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Restricted expression of Neuroglobin in the mouse retina and co-localization with Melanopsin and Tyrosine Hydroxylase

C.A. Hundahl [a,b,c,d,*], J. Fahrenkrug [a], H. Luuk [b,c], A. Hay-Schmidt [d], J. Hannibal [a]

[a] Department of Clinical Biochemistry, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark
[b] Centre of Excellence for Translational Medicine, University of Tartu, Tartu, Estonia
[c] Department of Physiology, University of Tartu, Tartu, Estonia
[d] Department of Neuroscience and Pharmacology, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

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ABSTRACT

Neuroglobin (Ngb), a neuronal specific oxygen binding heme-globin, reported to be expressed at high levels in most layers of the murine retina. Ngb's function is presently unknown, but based on its high expression level and oxygen binding capabilities Ngb was proposed to function as an oxygen reservoir facilitating oxygen metabolism in highly active neurons or to function as a neuroprotectant. In the present study, we re-examined the expression pattern of Ngb in the retina using a highly validated antibody. Furthermore, intactness of retino-hypothalamic projections and the retinal expression level of Melanopsin and Tyrosine Hydroxylase were investigated in Ngb-null mice. Ngb-immunoreactivity was found in a few neurons of the ganglion cell and inner nuclear layers co-expressing Melanopsin and Tyrosine Hydroxylase, respectively. Ngb deficiency neither affected the level of Melanopsin and Tyrosine Hydroxylase proteins nor the intactness of PACAP-positive retinohypothalamic projections in the suprachiasmatic nucleus. Based on the present results, it seems unlikely that Ngb could have a major role in retinal oxygen homeostasis and neuronal survival under normal conditions. The present study suggests that a number of previously published reports have relied on antibodies with dubious specificity.

1. Introduction

The retina has an even higher oxygen consumption rate per gram than the brain [1,2] leaving it very sensitive to fluctuations in oxygen level or inadequate blood supply [3]. As for the brain, the retina was believed to lack a globin-associated oxygen storage/diffusion system similar to myoglobin in the muscles. However, the discovery of Neuroglobin (Ngb) in neurons of the brain [4] and later in the retina [5] challenged that belief. Ngb is a monomeric heme-globin, which despite low sequence similarity to myoglobin and hemoglobin still displays the classic globin fold (for review [6]). Ngb was initially proposed to have a myoglobin-like function by facilitating oxygen diffusion or storage in the neurons despite very low overall concentration [4]. Later studies have challenged that belief based on Ngb's restricted expression pattern in the brain making it hard to reconcile with a broad oxygen storage/delivery function [7–10]. However, the situation is different in the retina where Ngb concentration has been reported to be up to 100 times higher than in the brain [5].

In the brain, the distribution of Ngb-immunoreactivity (IR) and mRNA is a matter of some controversy as it has been reported to be either ubiquitously expressed [4,11,12] or restricted to rather few areas [7–10,13–15] leading to fundamentally different functional interpretations and underlining the importance of proper antibody validation [16–21]. A number of papers have investigated Ngb protein expression in the retina and found it to be widespread and high [5,7,22–26]. Burmester and coworkers [5,22,26] described intense Ngb-IR in most layers of the mouse retina, which was, however, only partly supported by In Situ Hybridization (ISH). The discrepancy was ascribed to the hypothetical intracellular transport of Ngb protein after translation [5]. Based on the expression pattern and oxygen binding properties *in vitro* Ngb has been proposed to deliver oxygen to the highly metabolically active neurons of the retina [5,22,26]. This hypothesis was apparently supported by Ngb expression in the retinal layers with highest levels of mitochondria and oxygen consumption [5,26]. However, mathematical modeling of retinal oxygen consumption showed that Ngb has no significant role in this process [27].

In the light of the contradictory reports on Ngb expression and its proposed function in the brain it is likely that a similar situation may present itself in the retina. Therefore, and given the possible impact of Ngb on retinal physiology, the aim for this study was to re-examine Ngb expression in the retina. To this end, we have used Ngb-deficient mice (Ngb-null) to successfully validate our Ngb antibody and studied the effect of Ngb deficiency on the expression of marker proteins co-expressed with Ngb in the retina.

* Corresponding author. Address: Centre of Excellence for Translational Medicine, University of Tartu, Ravila 19, Tartu 50411, Estonia. Fax: +372 374 332.
E-mail address: c.hundahl@gmail.com (C.A. Hundahl).

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2. Materials and methods

2.1. Ngb deficient mice

The *Ngb*-null mouse model was created by GenOway (Lyon, France) under the project No. GenOway/SST/HSA1-*Ngb*/260307 and has been described previously [28,29].

2.2. Animals

For protein extraction, 15 wild type (wt) c57BL6 and 14 Ngb-null male mice (12–16 weeks old) were euthanized by decapitation and the eyes and brain were rapidly removed. Retina was dissected out on ice, snap-frozen on dry-ice and stored at −80 °C until protein extraction. For histology, six wt and six Ngb-null male mice were perfusion-fixed in Stefanini's fixative and brain and eyes were dissected out and postfixed in the same fixative for 24 h. The eyes and brains were cryoprotected by incubation in 30% sucrose in PBS for five days. The brains were frozen and sectioned in 40 μm thick sections in series of four. The eyes were sectioned in 14 μm thick sections and thaw-mounted on glass slides. Animal care and all experimental procedures were conducted in accordance to the principles of Laboratory Animal Care (Law on Animal Experiments in Denmark, publication 1306, November 23, 2007) and approved by Faculty of Health, University of Copenhagen, Denmark.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed according to previously described protocols [30]. The following primary antibodies were used for IHC: (1) rabbit anti-Ngb raised against purified recombinant mouse Ngb protein [8] (in-house, code# RbNGB 4836/5, in 1:100,000 dilution) (2) rabbit anti-Melanopsin (Mel) (in-house, code# 41K9-7 [31], in 1:1000 or 1:80,000 dilution), (3) rabbit anti-Calbindin (calb) (Swant, Marly, Switzerland. Cat. No. # CB-38a in 1:5000 dilution), (4) sheep anti-tyrosine hydroxylase (TH) (Novus Biologicals, Littelton, CO, USA Cat. No. # NB300-110 in 1:2000 dilution). The rabbit primary antibodies were detected either by donkey anti-rabbit Alexa-488 or Alexa-594 (Invitrogen, Carlsbad, CA, USA Cat. No. # A21206; A21207 in 1:800 dilution). The sheep antibody was detected by donkey anti-sheep Alexa-568 (Invitrogen, Carlsbad, CA, USA Cat. No. # A21099 in 1:800 dilution). When two rabbit primary antibodies were used in combination the tyramide signal amplification (TSA) (PerkinElmer, Waltham, MA, USA) was used as described in [30]. For detection of Pituitary Adenylate Cyclase-Activating Peptide (PACAP) and Ngb in the mouse brain of wt ($n = 3$) and Ngb-null mice ($n = 3$), a FITC-conjugated mouse anti-PACAP monoclonal antibody (in-house code# MabjHH1 [32], in 1:3000 dilution) and a guinea pig anti-Ngb (in-house, code# G [8], in 1:1000 dilution) were used. PACAP was detected as described in [33] and Ngb by a donkey-anti-guinea pig conjugated Dylight-594 (Jackson Immunoresearch Laboratories, Baltimore, PA, USA, Cat. No. # 706-516-148 in 1:500 dilution). The Ngb antibodies specificity was tested with pre-absorption with the immunizing antigen (recombinant Ngb protein) and by the lack of immunostaining in Ngb-null retina and brain [28,29].

2.4. Cell counts

Eyes from six and three wt mice were used for the quantification of Ngb co-expression with Mel and TH, respectively. The eyes were cut 14 μm thick sections with approximately ten sections per glass slid in series of ten. For quantification five series were used and counterstained with DAPI to visualize nuclei. Only cells with a clear nucleus were counted.

2.5. Extraction and immunoprecipitation of Melanopsin

Retina from wt and Ngb-null mice were homogenized with the aid of 10 strokes of a pellet pistel and a sterile scalpel in 200 μl ice-cold immunoprecipitation (IP) buffer. The IP was performed as described in [29]. Mel was IP by adding 4μL rabbit anti-Mel antiserum (in house, code# 41K9-3) to the lysate followed by incubation over night at 4 °C. Antibody-Mel complex was captured by incubation with 50% protein A-Sepharose slurry (Amersham, GE Healthcare, USA) for 1 h at 4 °C. Beads were washed three times with IP buffer and stored as wet pellets at −80 °C until Western blotting.

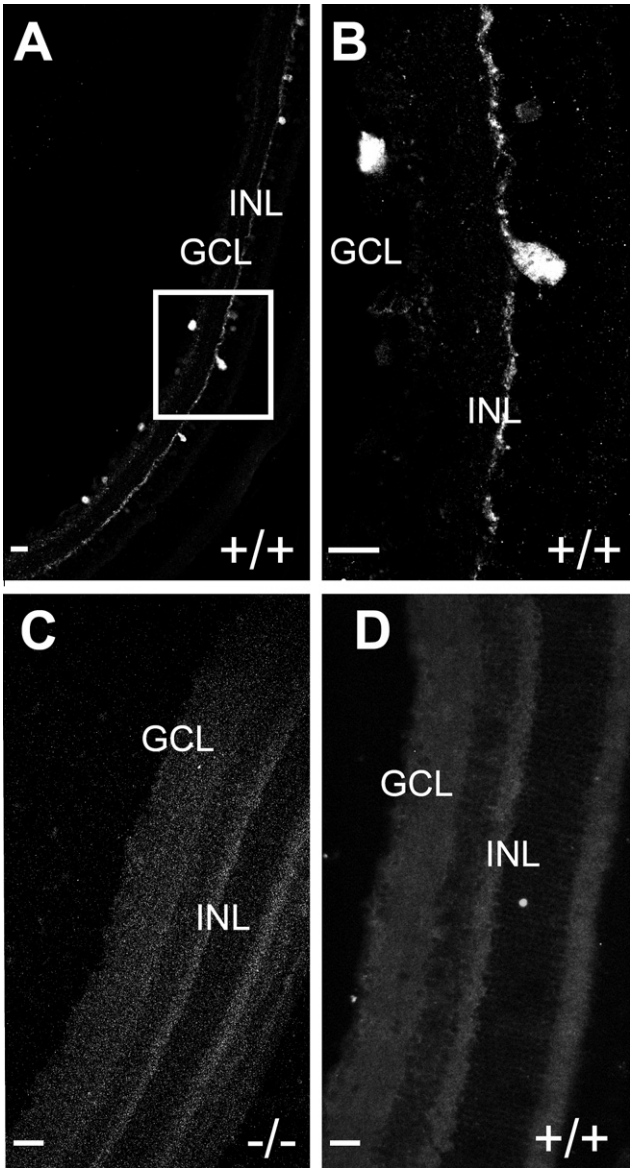

Fig. 1. Neuroglobin-immunoreactivity in the mouse retina. In (A) and (B) Ngb-IR can be seen in a subset of ganglion cell layer neurons (GCL) and amacrine neurons of inner nuclear layer (INL) of wild type mice (+/+). Strong Ngb-IR can be seen in cytoplasm, nucleus and processes. No Ngb-IR was observed in the retina of Ngb deficient mice (−/−) (C) and after pre-absorption with immunogen (D). Scale bar 50 μm.

2.6. Western blotting

The electrophoresis and western blotting was carried out as described in [28] using the following primary antibodies sheep anti-TH (Novus Biologicals, Littelton, CO, USA. Cat. No. # NB300-110 in 1:2000 dilution), rabbit anti-beta-actin (Cell Signaling Technology, Danvers, MA, USA. Cat. No. # 4970 in 1:2000 dilution), rabbit anti-calb (Swant, Marly, Switzerland, Cat. No. # CB-38a in 1:5000 dilution) and Mel (Thermo Scientific, Rockford, IL, USA, Cat. No. # PA1-780 in 1:5000 dilution). Immunoreactivity was detected with the following HRP-conjugated antibodies: swine anti-rabbit IgG (Dako, Glostrup, Denmark. Cat. No. # P0399 in 1:2500 dilution), donkey anti-sheep (Jackson Immunoresearch Laboratories, Baltimore, PA, USA. Cat. No. # 713-036-147 in 1:2000 dilution).

2.7. Statistics

Data were analyzed with Mann–Whitney test in GraphPad Prism software. Two-tailed $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ngb localization and co-localization

The expression of Ngb in the retina was sparse and restricted to two layers. Strong Ngb-IR was seen in both perikarya and processes of a subpopulation of neurons in the granule cell layer (GCL) and in amacrine cells of the inner nuclear layer (INL) (Fig. 1A and B). Processes of INL neurons were seen in the outermost part of the inner plexiform layer (IPL) (Fig. 1B). No Ngb-IR was observed in other retinal layers. The specificity of the Ngb antibody used in this study was confirmed by the lack of immunostaining both in the Ngb-null retina (Fig. 1C) and after pre-absorption with the immunizing antigen (Fig. 1D). Furthermore, in the brain, the expression pattern of the Ngb antibody completely overlaps with Ngb mRNA as reported in [8] and in the Allen Brain Atlas (www.brain-map.org).

Most (74 +/−6%) of the investigated Mel-IR cells were found to co-express Ngb-IR. This was true for all Mel cell types (Fig. 2A–E). Co-expression was observed in both the cell soma and processes. Mel-IR and Ngb-IR were clearly separated into the membrane and cytosolic/nuclear compartments, respectively (Fig. 2C and D). In the INL, almost all (82 +/−9%) TH-IR cells co-expressed Ngb-IR and the dense TH-IR fiber bundle seen in outermost part of the IPL was also Ngb-positive (Fig. 3A–C). Both Mel-IR (Fig. 2F) and TH-IR appeared normal in the Ngb-null retina (Fig. 3D). Very few Ngb-IR cells were found to co-express Calb-IR in the GCL and INL (Fig. 3E and F).

3.2. Effect of Ngb deficiency on TH, Mel, Calb and PACAP expression

Ngb-IR was present in 74% and 82% of the Mel and TH cells in the retina, respectively. It is therefore valid to use Mel and TH as surro-

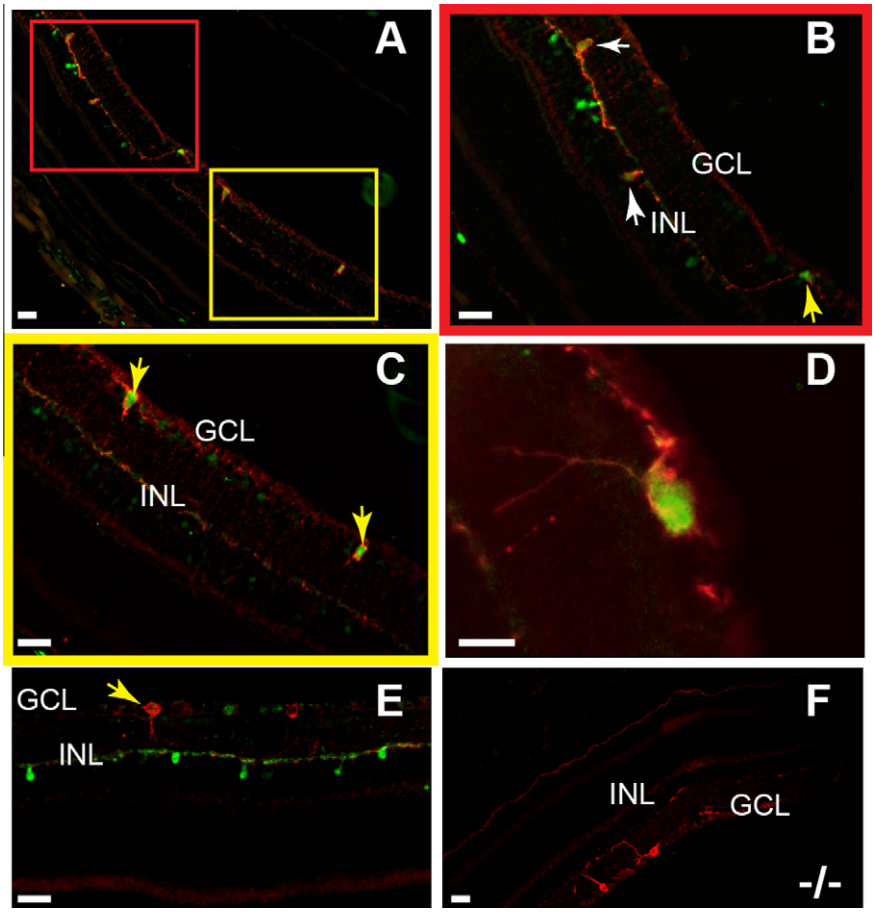

Fig. 2. Neuroglobin co-expression with Melanopsin. (A) Ngb-IR (green) and Melanopsin (Mel)-IR (red) are seen co-localized in both cells of the GCL and INL. The area within the red and yellow squares is magnified in (B) and (C) respectively. (B) Ngb-IR and Mel-IR was seen co-expressed in cells of the GCL (yellow arrows) and INL and in displaced INL cells (with arrow). (C) Show two GCL Mel-IR cells with clear co-expression of Ngb-IR and (D) a high magnification of a GCL cell where Ngb-IR can be seen in the cytoplasm, nucleus and processes and Mel-IR in the cell membrane. (E) Shows a Ngb-IR neurons co-expressin Mel-IR (arrow) and a neuron only expressing Mel-IR. (F) Shows Mel-IR GCL cells in retina from Ngb deficient mice. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

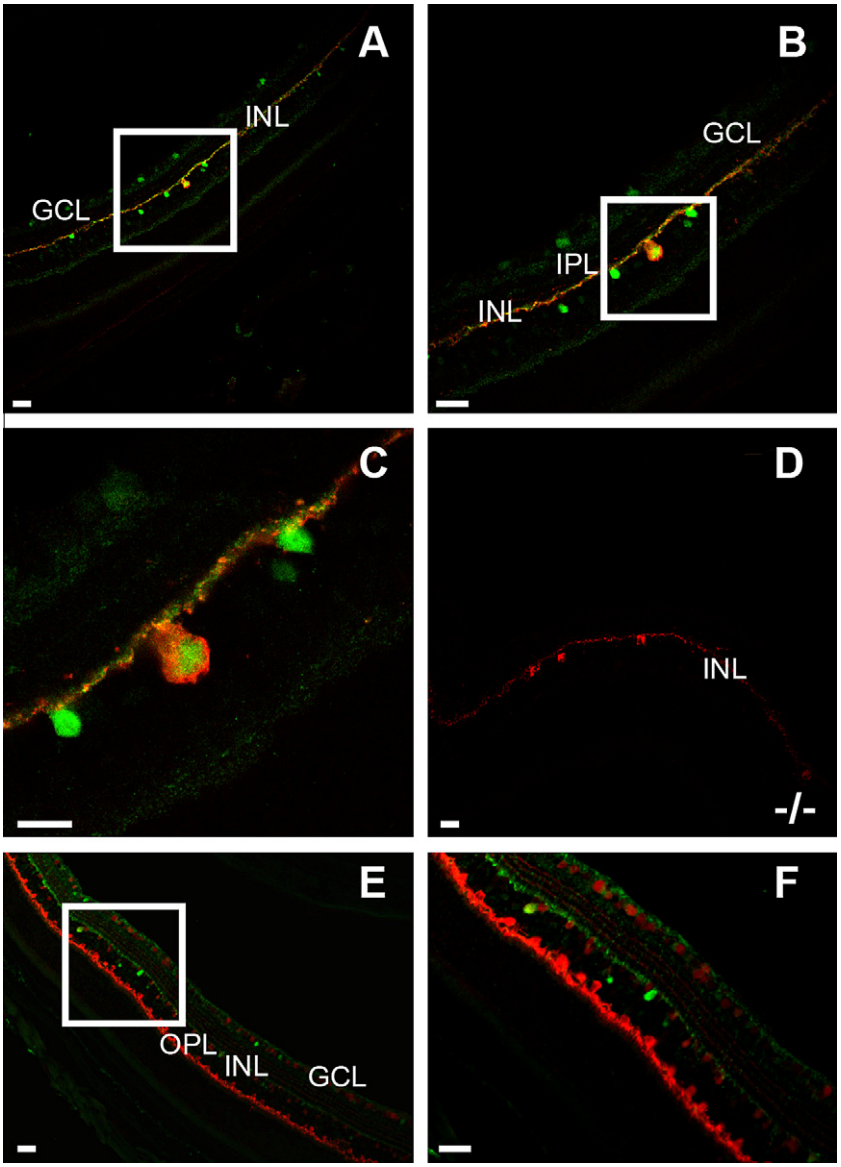

Fig. 3. Neuroglobin co-expression with Tyrosine Hydroxylase. (A) Shows co-expression of Ngb-IR (green) and Tyrosine Hydroxylase (TH)-IR (red) in a subset of Ngb-IR neurons in the INL. (B and C) Are higher magnifications showing Ngb-IR and TH-IR occur both in the perikariya and fibre bundle running horizontally along the inner plexiform layer (IPL). (D) Shows TH-IR in a retina from Ngb deficient mice. (E and F) Shows Ngb-IR (green) and Calbindin-IR (red) are expressed in different cells in the GCL and INL. Strong Calbindin-IR was also seen in the outer plexiform layer (OPL). Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gate markers for the cells in Ngb-null mice. Quantifying the levels of these two markers gives an estimate of the effect of Ngb-deficiency on Mel and TH. We found no significant difference in the levels of Mel and TH protein between wt and Ngb-null mice ($p > 0.05$, Mann Whitney test) (Fig. 4A) suggesting that the retinal populations of Mel and TH neurons are largely intact in Ngb-deficient mice. Calb was included since it is expressed in most of the retinal layers previously reported to express Ngb-IR [5]. No difference was seen in Calb levels between wt and Ngb deficient mice ($p > 0.05$, Mann Whitney test) (Fig. 4A). PACAP is a marker for retinal projections originating from Mel-IR cells and projecting to the suprachiasmatic nucleus (SCN) [31]. No alteration in PACAP innervation was apparent in Ngb-null mice when compared with wt (Fig. 4B).

4. Discussion

The discovery of high levels of Ngb in the mouse retina spurred a hypothetical explanation of how the retina can sustain a high metabolic rate [5]. The notion was further substantiated by a study showing Ngb expression in the retinal layers with the highest oxygen consumption rates and most mitochondria [26]. Others, finding a similar expression pattern of Ngb in the mammalian retina, suggested a role in neuronal protection [23–25,34,35]. So far, however, there is no functional evidence supporting the role of Ngb as either oxygen storage or in neuronal protection. In the present study we offer a contrasting view of Ngb expression pattern in the retina, indicating that Ngb-IR is restricted to a small number of GCL and INL neurons and their processes. The discrepancy between the Ngb-IR pattern in our study and in reports by other groups is likely due to differences in the specificity and validation methods of the antibodies involved. Incomplete antibody validation leading to the reporting of artifactual staining patterns is a major issue that has received attention in numerous editorials and review articles in leading IHC journals [16–21]. In this study, we verified the specificity of the Ngb antibody by pre-absorption and lack of staining in the retina (and brain) of Ngb deficient mice.

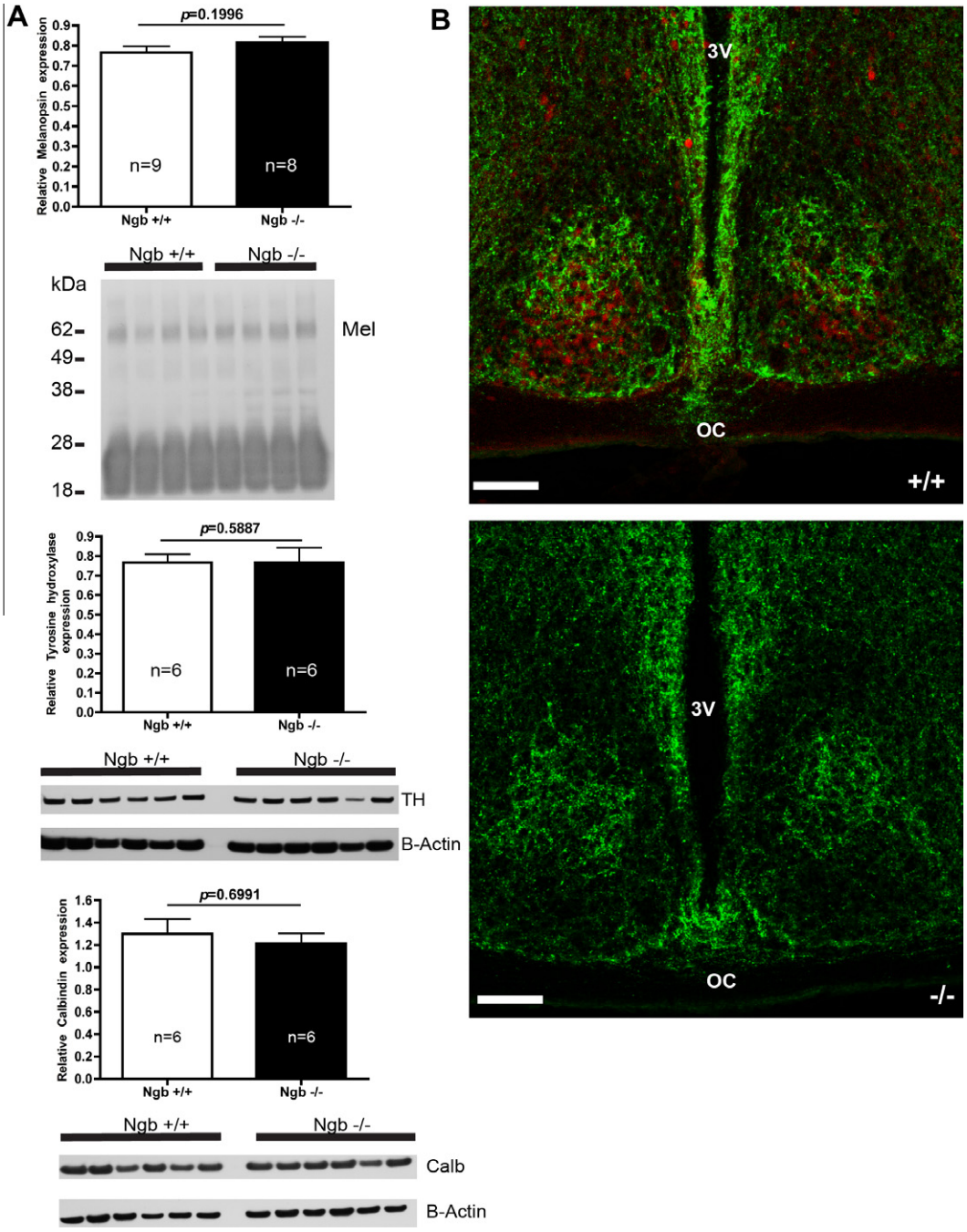

Fig. 4. Effect of Neuroglobin deficiency on expression levels of co-expressed protein. (A) Western blot analysis of the effect of Ngb deficiency on from the top immunoprecipitated Melanopsin (Mel), Tyrosine Hydroxylase (TH) and Calbindin (Calb) retina protein levels. Beta-actin was used as loading control for TH and Calb. No significant difference could be seen comparing Ngb deficient (−/−) and wild type (+/+) mice. (B) Pituitary adenylate cyclase-activating peptide (PACAP)-IR (green), a marker of retinal innervation, and Ngb-IR (red) in the suprachiasmatic nucleus (SCN) of Ngb +/+ and Ngb −/− mice. Apparent equal PACAP innervation was seen in both genotypes indicating that retinal innervation of the SCN is intact in Ngb −/− mice. Scale bar 50 μm. Third ventricle (3V), optic chiasm (OC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Importantly, lack of IHC signal in tissues from knock-out animals in which the antigen has been eliminated is recommended as the best and most stringent demonstration of antibody specificity [16–21] and none of the previous studies have used it to validate Ngb antibodies. It should be noted that the use of Ngb-null mice for antibody validation has only recently become an option (we are currently unaware of any other groups having published studies that use a Ngb-deficient mouse model) and, hence, previous reports have relied on other techniques to verify antibodies. In the initial study of Ngb-IR in the eye the primary antibody was successfully pre-absorbed with immunogen and ISH was used to verify the staining pattern [5]. As ISH did not yield a staining pattern identical to IHC the discrepancy was ascribed to the intracellular transport of the protein. Using the same antibody and mRNA probe in the mouse brain widespread Ngb-IR and mRNA distribution was also reported [11,12] in contrast to previous studies by us and others [8,14,15,28,29] (The Allen Brain Atlas (www.brain-map.org)). In the remaining studies of Ngb-IR in the retina antibody pre-absorption or omission of primary antibody were used for validation [7,23–26,34,36].

As is the case for the brain, functional conclusions about Ngb drawn from the various IHC studies are vastly different. When interpreting patterns of immunostaining it must be kept in mind that a staining pattern reports the location of the immunogen and as such it is devoid of any causal or mechanistic information. Consequently, a staining pattern should never be used as a major piece of evidence when drawing functional conclusions about the protein of interest. In the mouse retina, Ngb-IR is scarce and, hence, it is not suggestive of a major role by Ngb in oxygen delivery or storage as suggested in [5,22,26]. Of course, restricted retinal expression of Ngb-IR does not preclude involvement in less general oxygen-related processes. However, as is also the case for the Ngb's suggested role in neuroprotection, the question remains why only the few Ngb-IR neurons would benefit from a specific oxygen storing or protective mechanism. More information about the phenotype of Ngb expressing neurons and their functioning in Ngb-deficient mice may provide valuable clues as to Ngb's function(s). Having surrogate markers for Ngb expressing neurons such as Mel and TH can prove useful in this search.

Based on the morphology of the Ngb-IR neurons in the GCL and INL we chose to co-stain for Mel, TH and Calb. Very limited co-expression with Calb was found whereas a high percentage of Mel and TH expressing neurons co-expressed Ngb making them useful as markers of Ngb neurons. It must be noted however, that neurons co-expressing Ngb with Mel or TH constitute subpopulations of the Ngb-expressing neurons in the retina. In line with observations from the brain [28], no effect of Ngb-deficiency was found on the global level of Mel and TH proteins indicating that the lack of Ngb does not have major implications for retinal development. Retinal Mel-IR neurons have been shown to directly innervate the SCN via the retinohypothalamic tract [37] and function as circadian photoreceptors [38–40]. Mel neurons are also intrinsically photosensitive and knock-down of Mel results in altered light-induced phase shifting [41,42]. If Ngb was necessary for the normal functioning of the Mel neurons Ngb-deficient mice would be expected to display altered circadian behavior and changes in light induced cFOS (an immediate early gene, which is activated by light) expression in the SCN, target of Mel projections. However, Ngb deficient mice have normal circadian behavior and light induced expression of cFOS in the SCN is unaltered [29]. In addition, the density of PACAP-IR fibres in SCN as found in this study is normal.

The observations presented in this study emphasize the need for comprehensive antibody validation and caution against drawing functional conclusions from IHC studies. The function of Ngb in the retina and its relation to oxygen homeostasis remains unresolved.

Author contributions

Initiated the study: C.A.H. Designed the experiments: C.A.H., J.F., J.H. Performed the IHC, WB: C.A.H. Analyzed the data: C.A.H. Wrote the paper: C.A.H., H.L. Contributed reagents/material/equipment: C.A.H., A.H.S., J.F., J.H. Critically modified the manuscript: J.F., A.H.S., J.H. Approved the final manuscript: C.A.H., J.F., H.L., A.H.S., J.H.

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