimolar concentrations in order to be able to increase oxygen flux and raise its solubility, which would facilitate effective oxygen diffusion. Second, the kinetics of oxygen binding and release should not limit the diffusion process, and the overall affinity of the transporter must allow the binding of oxygen at the high concentration side of the gradient as well as its

release on the low concentration side³⁸ – the basic kinetic prerequisite for hemoglobins serving this role is an oxygen dissociation constant rate higher than 1 s^{-1} . Thus the concentration of Ngb and Cygb in the brain in the nanomolar to 1 micromolar range, appears to be insufficient for a role in oxygen transport in this organ. In contrast, a 100 times higher concentration in the retina could satisfy the concentration requirement for the oxygen delivery function¹⁶. However, both Ngb and Cygb oxygen dissociation constant rates ($< 1 \text{ s}^{-1}$) do not support an oxygen transporting role in normoxic conditions. Conflicting data exists in regard to oxygen affinity of Ngb. The previously reported P_{50} (P_{50} = O_2 tension at half-saturation) value for Ngb of 1 torr at $37 \text{ }^\circ\text{C}$ ³⁹ is consistent with a possible role in oxygen transport. However, it has been recently demonstrated⁴⁰ that the O_2 affinity of human Ngb falls in the lower end of the range of values for O_2 -binding heme proteins, with a P_{50} value at $37 \text{ }^\circ\text{C}$ of 7.5 torr at neutral pH. Myoglobin (Mb) has a P_{50} of 2-3 torr². While there is no available information about the intracellular neuronal O_2 tension, it has been speculated that given the neuronal high metabolic rate, oxygen tension could be compared to that of heavily working muscle⁴¹, or neurons could have even lower O_2 tensions than muscle cells. In the retina, cellular PO_2 values less than 1 torr have been measured during dark adaptation in cats³⁰. This means that under *in vivo* conditions the Ngb oxygen kinetics is not supportive of a role for Ngb as a significant source of the O_2 reserve in the retina. However, the physiological properties of Cygb, seem to be consistent with an O_2 supply function, as evident from its O_2 affinity and measured P_{50} values which lie within the range 0.7-1.8 torr calculated from kinetic experiments⁶.

b) Role in oxygen sensing and signal transduction

Ngb and Cygb may also function as O₂ sensors, and participate in a signal transduction pathway that modulates the activities of regulatory proteins in response to changes in O₂ concentration. It has been recently demonstrated⁴² that ferric Ngb binds to the GDP-bound form of the α subunit of heterotrimeric G protein (G α i). The interaction of GDP-bound G α i with ferric Ngb liberates G $\beta\gamma$, which might lead to protection against neuronal death. Furthermore, two Ngb-binding proteins were identified: flotillin-1⁴³ (a lipid raft protein) and cystatin C⁴⁴ (cysteine proteinase inhibitor, which is present in the retinal pigment epithelium, horizontal cells, bipolar and amacrine cells and retinal ganglion cells²⁶). Possible mechanisms of Ngb neuroprotection associated with oxygen-hypoxia sensing and signal transduction have recently been reviewed⁴⁵.

c) Role in free radical scavenging

It is possible that both globins (Ngb and Cygb) act as enzymes for detoxification of free oxidative radicals. It has been shown that Cygb has peroxidase activity and it was suggested that scavenging of radical-derived organic peroxides by Cygb could be an adaptive reaction to normalize the cellular redox status during the post-ischemic cell activation¹⁷. Furthermore, it was demonstrated that an oxygenated derivative of Ngb reacts very rapidly with free NO, yielding metNgb and thus scavenging NO⁴⁶. In addition, it has been shown that in the Fe(II)-NO form human Ngb reacts ~100 times more rapidly with peroxynitrite than does human hemoglobin under identical *in vitro* conditions⁴⁷. Thus, it has been suggested that Ngb retained ancestral function of globins, that presumably was to protect organisms against molecular oxygen, free radicals and reactive oxygen species⁴⁸.

Identification of the functional properties of Ngb and Cygb may significantly advance our understanding of retinal oxygenation in health and disease. Furthermore, the existence of

potential oxygen transporting 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.

3.6. Acknowledgements

The authors thank Yancy de Lathouder, Jeffrey Orasky, Matt Harper and Milan Joksimović for technical assistance during preparation of this manuscript. Also, we thank Daria Pospisilova, Petr Kasparek and Jiri Havlasek from BioVendor Laboratory Medicine, Inc. for providing us with mouse monoclonal anti-Ngb Ab and recombinant protein.

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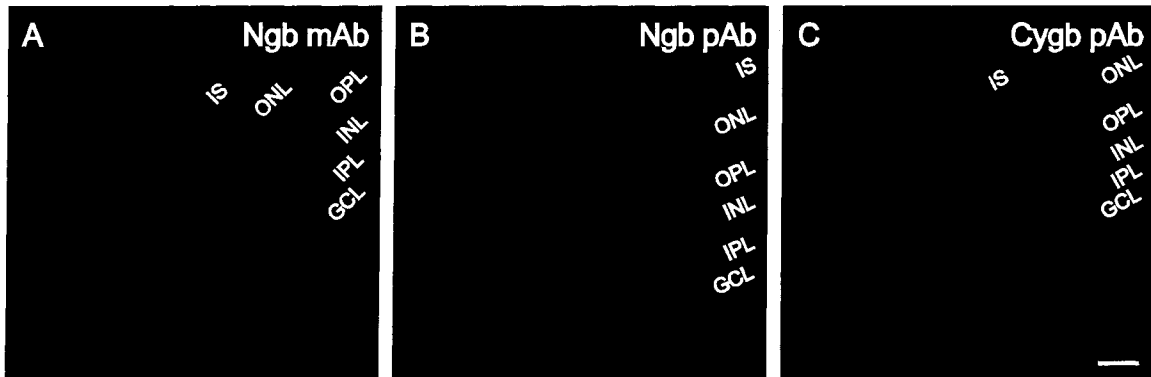
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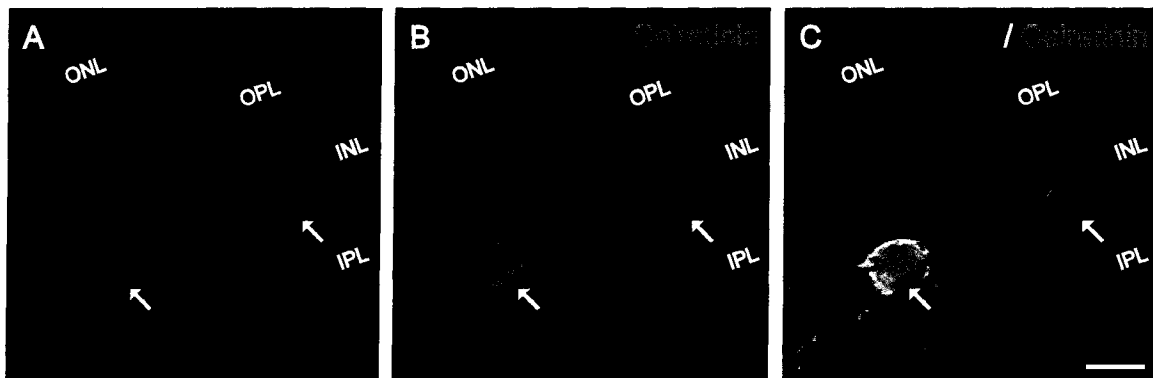
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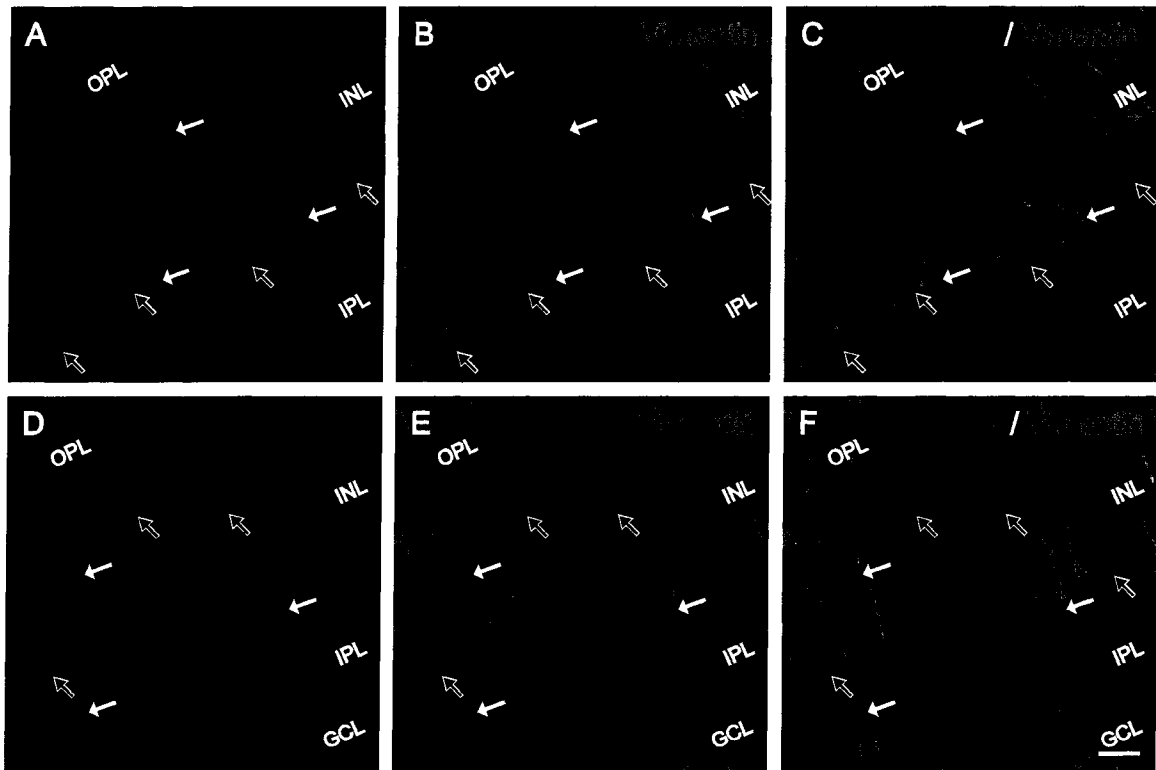
3.8. Supplemental Material



Supplemental Figure 1. Preadsorption controls confirmed the specificity of the anti-Ngb and anti-Cygb antibodies used in the study. Images were captured using confocal microscopy. (A) Mouse monoclonal anti-Ngb, (B) rabbit polyclonal anti-Ngb and (C) rabbit polyclonal anti-Cygb antibodies (red) were preadsorbed against their respective specific proteins before incubation on tissue sections. No specific immunoreactivity was observed. Cell nuclei (blue) were labeled with DAPI. Scale bar, 25 μm .



Supplemental Figure 2. Ngb co-localized with calretinin-IR cells. Images were captured using confocal microscopy. (A) Ngb- (red) and (B) calretinin-IR (green) co-localized in amacrine cells in the inner nuclear layer, as shown in the merged image (C). Arrows point to examples of amacrine cells. Cell nuclei (blue) were labeled with DAPI. Scale bar, 10 μm .



Supplemental Figure 3. Ngb and Cygb did not co-localize with vimentin-IR cells. Images were captured using confocal microscopy. No co-localization of (A) Ngb or (D) Cygb (red) with (B and E) vimentin (green) was detected on the merged images (C and F). Note also the absence of the radial pattern of immunoreactivity characteristic for Müller glia in images A and D. Arrows point to examples of Müller glial radial processes, open arrows point to examples of inner retinal neurons. Cell nuclei (blue) were labeled with DAPI. Scale bar, 10 μm .

CHAPTER 4: Neuroglobin and Cytochrome b – Novel Candidates for Oxygen Mediated Metabolism in Anterior Eye Segment

Submitted

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4.1. Abstract

Purpose: To describe detailed localization of two oxygen-binding proteins neuroglobin (Ngb) and cytochrome b (Cygb), in the anterior segment and non-vascular structures (cornea, lens) of healthy human and canine eyes.

Methods: Specific antibodies directed against Ngb and Cygb were used to examine the distribution patterns for Ngb and Cygb. The anterior segment structures examined included cornea, iris, trabecular meshwork, canal of Schlemm, ciliary body and lens. Antibody

binding patterns were captured with a confocal scanning laser microscope and conventional upright microscope.

Results: Immunohistochemical analysis of sections of healthy human and canine eye revealed Ngb and Cygb immunoreactivity (IR) in the corneal epithelium and endothelium. In the iris, Ngb and Cygb-IR was localized to the anterior border layer and the stroma, iridal sphincter and dilator muscle. In the iridocorneal angle, both Ngb and Cygb were detected in endothelial cells of the human and canine trabecular meshwork and canal of Schlemm in human. In the ciliary body, Ngb- and Cygb-IR was localized to the nonpigmented ciliary epithelium of the pars plana and pars plicata, as well as in ciliary body musculature. Ngb and Cygb distribution was consistent between human and canine anterior segments and was co-localized within the same cells in all structures.

Conclusions: Ngb and Cygb are co-localized within the same structures of the healthy human and canine anterior eye segment. Based on Ngb and Cygb immunolocalization and their previously reported biochemical features, we hypothesize that Ngb and Cygb may function as scavengers of reactive oxygen species and/or facilitators of oxygen diffusion.

4.2. Introduction

Globins are a family of heme-containing proteins that reversibly bind gaseous ligands such as oxygen (O₂), nitric oxide (NO) and carbon monoxide (CO). Their presence has been described in bacteria, fungi, protists, plants and animals¹. Four mammalian globins have been identified so far (hemoglobin, myoglobin, neuroglobin and cytoglobin)²⁻⁶. Neuroglobin has been identified in the brain^{2, 7-13} and in retinal neurons^{11, 12, 14}, but also in different endocrine tissues¹⁵. Functions proposed for Ngb include enhancement of oxygen delivery to

mitochondria^{12, 14, 16, 17}, detoxification of NO^{9, 18-20}, and hypoxia sensing²¹⁻²³. In addition, it has been demonstrated that Ngb expression is increased *in vivo* and *in vitro* during acute neuronal hypoxia, and that Ngb overexpression leads to enhanced survival of cortical neurons^{24, 25}. Cyoglobin was detected in virtually all tissues in humans, mice and zebrafish^{4, 5}. Proposed roles for Cygb include facilitation of oxygen diffusion, NO scavenging⁴ and O₂ supply for enzymatic reactions such as hydroxylation of proline residues during collagen synthesis²⁶.

The anatomical structures of the anterior segment of the mammalian eye are comprised of the cornea, iris, iridocorneal angle, ciliary body and lens²⁷. Some basic physiological requirements that need to be fulfilled in order for the anterior eye segment structures to function efficiently are sufficient O₂ supply²⁸⁻³², the existence of mechanisms for reactive oxygen species (ROS) scavenging³³⁻³⁸, and NO level regulation³⁹⁻⁴³. Based on Ngb and Cygb immunolocalization and their previously reported biochemical features, we hypothesize that Ngb and Cygb may serve important functions as scavengers of reactive oxygen species and/or facilitators of oxygen diffusion in the anterior eye segment and non-vascular eye structures. Therefore, the purpose of this study was to use immunohistochemical procedures and confocal microscopy in order to investigate detailed localization of the two gaseous ligand-binding globins, Ngb and Cygb, in these structures.

4.3. Materials and Methods

Human Tissue

Globes from six donors with no medical history of ocular diseases or histopathologic evidence of ocular disease (ages: 1.5 and 6 months, 60, 62, 78 and 82 years) were fixed for

48 hours in 10% neutral buffered formalin immediately after tissue procurement. They were then transferred to an ethanol-formaldehyde mixture (Penfix, Richard-Allan Scientific, Kalamazoo, MI), embedded in paraffin and sectioned at 4 μm thickness. The eyes were obtained through the Iowa Lions Eye Bank in Iowa City and from the F.C. Blodi Eye Pathology Laboratory at the University of Iowa. Tissue collection adhered to the tenets of the Declaration of Helsinki and University of Iowa guidelines.

Canine Tissue

Eyes from nine healthy adult beagles (2-4 years of age) were collected immediately after euthanasia. Each animal was euthanized for reasons other than use in this study. Prior to euthanasia all eyes were examined for signs of ocular abnormalities (slit lamp examination, fundus examination) and the presence of elevated intraocular pressure (tonometry). Eyes with detectable abnormalities of the anterior segment, lens or the fundus and/or presence of elevated intraocular pressure (>25 mmHg) were not collected for use in this study. Globes were fixed for 12 hours at 4°C in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Following fixation, globes were embedded in paraffin and sections were cut at 7 μm thickness. All research conducted in this study was in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and Iowa State University Committee on Animal Care regulations.

Antibodies

Full length human neuroglobin and cytoglobin recombinant proteins were synthesized and purified as described previously^{3,4}. Polyclonal antisera against synthesized Ngb or Cygb proteins were raised in rabbits and antibodies (Abs) were affinity purified from the serum using the recombinant proteins coupled to a SulfoLink column (Pierce Biotechnology,

Rockford, IL). Mouse monoclonal anti-Ngb antibody and the human recombinant Ngb protein used to produce this antibody were kindly provided by BioVendor Laboratory Medicine, Inc. Antibody specificity was confirmed on recombinant protein and canine tissue samples⁴⁴. Primary Abs used in this study and their dilutions are summarized in Supplemental Table 1.

Immunohistochemistry

Fluorescent immunohistochemistry was performed as described previously⁴⁴. Briefly, tissue sections were deparaffinized, rehydrated in a graded alcohol series, processed for antigen retrieval and incubated for 2 hours in blocking solution. Sections were double-labeled with primary Ab cocktail overnight, then incubated in one of the following secondary Ab cocktails: goat anti-mouse Cy5 (Jackson ImmunoResearch, West Grove, PA) and goat anti-rabbit Alexa 488 Ab (Molecular Probes, Eugene, OR), or goat anti-rabbit Cy5 (Jackson ImmunoResearch) and goat anti-mouse Alexa 488 Ab (Molecular Probes) diluted to 1:200. Following the 2 hour incubation, sections were washed in potassium phosphate buffered saline (KPBS) with Triton X-100. Finally, sections were counterstained with 1 µg/ml of 4',6-diamino-2-phenylindole (DAPI; Molecular Probes), washed in KPBS, and coverslipped. Negative controls were run in parallel during all processing and included the omission of the primary Ab or secondary Ab.

Analysis of Tissue Sections

Human and canine tissue sections labeled with fluorescent antibodies were visualized and images captured using a Leica confocal scanning laser microscope (TCS-NT; Leica Microsystems Inc., Exton, PA). A Sony DXC-S500 color digital camera (Labtek, Campbell, CA) was used for the brightfield images in Supplemental Fig. 2. All figures were prepared

using Photoshop (ver. 7.0, Adobe, San Jose, CA) and Freehand (ver.10.0, Macromedia, San Francisco, CA).

4.4. Results

To examine the distribution pattern of Ngb in the human and canine anterior eye segments we used mouse monoclonal and rabbit polyclonal anti-Ngb Abs. Both Abs displayed similar labeling patterns in all structures examined. Cygb distribution in human and canine anterior eye segments was determined using a rabbit polyclonal antibody. For our double-labeling studies, mouse monoclonal anti-Ngb antibody was used in combination with rabbit polyclonal anti-Cygb antibody.

Distribution of Neuroglobin and Cytoglobin in the Cornea

To investigate distribution of Ngb and Cygb within the anterior eye segment, double-labeling was performed on human and canine cornea. As illustrated in Fig. 1, Ngb-immunoreactivity (-IR) and Cygb-IR was detected in corneal epithelium and corneal endothelium. Weak immunoreactivity was detected in corneal stromal keratocytes. No immunoreactivity was present in human Bowman's membrane (Figs. 1A-D) and human and canine Descemet's membrane (Figs. 1E-H and 1M-P, respectively).

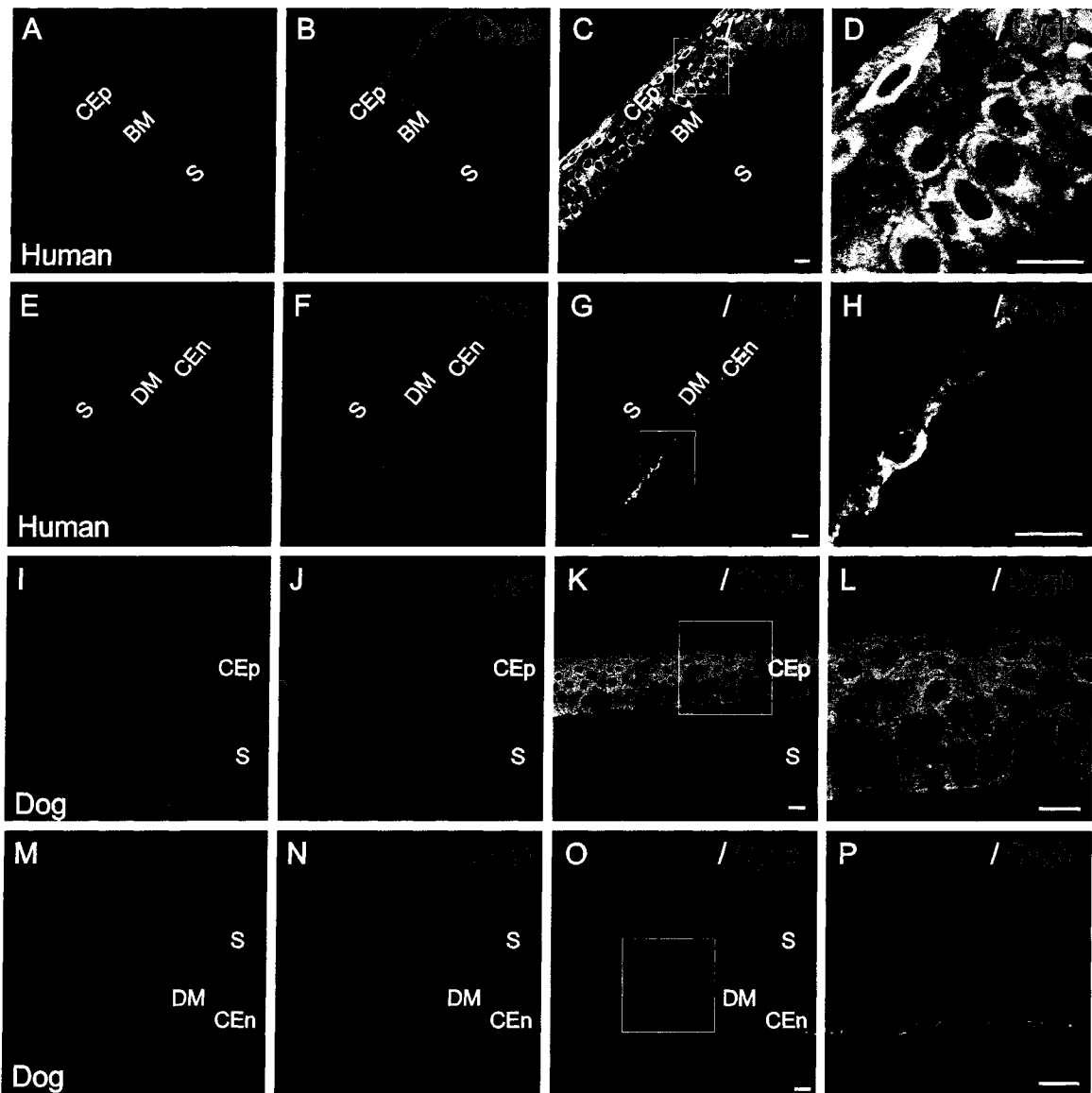


Figure 1. Ngb and Cygb immunolocalization in the human and canine cornea. (A) Confocal images of the human cornea double-labeled for (A) Ngb (red) and (B) Cygb (green) displayed immunoreactivity of both proteins in the corneal epithelium (C) and (D); note the absence of immunoreactivity in Bowman's membrane. (D) Enlarged region of the corneal epithelium indicated by the box from (C). (E) Ngb-IR (red) and (F) Cygb-IR (green) in human corneal endothelial cells co-localized, as shown in (G) and (H); note the absence of immunoreactivity in Descemet's membrane. (H) Enlarged region of the corneal endothelium indicated by the box from (G). (I) Confocal images of Ngb-IR (red) and (J) Cygb-IR (green) in the canine corneal epithelium; immunoreactivity co-localized, as shown in merged images (K) and (L). (L) Enlarged region of the corneal epithelium indicated by the box from (K). (M) Ngb-IR (red) and (N) Cygb-IR (green) in canine corneal endothelial cells co-localized in (O) and (P). (P) Enlarged region of the corneal endothelium indicated by the box from (O). In all images cell nuclei (blue) were labeled with DAPI. Scale bar 25 μ m; in D, H, L and

P 12.5 μm . Abbreviations: CEp (corneal epithelium), BM (Bowman's membrane), S (stroma), DM (Descemet's membrane), CEn (corneal endothelium).

Distribution of Neuroglobin and Cytoglobin in the Iris

Double-labeling with mouse monoclonal anti-Ngb and rabbit polyclonal anti-Cygb was performed in order to examine the distribution patterns of Ngb and Cygb in human and canine iris. As illustrated in Figs. 2A-F, Ngb-IR and Cygb-IR in the human iris was co-localized to the anterior border layer, stroma and iridal sphincter muscle. In addition, Ngb-IR and Cygb-IR was detected in the iridal dilator muscle (data not shown). In comparison with the human iris, similar patterns of distribution of Ngb and Cygb were observed in the canine iris, as shown in Figs 2G-L.

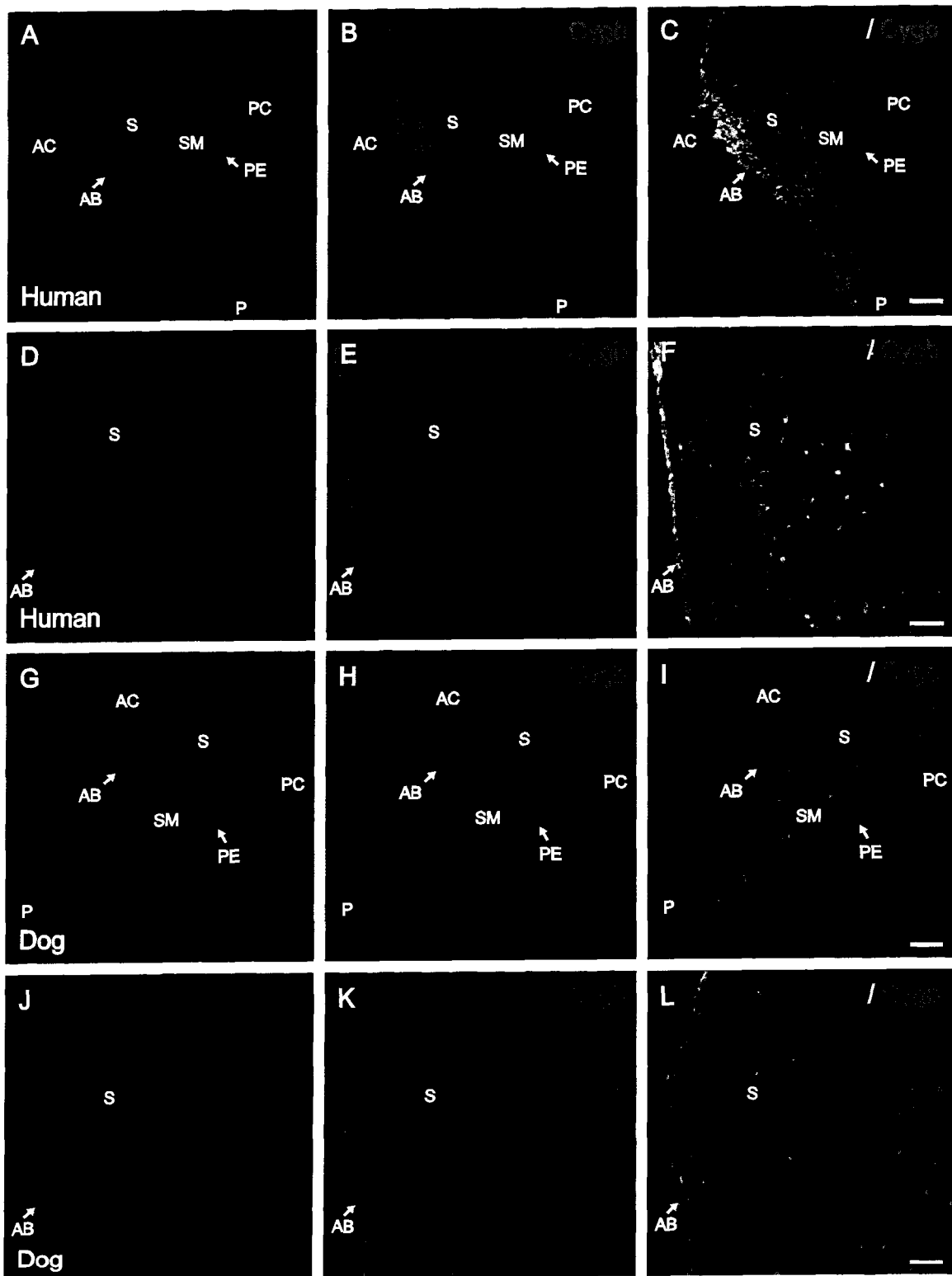


Figure 2. Ngb and Cygb immunolocalization in the human and canine iris. Confocal images of the human iris illustrating distribution patterns for (A) Ngb-IR (red) and (B) Cygb-IR

(green) in cells of the anterior border layer and the stroma, as well as in iridal sphincter. (C) Ngb and Cygb-IR co-localized within the same cells. Images of the area close to the root of the iris demonstrate (D) Ngb-IR (red) and (E) Cygb-IR (green) in cells of the anterior border layer and stroma; proteins are co-localized, as shown in merged image (F). Confocal images of the canine iris show (G) Ngb-IR (red) and (H) Cygb-IR (green) in cells of the anterior border layer and the stroma, as well as in iridal sphincter; proteins were found to co-localize, as illustrated in (I). Images of the area close to the root of the iris illustrating (J) Ngb-IR (red) and (K) Cygb-IR (green) in cells of the anterior border layer and stroma. Ngb and Cygb co-localize within the cells, as shown in the merged image (L). In all images the cell nuclei (blue) were labeled with DAPI. Scale bar 25 μ m. Abbreviations: AC (anterior chamber), AB (anterior border layer), S (stroma), SM (sphincter muscle), PE (pigmented epithelium), PC (posterior chamber), P (pupillary border).

The anterior border layer and stroma of the iris in both the human and canine eye is composed of two cell types: fibroblasts and melanocytes^{45, 46}. In order to determine if Ngb and Cygb are found in both cell types of the iris anterior border layer and stroma, double-labeling was performed with an anti-vimentin antibody, which labels both cell types, and light microscopic images which show melanin pigmentation were captured along with the confocal images of the fluorescent labeling (Supplemental Fig. 1).

Distribution of Neuroglobin and Cytoglobin in the Iridocorneal Angle

Iridocorneal angle of human and canine eyes was examined for the presence and possible co-localization of Ngb and Cygb. As illustrated in Fig.3, Ngb-IR and Cygb-IR were detected in the same cells of the trabecular meshwork in human and canine eyes. In addition, Ngb and Cygb co-localization was detected in human iridocorneal angle in the cells lining the canal of Schlemm, as illustrated in merged image 3C.

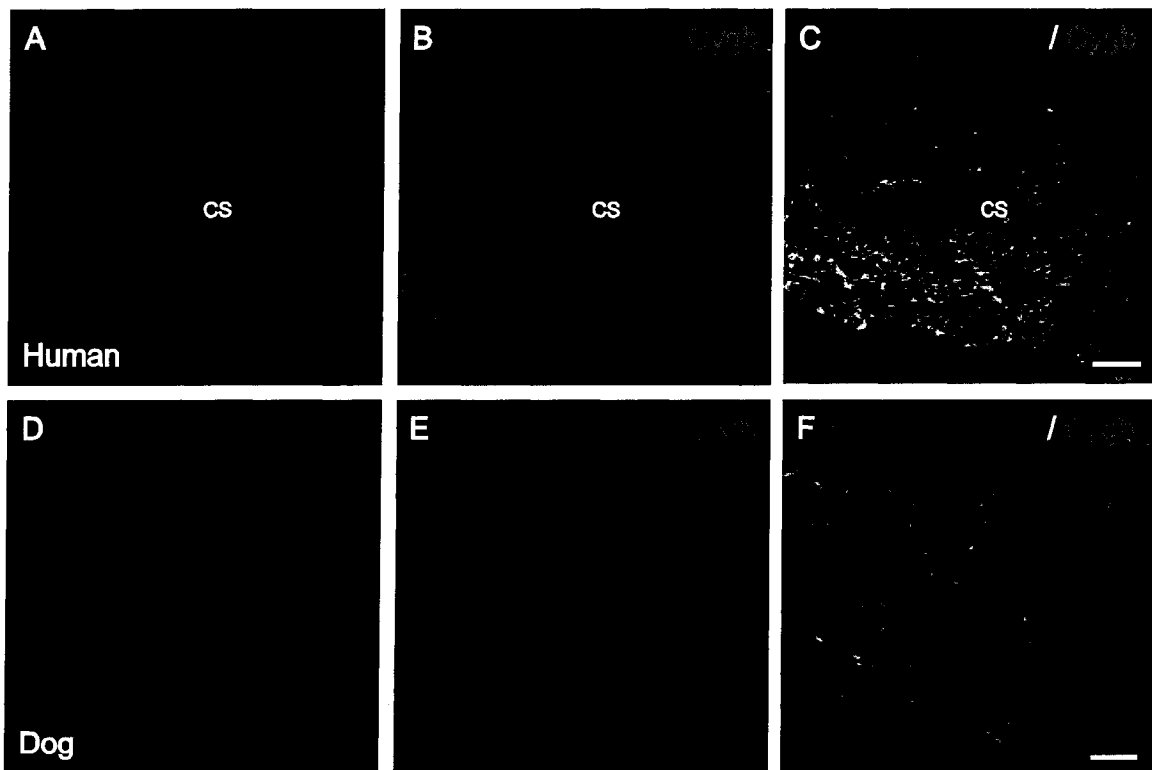


Figure 3. Ngb and Cygb immunolocalization in the human and canine iridocorneal angle. Images were captured with confocal microscopy. Human iridocorneal angle with (A) Ngb-IR (red) and (B) Cygb-IR (green) in the cells of the trabecular meshwork and the cells lining canal of Schlemm. Immunoreactivity for both proteins was found to co-localize within the same cells, as shown in (C). (D) Ngb-IR (red) and (E) Cygb-IR (green) co-localized within the cells of the canine trabecular meshwork (F). In all images cell nuclei (blue) were labeled with DAPI. Scale bar 12.5 μ m. Abbreviation: CS (canal of Schlemm).

Distribution of Neuroglobin and Cytoglobin in the Ciliary Body

To investigate distribution and possible co-localization of Ngb and Cygb within the ciliary body and lens of human and canine eyes, double-labeling was performed with mouse monoclonal anti-Ngb and rabbit polyclonal anti-Cygb antibodies. As illustrated in Fig. 4A-D, in the pars plana of the human ciliary body, Ngb-IR and Cygb-IR was co-localized to the nonpigmented ciliary epithelium. Similarly, as shown in Fig. 4E and F, in the pars plicata of the human ciliary body Ngb-IR and Cygb-IR was co-localized to the nonpigmented ciliary

epithelium. In addition, both Ngb-IR and Cygb-IR was detected in human ciliary muscle (Supplemental Fig. 2). In the canine ciliary body, as in human, Ngb-IR and Cygb-IR were localized in the nonpigmented ciliary epithelium of the pars plana and pars plicata and in the ciliary muscle (Figs. 4I-P). These results demonstrate extensive co-localization of Ngb and Cygb within ciliary body.

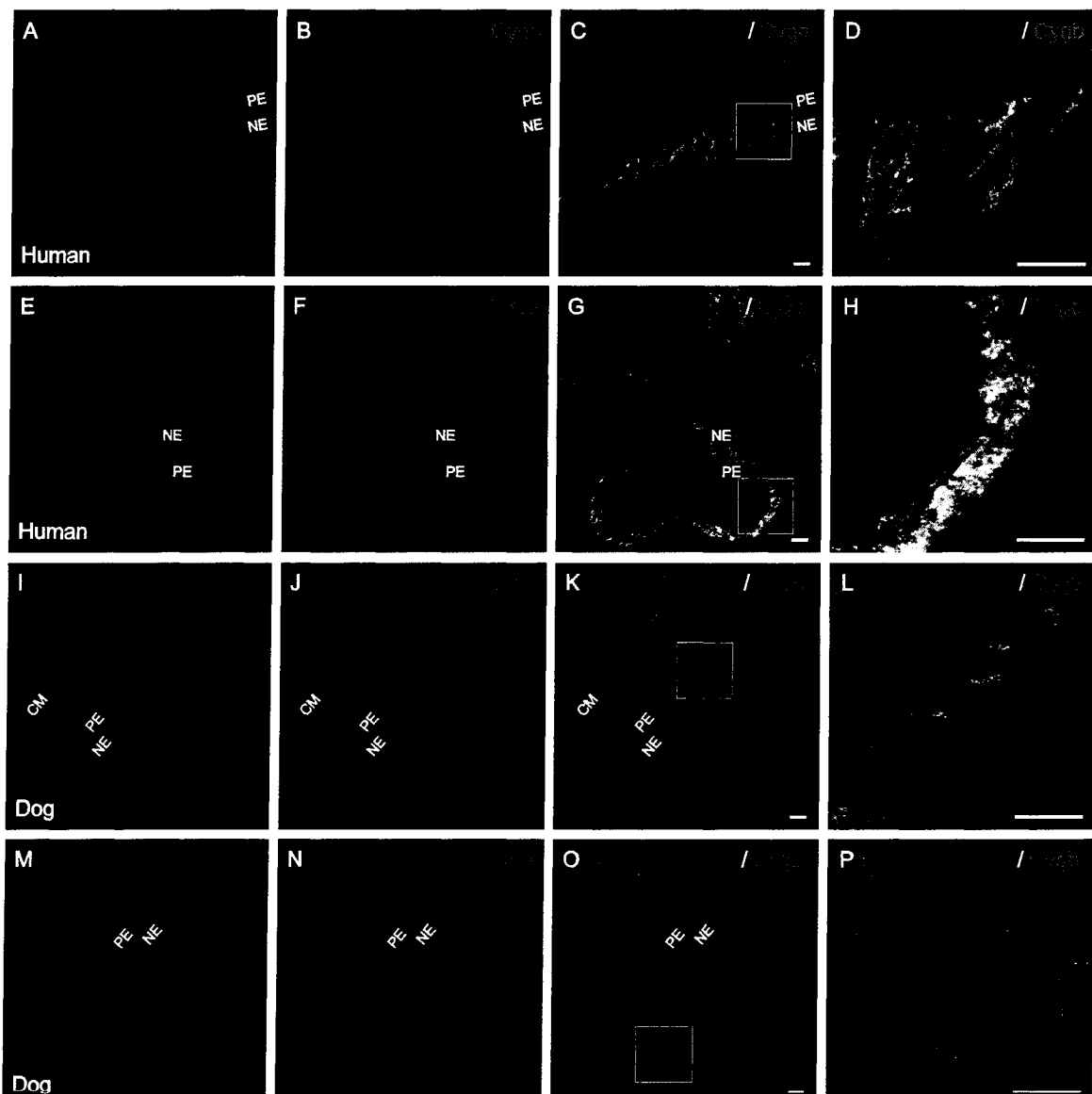


Figure 4. Ngb and Cygb immunolocalization in the human and canine ciliary body. Confocal images illustrating distribution patterns for (A) Ngb-IR (red) and (B) Cygb-IR (green) in nonpigmented epithelial cells of the human pars plana of the ciliary body. (C) and (D)

Merged images illustrate that Ngb and Cygb-IR co-localize within the same cells. **(D)** Enlarged region of nonpigmented epithelial cells indicated by the box from **(C)**. **(E)** Ngb-IR (red) and **(F)** Cygb-IR (green) in nonpigmented epithelial cells of the human pars plicata of the ciliary body co-localized in merged images **(G)** and **(H)**. **(H)** Enlarged region of nonpigmented epithelial cells indicated by the box from **(G)**. Confocal images illustrating **(I)** Ngb-IR (red) and **(J)** Cygb-IR (green) in nonpigmented epithelial cells of the canine pars plana of the ciliary body and ciliary muscle; immunoreactivity co-localized in **(K)** and **(L)**. **(L)** Enlarged region of nonpigmented epithelial cells indicated by the box from **(K)** **(M)** Ngb-IR (red) and **(N)** Cygb-IR (green) in nonpigmented epithelial cells of the canine pars plicata of the ciliary body co-localized in **(O)**. **(P)** Enlarged region of nonpigmented epithelial cells indicated by the box from **(O)** In all images cell nuclei (blue) were labeled with DAPI. Scale bar 25 μm ; in **D**, **H**, **L** and **P** 12.5 μm . Abbreviations: NE (nonpigmented epithelium), PE (pigmented epithelium), CM (ciliary muscle).

In the lens, as illustrated in Supplemental Fig. 3, weak Ngb-IR and Cygb-IR was co-localized to the lens epithelium.

Supplemental Table 2. summarizes the regional distribution of Ngb and Cygb in human and canine anterior segment.

4.5. Discussion

Highly differentiated tissues of the anterior eye segment, comprised of the ciliary body, iris, iridocorneal angle, cornea and lens, are critical for normal vision. In order to function properly, the anterior segment of the eye requires a sufficient supply of O_2 ²⁸⁻³¹, mechanisms for ROS scavenging³³⁻³⁵, and the ability to regulate levels of NO ³⁹⁻⁴³. A comparative study of the patterns of distribution of Ngb and Cygb in the anterior segment of the human and canine eye is warranted due to the advancements in canine research models for studying human disease. The recent completion of genome sequence in dogs (<http://www.ncbi.nih.gov/Genomes/>), together with long term inbreeding that has resulted in reduced genetic diversity within breeds, represents a good foundation for dogs to become

promising model for studying human genetic diseases. Furthermore, dogs appear to be a viable large-animal model for study of ocular disorders^{47, 48}, increasing the possibility that using a dog model in future studies would advance our current insight into key features of human ocular physiology and disease.

The presence of Ngb and Cygb has been studied in considerable detail in the retina of mouse, rat, dog and human^{14, 49, 50}, and both proteins were found in neuronal, but not in glial cells. In addition, Ngb has been detected in some endocrine tissues^{7, 15}, and Cygb has been reported in hepatic stellate cells⁵¹, connective tissue fibroblasts, osteoblasts and chondroblasts²⁶. Our study detected both Ngb and Cygb immunoreactivity in the diverse tissues of the anterior eye segment. To our knowledge, this is the first study to comprehensively investigate the presence and distribution of Ngb and Cygb, two recently discovered globins which have the ability to bind gaseous ligands, in the anterior segment of the eye. Previous to this report, a study by Schmidt et al.⁵⁰ reported the presence of Cygb immunoreactivity in the iris. The findings reported in the iris are similar to those obtained in this study. In contrast, these investigators did not detect Cygb immunoreactivity in the mouse ciliary epithelium. Although at present we have no explanation for the difference between their results and our finding of Cygb immunoreactivity in the cells of the nonpigmented ciliary epithelium, species specific differences and the different source of primary antibodies used might account for such results. While the anti-Cygb antibody used in our study was generated against whole recombinant human protein, Schmidt et al. used antibodies generated against different Cygb peptides⁵⁰.

Since the exact role of Ngb and Cygb has yet to be determined, it is difficult to hypothesize on the functional significance of the distribution pattern of these proteins. From

a developmental perspective, the presence of Ngb and Cygb in diverse tissues such as ciliary epithelium, ciliary muscle, iris stroma, sphincter and dilator muscle, endothelium of trabecular meshwork and corneal endothelium may be due to the neuroectoderm/neural crest^{52, 53} origin of these tissues. On the other hand, this does not explain presence of Ngb in corneal epithelium, which originates from surface ectoderm, nor in cells lining canal of Schlemm, which are of mesodermal origin^{52, 53}. Even though Ngb has been detected in tissues/cells other than neurons (β cells of the islets of Langerhans in pancreas¹⁵, spermatogonia and spermatocytes in testes, endocrine cells of the anterior pituitary, medulla and glomerular zone of the cortex of the adrenal gland⁷), it remains to be determined what role Ngb might have in these particular tissues.

It is possible that Ngb and Cygb function in the anterior segment of the eye is related to oxygen transport and free radical/NO scavenging. Oxygen supply to avascular tissues of the cornea and lens is thought to be accomplished from aqueous humor or diffusion from corneal surface, while the other anterior segment tissues are vascularised. Oxygen homeostasis in the anterior segment has not been studied in great detail^{30, 31}, and it is possible that these two oxygen-binding proteins, Ngb and Cygb, may play specific roles in oxygenation of avascular and vascularized structures of the anterior segment. Provided that Ngb and Cygb concentrations are high enough, their presence in two avascular tissues, cornea and lens, and in some cells of the highly metabolically active tissues such as ciliary body and iris could be linked to facilitation of oxygen metabolism.

The data from kinetic studies support the idea that Ngb is involved in scavenging of reactive oxygen and nitrogen species^{54, 55}. Also, Cygb has been shown to have peroxidase activity in stellate cells of the liver⁵¹. Based on these previous studies and our present

findings, we hypothesize that a similar scavenging role is maintained in the anterior eye segment. Indeed, anterior eye segment structures have been found to have diverse NOS activity under both physiological and pathological conditions⁴⁰. It has also been reported that NO acts as a vasodilator to regulate aqueous production in the ciliary body^{56, 57}, and can influence aqueous outflow in the trabecular meshwork and ciliary muscle^{43, 56, 58}. In addition, NOS activity was detected in corneal epithelium, stromal fibroblasts and endothelium during experimental inflammation^{40, 42}, and in iris due to endotoxin-induced uveitis⁴⁰. Furthermore, hydrogen peroxide (H₂O₂) is present in tissues of the anterior uvea and in mammalian aqueous humor at concentrations between 30 and 70 μM³³. In order to protect against oxidative stress induced by H₂O₂, different mechanisms are employed. Catalase, the enzyme that metabolizes H₂O₂ to water and oxygen, has been found in the cornea, aqueous, humor, trabecular meshwork, and ciliary body³³. It is possible that, in addition to other mechanisms, Ngb and/or Cygb could serve in protecting the eye from damage induced by reactive oxygen species and excess NO.

In summary, our double-labeling study describes the presence and co-localization of two gaseous ligand-binding molecules in anterior segment and non-vascular eye structures of human and canine eyes, laying the foundations for studying this feature of human ocular regulation of the oxidative metabolism.

4.6. Acknowledgements

The authors thank Jeffrey Orasky and Matt Harper for technical assistance during preparation of this manuscript. Also, we thank Daria Pospisilova, Petr Kasperek and Jiri Havlasek from BioVendor Laboratory Medicine, Inc. for providing us with mouse monoclonal anti-Ngb Ab.

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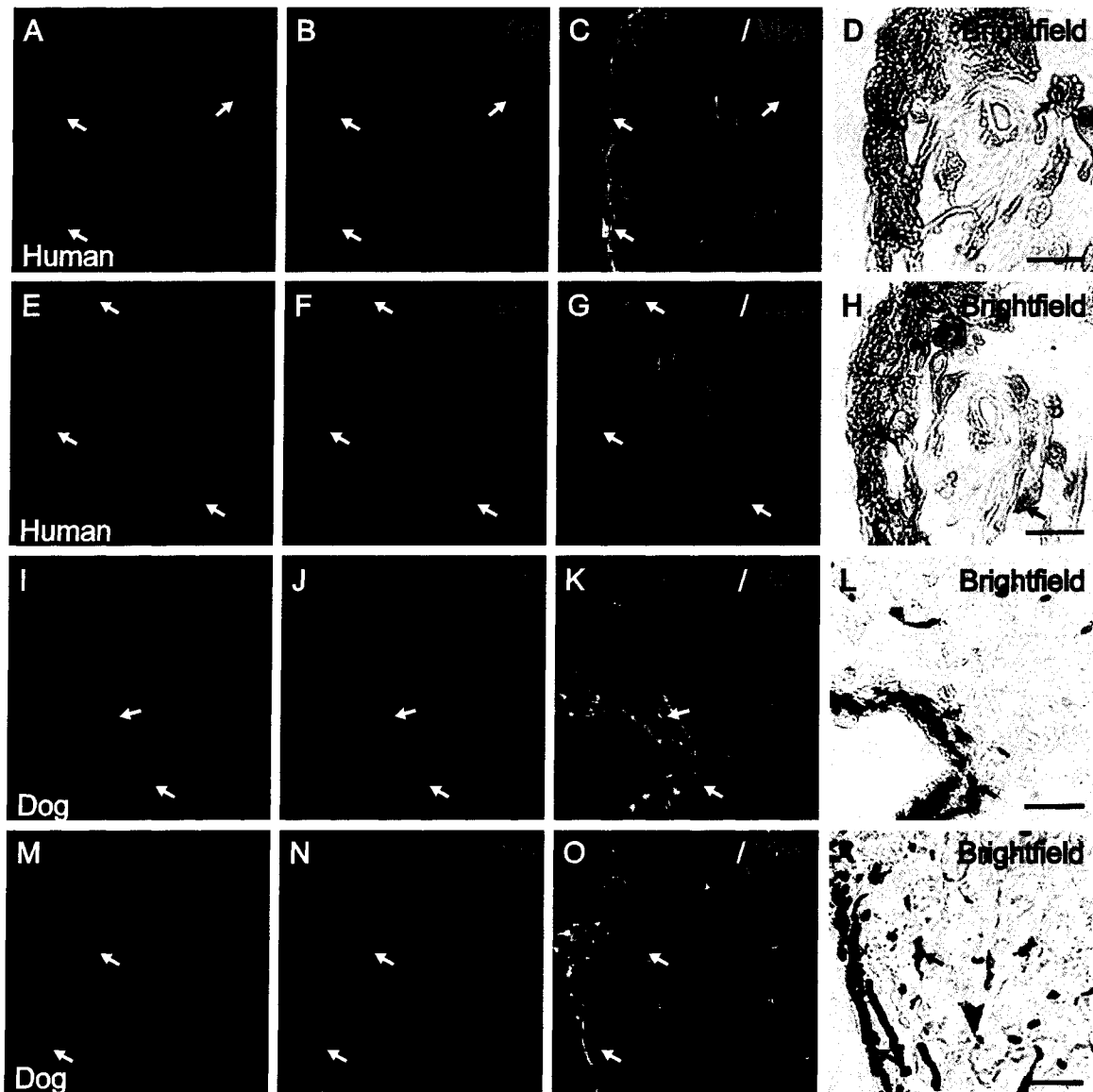
4.8. Supplemental Material

Supplemental Table 1. Primary Antibodies Used in the Study

Antibody	Specificity	Used for detecting:	Species	Dilution	Source
Anti-neuroglobin	Human recombinant neuroglobin	Ngb	mouse	1:100	BioVendor Laboratory Medicine, Inc.
Anti-neuroglobin	Human recombinant neuroglobin	Ngb	rabbit	1:100	M. Hargrove, Iowa State University, IA
Anti-cytoglobin	Human recombinant cytoglobin	Cygb	rabbit	1:100	M. Hargrove, Iowa State University, IA
Anti-Vimentin	Vimentin	Iris fibroblasts and melanocytes	mouse	1:200	DakoCytomation, Carpinteria, CA

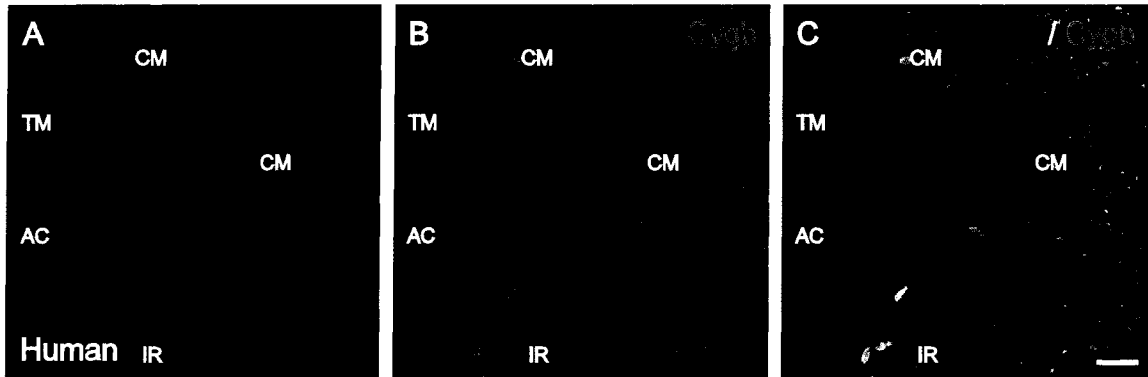
Supplemental Table 2. Summary of the distribution of Ngb and Cygb in the human and canine anterior eye segment

	Ngb	Cygb
Ciliary pigmented epithelium	-	-
Ciliary nonpigmented epithelium	+	+
Ciliary muscle	+	+
Iridal anterior border layer fibroblasts	+	+
Iridal anterior border layer melanocytes	+	+
Iridal stromal fibroblasts	+	+
Iris stromal melanocytes	+	+
Iridal sphincter muscle	+	+
Iridal dilator muscle	+	+
Iridal posterior epithelium	-	-
Trabecular meshwork	+	+
Corneal epithelium	+	+
Corneal stromal keratocytes	+	+
Corneal endothelium	+	+
Lens capsule	-	-
Lens anterior epithelium	+	+
Lens fibers	-	-

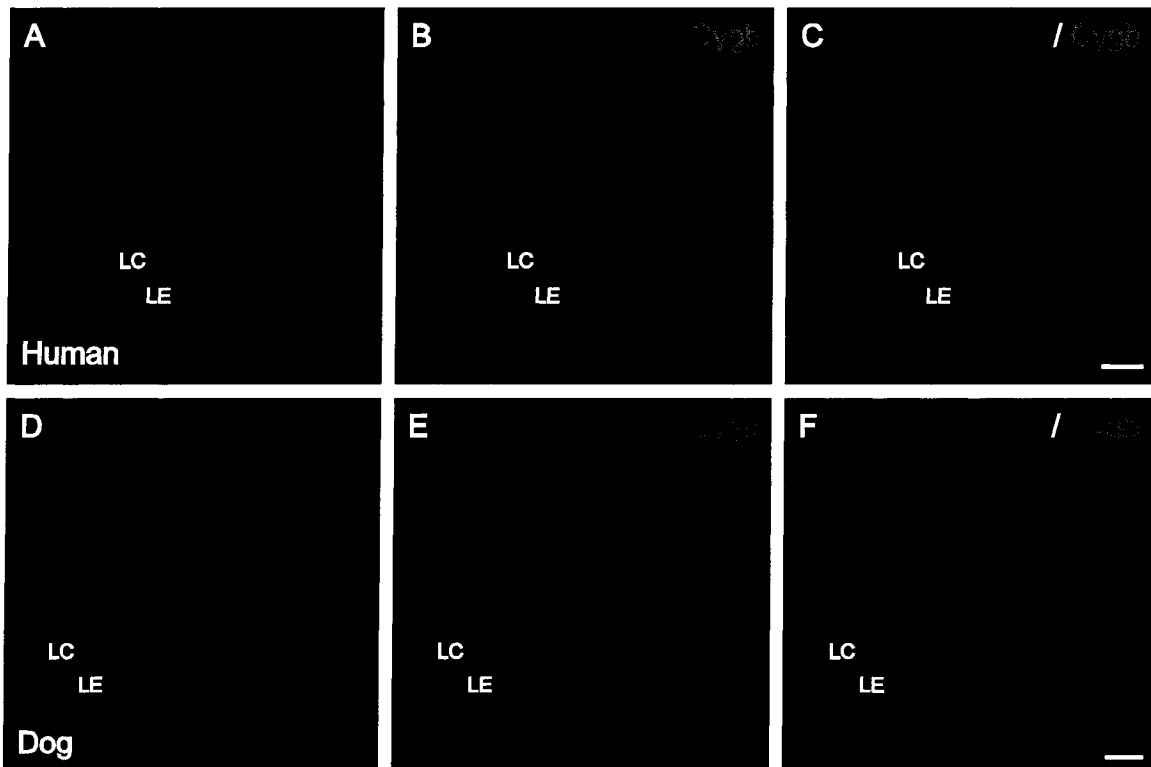


Supplemental Figure 1. Ngb and Cygb localization in human and canine iridal fibroblasts and melanocytes. Confocal images illustrating distribution patterns for (A) Ngb-IR (red) and (B) Vimentin-IR (green) in cells of the anterior border and stroma of the human iris. (C) Merged image illustrates colocalization of Ngb and vimentin within the same cells. (D) Corresponding brightfield image demonstrates pigmentation of some immunoreactive cells, as indicated with arrows. (E) Cygb-IR (red) and (F) Vimentin-IR (green) in cells of the anterior border and stroma of the human iris co-localized in (G). (H) Corresponding brightfield image showing pigmentation of some immunoreactive cells, as indicated with arrows. Confocal images illustrating (I) Ngb- IR (red) and (J) Vimentin-IR (green) in cells of the anterior border and stroma of the canine iris; immunoreactivity co-localized in (K). (L) Corresponding brightfield image; some immunoreactive cells are pigmented, as indicated with arrows. (M) Cygb- IR (red) and (N) Vimentin-IR (green) in cells of the anterior border and stroma of the canine iris are co-localized in (O). (P) Corresponding brightfield image

showing that some immunoreactive cells are also pigmented, as indicated with arrows. In all images cell nuclei (blue) were labeled with DAPI. Scale bar 25 μ m. Abbreviations: NE (nonpigmented epithelium), PE (pigmented epithelium), CM (ciliary muscle).



Supplemental Figure 2. Ngb and Cygb immunolocalization in the human ciliary muscle. Confocal images of the human ciliary muscle double-labeled for (A) Ngb (red) and (B) Cygb (green) shows immunoreactivity for both proteins in the ciliary muscle, trabecular meshwork and iris anterior border; immunostaining is co-localized, as illustrated in merged image (C). In all images cell nuclei (blue) were labeled with DAPI. Scale bar 25 μ m Abbreviations: AC (anterior chamber), TM (trabecular meshwork), CM (ciliary muscle), IR (iris root).

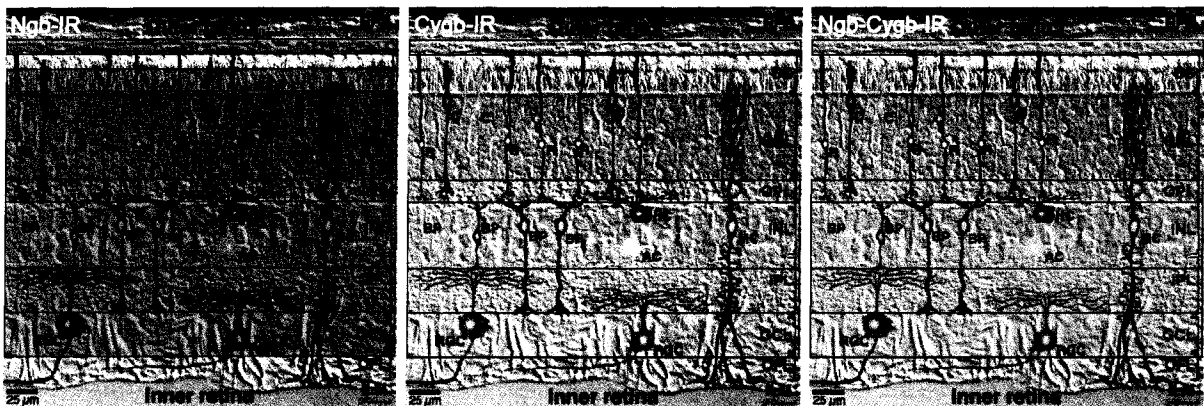


Supplemental Figure 3. Ngb and Cygb immunolocalization in the human and canine lens. (A) Confocal images of the human lens double-labeled for (A) Ngb (red) and (B) Cygb (green) showed weak immunoreactivity in the lens epithelium, which is co-localized in merged image (C). Confocal images illustrating (D) Ngb-IR (red) and (E) Cygb-IR (green) in canine lens co-localized in (F). In all images cell nuclei (blue) were labeled with DAPI. Scale bar 12.5 μm Abbreviations: LC (lens capsule), LE (lens epithelium).

CHAPTER 5: GENERAL CONCLUSIONS

5.1. General conclusions

Our studies are the first detailed description of Ngf and Cygf presence detected by immunohistochemical methods in different structures of the canine and human eye. Therefore, this dissertation has provided essential information about Ngf and Cygf distribution patterns in dog and human retina, including identification of retinal cell types in which these two proteins were present. The summary of the distribution pattern of Ngf immunoreactivity (IR), Cygf-IR and their co-localization in the canine and human retina is presented below:



Furthermore, this dissertation revealed Ngf and Cygf distribution pattern in anterior segment of human and canine eye. Based on the results of this dissertation, dog appears to be a viable “large eye” model for investigating roles that Ngf and Cygf play in the eye. Since the dog genome sequence has recently been completed (<http://www.ncbi.nih.gov/Genomes/>), we believe that using a dog as model organism in future studies would advance our current knowledge about the key features of human ocular physiology and disease.

In summary, work performed in this dissertation clearly demonstrated that:

1. Ngf immunoreactivity (IR) in the dog retina is located in the ganglion cell layer, inner and outer nuclear layers, inner and outer plexiform layers, photoreceptor inner segments and retinal pigment epithelium. Ngf-IR was localized within retinal neurons, but not within glial cells.
2. Cygf-IR in the dog retina was found in neurons and their processes in the ganglion cell layer, inner and outer nuclear layers, inner and outer plexiform layers, and retinal pigment epithelium. Cygf-IR was localized within retinal neurons, but not within glial cells.
3. Ngf and Cygf were expressed in the same cells in the ganglion cell layer and inner nuclear layer.
4. Ngf-IR in the human retina is distributed in the retinal ganglion cell layer, inner and outer nuclear layers, inner and outer plexiform layers, photoreceptor inner segments and retinal pigment epithelium. Ngf-IR was co-localized within neuronal, but not glial cells.
5. Cygf-IR was found in the retinal ganglion cell layer, inner nuclear layer, inner and outer plexiform layers, and retinal pigment epithelium. Cygf-IR was co-localized within neuronal, but not glial cells.
6. Ngf and Cygf were expressed in the same cells in the ganglion cell layer and inner nuclear layer; moreover, distribution pattern of Ngf and Cygf in human retina was similar to the distribution found in the dog retina.

7. Ngb and Cygb distribution patterns were the same for all dogs that were the subject of the study, as well as for all human subjects. In addition, no obvious difference in Ngb-IR and Cygb-IR in central vs. peripheral retina was observed.
8. Ngb and Cygb-IR was detected in the corneal epithelium and endothelium. In the iris, Ngb and Cygb-IR was localized to the anterior border layer and the stroma, iridal sphincter and dilator muscle. In the iridocorneal angle, both Ngb and Cygb were detected in endothelial cells of the human and canine trabecular meshwork and canal of Schlemm in human. In the ciliary body, Ngb- and Cygb-IR was localized to the nonpigmented ciliary epithelium of the pars plana and pars plicata, as well as in ciliary body musculature. Weak Ngb and Cygb-IR was detected in the lens epithelium.
9. Ngb and Cygb distribution was consistent between human and canine anterior segments and was co-localized within the same cells in all structures.

The final conclusion of the dissertation is that presence of neuroglobin and cytoglobin can be detected by immunohistochemical methods in different structures of the dog and human eye, and that there is similarity between distribution patterns of the two proteins in human and dog eyes. Based on Ngb and Cygb localization and their previously reported biochemical features, Ngb and Cygb may have important roles in scavenging reactive oxygen species and/or facilitating oxygen diffusion in the eye.

5.2. Recommendations for future research

The results and conclusions of this dissertation can be used as a framework for future Ngb and Cygb studies, including research at the cellular and molecular level, followed by *in vivo* studies to determine roles of these proteins in the eye, and potential for their therapeutic application in preventing retinal ganglion cell death¹.

One of the first steps that could be done would be the investigation of the cellular/subcellular localization of Ngb and Cygb in dog and human retinal ganglion cells, which could serve as a representative neuronal cell population found within the retina, and are the cell population most affected in glaucoma. Since it has been proposed that Ngb might facilitate oxygen diffusion to mitochondria, and that it interacts with some cellular and membrane-bound proteins²⁻⁴, electron microscopic analysis of subcellular localization of immunogold labeled Ngb is warranted. Similar investigation would also be beneficial for investigating Cygb localization, especially in clarifying the issue of Cygb nuclear localization that has been reported by one group of investigators⁵. In addition, subcellular Ngb and Cygb protein distribution could be compared to subcellular localization of Ngb and Cygb mRNA expression detected by electron microscope hybridization histochemistry⁶.

Furthermore, I would suggest a series of *in vitro* experiments performed on primary cultures of retinal ganglion cells isolated and purified by immunomagnetic selection of Thy-1.1-positive RGCs using antibody-coated magnetic beads⁷. A series of experiments should focus on possible neuroprotective role of Ngb and Cygb, and investigate if these proteins are upregulated during hypoxia (or hypoxia combined with increased hydrostatic pressure), and whether overexpression of Ngb or Cygb would rescue the cells from apoptosis. In addition,

molecular pathways involved in potential neuroprotection should be investigated, as well as the effect of hemin, which has been shown to induce Ngb expression in neural cells⁸.

Taking into account recent discovery of Mb in the brain⁹, in situ hybridization and immunohistochemical analysis will be necessary to investigate such possibility in the retina. Also, quantitative analysis of Ngb and Cygb protein expression in the retina, as well as in anterior eye segment is warranted in terms of additional information for the possible function of the two proteins. Furthermore, similar quantification procedure could be done for quantification of Ngb and Cygb within retinal ganglion cells isolated by immunomagnetic method from the dissected retina⁷. In addition, since it has been shown that Ngb concentration in the brain decreases with age¹⁰, a similar study investigating Ngb and Cygb concentration in retinas from young, middle-aged and aged animals would be indicative for the retina.

Further *in vivo* series of experiments are also necessary to determine Ngb and Cygb roles in the retina, and studies with knock-out mice, as well as double Ngb-Cygb knock-out mice are necessary to clarify and further delineate potential for the Ngb and Cygb in prevention and treatment of retinal neuronal damage. Knock-out animals could be exposed to short-term retinal ischemia, long-term retinal ischemia, and ocular hypertension. As noted earlier, before the above experiments, it should be determined if Mb is present in retina other than vascular wall. Tissue analysis should include RT-PCR and Western blot analysis, as well as in situ hybridization and immunohistochemistry of the tissue sections. Attention should be focused on investigating if both mRNA and protein levels increased, as well as determining if the change is qualitative or quantitative. Thus, double-labeling with cell specific markers, and

quantification of the immunogold labeled tissue sections (sampled, cut and processed under identical conditions), should be performed.

Finally, the combination of molecular techniques, genetic manipulation of the animals and *in vivo* experimentation using different research approaches and experimental strategies, should provide future investigations with essential information about physiological roles of Ngb and Cygb in the eye.

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ACKNOWLEDGMENTS

I would like to express deepest thanks to my Professor Dr Don Sakaguchi for the patience, help and just right amount of guidance. Dr Ed Powell gave strong intellectual support, and took me to wonderful trips that helped me maintain the balance. Also, I would like to thank Matt Harper and Jeff Orasky for essential everyday help. Many thanks to Dr Mark Hargrove and members of his lab, Dr Jan Buss, Dan Zamzow and Milan Joksimović. Finally, I would like to extend love and thanks to my family, especially my husband Dušan for his unlimited support and sound advices when I needed them the most.