

EXTENDED REPORT

Human ganglion cells express the alpha-2 adrenergic receptor: relevance to neuroprotection

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Background/aim: Alpha-2 α adrenergic receptor (α_2 -AR) agonists are thought to be neuroprotective, preventing retinal ganglion cell death independent of pressure reduction. Previous studies have identified α_2 -ARs in rat retina. The authors aimed to demonstrate the presence and localisation of α_2 -ARs in human and rat retina and on the rat retinal ganglion cell line, RGC-5.

Methods: Seven postmortem human and three postmortem rat eyes were paraformaldehyde fixed and frozen. RGC-5 cells were also paraformaldehyde fixed. The expression of α_2 -ARs was determined by antibody immunofluorescence.

Results: α_2 -AR expression was identified in the human retina, on ganglion cells, and cells in the inner and outer nuclear layers (INL, ONL). Differential α_2 -AR staining patterns in the INL and ONL suggest a further restriction to as yet unidentified neuronal subclasses. The RGC-5 cell line also expressed α_2 -ARs in undifferentiated cells and an increased expression upon fully differentiated cells.

Conclusion: α_2 -AR agonists in addition to their pressure lowering effects in the eye, may act directly upon retinal neurons, including retinal ganglion cells. The presence of α_2 -ARs on the RGC-5 cell line allows future investigation of these possible direct effects using in vitro glaucoma model systems.

In vivo studies have shown that in addition to lowering intraocular pressure, alpha-2 α adrenergic agonists, such as brimonidine, decrease retinal ganglion cell death subsequent to increases in intraocular pressure,^{1–4} retinal ischaemia,^{5–8} or optic nerve crush.⁹ Alpha-2 agonists, unlike other proposed neuroprotective agents—for example, β adrenergic antagonists, are thought to work directly via interaction with their receptor. This has been shown in studies where the protective effect, enabled in the presence of an α_2 agonist, is reversed or diminished in the presence of an α_2 selective antagonist, such as yohimbine^{6 10 11} or rauwolscine.^{5 6}

Studies evaluating brimonidine or other α_2 adrenergic receptor (α_2 -AR) agonist effectiveness as neuroprotective agents have used the drug applied either topically^{6–8 12 13} or systemically.^{1 3 5 7–9 14} These studies do not allow discrimination between whether the drug is acting directly to be neuroprotective, or indirectly by affecting surrounding mediators. These surrounding mediators include the vasculature systemically or locally, inflammatory mediators or other non-retinal ocular tissue such as in the anterior segment. An agent could be directly protective in glaucoma if it interferes with or blocks retinal ganglion cell apoptosis, the terminal outcome in glaucoma, and cause of irreversible loss of corresponding visual fields. A target for a neuroprotective agent in glaucoma would be the retinal ganglion cell, being both the output neuron for the retina and the cell that dies via apoptosis in glaucoma.^{15–19} Such a drug may thus have a direct effect on the cells involved in glaucoma pathogenesis.

There are at least three distinct α_2 -AR subtypes.²⁰ The α_{2A} -AR is the predominant subtype in the central nervous system and the receptor subtype for which brimonidine has highest affinity.²¹ Despite the knowledge that α_2 adrenergic agents exert their neuroprotective effect by receptor binding, little is known about the α_2 adrenergic binding sites within the retina. Table 1 reviews the current published data on localisation of α_2 -ARs to the eye, including knowledge of localisation to the retina. Previous evidence of the intraretinal localisation of α_2 -ARs comes from radioligand binding or

autoradiography studies, which have localised α_2 -AR to the mammalian (in particular to the human) retina without intraretinal receptor subtype localisation. Recently, Wheeler *et al*³ demonstrated that α_2 -ARs were present on rat ganglion cells as well as cells in the inner nuclear layer. Owing to the known difference in tissue expression of α_2 -ARs among different animal species,^{22–24} and the fact that the intraretinal α_2 -AR expression has not yet been shown in humans, we aimed to define the intraretinal localisation of α_2 -AR in the human retina. We also aimed to confirm previous rat intraretinal α_2 -AR localisation.³

In addition to human and rat intraretinal localisation, we looked for the presence of specific ganglion cell localisation using a novel retinal ganglion cell line, RGC-5. The RGC-5 cell line is a virally transformed rat retinal ganglion cell line and is currently the only retinal ganglion cell line.²⁵ It expresses Thy1-1 and Brn-3c, ganglion cell markers and also various neurotrophin receptors. The RGC-5 line morphologically differentiates with serum deprivation and succinyl concavalin A treatment, and also undergoes excitotoxic apoptosis with glutamate.²⁵

MATERIALS AND METHODS

Preparation of retinas for immunohistochemistry

Seven human postmortem donor eyes (ethics approval HREC 03225) from five different donors were obtained from the Lions Eye bank (Sydney, Australia). Patient age ranged from 26 to 84 years with no significant known ocular conditions besides cataract extractions. Human eyes were pre-fixed in 2% paraformaldehyde overnight. Retinal trephines were equilibrated in 30% sucrose, blocked, mounted, frozen, and sectioned onto sialinated coated slides.

A total of three freshly enucleated adult rat eyes (ethics approval ACEC 1997/101) were used for this study and were processed as per human retinas.

Abbreviations: α_2 -AR, alpha-2 adrenergic receptor; CRALBP, cellular retinaldehyde binding protein; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PBS, phosphate buffered saline

Table 1 Previous studies localising α_2 adrenergic receptors to the eye

Species	Tissue	Technique	α receptor subclass	Alpha adrenergic positive tissues	Reference
Human cadaveric	Whole eye cross sections	In vitro ligand binding and autoradiography	α_2	+++ iris and ciliary epithelium	26
Rats and rabbits	Whole eye cross sections	Autoradiography	α_2	++ciliary muscle, retina, RPE (and/or choroid) Ciliary processes	27, 28
Human	Cornea	Immunofluorescence	$\alpha_2A, \alpha_2B, \alpha_2C$	Ocular muscles Retina and RPE α_2A, α_2B and α_2C : cornea α_2B and α_2C : ciliary body α_2A : positive in rabbit ciliary body by immunofluorescence	29
Rabbit	Ciliary body	Immunofluorescence/ polymerase chain reaction/ dot blot hybridisation	$\alpha_2A, \alpha_2B, \alpha_2C$		
Human and bovine	Retinal homogenates	Radioligand binding	α -adrenergic	RNA encoding: $\alpha_2A, \alpha_2B, \alpha_2C$ all present in rabbit iris-ciliary body Retina α_2A	30–33 34
Human	Trabecular meshwork cells	Immunofluorescence	$\alpha_2A, \alpha_2B, \alpha_2C$		
Human	Ciliary body RPE-CC Iris Retina	Radioligand binding	$\alpha_2A, \alpha_2B, \alpha_2C$	α_2A : +++ iris ++ RPE-CC and ciliary body + retina	35
Bovine	Retina	Radioligand binding	$\alpha_2A, \alpha_2B, \alpha_2C, \alpha_2D$	α_2D +++ α_2A +	23, 36
Porcine	Iris Ciliary body Choroid Retina	Radioligand binding	$\alpha_2A, \alpha_2B, \alpha_2C$	α_2A in iris, ciliary body, and choroid α_2A and α_2C in retina	24
Rabbit	Iris-ciliary body membranes	Radioligand binding	α_1 and α_2 adrenergic	α_2 subtype in iris-ciliary body membrane	37
Rabbit	Ciliary body	Radioligand binding	α_1 and α_2 adrenergic	α_2 subtype in ciliary body	38
Rat	Retina	Radioligand binding	α_1 and α_2 adrenergic	α_1 subtype in OPL α_2 subtype ++ IPL, + INL and GCL	39
Rat	Retina	Immunohistochemistry	α_2A	α_2A in the GCL and INL	3

Levels are recorded as + with low or detectable levels measured, ++ with intermediate or moderate levels measured, and +++ correlating with high intensity or high levels measured. RPE, retinal pigmented epithelium, CC, choriocapillaris, GCL, ganglion cell layer, INL, inner nuclear layer, IPL, inner plexiform layer, OPL, outer plexiform layer.

Cell culture and preparation of RGC-5 cells

The transformed rat retinal ganglion cell line, RGC-5, (gift from N Agarwal) was grown as previously described⁴⁰ on poly-L-lysine coated glass coverslips. Adherent cells were differentiated as previously described for 3, 5, and 7 days.²⁵ Coverslips were fixed in 4% paraformaldehyde.

Antibodies

A chicken polyclonal antibody generated to recognise the α_2A -AR⁴¹ was generously supplied by J Regan. Primary antibody and control normal chicken IgY (R&D Systems, AB-101-C) were used at a dilution of 1:150. Secondary antibody goat anti-chicken IgG conjugated to alexa 546 (molecular probes, A-11040) was used at a dilution of 1:200. Cell nuclei were identified counterstaining with Hoechst 33258 (molecular probes, H1398).

Immunohistochemistry

Immunohistochemistry was performed using the indirect antibody method. Briefly, specimens were blocked with 5% normal goat serum, incubated overnight with primary antibody diluted into 0.4% saponin for permeabilisation. Following extensive washes with phosphate buffered saline (PBS), the immunoreactivities were revealed by secondary antibody goat anti-chicken conjugated to fluorochrome alexa 546.

Controls included omission of the primary antibody, normal chicken IgY substituted in place of the primary antibody, and staining of human cornea, a known positive control for this α_2AR antibody.²⁹ Preliminary co-localisation studies were performed by dual incubation with the α_2A -AR

antibody along with vimentin (Dako, M0725), cellular retinaldehyde binding protein (CRALBP) (gift from M Maddigan) and appropriate FITC secondary antibodies (Zymed 04–6111 and molecular probes A12373) (data not shown).

Analysis

Fluorescence was visualised and photographed using a confocal laser microscope (Leica TCS SP) equipped with Leica confocal software (version 2.5) attached to a two photon imaging system (Leica TCS MP). Images were taken with 20 \times (HC PL fluotar 20 \times 0.5 dry, Leica) and 63 \times water objectives (HC PL apo 63 \times 1.2Wcorr, Leica). Images collected with the 543 nm excitation laser were pseudocoloured red while those taken with the 790 nm excitation laser were pseudocoloured blue.

A total of 82 images of human retina stained with α_2A -AR antibody, 165 human retina negative controls (115 secondary only and 50 IgY negative controls), 73 human cornea, 25 of rat retina stained with α_2A -AR antibody, 34 rat negative controls (29 secondary only and four IgY negative controls). Total number of images stained with α_2A -AR antibody for the RGC-5 cells were 27 undifferentiated, 9 day three, 6 day five, and 28 day seven differentiated RGC-5 cells. Thirty six negative control images were taken (27 secondary only and 9 IgY negative controls). Intensity of the human tissue staining was graded with – representing no staining; + representing low or weak staining; ++ representing moderate or intermediate staining; and +++ representing high or strong levels of staining.

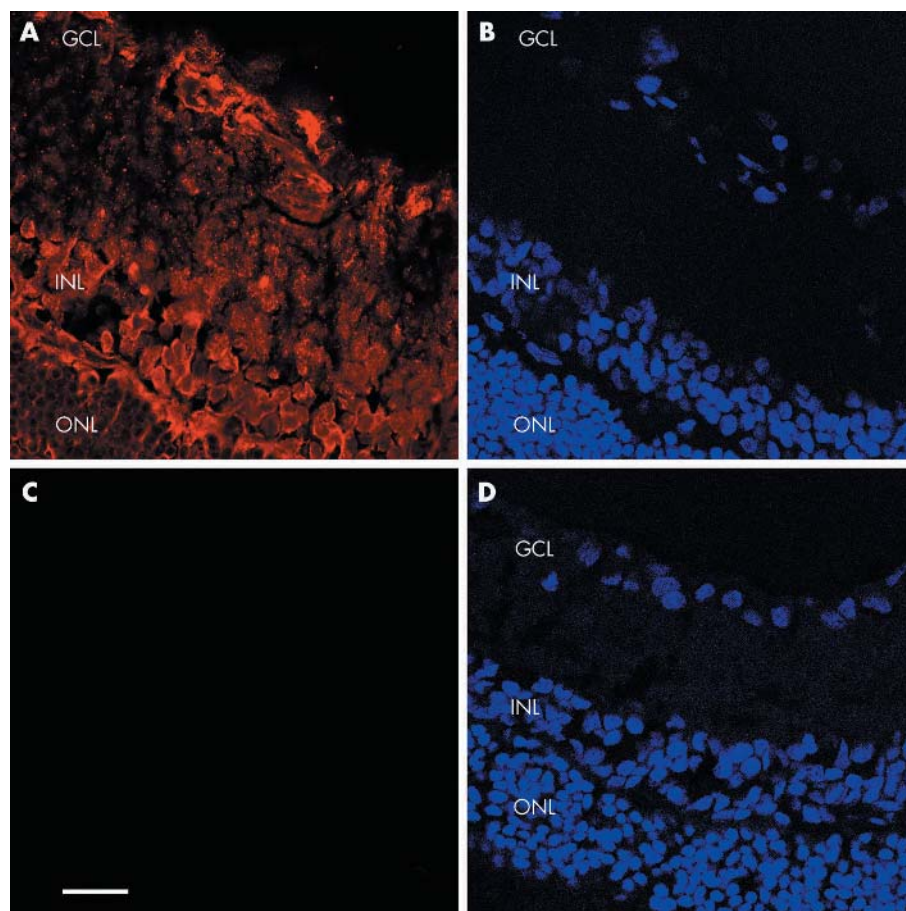


Figure 1 α_{2A} -AR expression in the rat retina. Confocal images of rat retina labelled with α_{2A} -AR antibody throughout retinal layers. (A) Shows positive immunostaining for the α_{2A} -AR on retinal ganglion cells, cells in the inner nuclear layer (INL) and less so, but also on the membrane of outer nuclear layer (ONL) cells. (C) A retina control with secondary antibody alone showing no non-specific staining. (B) and (D) are equivalent to (A) and (C) retinal sections, respectively, showing Hoechst nuclear counterstaining. All images are taken with the $\times 63$ objective, (C) shows 25 μm scale bar. GCL, ganglion cell layer.

RESULTS

Strong α_{2A} -AR staining was shown in human cornea, a known positive control (fig 2A).²⁹ In the rat retina, we confirmed the presence of α_2 -ARs on the somas of the ganglion cells and many of the cells in the inner nuclear layer. The immunopositive labelled cells all displayed uniform staining (fig 1A). In the human retina, α_2 -ARs were localised to the soma of ganglion cells, a subpopulation in the inner nuclear layer (INL) and a smaller subpopulation within the outer nuclear layer (ONL) (figs 2E and G). The intensity of receptor staining varied between layers in the same eye and between similar layers in different eyes, especially in both the INL and ONL as demonstrated by table 2 and figure 2. Within layers, there was variability in the staining levels seen between different eyes as demonstrated by figures 2E and G, images taken of different eyes. A sample quantitative count was carried out on two eyes using five images, with Hoechst counts defining total cell numbers. The percentage of positive cells staining in the ganglion cell layer was >80%, INL 40% and ONL only 20% positive. Of the positive staining cells in the ONL nearly all were weak + compared to the INL, which was mainly +++ (43%) and ++ (49%).

In the RGC-5 cell line, the α_{2A} -AR staining was seen on both undifferentiated (fig 3A) and differentiated cells (fig 3B, C, and D); however, staining was more intense on the day 7 differentiated RGC-5 cells (see fig 3D). Differentiated cells (fig 3D) cease to proliferate and display neurites unlike undifferentiated cells (fig 3A).

Preliminary tissue co-localisation studies did not reveal the positive alpha immunostaining to coincide with the staining

for CRALBP (a Muller cell marker)⁴²⁻⁴⁴ or vimentin,⁴² glial cell markers (results not shown).

The antibody was checked for any non-specific binding by incubation of retinas and RGC-5 cells with secondary antibody alone, to test for any non-specific binding of the secondary and with host normal chicken IgY to test for any non-specific binding of our chicken primary antibody. Both negative controls were negative (see figs 1C, 2C, and 3E), indicating no non-specific binding of our antibody for the α_{2A} -AR.

DISCUSSION

This is the first study immunochemically demonstrating the presence of α_2 -ARs on human retinal ganglion cells and other retinal neurons in both the inner and outer human retina. This study also demonstrated the presence of the α_2 -ARs on the RGC-5 cell line.

Our demonstration of α_2 -AR at the protein level in the retina is superior to both RNA and homogenate binding studies, offering increased anatomical resolution and sensitivity.⁴⁵ Previous studies have identified receptor localisation on rat retinal ganglion cells and other rat retinal neurons.³ This does not necessarily mean a similar expression in human retina. Specific receptor antibodies allow confirmation of adrenergic receptor subtype distribution as well as the possibility to test various pharmacological agents that interact specifically with these receptors.

Our current study confirmed previous immunochemical studies³ showing the presence of α_2 -ARs (specifically α_{2A} -ARs) on rat retinal ganglion and inner nuclear layer cells (fig 1), with even strong staining on all immunopositive cells.

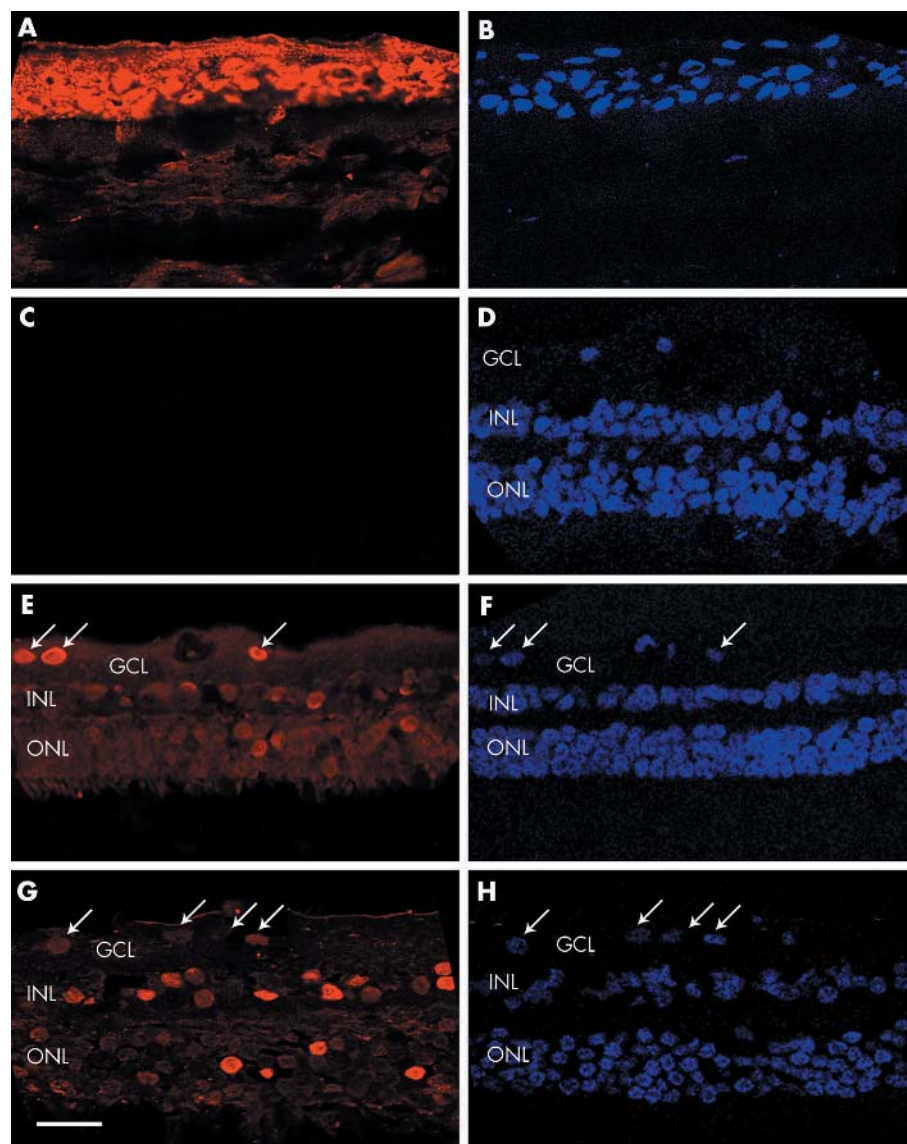


Figure 2 α_{2A} -AR expression in the human cornea and retina. Confocal images of human eye labelled with α_{2A} -AR antibody in cornea and retinal layers. (A), (E), and (G) Immunostained with α_{2A} -AR antibody and (C) with chicken IgY alone. (B), (F), (H), and (D) Corresponding Hoechst nuclear counterstaining. (A) The positive control, is human corneal epithelium showing specific strong staining for the 2AR. (C) A retina control with chicken IgY alone showing no non-specific staining. (E) and (G) Staining of two different human retinas with positive retinal ganglion cells (arrows) and variable staining in inner (INL) and outer nuclear layers (ONL). Between different retinas, there was some variability in overall intensity but a similar pattern of staining. All images are taken with the $\times 63$ objective. (G) Shows 25 μ m scale bar. GCL, ganglion cell layer.

In the human retina we found the presence of α_{2A} -ARs on human retinal ganglion cells and retinal cells in the inner and outer nuclear layers. In general, all eyes show a similar overall staining pattern, with some intensity variability

between eyes (fig 2E and G), possibly related to in vivo pharmacological interventions, different ages of eyes, and different time from enucleation to fixation. Differential staining occurred within the inner nuclear layer and outer nuclear layer and could reflect specific neuronal subtypes as these nuclear layers are composed of varying neuronal cell types. Preliminary studies using known glial cell markers,⁴²⁻⁴⁴ vimentin, and CRALBP did not co-localise with α_{2A} staining, suggesting that immunopositive cells in the inner and outer nuclear layers are neuronal and not glial. Previous studies in other species suggested that the subpopulation of cells staining positively in the inner nuclear layer are amacrine cells.³ Studies involving other retinal subclass markers could help to further discern the cell types staining positive for the α_{2A} receptor. The rat retina displays a less apparent differential staining than the human retina without a subpopulation of cells staining positive in the outer nuclear layer, reflecting interspecies variability and confirming the need for this current immunochemical study.

We found markedly enhanced α_{2A} -AR expression on day 7 differentiated RGC-5 cells compared to undifferentiated or early differentiated RGC-5 cells (fig 3). The changing receptor expression in the RGC-5 cell line with differentiation could reflect changes to in vivo expression levels with neuronal

Table 2 Summary of averaged intensity of immunostaining for the α_2 -AR (alpha-2 adrenergic receptor) in the human cornea and retina

Tissue	Layer	α_2 -AR staining
Cornea	Epithelium	+++
Retina	GCL	+++
	INL	3 populations, many positive
		-
		++
	ONL	+++
		3 populations, few positive
-		
		+ (most)
		+++ (few)

These are the averaged data from all images collected in the current study. Intensity of the human tissue staining was graded, with - representing no staining; + representing low or weak staining; ++ representing moderate or intermediate staining; and +++ representing high or strong levels of staining.

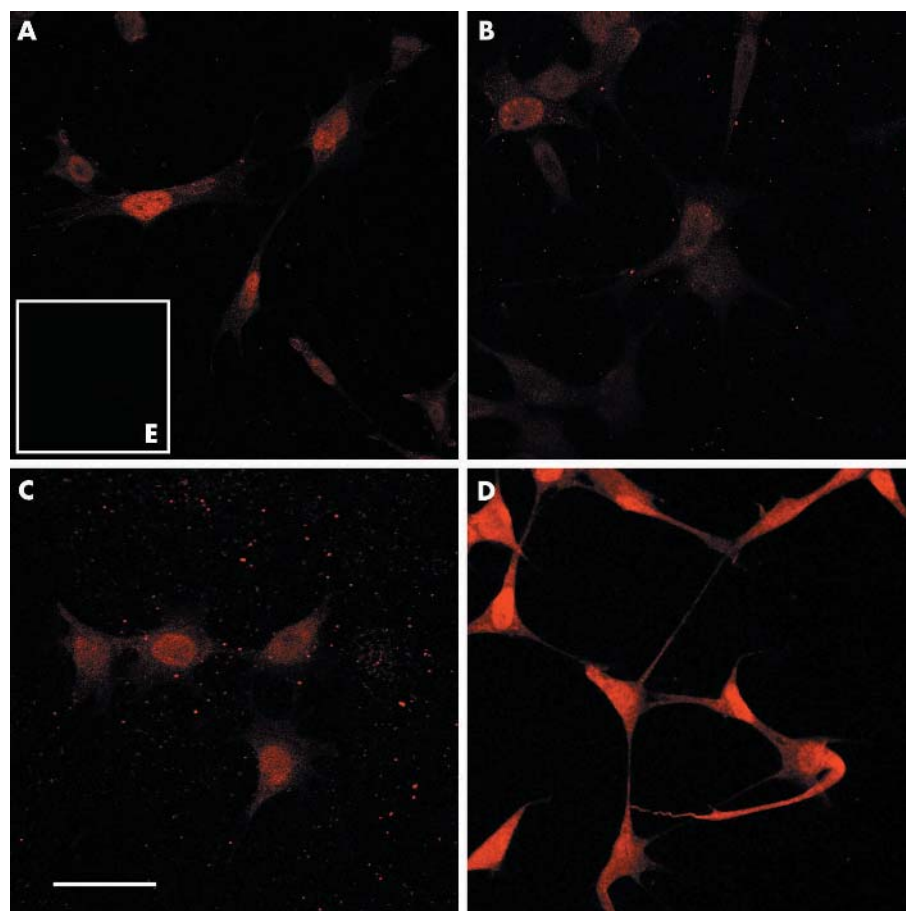


Figure 3 α_{2A} -AR expression in the rat retinal ganglion cell line, RGC-5. Confocal images of rat retinal ganglion cell line RGC-5 labelled with α_{2A} -AR antibody. (A) Undifferentiated RGC-5 cells have low, though positive, level of α_{2A} -AR expression. (B-D) 3, 5, and 7 days, respectively, of RGC-5 differentiation, showing positive α_{2A} -AR expression, with maximum expression at 7 days of differentiation. (E) (inset) RGC-5 cells with chicken IgY alone showing no non-specific staining. Identical staining and confocal imaging conditions used for all images. All images $\times 40$ objective. (C) Shows 25 μm scale bar.

development. The demonstration of α_{2A} -ARs on the RGC-5 cells substantiates positive staining found in the ganglion cell layers of human and rat retina, suggesting that positive immunoreactivity in this layer are on the ganglion cells rather than displaced amacrine cells. The presence of α_{2A} -ARs on the RGC-5 cell line allows the possibility of studying the direct effect of α_2 -AR agonists on retinal ganglion cells, the target cells of glaucoma. This eliminates the need for studying mixed primary cultures and then using ganglion cell markers in order to identify between the various cell types present. Thus, the direct effect of α_2 -AR agonists can be studied on retinal ganglion cells in culture, while holding relevance to potential human interactions.

Alpha-2 receptor activation has been implicated in enhanced neuronal survival in glaucomatous in vivo models¹⁻⁹ and hence alpha-2 agonists form one of the most studied neuroprotective agents. In contrast with some other proposed neuroprotective agents—for instance, betaxolol, alpha-2 agonists could exert their neuroprotective effect on retinal neurons via direct interaction with their receptor.^{6 10 11} Alpha-2-AR stimulation may protect retinal ganglion cells by inhibiting pro-apoptotic mitochondrial signalling, but yet the precise mechanism of neuroprotection is unknown.⁴⁶ Our demonstration of the α_{2A} -AR, the most common subtype of α_2 -ARs found in the central nervous system and the subtype for which brimonidine has highest affinity,²¹ on the human retinal ganglion cell as well as on other retinal neurons, importantly demonstrates a direct target for neuroprotection on and in the vicinity of the ganglion cells, which are dying by apoptosis in glaucoma.

While a direct relation, in neurons, between α_{2A} -ARs and apoptosis has not yet been demonstrated, preliminary data

from meetings (Wheeler *et al*, *Invest Ophthalmol Vis Sci* 2001;**42**:411) suggest that α_{2A} -AR agonists decrease apoptosis through an unknown mechanism that increases Bcl-2 expression. In contrast, the developing embryo expresses α_{2A} -ARs in regions of embryo apoptosis,⁴⁷ suggesting exactly the opposite relation with apoptosis. Further studies using α_{2A} -ARs agonists and retinal ganglion cells, potentially using the RGC-5 cell line, are required in order to ascertain the relevance of α_2 -ARs in apoptosis and neuroprotection.

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