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ORIGINAL PAPER

Immunocytochemical Analysis of Glycogen Phosphorylase Isozymes in the Developing and Adult Retina of the Domestic Chicken (*Gallus domesticus*)

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Abstract Glycogen is the major energy reserve in neural tissues including the retina. A key-enzyme in glycogen metabolism is glycogen phosphorylase (GP) which exists in three differentially regulated isoforms. By applying isozyme-specific antibodies it could be demonstrated that the GP BB (brain), but not the GP MM (muscle) isoform is expressed in the chicken retina in neuronal and glial (Müller) cells. In the embryonic chicken retina, GP showed a development-dependent expression pattern. Double-labeling experiments with cell type-specific antibodies revealed that GP is expressed in various layers of the retina some of which, e.g., the photoreceptor inner segments, are known to be sites of high energy consumption. This suggests important roles of GP BB, and therefore glycogen, in early differentiation, spontaneous wave generation and in formation and stabilization of synapses.

Keywords Chicken · Glycogen phosphorylase · Immunocytochemistry · Isozyme · Retina · Western blotting

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Introduction

Glycogen is the storage form of glucose for organisms from E. coli to primates. In mammalian tissues, the major depots are located in muscle and liver where its functions are well-known. Glycogen is also found in the brain, but a principal role as an energy reserve has been doubted due to its low concentration. Though, recently, neural glycogen has drawn increasing attention [1–6] and several roles have been suggested for it [7–11]. Nevertheless, many aspects of its function are still unclear.

The rate-limiting step in glycogen degradation is catalyzed by homodimeric glycogen phosphorylase (GP) which exists as three isozymes named according to the tissues they predominate in: MM (muscle), LL (liver) and BB (brain). These isozymes are regulated differentially by phosphorylation and allosteric control: GP LL is exclusively, GP MM mainly regulated by phosphorylation, while GP BB is predominantly sensitive to the cellular AMP level [12, 13]. Immunocytochemical studies with isozyme-specific antibodies revealed that rat neural tissues express the GP BB and MM isoforms [14]. In two parts of the central nervous system (CNS), the brain and spinal cord, GP is predominantly expressed in astrocytes, where both isoforms are present [14]. In contrast, peripheral nerves express only the GP BB isoform which is located exclusively in neuronal elements [15, 16]. It has been hypothesized that astrocytic glycogen could be degraded by GP BB for meeting the energy demand of the cells themselves, or, via GP MM, according to signals sent out by distant neurons, for the benefit of neighbouring cells, the polysaccharide thus fulfilling a more altruistic role of the astrocyte. In peripheral nerves, glycogen would serve as a local energy source for the metabolically isolated axon [16].

In the retina, which is also a part of the CNS, glycogen and its metabolizing enzymes have mainly been allocated to glial elements [17-20]. Only rarely have neuronal locations of glycogen been reported [21, 22]. Accordingly, in the rodent retina, Müller cells and astrocytes including those of the optic nerve express GP, but, in contrast to brain astrocytes, the BB isoform only [23, 24]. In addition, GP MM could be detected in some rare neurons in the inner nuclear layer (INL) of the rat retina, most probably bipolar cells [24], and it is found in mouse cones, from the pedicles to the outer segments (OS) [25, 26]. If the above hypothesis that GP MM is activated via far-distant signal pathways were correct, cells which were targets of cerebral projections to the retina should express GP MM. Such a centrifugal visual system has primarily been reported in birds, but appears to be an early evolutionary acquisition of the CNS of all vertebrates [27, 28]. To test this hypothesis and to gain more insight into glycogen metabolism during ontogeny, the spatio-temporal distribution pattern of GP isozymes was analyzed in the chicken retina during embryonic development and in the adult organ by applying isozyme-specific antibodies for Western blotting and immunocytochemistry. For the latter analysis antibodies against cell type-specific markers were applied in double-labeling immunocytochemical experiments in combination with antibodies against GP.

The developmental study was warranted by the fact that the development of neural tissues requires high amounts of energy, and therefore a special implication of glycogen can be expected. Indeed, in rat brain, GP activity and immunoreactivity have been reported to be elevated perinatally [29–31], and in the chicken brain glycogen concentration increases prior to hatching [32].

Experimental procedure

Antibodies

Rabbit and guinea pig antisera against the rat GP BB and MM isoforms were prepared as described [14]. The monoclonal antibody rho4D2 (generous gift of Dr David Hicks, Strasbourg) is highly specific for rods in several species including the chicken [33–36]. The polyclonal *CERN906* antibody was raised against purified chicken red and green opsins and is highly specific for these visual pigments ([35–38]; generous gift of Dr Willem DeGrip, University of Nijmegen). The amacrine, ganglion and horizontal cell specific monoclonal Pax-6 antibodies and Islet-1 antibodies [39–42], were purchased from Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA. The monoclonal acetylcholinesterase (AChE) antibody 3D10 is specific for cholinergic amacrine, ganglion and some horizontal cells ([43, 44]; generous gift of Dr K. Tsim, Hongkong). The monoclonal PKC_{α} antibody (Santa Cruz Biotechnology, Heidelberg, Germany) specifically recognizes rod bipolar cells and a sub-population of PKC_{α} immunoreactive amacrine cells [45]. The monoclonal antibody against vimentin (Millipore, Schwalbach, Germany) specifically stains Müller cells in the chicken retina. The mouse monoclonal β-actin antibody was purchased from Sigma-Aldrich (Deisenhofen, Germany). Cy3-conjugated goat anti-rabbit, anti-guinea pig and anti-mouse IgGs, as well as peroxidase-conjugated goat anti-rabbit, anti-guinea pig and anti-mouse IgGs were from Dianova (Hamburg, Germany).

Western blot analysis

To test the reactivity of the antisera raised against the rat GP isoforms toward chicken tissues, pieces of adult chicken brain, breast muscle, heart muscle and liver were kept frozen at -80°C. The preparation of supernatants from tissue homogenates, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were performed as described previously [15].

For Western blot analysis of embryonic and adult chicken retina, retinal tissues of appropriate stages were isolated, washed twice in PBS and stored at -70°C. Tissues were homogenized in 250 µl of buffer (100 mM $NaHCO_3$, 0.2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM spermidine, pH 7.5) by sonication on ice for 45 s. Protein concentrations were determined according to Bradford [46]. Protein samples (50 µg/lane) were separated by SDS-PAGE using 7.5% separating gels. Subsequently, proteins were transferred electrophoretically to a polyvinylidene fluoride membrane by a semi-dry apparatus (BioRad, Munich, Germany). The membrane was blocked for 1 h with 5% non-fat dry milk (BioRad, Munich, Germany) in TBST (50 mM Tris, 50 mM NaCl, 0.1% Tween 20, pH 7.4) and then incubated with the primary antibody diluted 1:1,000 in TBST for 1.5 h. After three washes in TBST the membrane was incubated with the peroxidase-conjugated secondary antibody diluted 1:5,000 in TBST. Detection was performed by chemiluminescence applying the NOVA solution kit (MoBiTec, Göttingen, Germany). All incubation steps were carried out at room temperature. For subsequent immunostaining of β-actin, the membrane was incubated at room temperature for 30 min in stripping solution (TBST, 2% SDS, 100 mM mercaptoethanol). After three washes in TBST, the membrane was blocked at 4°C overnight. The following steps were performed as described above.

Tissue fixation and production of cryo-sections

Eyes of embryonic and adult chicken were harvested at appropriate stages and fixed in 4% paraformaldehyde/PBS at 4°C for 24 h. After two washes in PBS eyes were soaked in 25% sucrose/PBS at 4°C for at least 24 h. Frozen sections of 12 µm thickness were cut on a cryostat (Leica, Bensheim, Germany) and mounted onto superfrost slides (VWR, Darmstadt, Germany).

Immunocytochemistry

Tissue sections were dried at 37°C and pre-incubated with a blocking solution (10% goat serum, 0.1% Triton X-100 in PBS) at room temperature for 30 min. Subsequently sections were incubated for 2 h with the GP BB- or GP MM-specific antisera diluted in blocking solution. For double-labeling experiments, the GP BB antiserum and one of the cell type-specific antibodies mentioned above were applied simultaneously. Cy3-conjugated secondary antibodies were diluted 1:200 in blocking solution and applied for 1 h. Cell nuclei were stained with 4,6-diamidine-2-phenylindole-dihydrochloride (DAPI, Roche, Mannheim, Germany; 0.1 mg/ml in PBS). Finally, sections were dried and embedded in Kaiser's Glycerol Gelatin (Sigma, Deisenhofen, Germany). Negative controls for GP were carried out by applying antisera pre-absorbed with the cognate peptides [14] instead of the GP BB- or GP MM-specific antisera only. In the case of monoclonal antibodies, negative controls were performed by omitting the primary antibody.

Fluorescence microscopy

All images were acquired with an upright Nikon E600 microscope equipped with epifluorescence optics or with an inverted Nikon TE 2000 microscope upgraded with an eC1 multi-mode confocal laser scanning system (Nikon, Düsseldorf, Germany) comprising an argon laser (488 nm) and a helium-neon laser (543 nm). Photomicrographs were processed with Lucia G, EZ-C1 software (Nikon) and Adobe Photoshop 7.0.

Results

GP isozyme pattern in adult chicken tissues

A rabbit anti-rat GP BB serum recognized a band of the appropriate molecular mass (97 kDa) in supernatants of homogenates from chicken brain and heart, but not from chicken skeletal muscle (Fig. 1A). An anti-rat GP MM serum raised in guinea pig revealed a band in chicken skeletal muscle homogenates, but not in chicken brain and heart, in contrast to rat where GP MM is also detected in brain and heart (Fig. 1B). None of the antisera reacted with supernatants from chicken liver homogenates (not shown).

GP isozyme pattern in embryonic and adult chicken retina

In the adult chicken retina, rabbit anti-rat GP BB serum recognized a band of the appropriate molecular mass; this band was also detectable during development starting at embryonic stage 10 (E10) with gradual increase, except at E16, where the intensity of the band was significantly and consistently lower (Fig. 2A). In contrast, with guinea pig anti-rat GP MM serum no band of the appropriate molecular mass was detected at any stage of development (Fig. 2B).

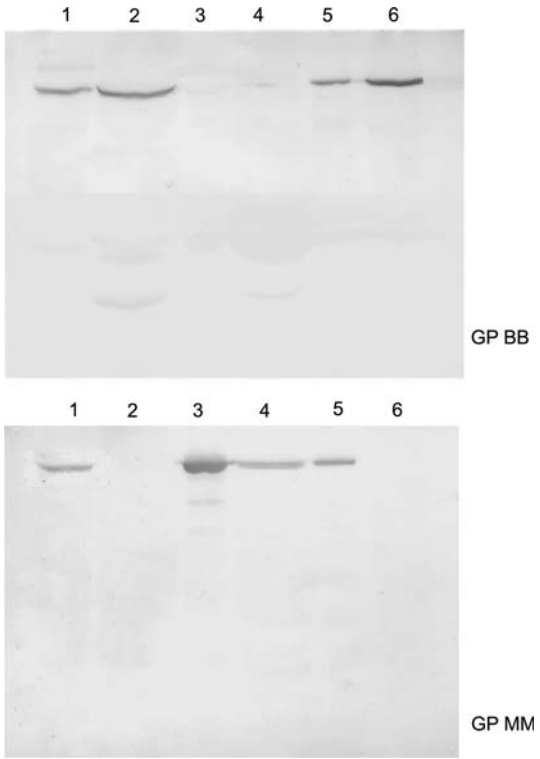

Fig. 1 Western blot analysis of GP BB and MM isozymes in supernatants of homogenates from adult chicken and rat brain, skeletal muscle and heart muscle. Alkaline phosphatase-based detection. 1, rat brain; 2, chicken brain; 3, rat skeletal muscle; 4, chicken skeletal muscle; 5, rat heart muscle; 6, chicken heart muscle. One hundred microgramms of protein were applied in *each lane* except for rat skeletal muscle (20 µg). **A** rabbit anti-GP BB, **B** guinea pig anti-GP MM

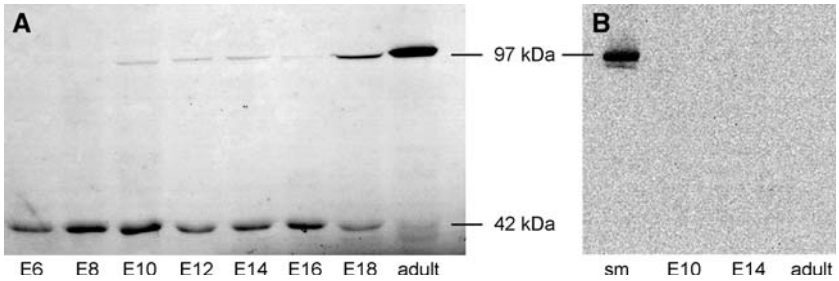

Fig. 2 Western blot analysis of chicken retina in various developmental stages from E6 to adult. Chemiluminescence detection. Fifty micrograms of protein were applied in *each lane*. The band of 97 kDa corresponds to both GP BB and MM, the band of 42 kDa in **A** refers to β-actin. **A** rabbit anti-GP BB, **B** guinea pig anti-GP MM; *sm* chicken skeletal muscle

Distribution pattern of GP BB during development of the chicken retina

To determine the spatial distribution of GP BB during development, cryo-sections of embryonic retina at sequenced stages were stained with anti-GP BB serum and DAPI. Nuclear staining with DAPI was carried out to facilitate the localization of GP BB-positive cells within the developing retina. The first pronounced immunolabeling was present at E6 and was mainly restricted to two narrow bands located in the outermost (scleral) and innermost (vitreal) parts of the yet unstratified retina (Fig. 3A–D). Comparison of Fig. 3C and Fig. 3D revealed that GP BB was also found in cells of the retinal pigment epithelium (RPE). Furthermore, weakly stained radial processes were also detectable (inset in Fig. 3B). During the following 2 days, further cells of the innermost half of the retina became immunoreactive (not shown). At E10, the outermost GP BB-positive band was still present, while a second, yet discontinuous narrow band appeared in the region of the prospective outer plexiform layer (OPL) and outer nuclear layer (ONL) (Fig. 3E–H). Moreover, an abundant number of GP BB-positive cells was found in the ganglion cell layer (GCL) whereas only a small number was located at the inner border of the INL (Fig. 3E–H, arrows). Within the inner plexiform layer (IPL), GP BB was present in displaced amacrine cells migrating from the INL toward the GCL (Fig. 3E–H, arrowheads). At E14 two intensely stained bands appeared at the outer part of the retina (Fig. 3I–L). One represented the OPL (Fig. 3I–L, arrows) whereas the second one marked the area of OS, proximal to cell nuclei of photoreceptors (Fig. 3I–L, arrowheads). Despite their significantly lower immunoreactivity, GP BB-positive cells could still be located in the GCL and INL of E14 retina. At E18, GP BB was present in two narrow sub-bands of the IPL (Fig. 3N–P, arrows). The first morphological signs of sub-bands in the IPL could be detected at E16 (not shown). Moreover, individual cells of the INL were now clearly detectable, whereby GP BB expression within these cells was restricted to a narrow rim of cytoplasm surrounding the nuclei (Fig. 3N–P, arrowheads) and to the apical tip where the processes originate and project into the IPL. Negative controls (Fig. 3Q–T) showed no staining for GP BB in E18 retina.

In contrast to sections of chicken skeletal muscle that were clearly stained (not shown), those of chicken retina in the various developmental stages were not stained by guinea pig anti-GP MM serum (not shown).

Allocation of GP BB to individual retinal cell types or layers

To identify retinal cell types that express GP BB, cryo-sections of E18 retina were double-labeled with antiserum against GP BB in combination with antibodies against well-known cell type-specific markers and subsequently analyzed with confocal laser scanning microscopy. First the staining pattern of AChE was compared with that of GP BB. In most vertebrate species, AChE is found in cholinergic amacrine cells and two sub-laminae of the IPL as well as in the outermost portion of the ONL [43, 47]. GP BB was found in a population of AChE-positive cholinergic amacrine cells located in the innermost two cell rows of the INL (Fig. 4A–C, arrowheads). Additionally, co-expression of GP BB and AChE was found in displaced cholinergic amacrine cells of the GCL (Fig. 4A–C, arrows) and in the outermost part of the ONL (Fig. 4A–C, triangles). Moreover, in two of the three GP BB-positive sub-laminae of the IPL, GP BB was prominently co-localized with the two AChE-positive cholinergic sub-laminae of the IPL (Fig. 4 A–C, *a*, *d*).

To analyze whether GP BB is expressed in Müller cells, GP BB antiserum was applied in combination with an antibody against vimentin, a marker for Müller cells. Although vimentin was present in a number of cell bodies in the INL (Fig. 4E), GP BB-positive cells of the INL (Fig. 4D, arrowheads) showed no co-localization with vimentin in these cells (Fig. 4F, arrowheads). However, processes of Müller cells located between cell bodies of the

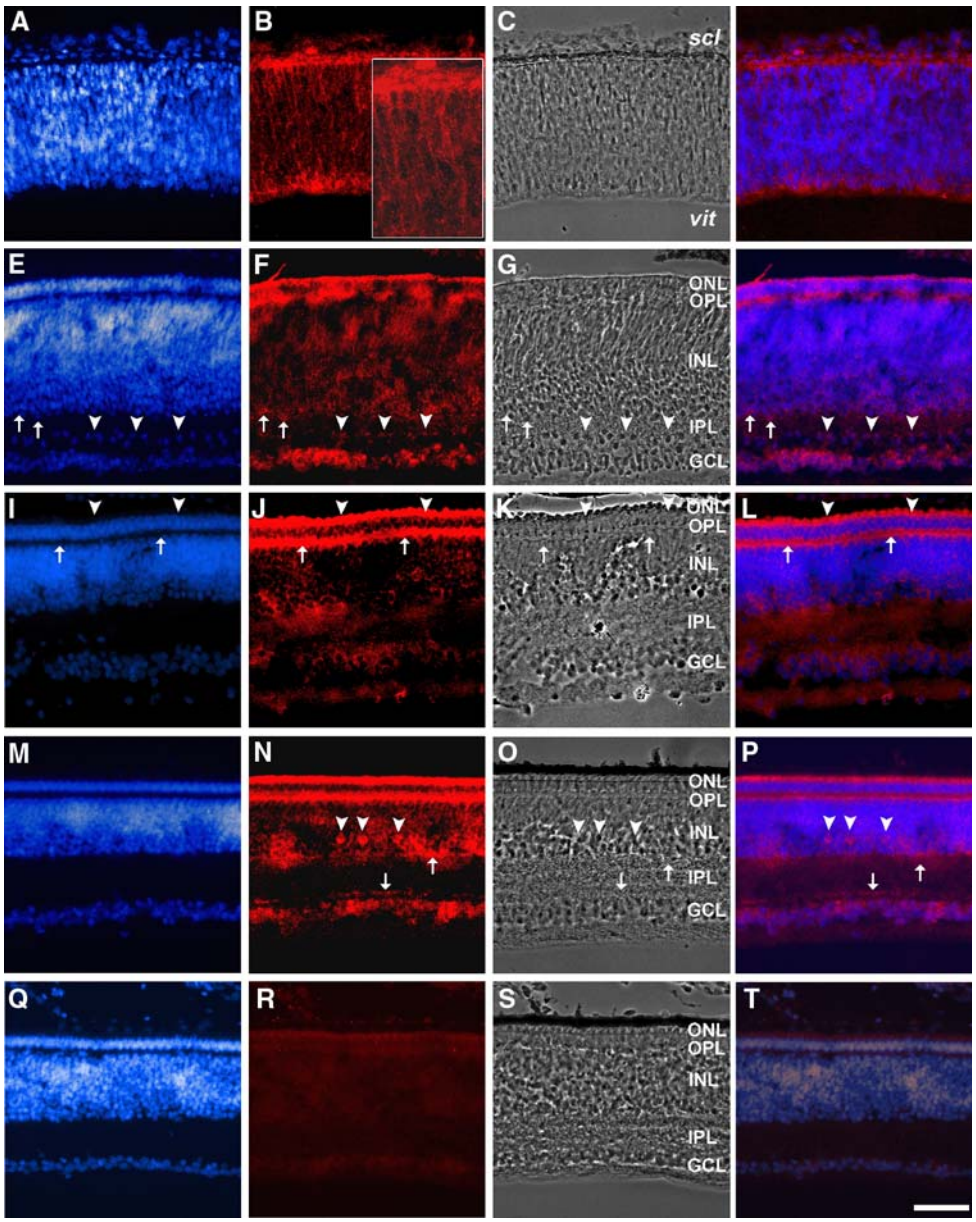

Fig. 3 Glycogen phosphorylase BB immunoreactivity in the developing chicken retina. Transversal sections of the retina obtained from eyes of different developmental stages stained with either DAPI (**A, E, I**, **M, Q**) or rabbit anti-GP BB serum (**B, F, J, N, R**). The corresponding phase contrast views are shown in **C, G, K, O** and **S**, respectively. The overlay of DAPI staining with GP BB immunostaining (**D, H, L, P, T**) demonstrates the location of GP BB in the retinal layers during development. At E6, GP BB is found in the outermost and innermost parts of the retina as well as at the apical edge of the RPE (**A–D**). Higher magnification (inset in **B**) reveals the presence of GP BB in radially oriented cellular processes. At E10 of embryonic development, the distribution of GP BB immunoreactivity has become more complex (**E–H**) and is detectable in the ONL, OPL, GCL, in cells of the innermost cell row of the INL (*arrows*), as well as in migrating amacrine cells of the IPL (*arrowheads*). Staining of 14-day-old retina shows a further increase of GP BB immunoreactivtiy in the OPL and ONL (**I–L**). At E18 (**M–P**), increased GP BB staining is evident in cells of the INL (*arrowheads*) and additional staining is observable in synaptic laminae of the IPL (*arrows*). Pre-absorption of the antiserum with the peptide antigen blocks immunostaining (**Q–T**). The *scale bar* in **T** represents 50 μm and applies to all frames

GCL showed occasional co-localization of vimentin and GP BB (Fig. 4F, arrows).

The transcription factor Pax-6 has been shown to be expressed in progenitor cells at very early stages of retinal development, whereas it becomes restricted to ganglion cells, amacrine cells and horizontal cells as retinal development proceeds [42, 48]. As shown in Fig. 5A–C, co-localization of Pax-6 and GP BB was found in cells of GCL and of the inner half of the INL. Within the INL, cells were detected that expressed (1) Pax-6 and GP BB, (2) GP BB only or (3) Pax-6 only (Fig. 5C, inset). In the first case, GP BB was located in the cytoplasm while Pax-6 was

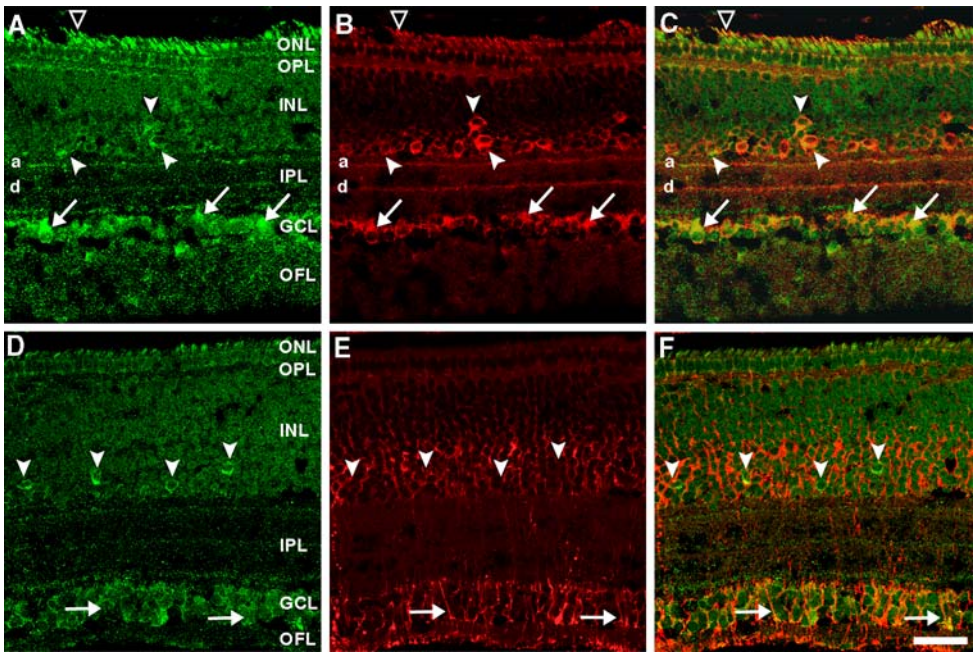

Fig. 4 Laser scanning microscopy of transversal sections of 18-day-old embryonic retina stained for GP BB in combination with either staining for AChE (**A–C**) or vimentin (**D–F**). **A–C**: Double-labeling for GP BB and AChE shows co-localization of the antigens in cholinergic amacrine cells (*arrowheads*), cholinergic cells of the GCL (*arrows*), in the cholinergic sub-laminae (*a*, *d*), and in AChE-positive inner segments of photoreceptors (*triangle*). **D–F**: Co-localization with vimentin is poor and can only be seen in Müller cell processes located in the GLC (*arrows*). GP BB-positive cell bodies in the INL (*arrowheads*) are vimentin-negative. The *scale bar* in **F** represents 25 μm and applies to **A–F**

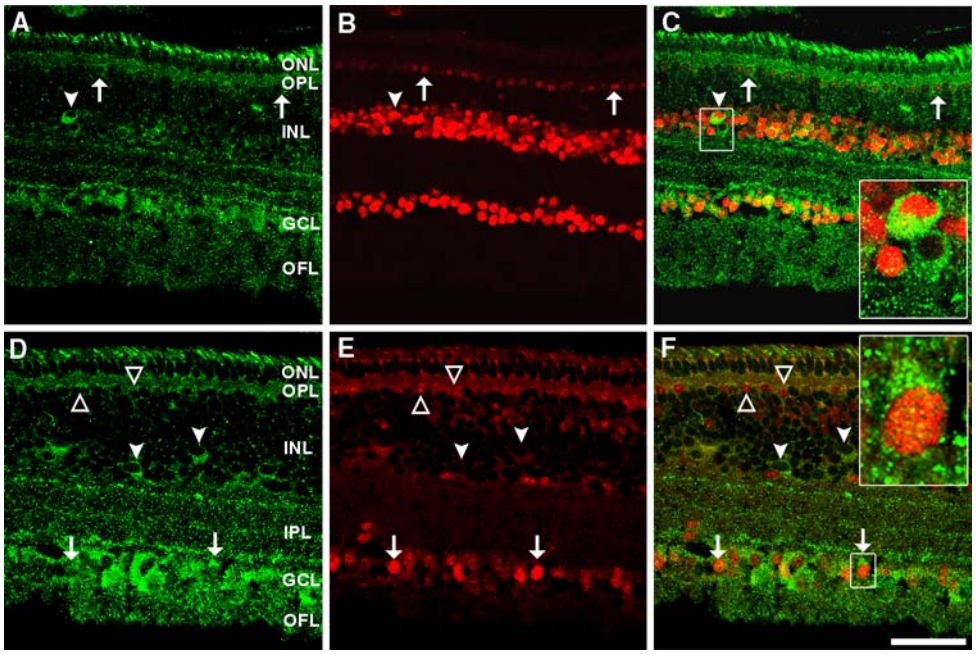

Fig. 5 Laser scanning microscopy of a transversal section of 18-day-old embryonic retina stained for GP BB (**A, D**) in combination with either staining for Pax-6 (**B**) or Islet-1 (**E**). **A–C**: Double-labeling for GP BB and *Pax-6* reveals co-localization in amacrine cells of the INL (*arrowhead*) and in cells of the GCL, as best seen in the overlays (**C, F**). Magnification of the frame in **C**, as shown in the inset in **C,** demonstrates that a population of Pax-6-positive cells in the INL does not express GP BB; also the other way around, a small number of GP BB-positive cells is Pax-6-negative. GP BB immunoreactivity closely linked to Pax-6-positive cells in the outermost part of the INL may be attributed to horizontal cells (*arrows*)**. D–F**: GP BB immunoreactivity is detectable in Islet-1-positive ganglion cells (*arrows*). Magnification of the frame in **F**, as shown in the inset in **F**, points out the location of Islet-1 in the nucleus while GP BB is restricted to the cytoplasm. GP BB-positive cells in the inner part of the INL are Islet-1-negative (*arrowheads*). At the outermost region of the INL, Islet-1-positive cells are in close proximity to the GP BB-positive OPL, indicating a possible expression of GP BB in horizontal cells (*triangles*). The *scale bar* in **F** represents 50 μm and applies to all frames

exclusively present in the nuclei (Fig. 5C, arrowhead, cf. inset). Moreover, co-localization of Pax-6 and GP BB was found in a single row of the INL at the border of the OPL (Fig. 5A–C, arrows). Here, Pax-6 positive nuclei were surrounded by GP BB-positive cytoplasm, marking a sub-population of horizontal cells.

Double-labeling with anti-GP BB serum and an Islet-1-specific antibody that recognizes ganglion cells, amacrine cells and horizontal cells in the chicken retina [41, 49] showed that GP BB was located in retinal ganglion cells (Fig. 5D–F, arrows). Here, GP BB was enriched at the apical side of the ganglion cell body where dendrites of ganglion cells originate (Fig. 5F). Furthermore, GP BB was found in the cells of the inner half of the INL which did not express Islet-1 (Fig. 5D–F, arrowheads). However, analogous to the Pax-6 expression pattern, GP BB could be detected in the outermost Islet-1-positive cell row of the INL (Fig. 5D–F, triangles), probably representing horizontal cells.

The possible expression of GP BB in bipolar cells was analyzed by applying an antibody directed against PKCα, which predominantly stains cell bodies of chicken bipolar cells and a sub-population of dopaminergic amacrine cells [45]. As expected, PKCα-positive bipolar cells were found in the INL (Fig. 6B, arrowheads) and in some amacrine cells (Fig. 6B, arrows). Both cell populations did not express GP BB (Fig. 6C). However, by comparing the expression of GP BB and PKCα within the OPL, a slight co-localization can be observed at the very border area of the OPL, indicated by a thin yellow band (Fig. 6C, triangles in large inset).

Expression of GB BB was prominent in the outermost part of the ONL (Fig. 4A). To analyze whether GP BB is restricted to a particular type of photoreceptor, antibodies against rod- or cone-specific markers were applied in combination with GP BB-specific antiserum. Comparison of the rod-specific rhodopsin immunoreactivity with the GP BB pattern showed no co-localization (Fig. 6D–F). However, GP BB could be detected in the inner segments (IS) of red- and green-sensitive cones (Fig. 6G–I) as revealed by comparing the appropriate visual pigment staining. As shown in Fig. 6I, the visual pigment staining of OS was in close proximity to the GP BB-positive IS indicating that both proteins were located within the same photoreceptor cells. Due to cryo-sectioning both the IS and OS of the photoreceptors are buckled to the right side.

Since it has been shown that GP BB is expressed in retinal Müller cells of adult rabbits, rats, guinea pigs and mice [24], GP BB antiserum was applied in combination with a vimentin-specific antibody on retinal sections of adult (15 months) chickens. In the adult chicken retina, GP BB was strongly expressed in the OFL, in the GCL, in cells and processes of the INL (Fig. 7A, arrow), in areas surrounding nuclei of photoreceptors (Fig. 7A, arrowhead), and in OS, whereas immunoreactivity was less pronounced in the OPL and two sub-laminae of the IPL. Vimentin-positive endfeet of Müller cells in the OFL expressed also GP BB, as is the case for Müller cell processes passing through the OFL, GCL, INL and OPL (Fig. 7C). Co-localization of vimentin and GP BB was also found in the region where cell bodies of photoreceptors are located.

Discussion

Glycogen phosphorylases are enzymes with highly conserved primary structures, the homology within a tissue isoform among species being higher than between two tissue isoforms within a single species [12]. In chicken, data base information is available for the liver (protein sequence data base at the NCBI, accession No. AAP33020) and brain (protein sequence data base at the NCBI, accession No. XP 419318) isoforms of GP which show high sequence homologies to the corresponding rat isozymes. Only partial sequences, deduced from sequencing of the isolated protein have been reported for the chicken skeletal muscle isoform [50]. The present study demonstrates that rabbit antibodies raised against a peptide out of the sequence of the brain isoform of rat GP cross-react with the chicken brain isozyme, and guinea pig antibodies raised against a peptide out of the sequence of the muscle isoform of rat GP cross-react with the chicken muscle isozyme, thus confirming the high degree of isoform conservation during evolution. As in rodent [24] and primate [21] retinae, GP BB is also expressed in the chicken retina. The presence of glycogen [51] and its starter protein glycogenin [52] has already been reported for the chicken retina.

In contrast to the rat brain where GP BB as well as GP MM are present [14], GP MM is not expressed in the chicken brain. In the rat, heart muscle is the only tissue with a similar 1:1 expression pattern of GP BB and MM (B. Pfeiffer-Guglielmi, unpublished data). In the chicken, GP MM is absent from heart muscle as it is from brain. GP MM could also not be detected in chicken retina. In addition to the BB isoform, the presence of GP MM has been reported for the primate retina [21]. In the rat and mouse retina, different cell populations express GP MM in a highly selective manner [24–26], pointing to a species-specific expression pattern. The absence of GP MM from the chicken retina must not necessarily exclude the influence of cerebral projections on retinal glycogen metabolism via GP MM activation for other species, since considerable morphological and functional variations concerning the nature of neurons involved, retinal target cells and neuroactive substances implied have been described [27]. These differences in the expression pattern of GP isoforms may point to a "basal" function of the BB isoform, whereas the presence of GP MM in tissues other than skeletal muscle could have been acquired later in evolution. This would imply that fine-tuning of metabolism by the presence in the same tissue or cell type of two isoforms

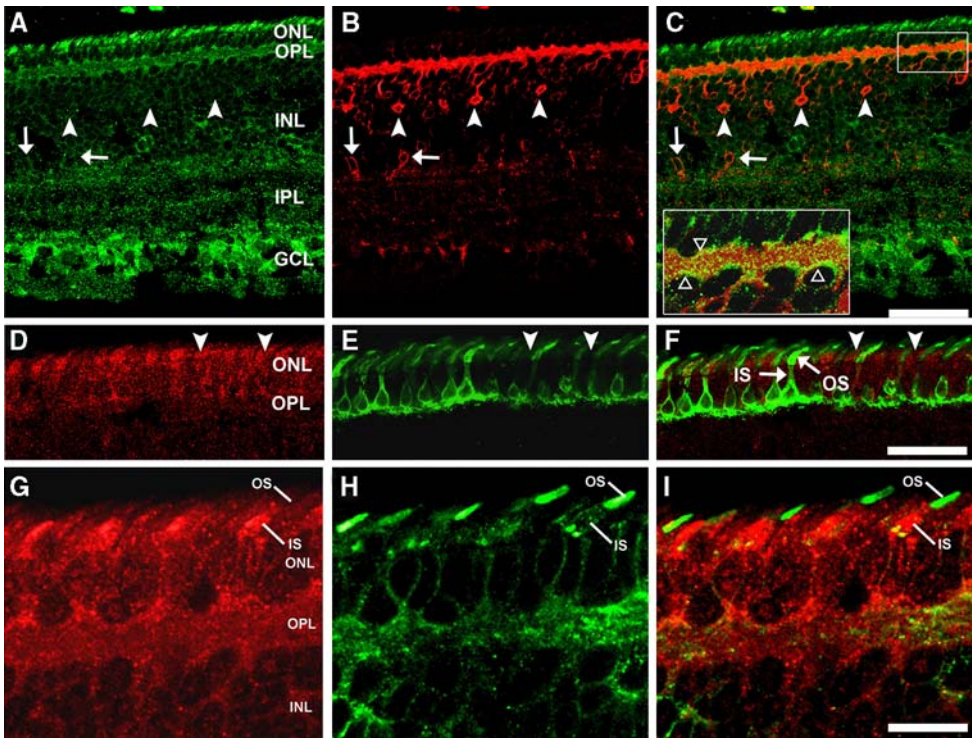

Fig. 6 Laser scanning microscopy of transversal sections of 18-day-old embryonic retina stained for GP BB (**A, D, G**) in combination with staining either for PKCα (**B**), rhodopsin (**E**), or red and green cone pigments (**H**). **A–C**: Double-labeling for GP BB and PKCα shows co-localization neither in cell bodies of bipolar cells (*arrowheads*) nor in amacrine cells (*arrows*), as best recognized in the overlay (**C**). Magnification of the frame in **C**, as shown in the inset in **C,** points out the co-localization of GP BB and PKCα (*triangles* in inset) in the OPL, indicating the appearance of GP BB in terminal endings of bipolar cell processes. Dendrites of amacrine cells in the INL (*arrowheads*) and cells in the GCL show co-localization. **D–F**: GP BB immunoreactivity is absent from outer segments (OS) and inner segments (IS) of rod photoreceptors (*green* in **E, F**). GP BB immunoreactivity (*arrowheads*) is clearly located between rhodopsin-positive OS. **G–H**: GP BB immunoreactivity (*red*) is located in IS of red and green opsin-positive cones (*green*). The *scale bar* in **C** corresponds to 50 μm and applies to frames **A–C**; the *scale bar* in **I** corresponds to 10 μm and applies to frames **G–I**

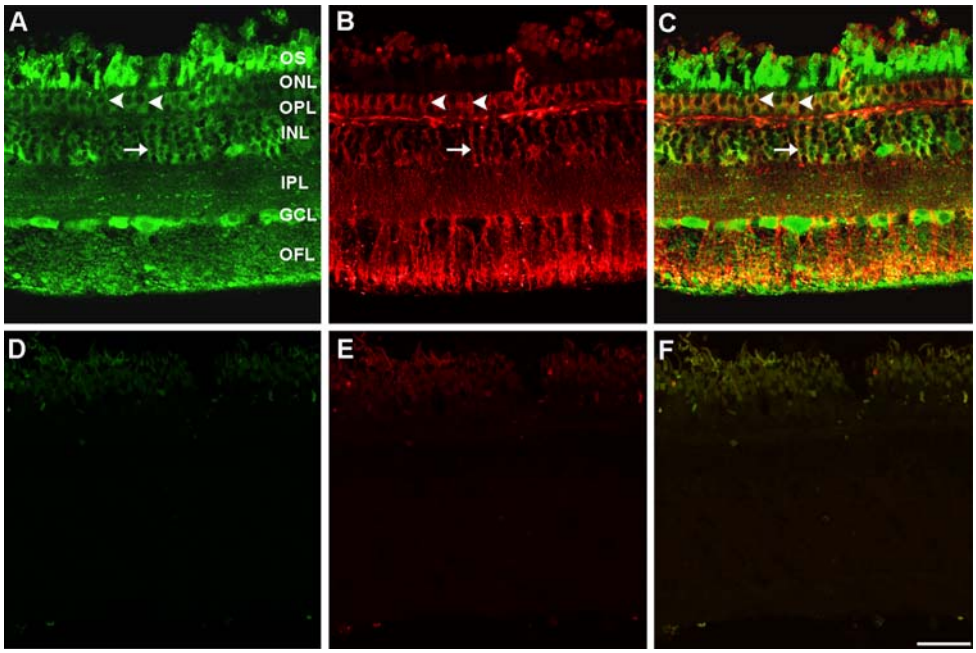

Fig. 7 **A–C**: Laser scanning microscopy of transversal section of adult chicken retina co-stained for GP BB (**A**) and vimentin (**B**). Prominent co-localization (**C**) can be seen in Müller cell processes and endfeet passing through the GCL and OFL. Additional GP BB immunoreactivity is present in areas of Müller cell bodies (*arrow*) and in microvilli surrounding the somata of photoreceptors (*arrowheads*). **D–F**: Negative controls to **A–C** applying anti-GP BB serum pre-absorbed with the peptide antigen (**D**) or omitting the antibody against vimentin (**E**). The *scale bar* in **F** (overlay) corresponds to 50 μm and applies to frames **A–F**

with different regulation patterns developed later in evolution.

GP BB is the prevailing fetal isoform in most species and tissues other than muscle and liver [53, 54]. The present study demonstrates that GP BB is expressed in discrete temporal and spatial patterns throughout the embryonic development of the chicken retina, indicating an important role of glycogen during retinogenesis.

The increase in GP BB during retinogenesis raises the question which cells this isozyme is expressed in. Immunocytochemical staining of retinal sections revealed that GP BB appears in a development-dependent manner across various cell and synaptic layers in the retina. The findings presented point to a potential role of glycogen during embryonic development of the retina.

Glycogen phosphorylase BB was found already as early as embryonic stage E6 in the outermost portion of the retina including the apical part of the RPE and in the presumptive GCL at the innermost retinal border. The presence of glycogen in RPE cells has already been demonstrated for the adult rat retina where it is most probably needed to meet the energy demand of the RPE itself and of neighbouring neurons, such as photoreceptors, especially if blood glucose levels are low [55]. However, the expression of GP BB at very early stages of development, when photoreceptors and other cells of the outer retina are still not born and differentiated, raises the question of the function of GP BB during this early embryonic period. It is conceivable that GP BB-mediated glycogen degradation in embryonic RPE and the outermost part of the retina is involved in the extended proliferative and migratory phase of early retinal development. This idea is supported by studies demonstrating that glycogen metabolism appears indeed to be required for an elevated cell proliferation as observed in various types of tumours [56, 57]. Hence, by glycogen degradation, GP BB in the outermost part of the retina including the RPE probably is able to supply dividing progenitors with a sufficient amount of energy. In this context it is supposable that maintaining an appropriate cellular energy level is an essential process preventing early retinal aberrations [58]. Admittedly, it appears to be generally accepted that degradation of glycogen and introduction of its glucosyl residues into the glycolytic pathway for substrate-chain generation of ATP is the fastest possible way for the intermittent, rapid generation of energy to meet suddenly appearing high demands in cellular energy metabolism. Nevertheless, rapidity of phasic provision of energy is most likely only one out of the set of versatile functions of glycogen, the common denominator of which would be the high—osmotically innocuous—local concentration of glucosyl residues. This high concentration could provide a cell not only with a high rate of availability of glucosyl residues but also with sustained availability for a protracted, though capacity-limited, period of time. This way the cell would become more independent of transient decreases in the concentrations of extracellular glucose. Examples for this aspect of glycogen function are most likely the roles of glycogen glucosyl residues as precursors for the synthesis of lung surfactant lipids [59] and in the detoxication of reactive oxygen species [9]. In view of these considerations the utilization of glycogen stores for fueling transient processes such as stages of cell proliferation and migration does not appear too farfetched.

In addition to the appearance of GP BB in this early phase of expression, the present study reports a gradual increase of GP BB immunoreactivity in the outer portion of the retina during further development. This appears to be closely linked to the differentiation of photoreceptors. The morphological differentiation of photoreceptors starts with the formation of the cilia and the blubbing of the IS around E11 [60, 61]. Already at E10, a prominent expression of GP BB was observed at the apical part of the ONL which most probably corresponds to the developing IS of photoreceptors. As development of photoreceptors proceeds, more and more GP BB accumulates in the IS of cone photoreceptors but not in the rods. This is in line with a previous study reporting that GP BB is exclusively expressed in cone photoreceptors of the human and monkey retina [21]. This might be a reason why cones are less sensitive to oxidative stress and programmed cell death than rods. It has been demonstrated that glycogen can be degraded for the generation of NADPH from glucose-6-phosphate during the disposal of peroxides in astroglial cultures [9]. Thus, glycogen degradation would become an essential process to meet the increased demand for glucose residues needed for energy production and disposal of reactive oxygen species that accompanies colour vision under daylight conditions of higher organisms.

Besides in cones, GP BB was also found in the OPL at a time when synapses have not yet been formed (E10). Therefore, GP BB is possibly involved in the pre-formation and stabilization of neuronal connections by providing neurons with the necessary energy for generating retinal waves. Pertinent in this context are recent reports that retinal wave generation also occurs in the outer (ventricular) part of the retina and appears to be regulated independently from waves of the inner retina [62, 63]. Retinal waves represent spontaneous bursts of coordinated neuronal activity that are most likely involved in synaptogenesis, neuronal differentiation as well as in migration, axonal refinement and formation of early networks [64–67]. In chicken embryos the first spontaneous retinal waves can be detected at E8 and become rhythmic at E9 [68–70]. Since local flashes of retinal waves are closely associated with acetylcholine-mediated transmission [70–72].

Amacrine cells and displaced amacrine cells, which both express GP BB, may play an important role in providing neighbouring cells and/or cholinergic amacrine cells themselves with energy for the generation of inner retinal waves by mobilizing glucosyl residues through the degradation of glycogen. Nevertheless, it is still a matter of debate where the energy for the generation of retinal waves originates from [67, 72]. In this context it is important to note that the adenosine-triggered cAMP/proteinkinase second messenger cascade is involved in the retinal wave propagation [73]. Since GP is a downstream target of this cascade, activation of this pathway could be crucial for generating and maintaining retinal waves by glycogen degradation. An involvement in synaptogenesis is also supported by the expression of GP BB in three sub-laminae of the IPL. Two of them can be allocated to cholinergic synapses mainly innervated by "starburst" amacrine cells located in the INL and GCL [74, 75]. The neurotransmitter acting in the third GP BB-positive sub-lamina remains to be identified.

As retinal development proceeds, GP BB distribution resembles more and more the expression pattern that is found in the adult chicken retina. The present studies have demonstrated that in adult retina GP BB is expressed in cone photoreceptor IS, in synapses within the OPL and IPL as well as in cells of the GCL, layers known to be metabolically highly active [76, 77]. GP BB expression in these layers already at E18 is consistent with the fact that chicken belong to the group of precocial birds which highly depend on a functioning visual system immediately after hatching around E21.

Müller cells did not express GP BB in the embryonic chicken retina. First evidence of GP BB immunoreactivity in Müller cells could be demonstrated in the mature chicken retina at E18. In the adult chicken (15 months of age), the GP BB expression pattern in Müller cells resembles strongly that of adult rodent retina [24]. This is in line with the fact that Müller cells are born very late during retinogenesis [78].

In conclusion, the present study reveals the expression of GP BB, but not MM, in the chicken retina. GP BB is expressed in neuronal cells and, to a lesser extent, in Müller cells. In the embryonic chicken retina, GP BB is expressed in a development-dependent manner in different cell types and the two plexiform layers. The temporal and spatial expression patterns suggest an important role of glycogen metabolism during retinogenesis. The localization of GP BB in both undifferentiated and differentiated neurons suggests that glycogen-derived energy is involved in the proliferation of progenitors, in retinal wave generation and in synaptogenesis.

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