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Identification of GABA receptors in chick retinal pigment epithelium

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HIGHLIGHTS

- GABA receptor mRNA is expressed in isolated chick RPE.
- GABA receptor protein is expressed in isolated chick RPE.
- GABA receptor protein is localized to the cell membrane and plasma of chick RPE.

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ABSTRACT

Purpose: The retinal pigment epithelium (RPE) is a multifunctional, monolayer of cells located between the neural retina and the choroicapillaris. γ-Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the retina and GABA receptors are known to be present in chick retina, sclera and cornea. There is a report of genes involved in GABA receptor signaling being expressed in human RPE, however, whether GABA receptors are present in chick RPE is unknown.
Methods: Real time PCR and western blot were used to determine the expression of GABA receptors (alpha$_1$ GABA$_A$, GABA$_B$R$_2$, and rho$_1$ GABA$_C$ receptors) in isolated chicken RPE. Immunofluorescence using antibodies against one of the GABA receptor sub-types was used to determine receptor localization.
Results: Both real-time PCR and western blot demonstrated that alpha$_1$ GABA$_A$, GABA$_B$R$_2$ and rho$_1$ GABA$_C$ receptors were expressed in isolated chick RPE. Immunofluorescence further demonstrated that GABA receptors were localized to the cell membrane and plasma of RPE cells.
Conclusions: Alpha$_1$ GABA$_A$, GABA$_B$R$_2$ and rho$_1$ GABA$_C$ receptors were expressed in chick RPE. The purpose of the GABA receptors within the RPE remains to be explored.

1. Introduction

The retinal pigment epithelium (RPE) is a single layer of predominantly hexagonal, pigmented cells located between the neural retina and the choroicapillaris [3]. The RPE has many important functions including transporting ions, metabolites and fluid across the subretinal space [25], forming the outer blood-retinal barrier [32], phagocytoses of shed photoreceptor outer segment discs and metabolism and storage of vitamin A and other molecules with important roles in vision [39,40]. The chick RPE has been suggested to be an excellent model for the study of wound healing of the RPE [14], photoreceptor regeneration [20], retinal degenerations [19], and eye growth regulation [36,47].

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the retina (including for both neurons and glia) and the central nervous system. It exerts effects through three classes of membrane receptors, GABA$_A$, GABA$_B$ and GABA$_C$ [5]; GABA$_C$ receptors have been re-classified as GABA$_{A0r}$, a subset of GABA$_A$ rather than an individual GABA receptor subtype [30]. GABA$_A$ receptors encompass 16 subunits (alpha1-6, beta1-3, gamma1-3, delta, epsilon, theta and pi) [4], GABA$_C$ receptors consist of 3 rho subunits (rho1-3) [2], and the GABA$_B$ receptor is a dimer comprising GABA$_B$R$_1$ and GABA$_B$R$_2$ components [48]. GABA$_A$ and GABA$_C$ receptors are ligand-gated chloride channels that mediate fast synaptic inhibition when activated by GABA [6], whereas GABA$_B$ receptors

Abbreviations: GABA, γ-amino-butyric acid; RPE, retinal pigment epithelium; PCR, polymerase chain reaction.

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regulate potassium and calcium channels through G-protein and intracellular second-messenger pathways, when activated they mediate slow synaptic inhibition [26].

Reports of GABA receptor localization within the eye predominantly involve the retina [24,46], and, as for many other vertebrates, the chick retina contains many GABAergic neurons [27]. There are also some reports of GABA receptor expression in non-retinal ocular tissues [7,8,38]. $GABA_A$ receptors have been reported in a small number of cultured human corneal stem cells [38]. We have recently shown that $alpha_1$ $GABA_A$ and rho_1 $GABA_C$ receptors, but not $GABA_B$ receptors are expressed in chick cornea and located in corneal epithelium [8], and that rho_1 $GABA_C$ receptors are present in chick sclera [7]. However, we could not find any reports of studies of GABA receptor expression in RPE in any animal species. Although there is a report GABA transporters in the bullfrog RPE [31], with the RPE thought to play a role in clearing excess GABA from the subretinal space [31]. The RPE has many "glial-like" functions [25,39,40], and GABA receptor expression shown in both peripheral and central glial cells [42,44]. There is also a report of RPE-specific gene expression for GABA receptor signaling in human RPE [1]. We thus proposed that GABA receptors may be present in chick RPE.

In addition, a range of other neurotransmitter receptors have been reported in the RPE of a diverse range of animal species and also human tissue. Muscarinic receptors are expressed in rat RPE [37] and in cultured human RPE cells [13]. Dopamine D1 receptors have been localized to cultured bovine RPE [43] and dopamine D2 receptors observed in chick RPE [33]. Alpha-1 adrenergic receptors have been detected in bovine RPE [16] and beta adrenergic receptors identified on cultured human RPE cells [12]. Glutamate and 5-HT1A receptors have been visualized on cultured human RPE cells [23,29] and serotonin-2A receptors on cultured rat RPE [28].

The expression or location of GABA receptors in chick RPE remains unexplored. Based on reports that GABA receptors are located in other ocular tissues of the chick [7,8,24], that there is GABA receptor signaling in human RPE [1], that the RPE may have a role in clearing GABA from the subretinal space [31], and other neurotransmitter receptors are expressed in RPE, we predicted that chick RPE would contain GABA receptors. The aims of this study were to determine if GABA receptors are expressed in chick RPE and if so the localization.

2. Materials and methods

2.1. Animals and tissue preparation

Chick RPE were isolated and collected as previously described [7,8,12]. Twenty-five 12-day-old White Leghorn cockerels (Gallus gallus) were used. Animals were administered a lethal dose of pentobarbital sodium and the eyes were enucleated. All experimental procedures were in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

Right eyes of 5 chicks were placed in sterilized ice-cold PBS, hemisected behind the ossicles, and the anterior segment, vitreous, and retina were removed under a dissection scope. The RPE–choroid complex was separated from the sclera and put in sterilized ice-cold PBS with the RPE facing up. Using forceps and a scalpel, in sterilized ice-cold PBS, the RPE was mechanically split from Bruch's membrane. Using microscopy it was verified that the RPE sheets comprised a single layer of RPE cells with no cellular contamination. The RPE sheets were collected, washed 3 times in sterilized ice-cold PBS, then put in a 1.5 ml microtube with 1 ml Trizol Reagent (Invitrogen, Carlsbad, CA, USA.) for PCR ($n = 5$).

The RPE from both eyes of 2 chicks were collected in a 1.5 ml microtube with 200 μl ice-cold lysis buffer for western blot and were considered as 1 sample ($n = 5$, 10 chicks were used). RPE from both eyes of 2 chicks were collected for immunochemistry and considered 1 sample ($n = 5$, 10 chicks were used). The retinal samples were used as the positive control as previously described [7,8].

2.2. Real-time PCR

RNA isolation and reverse transcription were performed as previously described [7,8]. RNA concentration and purity were determined at an optical density ratio of 260:280 using a spectrophotometer. The primer sequences of $alpha_1$ $GABA_A$ were CTCCCTAAGGTGGCCTACGCC forward and AATGGTTGCCAGCCCAGGGTC reverse. The sequences of $GABA_BR_2$ were TCGGGACCAACCCAACGTGC forward and CGTGCTGGCCTGATTGACGCT reverse. The sequences of rho_1 $GABA_C$ were TCGGTGCTGGAATACGCGGC forward and GGGCTGAGGAAGGCTGCACG reverse. SYBR Green real-time PCR was performed by use of a Real-time PCR Detection System, LightCycler (Roche Applied Science, IN, USA). Denaturation was performed for 10 s at 95.0 °C, annealing for 10 s at 60 °C, and extension for 10 s at 72 °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 for sequence analysis.

2.3. Western blot

Western blot was performed as previously described [7,8]. Total protein was extracted separately from each RPE sample. The protein concentration was detected using BAC kits. Proteins were separated by 7.5% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes. After 60 min milk block, the membranes were exposed to either goat anti-human $alpha_1$ $GABA_A$, $GABA_BR_2$, or rho_1 $GABA_C$ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution in blocking buffer and incubated overnight at 4 °C, then horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000). Protein bands were exposed to a negative film, developed, and fixed. The bands were scanned and analyzed with Fluorchem™ 9900 Analyzer Software. GAPDH (Zhongshan Goldenbridge Biotechnology Co. Ltd. Beijing, China) was used as a housekeeping protein to normalize the protein load. Samples were analyzed in triplicate.

2.4. Immunofluorescence

Immunofluorescence was performed as previously reported [45]; standard staining methods [7,8] could not be used due to the pigmented (dark colored) nature of the RPE tissue. Briefly, the isolated RPE were fixed with 4% paraformaldehyde, blocked with 10% normal donkey serum and were incubated with either goat anti-human $alpha_1$ $GABA_A$, $GABA_BR_2$, or rho_1 $GABA_C$ receptor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100) overnight at 4 °C. Other sections were incubated in PBS without primary antibody as a negative control. Then subsequent secondary antibodies conjugated with Alexa Fluo 488 (1:2000, Invitrogen, CA, USA) were added. A drop of Prolong Gold anti-fade reagent with DAPI (Invitrogen, CA, USA) was applied before cell images were acquired using a LSM 710 laser confocal microscope (Zeiss, Germany).

3. Results

3.1. $Alpha_1$ $GABA_A$, $GABA_BR_2$, and rho_1 $GABA_C$ receptor mRNA expression in chick RPE

The mRNA of $alpha_1$ $GABA_A$, $GABA_BR_2$ and rho_1 $GABA_C$ receptors were detected in isolated chick RPE and in retina (positive control),