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The pattern of expression of guanine nucleotide-binding protein $\beta 3$ (GNB3) in the retina is conserved across vertebrate species

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Abstract

Guanine nucleotide-binding protein $\beta 3$ (GNB3) is an isoform of the β subunit of the heterotrimeric G protein second messenger complex that is commonly associated with transmembrane receptors. The presence of GNB3 in photoreceptors, and possibly bipolar cells, has been confirmed in murine, bovine and primate retinas (Lee et al., 1992, Peng et al., 1992, Huang et al., 2003). Studies have indicated that a mutation in the GNB3 gene causes progressive retinopathy and globe enlargement (RGE) in chickens. The goals of this study were to 1) examine the expression pattern of GNB3 in wild-type and RGE mutant chickens, 2) characterize the types of bipolar cells that express GNB3 and 3) examine whether the expression of GNB3 in the retina is conserved across vertebrate species. We find that chickens homozygous for the RGE allele completely lack GNB3 protein. We find that the pattern of expression of GNB3 in the retina is highly conserved across vertebrate species, including teleost fish (*Carassius auratus*), frogs (*Xenopus laevis*), chickens (*Gallus domesticus*), mice (*Mus musculata*), guinea pigs (*Cavia porcellus*), dogs (*Canis familiaris*) and non-human primates (*Macaca fascicularis*). Regardless of the species, we find that GNB3 is expressed by Islet1-positive cone ON-bipolar cells and by cone photoreceptors. In some vertebrates, GNB3-immunoreactivity was observed in both rod and cone photoreceptors. A protein-protein alignment of GNB3 across different vertebrates, from fish to humans, indicates a high degree (>92%) of sequence conservation. Given that analogous types of retinal neurons express GNB3 in different species, we propose that the functions and the mechanisms that regulate the expression of GNB3 are highly conserved.

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Keywords

Photoreceptor; Bipolar cell; Transducin; Retinopathy

Guanine nucleotide-binding proteins (G-proteins) are heterotrimeric proteins, composed of alpha (G_α), beta (G_β) and gamma (G_γ) subunits. G-proteins are second messengers that interact with 7-transmembrane domain metabotropic receptors, also known as G-protein-coupled receptors (GPCRs) (Cabrera-Vera et al., 2003, McCudden et al., 2005, Oldham and Hamm, 2008). Of the 5 different isoforms of the beta subunit that have been recognized, the $\beta 1$ and $\beta 3$ subunits are known to be expressed by photoreceptors in the retina; with $\beta 1$ (GNB1) associated with rod photoreceptors and $\beta 3$ (GNB3), also known as β -transducin, associated with cone photoreceptors (Lee et al., 1992, Peng et al., 1992). Furthermore, there is some evidence that suggests that a subset of bipolar cells express GNB3 (Peng et al., 1992, Huang et al., 2003).

Mutations in GNB3 have been implicated in the development of multiple systemic diseases in humans. A polymorphism in the GNB3 gene, C825T, has been associated with hypertension, obesity and depression (Roskopf et al., 2000, Zill et al., 2000). In the retina, a mutation in the GNB3 gene has been shown to impact vision. This was first observed in commercial chicken breeding stocks in the United Kingdom in the 1980's with the discovery of the retinopathy, globe enlarged (RGE) chicken (Curtis et al., 1987). The RGE phenotype is known to result from a single codon deletion leading to the loss of a single aspartic acid residue (Tummala et al., 2006). The loss of the aspartic acid is believed to destabilize the GNB3 protein and, consequently, reduce expression levels (Tummala et al., 2006). Putative decreases in GNB3 expression levels results in severely reduced visual acuity at hatching that progressively worsens to complete vision loss in adult animals (Montiani-Ferreira et al., 2003).

Corresponding to the loss of visual acuity, electroretinograms (ERGs) of RGE chickens revealed abnormalities compared to wild-type counterparts. These ERG recordings had an elevated response threshold under dark- and light-adapted conditions, delayed onset of the a-wave and elevated b-wave amplitudes with bright light (Montiani-Ferreira et al., 2003, Montiani-Ferreira et al., 2007). Despite the reduction in visual acuity at hatching, fundusoscopic and histological examination of the RGE retinas revealed no overt retinal abnormalities during the first 6 weeks of life (Montiani-Ferreira et al., 2005). Beyond the first 6 weeks, the eyes of RGE chickens undergo a progressive globe enlargement involving an increase in vitreous chamber depth, increased axial length, decreased anterior chamber depth and flattening of the cornea. Concurrent with the globe enlargement, the retina slowly degenerates; this degeneration involves the loss of photoreceptors (Montiani-Ferreira et al., 2003). The precise mechanisms underlying the loss of visual acuity in young (<P45) RGE chickens, with apparently normal retinas, remains uncertain.

Given the retinopathy that occurs in adult RGE chickens, proper function of GNB3 is required not only for photoreceptor signal transduction, but also in maintaining the integrity of the photoreceptors and the maintenance of proper eye size in adult animals. Although there is compelling evidence for the expression of GNB3 in cone photoreceptors in murine, bovine and primate retinas, little is known about the expression of GNB3 in the retinas of other species. Moreover, expression of GNB3 in bipolar cells has not been well studied. Thus, the purpose of this study was to examine the expression pattern of GNB3 in wild-type and RGE mutant chickens, characterize the types of bipolar cells that express GNB3 and to test whether the expression of GNB3 in the retina is conserved across vertebrate species.

Experimental procedures

Animals

The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health and the Ohio State University. Fertilized eggs and newly hatched wild type leghorn chickens (*Gallus gallus domesticus*) were obtained from the Department of Animal Sciences at the Ohio State University. The stage of the chick embryos was determined according to the guidelines established by Hamburger and Hamilton in 1951 (Hamburger and Hamilton, 1992). Postnatal chicks were kept on a cycle of 12 hours light, 12 hours dark (lights on at 8:00 AM). RGE chickens were hatched from fertilized eggs obtained from a cross of RGE homozygous (RGE^{-/-}) chickens from the Department of Small Animal Clinical Sciences, Michigan State University. Chicks were housed in a stainless steel brooder at about 25°C and received water and Purina™ chick starter *ad libitum*.

Eyes were obtained post-mortem from goldfish (*Carassius auratus*; Dr. Christophe Ribelyaga, Department of Neuroscience, The Ohio State University), frogs (*Xenopus laevis*; Dr. Candice Askwith, Department of Neuroscience, The Ohio State University), mice (*Mus musculata*; Dr. Karl Obrietan, Department of Neuroscience, The Ohio State University), guinea pigs (*Cavia porcellus*; Dr. Jackie Wood, Department of Physiology and Cell Biology, Ohio State University), dogs (*Canis familiaris*; Simon Petersen-Jones, Veterinary Sciences, Michigan State University) and monkeys (*Macaca fascicularis*; Dr. John Buford, Department of Physiology and Cell Biology, The Ohio State University).

Reverse transcriptase PCR

Retinas from 2 P7 chicks were pooled and placed in 1.5 ml of Trizol Reagent (Invitrogen) and total RNA was isolated according to the Trizol protocol and resuspended in 50 µl RNase free water. Genomic DNA was removed by using the *DNA FREE* kit provided by Ambion. cDNA was synthesized from mRNA by using Superscript™ III First Strand Synthesis System (Invitrogen) and oligo dT primers according to the manufacturer's protocol. Control reactions were performed using all components with the exception of the reverse transcriptase to exclude the possibility that primers were amplifying genomic DNA.

PCR primers were designed by using the Primer-BLAST primer design tool at NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer sequences are as follows: GNB3 – forward 5' GCC CAC GTG GAG AAG CCA CC 3' – reverse 5' CCT GGT CTG CCC GGA GGT CA 3'; GAPDH – forward 5' CAT CCA AGG AGT GAG CCA AG 3' – reverse 5' TGG AGG AAA TTG GAG GA 3'. The predicted product size was 812 base pairs for GNB3 and 134 base pairs for GAPDH. PCR reactions were performed by using standard protocols, Platinum™ Taq (Invitrogen) and an Eppendorf thermal cycler. PCR products were run on an agarose gel to verify the predicted product sizes.

Western Blotting

Retinas from 2 P7 wild-type and 2 RGE chicks were harvested on ice in HBSS+ and immediately sonicated in extraction buffer (Bio-Rad) added with a protease inhibitor cocktail tablet (Roche). After 5 minute ice incubation, the sample was centrifuged and the supernatant collected. Protein concentration was determined using a BCA Protein Assay (Thermo Scientific). Samples were loaded into 10-well, 4-15% Tris-HCL acrylamide gels (Bio Rad) with Precision Plus Protein Standard (Bio Rad) for electrophoresis at 95V. Protein transfer was performed via electrophoresis overnight at 20V onto a nitrocellulose membrane (162-0117; BioRad). After protein transfer, the membrane was blocked in Tris-buffered saline with 5% (w/v) milk powder and incubated in primary antibodies for anti-mouse

GAPDH at 1:2500 (IMG-5019A-1; Imgenex) or anti-rabbit GNB3 at 1:500 (HPA005645; Sigma-Aldrich) at room temperature overnight. The membrane was washed in Tris-buffered saline and incubated under horseradish-peroxidase conjugated secondary antibodies at 1:4000 (Amersham GE Healthcare; anti-mouse IgG NA931V; anti-rabbit IgG NA934V) applied for 60 minutes at room temperature. The membranes were washed in Tris-buffered saline and developed using an ECLTM Western Blotting Detection Reagents (Amersham GE Healthcare; RPN2106) and UVP BioSpectrum 500 imaging system.

Fixation, sectioning and immunocytochemistry

Tissues were fixed, sectioned and immunolabeled as described previously (Fischer et al., 2008b, Fischer et al., 2009). A summary of the antibodies used in this study is provided in table 1. Working dilutions and sources of antibodies used in this study included the following. (1) The Islet1 mouse monoclonal antibody was raised to the C-terminus (amino acids 247-349) of rat Islet1 and used at 1:50 (40.2D6; Developmental Studies Hybridoma Bank – DSHB; University of Iowa). (2) mouse anti-Lim3 was raised to recombinant full-length murine Lim3 fused to GST and used at 1:50 (67.4E12; DSHB). (3) mouse anti-visinin was raised to purified bovine visinin and used at 1:100 (7G4; DSHB). (4) mouse anti-calbindin was raised to calbindin D28k purified from chicken gut and used at 1:400 (300; Swant Immunochemicals; Bellinzona, Switzerland). (5) rabbit anti-red/green opsin was raised to recombinant human red/green opsin and used at 1:400 (AB5405; Chemicon; Temecula, CA). (6) mouse anti-rhodopsin was raised to purified bovine rhodopsin and used at 1:200 (rho4D2; Dr. R. Molday; University of British Columbia). (7) mouse anti-PSD-95 was raised to amino acids 77-299 of human PSD-95/SAP-90 and used at 1:50 (K28/43; NeuroMab). (8) RetP1, a mouse anti-rhodopsin monoclonal antibody (MAB5316; Chemicon; Temecula, CA). (9) Rabbit anti-GNB3 antibody was used at 1:400. The antibody to GNB3 was raised to amino acids 172-317 of human guanine nucleotide-binding protein β 3 subunit and was used at 1:400 (HPA005645; Sigma-Aldrich). In the current study, we demonstrate the specificity of the GNB3 antibody by using Western blot analysis and absence labeling in a GNB3-mutant retina.

We evaluated the specificity of primary antibodies by comparison with published examples of results and assays for specificity. None of the observed labeling was due to non-specific labeling of secondary antibodies or autofluorescence because sections labeled with secondary antibodies alone were devoid of fluorescence. Secondary antibodies included donkey-anti-goat-Alexa488/568, goat-anti-rabbit-Alexa488/568/647, goat-anti-mouse-Alexa488/568/647, goat anti-rat-Alexa488 and goat-anti-mouse-IgM-Alexa568 (Invitrogen) diluted to 1:1000 in PBS plus 0.2% Triton X-100.

Photography, measurements and cell counts

Photomicrographs were obtained using a Leica DM5000B microscope equipped with epifluorescence and Leica DC500 digital camera. Confocal images were obtained using a Zeiss LSM 510 imaging system at the Hunt-Curtis Imaging Facility at the Ohio State University. Images were optimized for color, brightness and contrast, multiple channels overlaid and figures constructed by using Adobe PhotoshopTM_{6.0}. Cell counts were performed on representative images. To avoid the possibility of region-specific differences within the retina, cell counts were consistently made from the same region of retina for each data set.

Results

Expression of GNB3 in wild-type and RGE chicken retinas

Reverse-transcriptase PCR (RT-PCR) was used to detect GNB3 mRNA in retinas from P7 wild-type and RGE chicks. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control. GNB3 mRNA was detected in both in wild-type and RGE chicks (Fig. 1a). We probed for GNB3 protein in retinal homogenates using Western blotting. GNB3 protein was detected, as a single band at a molecular mass of 40kDa, in wild-type retinas but not in RGE retinas (Fig. 1b). In wild-type retinas, GNB3 immunofluorescence was detected in the cytoplasm of photoreceptors and bipolar cells, but was completely absent in RGE retinas (Fig. 1c, d). Interestingly, markers for bipolar cells (Islet1 and PKC) and photoreceptors (calbindin) have patterns of expression that were similar in both wild-type and RGE chicks (Fig. 1e). This indicates that the loss of GNB3 from bipolar cells and photoreceptors does not overtly affect the morphology and phenotype of these cells, consistent with previous reports (Montiani-Ferreira et al., 2005).

GNB3 in the developing chick retina

RGE mutant chicks have reduced visual acuity at the time of hatching (Montiani-Ferreira et al., 2003) even though the retina appears normal at this stage of development (Montiani-Ferreira et al., 2005). Thus, it seems unlikely that GNB3 is expressed during early embryonic stages to significantly impact retinal development. To test this hypothesis we examined when GNB3 was first expressed during embryonic retinal development. Accordingly, we labeled sections of embryonic chicken retina at various stages beginning at embryonic day 5 (E5), shortly after the onset of neuronal differentiation (Prada et al., 1991). At E5 and E8 we failed to detect GNB3 expression in wild-type retinas (data not shown). GNB3 expression was not detectable in the retina until E13 where immunoreactivity was observed in both the inner plexiform layer (IPL) and the outer plexiform layer (OPL) (Fig. 2a-c). In order to identify the cell populations that express GNB3 we probed for the expression of the LIM-domain transcription factor Islet1. This factor is expressed by ganglion cells, cholinergic amacrine cells, many bipolar cells and about half of all horizontal cells shortly after fate specification (Edqvist et al., 2006, Fischer et al., 2008a, Stanke et al., 2008). We observed transient Islet1-immunofluorescence in the nuclei of cells in the ONL, consistent with a previous report that the Islet1 antibody cross-reacts with Islet2 in immature photoreceptors (Fischer et al., 2008a). In addition, Islet1 was detected in the nuclei of presumptive horizontal cells, bipolar cells and cholinergic amacrine cells in INL (Fig. 2b, e, h, k) consistent with previous observations (Fischer et al., 2007). As development progresses, GNB3 appears in the peri-nuclear cytoplasm of bipolar cells at E15 (Fig. 2d-f). By E17, immunoreactivity for GNB3 was observed in the cytoplasm of photoreceptors as expression of Islet2 declines (Fig. 2g-i). By E18.5, GNB3 immunoreactivity was concentrated in the outer segments (Fig. 2k). We compared GNB3 expression to that of visinin, the avian homologue of mammalian recoverin, which is expressed by all types of rod and cone photoreceptors shortly after terminal mitosis (Yamagata et al., 1990). In E13 chick embryos, visinin was present in the photoreceptors, concentrated in the immature outer segments, whereas GNB3 immunoreactivity remained below detectable levels in the photoreceptors (Fig. 2c). As photoreceptors mature and become morphologically distinct, GNB3 immunoreactivity becomes concentrated in the cone pedicles and the outer segments, whereas levels of GNB3 remained low in the peri-nuclear cytoplasm (Figs. 2f,i and l).

Distribution of GNB3 in the postnatal chick retina (*Gallus gallus domesticus*)

We sought to better characterize the types of cells in the mature chick retina that express GNB3. Accordingly, we labeled retinal sections with antibodies to different cell-distinguishing markers. We applied antibodies to PKC, which is known to be expressed at

high levels in rod bipolar cells and at lower levels in cone ON-bipolar cells in the avian retina (Negishi et al., 1988, Young and Vaney, 1990, Fischer et al., 1998, Caminos et al., 1999) and in other species (Negishi et al., 1988, Greferath et al., 1990, McCord et al., 1996, Vaquero et al., 1996, Caminos et al., 1999). We found that PKC was co-localized to some of the GNB3-positive bipolar cells (Fig. 3a-c). The rod bipolar cells were intensely immunoreactive for PKC and weakly immunoreactive for GNB3. Consistent with the finding of Negishi and colleagues (1988) the axon terminals of the rod bipolar cells were tristratified, with endings concentrated in layers 1, 3 and 5 of the IPL (Negishi et al., 1988). In addition, GNB3-immunoreactivity was detected in presumptive cone ON-bipolar cells that were weakly immunoreactive for PKC (Fig. 3a-c). The GNB3-positive bipolar cells appeared to produce axons that are bundled together and fasciculate within the inner nuclear layer (INL), and branch apart upon entry into the IPL (Fig. 3a & d). Consistent with the notion that GNB3 is expressed by cone ON-bipolar cells, the axon terminals of these cells were densely ramified in the ON sub-lamina of the IPL with endings concentrated in sub-laminae 3 and 4 (Fig. 3e). In addition, we found GNB3-positive bipolar cells with somata in the scleral INL that were PKC-negative (Fig. 3a-c). We found a high degree of overlap between Islet1 and GNB3 in bipolar cells (Fig. 3d-f). Although there was no overlap of GNB3 and Islet1 in the horizontal cells, there was a complete overlap of GNB3 and Islet1 in the bipolar cells (Fig. 3f). In other words, all of the Islet1-positive bipolar cells were co-labeled for GNB3 and all of the GNB3-positive bipolar cells were labeled for Islet1. We next assayed for the co-expression of GNB3 and Lim homeobox gene 3 (Lim3), a transcription factor that is known to be expressed by many bipolar cells and immature photoreceptors in the chick retina (Edqvist et al., 2006, Fischer et al., 2008a). We found weak immunofluorescence for Lim3 in the nuclei of many bipolar cells that were GNB3-positive (Figs. 3g-i). In addition, we found strong immunofluorescence for Lim3 in the nuclei of bipolar cells that were GNB3-negative (Figs. 3g-i).

GNB3 was expressed by most, if not all, types of photoreceptors in the chick retina. We found that all of the calbindin-positive cone photoreceptors were immunoreactive for GNB3 which was concentrated in the outer segments (Figs. 4a-d). However, we identified many GNB3-positive photoreceptor outersegments that were negative for calbindin (Figs. 4b-d). The identity of the GNB3-positive/calbindin-negative photoreceptors remains uncertain. Examination of the OPL revealed 2 strata of axon terminals, with the most distal strata containing the terminals of calbindin-positive cone photoreceptors, consistent with prior findings (Fischer et al., 2008a). Immunolabeling for visinin and GNB3 revealed significant overlap in outersegments (Figs. 4e-g), suggesting that GNB3 is expressed by both rod and cone photoreceptors. Accordingly, rod photoreceptors were examined with antibodies to rhodopsin and GNB3. We found some overlap of rhodopsin- and GNB3-immunoreactivity in the outer segments of photoreceptors (Figs. 4h-j), consistent with the notion that GNB3 is expressed by rods in addition to cone photoreceptors.

GNB3 in teleost fish retina (*Carassius auratus*)

We next assayed for the expression of GNB3 in the fish retina. Unlike the pattern of GNB3 expression in the chick retina, there was little immunoreactivity for GNB3 in bipolar cells in the fish retina (Fig. 5d). We found immunoreactivity for GNB3 in the outer retina, in putative cone photoreceptors (Figs. 5a and d). Immunolabeling for GNB3 was present throughout the cytoplasm of cone photoreceptors, with a concentration in the outer segments (Fig. 5a). The GNB3-positive photoreceptors appeared to be cones based on their distinctive morphology. To identify the types of photoreceptors that express GNB3 we used the photoreceptor-specific markers peanut agglutinin (PNA lectin), RetP1 and immunoreactivity to rhodopsin, which have been shown to label photoreceptors in a number of species, including several varieties of fish (Xu and Tian, 2008). There was no overlap of labeling for

GNB3 and rhodopsin (Figs. 5a-c). However, labeling with the RetP1 monoclonal indicated co-localization with GNB3 in photoreceptors (Figs. 5d-f). Labeling for PNA lectin and GNB3 overlapped in the cone outer segments, with GNB3 concentrated in the distal aspects of the outer segments (Figs. 5g-i).

GNB3 in frog retina (*Xenopus laevis*)

Patterns of GNB3-immunoreactivity in the frog retina were similar to those seen in fish retina. In *Xenopus* retinas, intense immunoreactivity for GNB3 was observed in cone photoreceptors (Figs. 6a-c), based on the morphology of these cells. In addition, weak GNB3-immunoreactivity was observed in the outer segments of rod photoreceptors that were labeled by the RetP1 monoclonal antibody (Figs. 6a-c). Further, GNB3-immunoreactivity was localized to Islet1-positive nuclei of bipolar cells (Figs. 6d-f). However, many of the GNB3-immunoreactive bipolar cells were negative for Islet1 (Fig. 6d-f).

GNB3 in mouse retina (*Mus musculata*)

We next sought to determine whether the patterns of expression of GNB3 observed in birds, fish and frogs were preserved in mammals. GNB3-immunoreactivity was concentrated in the outer segments of photoreceptors in mouse retinas (Fig. 7a), consistent with a previous report (Huang et al., 2003). In central regions of the retina, the majority of GNB3-positive outer segments were immunoreactive for red/green opsin, consistent with the notion that GNB3 is expressed by cone photoreceptors (Figs. 7b-d). A minority of GNB3-positive cone outer segments were negative for red/green opsin in the photoreceptor layer (PRL), suggesting that these were blue-sensitive cones (Figs. 7a-d).

GNB3-immunolabeling was observed in the INL in bipolar cells (Fig. 7e). Co-labeling for PKC and GNB3 was observed in many bipolar cells (Fig. 7e-g). A minority of the GNB3-positive bipolar cells were negative for PKC (Fig. 7g). The axons terminals of the PKC-positive bipolar cells overlapped with GNB3-immunofluorescence in the vitread IPL (Fig. 7e-g). However, GNB3-positive/PKC-negative bipolar cell terminals were observed in the middle stratum of the IPL (Fig. 7e-g). The majority of GNB3-immunoreactive bipolar cells were positive for Islet1, whereas a minority of the GNB-positive bipolar cells did not contain detectable levels of Islet1 (Figs. 7h-j). Our observations are consistent with a recent report that GNB3 is expressed by a subset of bipolar cells in mouse retina (Huang et al., 2003). To determine whether GNB3-immunoreactivity in the OPL was present in the dendrites of bipolar cells or axon terminals of photoreceptors, we labeled sections with antibodies to PSD-95, a MAGUK-family synaptic protein. In the rodent retina, PSD-95 is known to be present in the axon terminals of photoreceptors (Aartsen et al., 2006). There was little overlap of PSD-95 and GNB3 immunofluorescence in the OPL (Figs. 7k-m), suggesting that the PSD-95 was confined to the axon terminals of the photoreceptors and the GNB3 was confined to the dendrites of bipolar cells.

GNB3 in guinea pig retina (*Cavia porcellus*)

Examination of the guinea pig retina revealed widespread GNB3 expression in photoreceptors and bipolar cells. GNB3-immunolabeling was concentrated in the outer segments of presumptive cone photoreceptors (Fig. 8a-c). Immunolabeling for GNB3 and red/green opsin revealed overlap in the outer segment in most cones (Fig. 8c). A minority of GNB3-positive outer segments were negative for red/green opsin (Figs. 8a-c). Examination of rod photoreceptors with antibodies to rhodopsin revealed weak GNB3-immunofluorescence in the outer segments of rod photoreceptors (Fig. 8f). By increasing the detector gain, GNB3-immunofluorescence was detected in rod outer segments and in cone inner segments (Fig. 8d). In addition, we found some immunolabeling for rhodopsin in

GNB3-positive cone photoreceptors (Figs. 8d-f), suggesting that the rhodopsin antibody may cross-react with opsin.

We found significant overlap of GNB3 and Islet1 in the bipolar cells of the guinea pig retina (Figs. 8g-l). We found that nearly all (~92%) Islet1-positive nuclei in the distal INL were rimmed with GNB3-immunofluorescence (Figs. 8g-l). Many (~35%) of the GNB3-positive bipolar cells were intensely immunoreactive for PKC (Figs. 8m-p). However, numerous GNB3-positive bipolar cells were negative for PKC. Examination of the OPL revealed stratification of SV2-positive photoreceptor terminals and GNB3-labeling, suggesting that GNB3 is present in bipolar cell dendrites, not the axon terminals of photoreceptors (Fig. 8q-s).

GNB3 in dog retina (*Canis familiaris*)

We next examined the distribution of GNB3 in the canine retina. In the distal retina, GNB3-immunoreactivity was observed in presumptive cone photoreceptors (Figs. 9a and d). Labeling for cone arrestin and GNB3, combined with cell morphology, confirmed that GNB3 was expressed by cone photoreceptors (Fig. 9b and c). Weak Islet1/2-immunolabeling was observed in the nuclei of cone photoreceptors, consistent with reports that Islet2 is expressed by developing photoreceptors in the chick retina (Fischer et al., 2008a) and is maintained at low levels in mature photoreceptors (unpublished observations). Similar to the distribution seen in other species, GNB3 was concentrated in the outer segments of the canine cone photoreceptors (Figs. 9a-c).

Examination of the INL revealed Islet1-positive bipolar cell nuclei surrounded by a rim of GNB3-immunofluorescence cytoplasm (Figs. 9e and 9f). All of the Islet1-positive nuclei in the distal INL were associated with GNB3-labeling. These GNB3-positive bipolar cells had axons that terminated in the proximal, ON sub-lamina, of the IPL. Furthermore, most of the GNB3-positive bipolar cells were labeled for PKC (Figs. 9g-i). However, a minority (<25%) of the GNB3-positive bipolar cells were negative for PKC (Figs. 9g-i).

GNB3 in the primate retina (*Macaca fascicularis*)

We found that the pattern of expression of GNB3 in the macaque retina was similar to that observed in the retinas of other vertebrates. Immunolabeling for Islet1 was co-localized to GNB3-positive bipolar cells within the INL (Fig. 10d). Consistent with previous reports (Fischer et al., 2001), Islet1 was detected in the nuclei of presumptive ganglion cells and cholinergic amacrine cells, in addition to the nuclei of bipolar cells (Figs. 10d and 10e). A majority of the Islet1-positive bipolar cell nuclei overlapped with GNB3 immunofluorescence. A minority of the Islet1-positive nuclei in the distal INL was not associated with detectable levels of GNB3 (Figs. 10e-g). GNB3-labeling was also prominent in the distal portion of the outer segments of cones in the PRL, but was detected at lower levels in the inner segments, including the axon terminals (Fig. 10 c), consistent with a previous report (Peng et al., 1992). Labeling with PNA lectin overlapped with GNB3 in the outer segments of photoreceptors (Figs. 10a-d). PNA lectin is known to label cone photoreceptors in the primate retina (Blanks and Johnson, 1984).

GNB3 protein sequence homology among species

The near-identical immunolabeling patterns for GNB3 in the retinas of different species suggest that the primary amino acid sequence is highly conserved across species. To test this notion, we examined the homology of the primary amino acid sequences of GNB3 in different species by using a protein-protein alignment search tool (BLASTp; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). We found a very high level (83%-100%) of sequence identity among vertebrate species when compared to human GNB3 protein (Table

2). Accounting for conservative amino acid substitutions, we found very high sequence conservation, ranging from 92% to 100%, among GNB3 sequences from different vertebrates. The highly conserved sequence homology observed among species, as well as the similar expression patterns among retinal cell types, suggests the importance of GNB3 function in the retina.

Discussion

In the retinopathy, globe enlarged (RGE) chicken, the mutation of GNB3 results in a profound phenotype featuring a significant loss of visual acuity beginning at the time of hatching, and photoreceptor degeneration and globe enlargement in adults (Montiani-Ferreira et al., 2003, Montiani-Ferreira et al., 2005, Montiani-Ferreira et al., 2007). Given the significance of this presentation, we sought to characterize the expression pattern of GNB3 in the retinas of chicks, as well as in the retinas of different non-avian vertebrates. The expression of GNB3 in cone photoreceptors was consistent across species from teleost fish to primates. Furthermore, GNB3 expression was observed in a subset of bipolar cells, which express Islet1 and PKC in most species. Taken together, our findings suggest that patterns of GNB3 expression are highly conserved in cones and bipolar cells across vertebrate species.

GNB3 has been consistently observed in the outer segments of cone photoreceptors in different mammalian species, including mice, cows and monkeys (Lee et al., 1992, Peng et al., 1992, Huang et al., 2003). However there have been no reports of GNB3 expression in rod photoreceptors. Similar to previous reports, we found GNB3 was exclusively expressed by cones among primate, murine and canine photoreceptors. However, in chicken, guinea pig, goldfish and frog retinas, GNB3 was detected in rod photoreceptors; albeit at levels lower than those observed in cone photoreceptors. The expression of GNB3 in rod photoreceptors was unexpected given previous literature indicating GNB3 expression is restricted to cone photoreceptors (Lee et al., 1992, Peng et al., 1992, Tummala et al., 2006, Dutt and Cao, 2009, Haider et al., 2009). It is possible that GNB3 expression occurs in both rods and cones; however, the GNB3 antibody utilized in this study may lack specificity to cone β -transducin and could recognize rod β -transducin. Alternatively, GNB3 may be expressed by both types of photoreceptors. In the chick retina, GNB3 was detected in rod photoreceptors labeled with the 4D2 rhodopsin monoclonal antibody. This antibody has been shown to label mid-wavelength green-sensitive cones where the photopigment shares the closest sequence homology with rhodopsin (Xie and Adler, 2000). However, all GNB3-immunolabeling disappeared in RGE retinas, indicating that the antibody was not cross-reacting with rod β -transducin in the chick. In guinea pigs, cross-reactivity of the 4D2 antibody with green-opsin in cones may explain the observation of GNB3 in the outer segments of some rhodopsin-positive photoreceptors. Despite the presence of GNB3 labeling in the rod photoreceptors of some species, findings that GNB3 is expressed in cone photoreceptors in all species examined indicates the highly conserved nature of the protein and suggests the importance of GNB3 in cone-driven vision.

Examination of the photoreceptor axon terminals revealed the presence of GNB3 in cone pedicles in most species with the exception of rodents. In mice and guinea pigs, there was little or no labeling for GNB3 in the cone pedicles, compared to labeling observed in the outer segments. It is possible that the differential distribution of GNB3 in the axon terminals of the photoreceptors may result from light-dependent trafficking of GNB3. Examples of light-dependent trafficking of transducin have been documented in rod photoreceptors, where rod α -transducin translocates from the outer segments under dark-adapted conditions to the axon terminal in the OPL following light exposure (Brann and Cohen, 1987). There is also evidence of light-dependent translocation of rod β -transducin in

the retinas of mice and rats (Sokolov et al., 2002, Lobanova et al., 2007). By comparison, there is little evidence of light-dependent translocation of phototransduction-related proteins in cone photoreceptors. Elias and colleagues reported that, unlike rod α -transducin, cone α -transducin does not translocate with varying light levels (Elias et al., 2004). Rosenzweig and colleagues showed that the subcellular distribution of transducin was correlated to light-dependent changes in membrane affinity, with cone α -transducin remaining bound to the outer segment during light activation (Rosenzweig et al., 2007). Furthermore, when cone α -transducin is ectopically expressed in rod photoreceptors this protein is translocated to inner segments with changes in light levels, indicating differences in the cell-intrinsic trafficking mechanisms can (Rosenzweig et al., 2007). Chen and colleagues reported that cone α -transducin partially translocates in rat retina after a brief exposure to high-intensity light (Chen et al., 2007). Reports of cone β -transducin translocation are rare; however, McGinnis and colleagues reported migration of cone β -transducin with light exposure of dark-adapted retinas in mice (McGinnis et al., 2002). Given this evidence, it is possible that we failed to detect GNB3 in the axon terminals of cone photoreceptors in mice and guinea pigs because of light-dependent trafficking and harvesting conditions.

In the inner retina, GNB3 expression was observed in presumptive cone ON-bipolar cells across species with one exception; goldfish retinas had low levels of GNB3 expression in bipolar cells. There is some prior evidence for GNB3 expression in bipolar cells. Peng and colleagues reported, in primate retina, that GNB3 is expressed in presumptive rod bipolar cells that were co-labeled for PKC. This study also noted that the synaptic terminals of the GNB3-positive bipolar cells terminated in sublamina b of the INL, suggesting that some these bipolar cells were cone ON-bipolar cells (Peng et al., 1992). Furthermore, Huang and colleagues demonstrated GNB3 in the dendrites of cone bipolar cells and colocalization of GNB3 with PKC in rod bipolar cells in mouse retina (Huang et al., 2003). In our study, cone ON-bipolar and rod bipolar cells were identified using antibodies to Islet1 in chicken, macaque, mouse, canine and guinea pig retinas. It has been previously reported that Islet1, a LIM-domain transcription factor, is required for the normal differentiation of ON- and OFF-bipolar cells in the rodent retina (Elshatory et al., 2007). In chicken, macaque, mouse, canine and guinea pig, the GNB3-positive bipolar cells expressed Islet1. Supporting the notion that most of the GNB3-positive bipolar cells are cone ON-bipolars, the axonal processes of these cells terminate within the ON layer of the IPL, sub-lamina B. In *Xenopus* retina, GNB3 and Islet1 did not overlap completely within bipolar cells, suggesting that GNB3 may be expressed by types of bipolar cells in addition to the cone ON-bipolar cells or that Islet1 is not exclusively expressed by cone ON-bipolar cells.

Consistent with the hypothesis that GNB3 is expressed by cone ON-bipolar cells, we observed significant overlap of GNB3 and PKC. PKC has been shown to be expressed by both rod bipolar cells and cone ON-bipolar cells in multiple species (Negishi et al., 1988, Greferath et al., 1990, McCord et al., 1996, Vaquero et al., 1996, Caminos et al., 1999). In the chicken retina, labeling for PKC and GNB3 revealed 3 different sets of bipolar cells; (1) PKC-negative/GNB3-positive cells, (2) intense PKC-positive/GNB3-positive cells, and (3) weak PKC-positive/GNB3-positive bipolar cells. These findings suggest that these bipolar cells were presumptive cone ON-bipolar (weak PKC-positive/GNB3-positive cells) and rod ON-bipolar cells (intense PKC-positive/GNB3-positive), whereas the identity of the PKC-negative/GNB3-positive bipolar cells remains uncertain. In the mammalian retinas, GNB3 was observed in PKC-immunoreactive bipolar cells. The overlap of expression of PKC and GNB3 in bipolar cells suggests that cone ON-bipolar and rod bipolar cells utilize GNB3 for signal transduction. In the rodent retina, some of the axon terminals of the GNB3-positive bipolar cells were clearly segregated from some of PKC-positive terminals in the IPL, supporting the notion that GNB3 is expressed by more than one type of bipolar cell. Similar results were observed in guinea pig and dog retinas, where GNB3-positive/PKC-negative

and GNB3/PKC-positive bipolar cells were observed. Our observations indicate that the GNB3-positive bipolar cells in the chick retina express Islet1 and many of these cells express low levels of Lim3. The GNB3-negative bipolar cells that express high levels of Lim3 are likely to be OFF-cone bipolar cells. Taken together, these findings suggest that GNB3 is expressed by cone ON-bipolar cells and in some rod ON-bipolar cells, indicating the importance of GNB3 signal transduction in the ON pathway.

In the current study, immunofluorescence combined with Western blot analysis, clearly indicate that GNB3 protein is entirely lost from the retinas of RGE mutant chicks. The presence of mRNA in both WT and RGE retinas indicates that the transcriptional mechanisms for the GNB3 expression are intact in the RGE chicken. A previous report has suggested that the deletion of a single aspartic acid (D153del) from GNB3 in the RGE chicken reduces levels of expression by about 70%, as determined by an immuno-slot-blot preparation (Tummala et al., 2006). The discrepancy between these studies likely resulted from the use of different GNB3 antibodies and the application of different immunological techniques. Our findings indicate that the loss of GNB3 in the RGE chicken does not overtly affect the formation and maturation of the retina during embryonic and early postnatal development. This is consistent with a previous report demonstrating the normal immunohistological profile of retinas in young (<P30) RGE chicks (Montiani-Ferreira et al., 2005).

Clearly, the loss of GNB3 underlies the phenotype of the RGE^{-/-} chicken which involves the loss of visual acuity at the time of hatching, and eventually (after P45) globe enlargement and retinal degeneration (Montiani-Ferreira et al., 2003, Montiani-Ferreira et al., 2005, Tummala et al., 2006). The mechanisms underlying the progressive retinopathy and subsequent excessive ocular growth in this animal model remains unknown. We failed to find GNB3 expression in ocular tissues, with the exception of the retina. Thus, the ocular phenotypes of the RGE chicken, including uncontrolled eye growth, flattening of the anterior chamber and progressive retinopathy, result from a complete loss of GNB3 from retinal bipolar cells and photoreceptors.

The onset of expression of GNB3 during embryonic development coincides with the late-onset of genes in both photoreceptors and bipolar cells. The maturation of photoreceptors is delayed for at least 1 week after terminal mitosis and fate specification in the chick retina (Fischer et al., 2008a, Ghai et al., 2008). The onset of GNB3 expression in bipolar cells occurs before the onset of PKC expression (Caminos et al., 1999), and GNB3 expression in photoreceptors appears before the onset of cone opsin expression, at about E15 (Bruhn and Cepko, 1996). Despite an absence of GNB3 expression, the retinas of RGE^{-/-} chicks show no gross retinal abnormalities, with the exception of subtle synaptic changes in the OPL (Montiani-Ferreira et al., 2005). Moreover, the late onset of GNB3 in the embryonic chick retina may explain why no severe ocular abnormalities are observed at hatching in the RGE phenotype; an early onset of expression might be expected to have more severe developmental impact. However, the survival of photoreceptors is compromised in RGE retinas beginning at about P45, indicating that proper GNB3 function is required to maintain the photoreceptors in adult animals (Montiani-Ferreira et al., 2005).

Conclusions

The pattern of GNB3 expression is very similar in the retinas of multiple species, suggesting the highly conserved nature of the protein and its importance for normal visual function. Given the remarkably similar expression patterns of GNB3 in the retina across species, we propose that the functions and mechanisms of expression for GNB3 within the retina are highly conserved. Furthermore, normal GNB3 protein expression is critical for normal

signal transduction in cone photoreceptors and ON-bipolar cells, allowing for proper retinal function. We propose that the loss of GNB3 from bipolar cells and photoreceptors in the RGE retina underlies the loss of vision at the time hatching, and the progressive degeneration and abnormal ocular growth in adult chickens.

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List of Abbreviations

GNB3	Guanine nucleotide-binding protein β 3
RGE	Retinopathy, globe enlarged
G-proteins	Guanine nucleotide-binding proteins
GPCRs	G-protein-coupled receptors/7-transmembrane domain metabotropic receptors
GNB1	Guanine nucleotide-binding protein β 1
ERG	Electroretinogram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
RT-PCR	Reverse-transcriptase polymerase chain reaction
PKC	Phosphokinase C
Lim3	Lim homeobox gene 3
PNA	Peanut agglutinin
INL	Inner nuclear layer
ONL	Outer nuclear Layer
PRL	Photoreceptor layer
OPL	Outer plexiform layer
WT	Wild-type
mGluR6	Metabotropic glutamate receptor 6
GCL	Ganglion cell layer

References

- Aartsen WM, Kantardzhieva A, Klooster J, van Rossum AG, van de Pavert SA, Versteeg I, Cardozo BN, Tonagel F, Beck SC, Tanimoto N, Seeliger MW, Wijnholds J. Mpp4 recruits Psd95 and Veli3 towards the photoreceptor synapse. *Hum Mol Genet* 2006;15:1291–1302. [PubMed: 16520334]
- Blanks JC, Johnson LV. Specific binding of peanut lectin to a class of retinal photoreceptor cells. A species comparison. *Invest Ophthalmol Vis Sci* 1984;25:546–557. [PubMed: 6715128]
- Brann MR, Cohen LV. Diurnal expression of transducin mRNA and translocation of transducin in rods of rat retina. *Science* 1987;235:585–587. [PubMed: 3101175]
- Bruhn SL, Cepko CL. Development of the pattern of photoreceptors in the chick retina. *J Neurosci* 1996;16:1430–1439. [PubMed: 8778294]

- Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE. Insights into G protein structure, function, and regulation. *Endocr Rev* 2003;24:765–781. [PubMed: 14671004]
- Caminos E, Velasco A, Jarrin M, Aijon J, Lara JM. Protein kinase C-like immunoreactive cells in embryo and adult chicken retinas. *Brain Res Dev Brain Res* 1999;118:227–230.
- Chen J, Wu M, Sezate SA, McGinnis JF. Light threshold-controlled cone alpha-transducin translocation. *Invest Ophthalmol Vis Sci* 2007;48:3350–3355. [PubMed: 17591908]
- Curtis PE, Baker JR, Curtis R, Johnston A. Impaired vision in chickens associated with retinal defects. *Vet Rec* 1987;120:113–114. [PubMed: 3604018]
- Dutt K, Cao Y. Engineering retina from human retinal progenitors (cell lines). *Tissue Eng Part A* 2009;15:1401–1413. [PubMed: 19113950]
- Edqvist PH, Myers SM, Hallbook F. Early identification of retinal subtypes in the developing, pre-laminated chick retina using the transcription factors Prox1, Lim1, Ap2alpha, Pax6, Isl1, Isl2, Lim3 and Chx10. *Eur J Histochem* 2006;50:147–154. [PubMed: 16864127]
- Elias RV, Sezate SS, Cao W, McGinnis JF. Temporal kinetics of the light/dark translocation and compartmentation of arrestin and alpha-transducin in mouse photoreceptor cells. *Mol Vis* 2004;10:672–681. [PubMed: 15467522]
- Elshatory Y, Everhart D, Deng M, Xie X, Barlow RB, Gan L. Islet-1 controls the differentiation of retinal bipolar and cholinergic amacrine cells. *J Neurosci* 2007;27:12707–12720. [PubMed: 18003851]
- Fischer AJ, Foster S, Scott MA, Sherwood P. Transient expression of LIM-domain transcription factors is coincident with delayed maturation of photoreceptors in the chicken retina. *J Comp Neurol* 2008a;506:584–603. [PubMed: 18072193]
- Fischer AJ, Hendrickson A, Reh TA. Immunocytochemical characterization of cysts in the peripheral retina and pars plana of the adult primate. *Invest Ophthalmol Vis Sci* 2001;42:3256–3263. [PubMed: 11726631]
- Fischer AJ, Ritchey ER, Scott MA, Wynne A. Bullwhip neurons in the retina regulate the size and shape of the eye. *Dev Biol* 2008b;317:196–212. [PubMed: 18358467]
- Fischer AJ, Scott MA, Tuten W. Mitogen-activated protein kinase-signaling stimulates Muller glia to proliferate in acutely damaged chicken retina. *Glia* 2009;57:166–181. [PubMed: 18709648]
- Fischer AJ, Seltner RL, Poon J, Stell WK. Immunocytochemical characterization of quisqualic acid- and N-methyl-D-aspartate-induced excitotoxicity in the retina of chicks. *J Comp Neurol* 1998;393:1–15. [PubMed: 9520096]
- Fischer AJ, Stanke JJ, Aloisio G, Hoy H, Stell WK. Heterogeneity of horizontal cells in the chicken retina. *J Comp Neurol* 2007;500:1154–1171. [PubMed: 17183536]
- Ghai K, Stanke JJ, Fischer AJ. Patterning of the circumferential marginal zone of progenitors in the chicken retina. *Brain Res* 2008;1192:76–89. [PubMed: 17320838]
- Greferath U, Grunert U, Wassle H. Rod bipolar cells in the mammalian retina show protein kinase C-like immunoreactivity. *J Comp Neurol* 1990;301:433–442. [PubMed: 2262600]
- Haider NB, Mollema N, Gaule M, Yuan Y, Sachs AJ, Nystuen AM, Naggert JK, Nishina PM. Nr2e3-directed transcriptional regulation of genes involved in photoreceptor development and cell-type specific phototransduction. *Exp Eye Res* 2009;89:365–372. [PubMed: 19379737]
- Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn* 1992;195:231–272. [PubMed: 1304821]
- Huang L, Max M, Margolske RF, Su H, Masland RH, Euler T. G protein subunit G gamma 13 is coexpressed with G alpha o, G beta 3, and G beta 4 in retinal ON bipolar cells. *J Comp Neurol* 2003;455:1–10. [PubMed: 12454992]
- Lee RH, Lieberman BS, Yamane HK, Bok D, Fung BK. A third form of the G protein beta subunit. 1. Immunochemical identification and localization to cone photoreceptors. *J Biol Chem* 1992;267:24776–24781. [PubMed: 1447215]
- Lobanova ES, Finkelstein S, Song H, Tsang SH, Chen CK, Sokolov M, Skiba NP, Arshavsky VY. Transducin translocation in rods is triggered by saturation of the GTPase-activating complex. *J Neurosci* 2007;27:1151–1160. [PubMed: 17267570]

- McCord R, Klein A, Osborne NN. The occurrence of protein kinase C theta and lambda isoforms in retina of different species. *Neurochem Res* 1996;21:259–266. [PubMed: 9182251]
- McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS. G-protein signaling: back to the future. *Cell Mol Life Sci* 2005;62:551–577. [PubMed: 15747061]
- McGinnis JF, Matsumoto B, Whelan JP, Cao W. Cytoskeleton participation in subcellular trafficking of signal transduction proteins in rod photoreceptor cells. *J Neurosci Res* 2002;67:290–297. [PubMed: 11813233]
- Montiani-Ferreira F, Fischer A, Cernuda-Cernuda R, Kiupel M, DeGrip WJ, Sherry D, Cho SS, Shaw GC, Evans MG, Hocking PM, Petersen-Jones SM. Detailed histopathologic characterization of the retinopathy, globe enlarged (rge) chick phenotype. *Mol Vis* 2005;11:11–27. [PubMed: 15660021]
- Montiani-Ferreira F, Li T, Kiupel M, Howland H, Hocking P, Curtis R, Petersen-Jones S. Clinical features of the retinopathy, globe enlarged (rge) chick phenotype. *Vision Res* 2003;43:2009–2018. [PubMed: 12842154]
- Montiani-Ferreira F, Shaw GC, Geller AM, Petersen-Jones SM. Electroretinographic features of the retinopathy, globe enlarged (rge) chick phenotype. *Mol Vis* 2007;13:553–565. [PubMed: 17438521]
- Negishi K, Kato S, Teranishi T. Dopamine cells and rod bipolar cells contain protein kinase C-like immunoreactivity in some vertebrate retinas. *Neurosci Lett* 1988;94:247–252. [PubMed: 3205402]
- Oldham WM, Hamm HE. Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 2008;9:60–71. [PubMed: 18043707]
- Peng YW, Robishaw JD, Levine MA, Yau KW. Retinal rods and cones have distinct G protein beta and gamma subunits. *Proc Natl Acad Sci U S A* 1992;89:10882–10886. [PubMed: 1438293]
- Prada C, Puga J, Perez-Mendez L, Lopez R, Ramirez G. Spatial and Temporal Patterns of Neurogenesis in the Chick Retina. *Eur J Neurosci* 1991;3:559–569. [PubMed: 12106488]
- Rosenzweig DH, Nair KS, Wei J, Wang Q, Garwin G, Saari JC, Chen CK, Smrcka AV, Swaroop A, Lem J, Hurley JB, Slepak VZ. Subunit dissociation and diffusion determine the subcellular localization of rod and cone transducins. *J Neurosci* 2007;27:5484–5494. [PubMed: 17507570]
- Roskopf D, Busch S, Manthey I, Siffert W. G protein beta 3 gene: structure, promoter, and additional polymorphisms. *Hypertension* 2000;36:33–41. [PubMed: 10904009]
- Sokolov M, Lyubarsky AL, Strissel KJ, Savchenko AB, Govardovskii VI, Pugh EN Jr. Arshavsky VY. Massive light-driven translocation of transducin between the two major compartments of rod cells: a novel mechanism of light adaptation. *Neuron* 2002;34:95–106. [PubMed: 11931744]
- Stanke JJ, Lehman B, Fischer AJ. Muscarinic signaling influences the patterning and phenotype of cholinergic amacrine cells in the developing chick retina. *BMC Dev Biol* 2008;8:13. [PubMed: 18254959]
- Tummala H, Ali M, Getty P, Hocking PM, Burt DW, Inglehearn CF, Lester DH. Mutation in the guanine nucleotide-binding protein beta-3 causes retinal degeneration and embryonic mortality in chickens. *Invest Ophthalmol Vis Sci* 2006;47:4714–4718. [PubMed: 17065478]
- Vaquero CF, Velasco A, de la Villa P. Protein kinase C localization in the synaptic terminal of rod bipolar cells. *Neuroreport* 1996;7:2176–2180. [PubMed: 8930984]
- Xie HQ, Adler R. Green cone opsin and rhodopsin regulation by CNTF and staurosporine in cultured chick photoreceptors. *Invest Ophthalmol Vis Sci* 2000;41:4317–4323. [PubMed: 11095633]
- Xu HP, Tian N. Glycine receptor-mediated synaptic transmission regulates the maturation of ganglion cell synaptic connectivity. *J Comp Neurol* 2008;509:53–71. [PubMed: 18425804]
- Yamagata K, Goto K, Kuo CH, Kondo H, Miki N. Visinin: a novel calcium binding protein expressed in retinal cone cells. *Neuron* 1990;4:469–476. [PubMed: 2317380]
- Young HM, Vaney DI. The retinæ of Prototherian mammals possess neuronal types that are characteristic of non-mammalian retinæ. *Vis Neurosci* 1990;5:61–66. [PubMed: 2271460]
- Zill P, Baghai TC, Zwanzger P, Schule C, Minov C, Riedel M, Neumeier K, Rupprecht R, Bondy B. Evidence for an association between a G-protein beta3-gene variant with depression and response to antidepressant treatment. *Neuroreport* 2000;11:1893–1897. [PubMed: 10884039]

Figure 1. Expression of GNB3 mRNA and protein in wild-type and RGE $-/-$ chickens
 mRNA for GNB3 was detected in P7 wild-type and RGE $-/-$ retinas by using RT-PCR. Each lane represents pools of retinal cDNA from different animals (**a**). Protein for GNB3 was detected in P7 wild-type and RGE $-/-$ retinas by using Western blot analysis (**b**) and immunofluorescence(**c**). Vertical sections of wild-type and RGE $-/-$ retinas were labeled with antibodies to GNB3 (**c** and **d**), Islet1, PKC or calbindin (**e**). Expression of bipolar cell markers (Islet1 and PKC) and the cone marker calbindin are identical at P7 in wild-type and RGE $-/-$ retinas (**e**). The calibration bar (50 μ m) in panel **d** applies to panels **c**, **d** and **e**. Abbreviations: RGE - retinopathy, globe enlargement, GAPDH - Glyceraldehyde 3-phosphate dehydrogenase, ONL - outer nuclear layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer.

Figure 2. GNB3 expression in the developing chicken retina

Vertical sections of retina were labeled with antibodies to GNB3 (green; **a-l**) and Islet1 (red; **b, e, h** and **k**) and Visinin (red; **c, f, i**, and **l**). Retinas were obtained from embryos at E13 (**a-c**), E15 (**d-f**), E17 (**g-i**) and E18.5 (**j-l**). The arrowheads indicate co-labeling for GNB3 and Islet1 in bipolar cells (**k**). Areas outlined in yellow magnified approximately 2.5 times in the lower right corner (**k** and **l**). The calibration bar (50 μ m) in panel **l** applies to all panels. Abbreviations: OPL - outer plexiform layer, IPL - inner plexiform layer.

Figure 3. GNB3 is expressed by photoreceptors and bipolar cells in the retinas of wildtype chicks
 Vertical sections of P7 chicken retinas were labeled with antibodies to GNB3 (green), PKC (red, **b** and **c**), Islet1 (red, **d-f**) and Lim3 (red, **h, i**). DRAQ5 (magenta) was applied as a nuclear counter-stain. Images were obtained using confocal microscopy. Panel **e'** is an approximate 3-fold magnification of the INL. Panel **f** is an orthogonal reconstruction of a Z-stack of optical sections through central retina labeled for Islet1 and GNB3. Arrows indicate GNB3-positive bipolar cells that are co-labeled for PKC or Islet1 (**a-f**). Arrowheads indicate horizontal cells that are labeled for Islet1 alone (**d, f**). Carets indicate bipolar cells that are GNB3-negative and intensely immunoreactive for Lim3 (**g-i**). Small double-arrows indicate cholinergic amacrine cells that are labeled for Islet1 (**d**). The calibration bar (50 μ m) in panel **e** applies to **a-c** and **e**, the bar in **i** applies to **g-i** and the bar in **d** applies to **d** alone. Abbreviations: PRL - photoreceptor layer, ONL - outer nuclear layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer.

Figure 4. GNB3 is expressed by all photoreceptors in retinas of wildtype chicken

Vertical sections of P7 chicken retinas were labeled with antibodies for GNB3 (green), calbindin (red, **a**, **c** & **d**), visinin (red, **f** and **g**) and rhodopsin (red, **i** and **j**). Images were obtained using confocal microscopy. Arrows indicate photoreceptors labeled for GNB3 and calbindin, visinin or rhodopsin. Arrowheads indicate photoreceptors labeled for GNB3 alone. The calibration bar (50 μ m) in panel **a** applies to **a**, the bar in **d** applies to **b-d** and the bar in **g** applies to **e-j**. Abbreviations: PRL - photoreceptor layer, ONL - outer nuclear layer, INL - inner nuclear layer, IPL - inner plexiform layer.

Figure 5. GNB3 is expressed by cone photoreceptors and bipolar cells in the goldfish retina
 Vertical sections of goldfish retinas were labeled with antibodies to GNB3 (green), rhodopsin (magenta, **b** and **c**), RetP1 (red, **e** and **f**), and PNA-lectin (red, **h** and **i**). Images were obtained using confocal microscopy. Arrows indicate photoreceptors labeled for GNB3 and RetP1 (**d-f**) or PNA-lectin (**g-i**). Arrowheads indicate bipolar cells labeled for GNB3 alone (**d-f**). The calibration bar (50 μ m) in panel **b** applies to **a-c**, the bar in **f** applies to **d-f** and the bar in **i** applies to **g-i**. Abbreviations: PRL - photoreceptor layer, ONL - outer nuclear layer, INL - inner nuclear layer, IPL - inner plexiform layer.

Figure 6. GNB3 is expressed by photoreceptors and bipolar cells in the *Xenopus* retina
 Vertical sections of *Xenopus* retinas were labeled with antibodies to GNB3 (green), RetP1 (red, **b** and **c**) and Islet1 (red, **e** and **f**). Small triple arrows indicate the outer segments of rod photoreceptors (**a-c**). Arrows in panels **a-c** indicate cone photoreceptors, and arrows in panels **d-f** indicate bipolar cells labeled for Islet 1 and GNB3. Arrowheads indicate bipolar cells labeled for GNB3 alone (**d-f**). The inset in panel **f** is enlarged approximately 3-fold. The calibration bar (50 μm) in panel **f** applies to **a-f**. Abbreviations: PRL - photoreceptor layer, ONL - outer nuclear layer, INL - inner nuclear layer, IPL - inner plexiform layer.

Figure 7. GNB3 is expressed by cone photoreceptors and bipolar cells in the mouse retina
 Vertical sections of mouse retinas were labeled with antibodies to GNB3 (green), red/ green opsin (red, **a**, **c** and **d**), PKC (red, **f** and **g**), Islet1 (red, **i** and **j**) and PSD-95 (red, **l** and **m**). Arrows indicate cone photoreceptors labeled for GNB3 and red/ green opsin (**b-d**) or bipolar cells labeled for GNB3 and Islet1 (**h-j**). Arrowheads indicate cholinergic amacrine cells labeled for Islet1 (**h-j**). Small double arrows indicate capillaries within the retina that are labeled with secondary alone (**h-m**). The calibration bar (50 µm) in panel (**g**) applies to (**b-g**), the bar in (**m**) applies to (**h-m**) and the bar in (**a**) applies to (**a**) alone. Abbreviations: PRL - photoreceptor layer, ONL - outer nuclear layer, INL - inner nuclear layer, IPL - inner plexiform layer.

Figure 8. GNB3 is expressed by cone photoreceptors and bipolar cells in the guinea pig retina
 GNB3 immunoreactivity is found in the OPL, IPL, bipolar cells and photoreceptors. Vertical sections of guinea pig retinas were labeled with antibodies for GNB3 (green), red/ green opsin (red, **b** and **c**), rhodopsin (red, **e** and **f**), Islet1 (red, **h-i**), PKC (red, **m-p**) and SV2 (red, **r** and **s**). Arrows in panels **a-c** indicate cones double positive for red/ green opsin and GNB3, whereas arrowheads point to GNB3 positive, red/ green opsin negative cones. Insets in panels **c-f** are enlarged approximately 3-fold. The areas boxed-out (magenta) in panels **i** and **m** are enlarged approximately 2-fold in panels **j-l** and **n-p**, respectively. Small double arrows indicate Islet1-positive, GNB3-negative ganglion cells (**g-i**). Arrows in panels **g-l** indicate bipolar cells that are labeled for Islet1 and GNB3, and the arrowheads indicate GNB3 positive bipolar cells. Hollow arrowheads (**q-s**) point to the presynaptic terminals of photoreceptors in the OPL. The calibration bar (50 μ m) in panel **f** applies to panels **a-i**, bar in **s** applies to panels **q-s**. Abbreviations: ONL - outer nuclear layer; INL - inner nuclear layer; IPL - inner plexiform layer, GCL - ganglion cell layer.

Figure 9. GNB3 is expressed by cone photoreceptors and bipolar cells in the dog retina
 Vertical sections of canine retinas were labeled with antibodies to GNB3 (green), human cone arrestin (hCAR; red, **b** and **c**), Islet1 (red, **e** and **f**) and PKC (red, **h** and **i**). Arrows indicate cone photoreceptors labeled for GNB3 and hCAR (**a-c**) or Islet1 (**d-f**), arrowheads indicate bipolar cells labeled for GNB3 and Islet1 (**d-f**) or PKC (**g-i**). The calibration bar (50 μ m) in panel **i** applies to panels **a-i**. Small double arrows (**d-f**) indicate Islet1 positive ganglion cells. Abbreviations: ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer.

Figure 10. GNB3 is expressed by cone photoreceptors and bipolar cells in the primate retina (*Macaca fascicularis*)

Vertical sections of retina were labeled with antibodies to PNA-Lectin (red; **a** and **d**), GNB3 (green; **c**, **d**, **f** and **g**) and Islet1 (blue; **b**, **d** and **e**). The large arrows indicate co-labeling for GNB3 and PNA-568 in cone outer segments. The arrow heads indicate co-labeling for GNB3 and Islet1 in bipolar cells. The calibration bar (50 μ m) in **d** applies to panels **a-d**. Panels **e-g** are enlarged approximately 3-fold. Abbreviations: ONL - outer nuclear layer; INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer.

Table 1

Table of Antibodies used in immunohistochemistry and Western Blotting

Antigen	Species	Concentration	Catalog or Clone No.	Source
Islet1	mouse	1:50	40.2D6	DSHB
Lim3	mouse	1:50	67.4E12	DSHB
Visinin	mouse	1:100	7G4	DSHB
Calbindin	mouse	1:400	300	Swant Immunochemicals
Red/Green Opsin	rabbit	1:400	AB5405	Chemicon
Rhodopsin	mouse	1:200	rho4D2	Dr. R. Molday
PSD-95	mouse	1:50	K28/43	NeuroMab
RetP1	mouse	1:1000	MAB5316	Chemicon
GNB3	rabbit	1:400	HPA005645	Sigma-Aldrich
GAPDH	mouse	1:2500	IMG-5019A-1	Imgenex

Table 2

GNB3 protein sequence homology across species referenced to *Homo Sapiens*: protein-protein BLAST. Identity refers to identically matched amino acids. Positives accounts for substitutions with similar amino acids.

Species	Identity	%	Positives	%
Zebrafish	284/340	83	315/340	92
Goldfish	N/A	N/A	N/A	N/A
Gallus Gallus	314/337	93	332/337	98
Xenopus	299/340	87	326/340	95
Mouse	330/340	97	335/340	98
Guinea Pig	283/340	83	315/340	92
Dog	329/340	96	335/340	98
Macaca	340/340	100	340/340	100