

Identification of GABA receptors in chick cornea

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Purpose: The cornea has an important role in vision, is highly innervated and many neurotransmitter receptors are present, e.g., muscarine, melatonin, and dopamine receptors. γ -aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the retina and central nervous system, but it is unknown whether GABA receptors are present in cornea. The aim of this study was to determine if GABA receptors are located in chick cornea.

Methods: Corneal tissues were collected from 25, 12-day-old chicks. Real time PCR, western blot, and immunohistochemistry were used to determine whether α_1 GABA_A, GABA_B, and ρ_1 GABA_C receptors were expressed and located in chick cornea.

Results: Corneal tissue was positive for α_1 GABA_A and ρ_1 GABA_C receptor mRNA (PCR) and protein (western blot) expression but was negative for GABA_B receptor mRNA and protein. α_1 GABA_A and ρ_1 GABA_C receptor protein labeling was observed in the corneal epithelium using immunohistochemistry.

Conclusions: These investigations clearly show that chick cornea possesses α_1 GABA_A, and ρ_1 GABA_C receptors, but not GABA_B receptors. The purpose of the α_1 GABA_A and ρ_1 GABA_C receptors in cornea is a fascinating unexplored question.

The cornea, as the eye's first refracting surface, plays an important function in vision, is highly innervated and has precise mechanisms for maintaining optical clarity. The chick cornea has been suggested to be an excellent model for the study of wound healing, scar tissue formation and neuronal re-innervation [1] and thus it is important to learn more about chick corneal anatomy and physiology. Many neurotransmitter receptors have been shown to be present in the cornea, e.g., muscarinic [2], melatonin [3], and dopamine receptors [4], but others appear to have not been studied to any significant degree (e.g., γ -aminobutyric acid [GABA] receptors). In the literature there is a sole report of GABA receptor expression in cultured human stem cells [5].

GABA is the major inhibitory neurotransmitter of the retina and central nervous system. It exerts its effects through three classes of membrane receptors, GABA_A, GABA_B, and GABA_C which contain multiple sub-units [6-14]. These include 16 subunits (α_1 -6, β_1 -3, γ_1 -3, δ , ϵ , θ , and π) combined as GABA_A, and 3 ρ subunits (ρ_1 -3), which form homomeric receptors composed of ρ subunits, and are commonly called GABA_C receptors. GABA_A and GABA_C are ionotropic receptors involving chloride channels that mediate fast synaptic inhibition when activated by GABA [13]. GABA_B receptors are members of

the metabotropic receptor family that via G proteins interact with neuronal inwardly rectifying potassium and voltage-gated calcium channels and when activated mediate slow synaptic inhibition [14].

Knowledge regarding the distribution of GABA receptors in the eye is very limited and information on GABA receptors in the cornea even more sparse. GABA_A [6-8], GABA_B [9-11], and GABA_C [12] receptors have been reported in the retina. We have recently shown that ρ_1 GABA_C receptors are present in chick sclera [15]. A small number of cultured human corneal stem cells have been shown to exhibit GABA_A receptor immunoreactivity [5]. We found no reports of studies of GABA receptor expression in any animal species, although there is a report of GABA modification of the rabbit corneal endothelial fluid pump [16] and an observation of activity of the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD), in rat cornea [17].

A range of other neurotransmitter receptors have been reported in corneal tissues of a diverse animal species [2-4, 18] and human tissue [19,20]. The muscarinic receptor subtypes M1 and M2 are expressed in bovine corneal epithelial cells [2]. In the rabbit cornea, D1 and D2 dopamine receptors have been localized to both epithelial and endothelial layers [4]. In the mouse cornea, retinoic acid (RA) receptors have been detected in the epithelium and stroma [18]. In the *Xenopus laevis* eye, melatonin receptors have been visualized in the corneal epithelium, fibroblasts, and endothelium [3]. In the human cornea, muscarinic M1-5 receptors have been observed on corneal epithelial cells [19],

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and M2, M4, and M5 are expressed in corneal endothelium [20].

Although GABA receptors are located primarily in retina and the central nervous system, reports have shown the presence of GABA receptors in non-neural tissues [21-36]. For example, GABA_A receptors have been shown to be located in murine gut [21], in cat carotid body [22], in rat taste bud [23], in human thyroid [24], human hepatocellular [25], human peripheral blood mononuclear cells [26], and human prostate [27]. GABA_B receptors have been reported in spider leg mechanosensilla [28], rat testis and sperm [29], the rat olfactory bulb [30], the rat taste bud [23], the rat gastrointestinal tract [31], and rat cardiomyocytes [32]. In human, GABA_B receptors are reported in airway epithelium [33] and fallopian tube [34], and GABA_C receptors have been reported in gut [35], testis and spermatozoa [36].

The fact that human corneal stem cells express GABA_A receptors [5], that there are reports of some GABA actions in the cornea of animal species [16,17], that GABA receptors are present in a range of non-neuronal tissues [21-36], and that the chick sclera possess GABA_C receptors [15], lead to the hypothesis that GABA receptors may be present in chick cornea. The aim of this study was to determine if GABA_A, GABA_B, and/or GABA_C receptors are located in the chick cornea. The secondary aim was to determine if expression occurred in the corneal endothelium, stroma or epithelium.

METHODS

This research comprised: (1) study of the expression of alpha₁ GABA_A, GABA_B, and rho₁ GABA_C receptors mRNA in chick cornea, and (2) identification and distribution of alpha₁ GABA_A, GABA_B, and rho₁ GABA_C receptors protein in chick cornea.

Animals and tissue preparation: Animals and tissue were prepared as previously described [15]. Twenty-five 12-day-old White Leghorn cockerels (*Gallus gallus*) were obtained from Jinan Spafury Poultry Farms (Jinan, China). Animals were administered a lethal dose of pentobarbital sodium (3%, 5ml/kg; Beijing Chemistry Com., Beijing, China) and the eyes enucleated. Eyes (n=5, 1 eye from 5 chicks) for immunohistochemistry were placed whole into microtubes, snap frozen in liquid nitrogen and stored at -80 °C. Eyes for real time PCR (n=5 different samples, 4 eyes of 2 chicks were used as 1 sample, 20 eyes of 10 chicks were used) and western Blot (n=5 different samples, 4 eyes of 2 chicks were used as 1 sample, 20 eyes of 10 chicks were used) were placed on a cold plate (8 °C) and the corneal tissue was removed along the limbus, and was separated from the sclera, conjunctiva, and iris using surgical scissors and forceps under a dissection scope. Corneal tissues from 4 eyes of 2 chicks formed one sample and were placed in separate microtubes, snap frozen in liquid nitrogen and stored at -80 °C until processing. Retina from these eyes were obtained and used as the positive control;

the retina has been previously shown to possess alpha₁ GABA_A [6-8], GABA_B [9-11], and rho₁ GABA_C [12] receptors.

Experiments were conducted with ethics approval in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, published by the National Health and Medical Research Council of Australia. All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China (document No 55, 2001).

Real-time PCR: Real-time PCR was performed as previously described [15,37] with minor modification according to the manufacturer's instructions. Total RNA was extracted from chick cornea and retina using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA concentration and purity were determined at an optical density ratio of 260:280 using a spectrophotometer. cDNAs (cDNAs) were synthesized with 1 µg of total RNA, 1 µl random primer, 1 µl dNTPs, 2 µl DTT, and 200 U MMLV reverse transcriptase, 5× RT buffer (4 µl) at 37 °C for 50 min, followed by 70 °C for 15 min, using a TaqMan Reverse-Transcription kit from Invitrogen.

Samples were analyzed in triplicate using gene-specific chicken primers together with SYBR Green (TaKaRa Biotechnology Co. Ltd., Dalian, China) using a Real-time PCR Detection System, LightCycler (Roche Applied Science, Indianapolis, IN). Based on the sequences reported in the GenBank database, primers were selected from chick sequences of alpha₁ GABA_A (NCBI Reference Sequence: [NM_204318.2](#)), GABA_B (NCBI Reference Sequence: [XM_419066.3](#)) and rho₁ GABA_C (NCBI Reference Sequence: [XM_426190.2](#)) receptors, using NCBI primer-BLAST, targeting at areas non-homologous to the other mRNA sequence, and ordered from Shanghai Biosune Biotechnology Company (Shanghai, China). The sequences of alpha₁ GABA_A were 5'-CTC CCT AAG GTG GCC TAC GCC-3' forward and 5'-AAT GGT TGC CAG CCC AGG GTC-3' reverse. The sequences of GABA_B were 5'-TCG GGA CCA ACC CAA CGT GC-3' forward and 5'-CGT GCT GGC CTG ATT GAC GCT-3' reverse. The sequences of rho₁ GABA_C were 5'-TCG GTG CTG GAA TAC GCG GC-3' forward and 5'-GGG CTG AGG AAG GCT GCA CG-3' reverse.

A typical reaction was performed in 20 µl, consisting of 1 µl of cDNA and 10 µl of 2× SYBR Green I PCR mix, containing the specific primer pairs (final 10 pmol each). Denaturation was performed for 10 s at 95.0 °C, primer annealing for 10 s at 60 °C, and extension was performed for 10 s at 72.0 °C. Correct product size was confirmed by DNA agarose gel, and lack of primer dimer formation was verified by melt curve analysis, and the real time PCR products were sent to Shanghai Biosune Biotechnology Company (Shanghai, China) for sequence analysis. For real time PCR, comparing to the cornea, the samples with cDNA from the

retina were used as the positive control, and the samples without cDNA were used as the negative control. Samples were analyzed in triplicate.

Western Blot: Western blot was performed as previously described [15]. For ρ_1 GABA_C, we used the antibody as previously described [15]. For α_1 GABA_A and GABA_B, we used the goat anti-human α_1 GABA_A and GABA_B receptor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The homology for the antibody sequence between chicken and human was more than 90% for the α_1 GABA_A (Homologene Blast comparison of human α_1 GABA_A, accession number: [NP_001121120.1](#), with *Gallus gallus* α_1 GABA_A receptor, accession Number, [P19150.1](#)), and 100% for the GABA_B (Homologene Blast comparison of human GABA_B, accession number: [NP_005449.5](#), with *Gallus gallus* GABA_B receptor, accession Number, [XP_419066.3](#)).

Total protein was extracted separately from each tissue sample by lysing the cornea and retina in ice-cold lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), including 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Samples were centrifuged at 15,000× g at 4 °C for 15 min. The protein concentration was detected using BAC kits (Beyotime Institute of Biotechnology). Aliquots of protein extracts were loaded in each lane of 7.5% sodium dodecyl sulfate-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes for electrophoresis, and blocked in Tris Buffered Saline with Tween (TBST; 5% fat-free dry milk, 0.1% Tween-20, 150 mM NaCl, and 50 mM Tris at pH 7.5) for 2 h. The membranes were exposed to goat anti-human α_1 GABA_A, GABA_B, and ρ_1 GABA_C polyclonal antibody (Santa Cruz Biotechnology) at a 1:100 dilution in blocking buffer and incubated overnight at 4 °C. This was followed by incubation with a rabbit anti-goat secondary horseradish peroxidase (HRP)-labeled antibody (Zhongshan Goldenbridge Biotechnology Co. Ltd. Beijing, China) at a dilution of 1:5,000 for 1 h at 37 °C. Protein bands were exposed to a negative film, developed, and fixed. The film was scanned and analyzed with Fluorchem™ 9900 Analyzer Software. β -actin (Zhongshan Goldenbridge Biotechnology Co. Ltd.) was used as a housekeeping protein to normalize the protein load. Samples were analyzed in triplicate.

Immunohistochemistry: Immunohistochemistry was used to investigate the expression and distribution of α_1 GABA_A, GABA_B, and ρ_1 GABA_C receptors in chick cornea at the protein level, and was performed as has been described previously [15,38]. Whole eyes were freeze-mounted onto sectioning blocks. Vertical sections (8 μ m thick) were cut from the anterior pole of the eye on a Leica RM2125 microtome (Leica Microsystems, Muenster, Germany) and thaw-mounted onto gelatin-coated glass slides. Sections were fixed for 10 min in acetone. Fixed sections were washed three

times with PBS, covered with 10% BSA (Sigma, St Louis, MO) diluted in PBS, and incubated for 20 min at 37 °C. The slides were incubated at 4 °C overnight with primary antibodies (goat anti-human α_1 GABA_A, GABA_B, and ρ_1 GABA_C receptors polyclonal antibody, Santa Cruz Biotechnology) at a 1:50 dilution in blocking buffer. Sections were incubated in PBS without primary antibodies as a negative control. The antibody-treated and negative control sample slides were washed with PBS and exposed to a rabbit anti-goat secondary horseradish peroxidase (HRP)-labeled antibody (Zhongshan Goldenbridge Biotechnology Co. Ltd.) at a dilution of 1:500 for 1 h at 37 °C. The slides were washed in PBS three times. A nickel solution of DAB (3,3'-diaminobenzidine tetrahydrochloride; Zhongshan Goldenbridge Biotechnology Co. Ltd.) was prepared and applied to the sections for less than 30 s. The DAB was then thoroughly rinsed from the sections using water. Sections were then stained with Hematoxylin and Eosin. The sections were dehydrated three times for 1 min in 100% ethanol, and then cleared in Histoclear (Huntz Biotechnology Co. Ltd. Shanghai, China) three times for 1 min. The sections were examined with a light microscope (40×) and the images were digitized using a camera. Samples were analyzed in triplicate.

RESULTS

Alpha₁ GABA_A, GABA_B, and rho₁ GABA_C receptor mRNA expression in chick cornea: We observed the mRNA expression of α_1 GABA_A and ρ_1 GABA_C receptors in all chick cornea and retina, but not in the negative control. We observed the mRNA expression of GABA_B receptors only in chick retina, but not in the cornea and not in the negative control. Ethidium bromide-stained agarose gels of real time PCR products were positive for mRNA expression of α_1 GABA_A and ρ_1 GABA_C receptors in chick cornea and retina, but not in the negative control run, and for mRNA expression of GABA_B receptors only in samples of chick retina, but not in the cornea and negative control run. Products corresponding to α_1 GABA_A, GABA_B, and ρ_1 GABA_C receptors were amplified and expression occurred at the nucleic acid size marker base pair consistent with that of the α_1 GABA_A (255 bp), GABA_B (217 bp), and ρ_1 GABA_C (105 bp) receptors (Figure 1). The sequence analysis of the real time PCR products revealed that the sequence of the products corresponded to the targeted sequence of the mRNA of the α_1 GABA_A, GABA_B, and ρ_1 GABA_C receptors with the primers.

Alpha₁ GABA_A, GABA_B, and rho₁ GABA_C receptor protein expression in chick cornea: Using antibodies for α_1 GABA_A receptors, one intense band (approximately 51 kDa) was detected in all chick corneal and retinal samples. Using antibodies for GABA_B receptors, one intense band (approximately 130 kDa) was detected in the chick retina samples but was not present in the corneal samples. Using antibodies for ρ_1 GABA_C receptors, one intense band

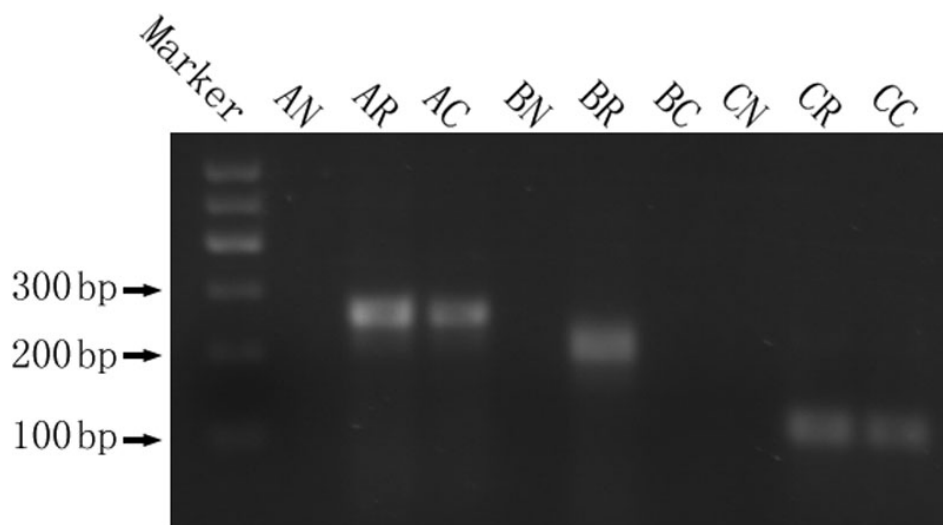


Figure 1. Sample ethidium bromide gel of real-time PCR products of α_1 GABA_A, GABA_B and ρ_1 GABA_C receptors in chick cornea and retina. Lane 1: molecular weight ladder (Marker), Lane 2: negative control for α_1 GABA_A (AN), Lane 3: chick retina for α_1 GABA_A (AR), Lane 4: chick cornea for α_1 GABA_A (AC), Lane 5: negative control for GABA_B (BN), Lane 6: chick retina for GABA_B (BR), Lane 7: chick cornea for GABA_B (BC), Lane 8: negative control for ρ_1 GABA_C (CN), Lane 9: chick retina for ρ_1 GABA_C (CR), Lane 10: chick cornea for ρ_1 GABA_C (CC).

(approximately 48 kDa) was detected in the chick corneal and retinal samples. Using antibodies for β -actin, one intense band (approximately 43 kDa) was detected in all the chick corneal and retinal samples (Figure 2).

Alpha₁ GABA_A, GABA_B, and rho₁ GABA_C receptor localization in chick cornea: Alpha₁ GABA_A, and ρ_1 GABA_C immunoreactivity was observed in chick corneal epithelium, was not observed in the stroma and not in the endothelium. No corneal layer displayed immunoreactivity to GABA_B antibodies. In the retina, immunoreactivity for each of the antibodies was observed in the inner plexiform layer, outer plexiform layer, inner nuclear layer and ganglion cell layer, corresponding with previous published data [6-11,15]. There was essentially no immunoreactivity observed in the negative controls tissues (Figure 3).

DISCUSSION

Our results add new information on the distribution of GABA receptors within ocular tissues; GABA_A receptors have been previously reported to be localized to a few cultured human corneal stem cells [5], here we clearly show that the chick cornea is positive for mRNA and protein specific for α_1 GABA_A and ρ_1 GABA_C receptors, but negative for GABA_B receptors. It would be of great interest to know if the corneas of other animal species also express GABA receptors and if so which sub-receptor types and in which corneal tissues. Staining density suggests that the α_1 GABA_A and ρ_1 GABA_C receptors occur throughout the entire chick corneal epithelial layer, and are located on epithelial cells. The finding suggests a possible, but untested, role of the α_1 GABA_A and ρ_1 GABA_C receptors in control of corneal functions.

Receptor types that are generally thought of as having primarily retinal localizations and function have been observed in corneal tissue. This includes muscarinic receptors

[2,19,20,37], dopamine receptors [4], melatonin receptors [3], and retinoic acid receptors [18], here we show that the α_1 GABA_A and ρ_1 GABA_C receptors, but not GABA_B receptors are located in chick corneal epithelium. The functions mediated through these different receptors are diverse, eg sensation and epithelial cell proliferation (muscarinic receptors [19,20]), ion transport (dopamine receptors [39]), diurnal variations in corneal hydration and thickness (melatonin [40]), and maintenance of the ocular surface (retinoic acid receptors [18]).

Muscarinic receptor subtypes have been observed on bovine corneal epithelial cells [2], and on human corneal epithelium and endothelium [19,20]. The muscarinic receptor agonist, carbachol (0.001–100 μ M) can increase the intracellular Ca²⁺ concentration in bovine corneal epithelial cells, and if the cells were preincubated with either 1 μ M atropine or 1 μ M pirenzepine [2] this was suppressed. When rats were fed a muscarinic agonist, corneal opacities with histopathological features including neovascularization, acanthosis, and stromal proliferation were observed in a dose-related fashion at 100 and 200 mg/kg/day [41]. Dopamine receptors, the D2 subtype, are located in rabbit cornea epithelial and endothelial layers [4]. Dopamine increase Cl⁻ secretion by the activation of specific dopamine receptors, and stimulate ion transport within the rabbit corneal epithelium [39]. Melatonin receptors (Mel_{1a} [40]) are expressed in chick corneal epithelium, stroma and endothelium. It has been suggested [40] that melatonin may modulate daily rhythms in corneal hydration/thickness via melatonin receptors on the corneal endothelium. Unlike dopamine, muscarinic and melatonin receptors that are localized in both epithelium and endothelium, here α_1 GABA_A and ρ_1 GABA_C are only located in epithelium.

One of the important functions of the cornea is maintaining its optical transparency which is crucial for high

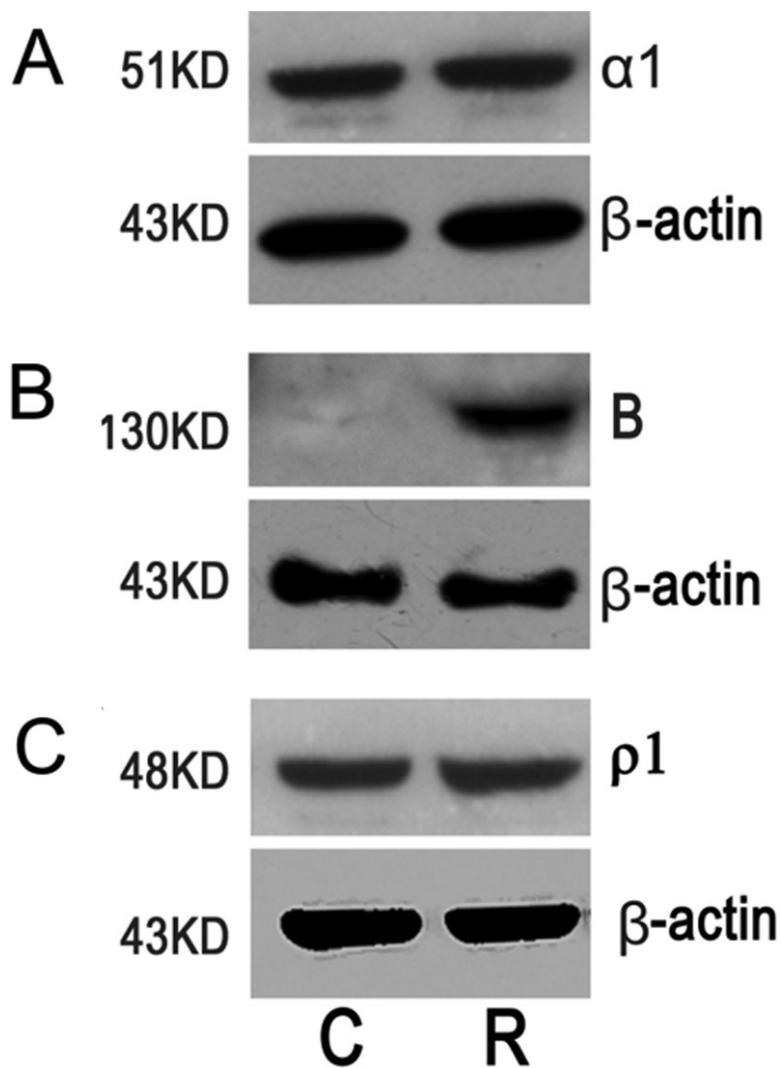


Figure 2. Western blot analysis of $\alpha 1$ GABA_A, GABA_B and $\rho 1$ GABA_C receptors in the chick cornea and retina. **A:** This panel shows one intense band (approximately 43 kDa) for β -actin and one intense band (approximately 51 kDa) for $\alpha 1$ GABA_A receptors ($\alpha 1$) both in cornea (C) and in retina (R). **B:** This panel shows one intense band (approximately 43 kDa) for β -actin both in cornea (C) and in retina (R), and one intense band (approximately 130 kDa) for GABA_B receptors (B) in retina (R) but not in cornea (C). **C:** This panel shows one intense band (approximately 43 kDa) for β -actin, and one intense band (approximately 48 kDa) for $\rho 1$ GABA_C receptors ($\rho 1$) both in cornea (C) and in retina (R).

quality visual performance. Corneal transparency is dependent on regulation of the hydration of the cornea, and the Cl⁻ ion channel is involved in fluid transportation within the corneal epithelium and endothelium [42-44]. The presence of Cl⁻ ion channels has been reported in human and rabbit corneal epithelium [45,46], in rabbit endothelium [45], and in rabbit corneal keratocytes [47,48]. GABA and its analogs have been shown to activate the rabbit corneal endothelial fluid pump, and this stimulation was abolished by the GABA_A antagonists, bicuculline and chlorpromazine [16]. In this study we found $\alpha 1$ GABA_A and $\rho 1$ GABA_C receptors

in chick corneal epithelium, but not the endothelium, and thus it seems unlikely that GABA is involved in regulating the fluid pumps, at least within the chick corneal endothelium. Whether $\alpha 1$ GABA_A and $\rho 1$ GABA_C receptors regulate Cl⁻ conductance, and then influence transparency within the chick cornea requires further investigation. Determination of the effect of GABA agents on corneal hydration would resolve this.

To maintain corneal composition, organization and clarity the cornea has the greatest density of peripheral sensory nerves of any ocular tissue. Nerve terminals in the cornea are

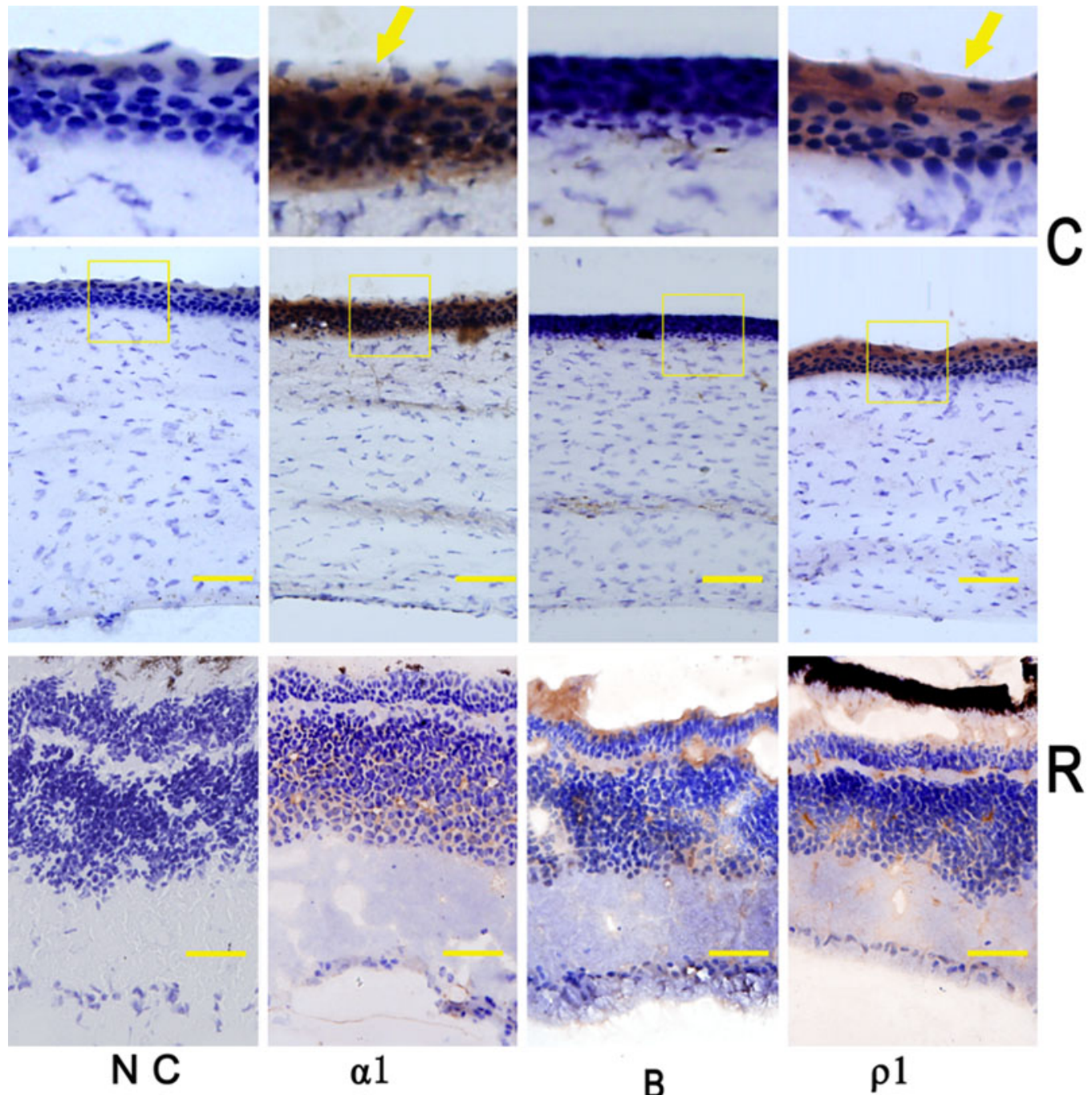


Figure 3. Alpha₁ GABA_A (α_1), GABA_B (B) and rho₁ GABA_C (ρ_1) receptor protein distribution in chick cornea (C) and retina (R). With antibodies for alpha₁ GABA_A, GABA_B and rho₁ GABA_C receptors, immunoreactivity was observed in corneal epithelium cell for alpha₁ GABA_A (α_1) and rho₁ GABA_C (ρ_1 ; yellow arrow), but not for GABA_B receptor (B) in the cornea (C). In the retina (R), immunoreactivity was found in the inner plexiform layer, outer plexiform layer, inner nuclear layer, and ganglion cell layer for alpha₁ GABA_A (α_1), GABA_B (B) and rho₁ GABA_C (ρ_1) receptors, corresponding to previous reports [6-11,15]. There was essentially no immunoreactivity observed for the negative controls (N.C). The scale bar is 10 μ m. Photographs were taken at 40 \times magnification.

almost exclusively nociceptive A δ and C fibers originating from the ophthalmic branch of the trigeminal ganglion [49], and structural and functional specialization of A δ and C fiber free nerve endings innervate the corneal epithelium [50] across the entire corneal surface [1]. In this study we found

alpha₁ GABA_A and rho₁ GABA_C receptor within the chick corneal epithelium. A potential role for these GABA receptors might involve regulating corneal sensitivity or other neural functions and this requires further investigation.

If the GABA receptors are to have a functional role within the cornea then there must be a supply of GABA either within the cornea, aqueous humor or tears to interact with the receptors. GABA-synthesizing enzyme was observed in the rat cornea [17], which implies that there is endogenous synthesis of GABA in rat cornea. It may be possible that cholinergic neurons within the cornea release GABA, like they do within retinal tissue [51]. We could not locate any articles that mention the presence of GABA within the eye's aqueous humor or tears. All of these potential sources of GABA are yet to be tested in the chick eye.

In conclusion, we found that both α_1 GABA_A and ρ_1 GABA_C receptor were located within the chick corneal epithelium; the GABA_B receptor was not present. The presence of GABA receptors within chick corneal epithelium suggests that some epithelial cell functions can be modified by GABA. Research is underway to determine how activation of these receptors alters corneal functions and where GABA or other substances that modifies them are expressed.

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