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Neural Mechanisms Underlying Brimonidine's Protection of Retinal Ganglion Cells in Experimental Glaucoma

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1. Introduction

Glaucoma is a neurodegenerative disease characterized by a progressive loss of retinal ganglion cells (RGCs), the output neurons of the retina. Elevated intraocular pressure (IOP) has long been recognized as a major risk factor for human glaucoma (Kass et al., 1980; Quigley et al., 1994; Tsai & Kanner, 2005). Indeed, in animal models of glaucoma, ranging from rodents (Johnson & Tomarev, 2010) to primates (Gaasterland & Kupfer, 1974; Hare et al., 2004), elevated IOP produced either biophysically (Gaasterland & Kupfer, 1974; WoldeMussie et al., 2001) or genetically (Anderson et al., 2001; Ju et al., 2009) can lead to RGC degeneration similar to that found in human glaucoma (Quigley, 2005).

A common and effective treatment for glaucoma is the use of IOP lowering topical drugs that act at a variety of cellular targets, such as the α2 and β adrenergic receptors (Tsai & Kanner, 2005). However, in many patients, the disease continues to progress despite successful IOP reduction with topical drugs (Heijl et al., 2002; Vasudevan et al., 2011).

Brimonidine, a selective α2 receptor agonist, is the active ingredient in one class of topical IOP lowering drugs, such as Alphagan® and Alphagn-P®. Brimonidine has been shown to protect RGCs in experimental glaucoma (WoldeMussie et al., 2001; Dong et al., 2008), retinal ischemia (Donello et al., 2001; Lai et al., 2002), optic nerve injury (Yoles et a., 1999), and retinal excitotoxicity (Dong et al., 2008). In experimental glaucoma, brimonidine's neuroprotective effect appears to be independent of its IOP lowering action (Dong et al., 2008; Hernandez et al., 2008). More recently, in a randomized, double-masked, multicenter clinical trial, brimonidine has been shown to be more effective in slowing disease progression (visual field loss), compared with timolol (a β blocker), despite the fact that the mean treated IOP was similar in both treatment groups at all time points (Krupin 2011). These clinical data suggest that brimonidine may have a *direct* RGC protective effect that is independent of its IOP lowering action in human low-pressure glaucoma, similar to that found in experimental glaucoma (Dong et al., 2008; Hernandez et al., 2008).

In this chapter, we will summarize the results of our recent studies on the mechanisms that underlie brimonidine's protection of RGCs in experimental glaucoma and retinal excitotoxicity. We will first describe the properties of RGCs and the *ex vivo* and *in vivo* models used in our studies on the mechanisms of RGC injury and protection. Then we will

discuss neuronal Ca^{++} signaling in health and disease, as well as the properties of the α2 receptor and its intracellular signaling pathways responsible for brimonidine mediated neuroprotection. A particular emphasis will be placed on the role of the α2 receptor in modulation of retinal Ca^{++} signaling pre- and postsynaptic to RGCs. Finally, we will briefly discuss other beneficial effects of brimonidine in retinal disease models reported in the literature.

2. Properties of RGCs

The vertebrate retina is a part of the central nervous system (CNS) and RGCs are a type of CNS projection neuron (Dowling, 1987; Rodieck, 1973). Unlike all other types of retinal neurons that receive their synaptic inputs and send the outputs within the retina, RGCs receive synaptic input at the inner plexiform layer (IPL) of the retina, but transmit their output at locations far away from the retina via long axons (a major component of the optic nerve). Also unlike the majority of the retinal neurons, such as photoreceptors, bipolar cells, and horizontal cells, that use only graded potentials to transmit the visual signal, RGCs are the only retinal neurons that use *exclusively* action potentials (nerve spikes) to transmit signals to their postsynaptic neurons at higher brain centers, such as lateral geniculate nucleus and superior colliculus (Dowling, 1987; Ito et al., 2008; Rodieck & Watanabe, 1993).

While these unique functional and structural properties of RGCs are required for long-range signaling as projection neurons, they also cause significantly elevated metabolic demand due to increased energy (ATP) usage to prevent intracellular ionic imbalance resulting from the high frequency spiking activity and for long-range bidirectional axonal transport. This elevated metabolic demand likely makes RGCs more susceptible to various stresses under pathophysiological conditions, such as intracellular Ca^{++} dysregulation (Dong et al., 2008), metabolic challenge caused by elevated IOP (Baltan et al., 2010) or vascular abnormality (Moore et al., 2008), and axonal ionic imbalance such as Na^{+} overload (Dong & Hare, 2005; Waxman et al., 1994).

3. *Ex vivo* and *in vivo* models used in determination of the mechanisms of RGC injury and α2 protection

In order to determine the mechanisms that underlie high IOP induced RGC degeneration in experimental glaucoma and protection by brimonidine, we used two *ex vivo* models: live rat retinal slice (Dong et al., 2007) and the isolated, flat-mount rat and rabbit retina (Dong et al., 2008) and two *in vivo* models: a rat chronic ocular hypertensive glaucoma model (WoldeMussie et al., 2001; Dong et al., 2008) and a rabbit retinal excitotoxicity model (Dong et al., 2008). We use the *ex vivo* models to study intracellular signaling pathways of the α2 receptor and its interactions with the voltage- and transmitter-gated Ca^{++} channels, namely the L-type Ca^{++} channel and the NMDA receptor. We use *in vivo* models to test our hypotheses based on our findings from the *ex vivo* models. Rodent ocular hypertensive models of various kinds have been widely used in glaucoma research (Johnson & Tomarev, 2010). However, the other three models, namely the retinal slice, *in situ* RGCs in the isolated retina, and the rabbit retinal excitotoxicity model, are not commonly used. We therefore will briefly describe their unique features and utility in glaucoma research.

3.1 Live retinal slice

Both the retinal slice and isolated flat-mount retina are *ex vivo* preparations that retain rather well natural intercellular neural connections/interactions and have neuronal gene express patterns that are very similar to those under *in vivo* conditions. And yet, they allow experimentation under more controlled conditions than possible under *in vivo* conditions. For example, the compositions of extracellular media can be controlled precisely and changed rapidly and retinal neurons can be accessed readily for electrophysiological recording and optical imaging/labeling while still maintaining connections with their neighboring neurons and other supporting cells such as glial cells. Therefore, these *ex vivo* preparations are particularly useful for studies aiming at understanding the mechanisms of eye diseases and drug action.

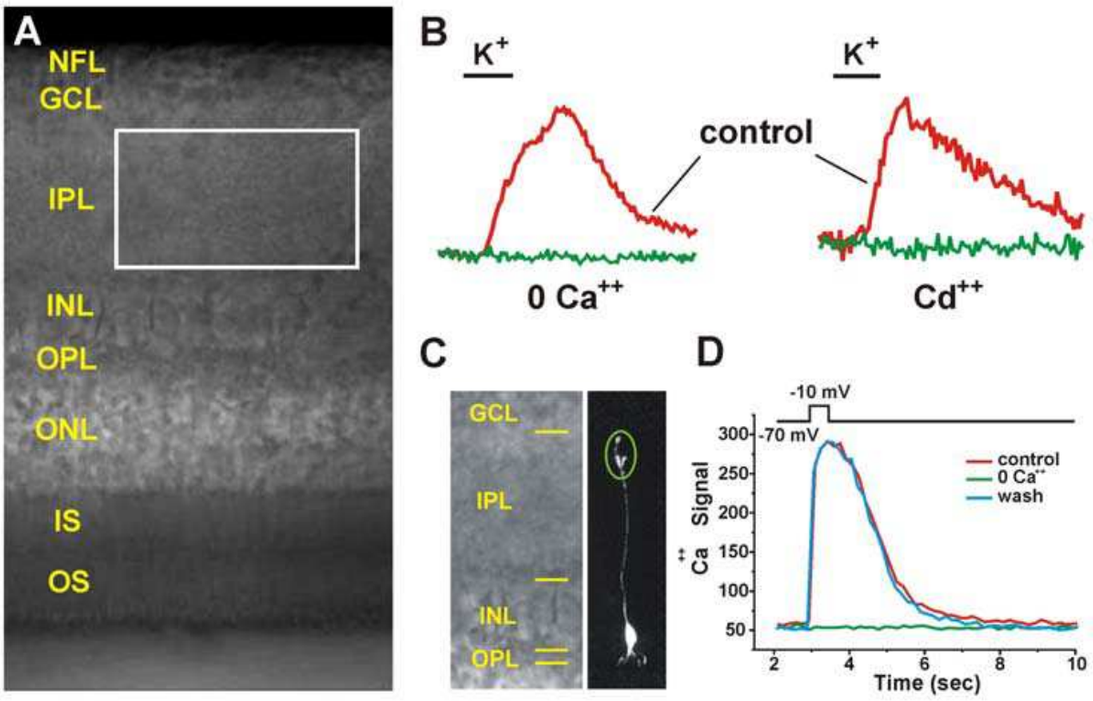

Fig. 1. The live rat retinal slice preparation. See the text for details

Intracellular Ca^{++} plays a critical role in neuronal signal processing and communication, such as neurotransmitter release from presynaptic axon terminals. It is also a key signaling molecule to trigger cell apoptosis/death under pathophysiological conditions (see the next section below). Figure 1A shows an acutely cut and superfused rat retinal slice in which the major retinal layers (nerve fiber layer, NFL; ganglion cell layer, GCL; inner plexiform layer, IPL; inner nuclear layer, INL; outer plexiform layer, OPL; outer nuclear layer, ONL; photoreceptor inner segment, IS; photoreceptor outer segment, OS) are easily visible under the microscope with a water immersion objective. When retinal slices are loaded with a membrane permeable fluorescent Ca^{++} dye (Fluo-4 AM), changes in cytosolic free Ca^{++} under various experimental conditions can be recorded with a confocal imaging system (for technical details see Dong et al., 2007). Membrane depolarization induced by a 5-8 sec rapid perfusion of high K^+ Ringer solution elicited a robust Ca^{++} signal (red traces in Fig. 1B) at

IPL where RGCs communicate with their presynaptic neurons, such as bipolar and amacrine cells. A representative area for the Ca^{++} measurement at IPL is indicated by the white rectangle in Fig. 1A. The high K^+ induced Ca^{++} signals were abolished completely after perfusing with either a Ca^{++}-free Ringer solution or normal Ringer solution that contained 100 μM Cd^{++}, a broad-spectrum Ca^{++} channel blocker (green traces in Fig. 1B). This indicates that the signal is generated by Ca^{++} influx through depolarization activated Ca^{++} channels at IPL.

The Ca^{++} influx into bipolar cell synaptic terminals (located at IPL) is likely a major contributor to the depolarization induced Ca^{++} signal shown in Fig. 1B. Bipolar cells are key presynaptic partners of RGCs. Bipolar cells release glutamate (Matsui et al., 1998) as their neurotransmitter at the synaptic terminals to communicate with RGCs and amacrine cells and Ca^{++} influx is needed to trigger the release. Therefore, it is not surprising that voltage-gated Ca^{++} channels are highly concentrated at bipolar cell terminals for glutamate release (Pan, 2000, 2001). When these Ca^{++} channels are over activated under pathophysiological conditions, such as retinal ischemia, glutamate released from bipolar terminals could be a major contributor to the significantly elevated extracellular glutamate that can cause excessive activation of the NMDA receptor on RGCs and lead to RGC dysfunction/death.

The right panel of Fig. 1C shows the confocal image of a Ca^{++} dye labeled bipolar cell from a live rat retinal slice. The major parts of the cell, the dendrites, soma, axon, and synaptic terminals (indicated by the green oval), are clearly visible. The Ca^{++} dye (a cell membrane-impermeable version of Fluo-4) was delivered to the bipolar cells intracellularly via a patch-clamp electrode (not shown). Membrane depolarization induced by a 0.5 sec voltage step from the holding potential of -70 mV to -10 mV through the recording electrode elicited at the bipolar cell terminals a large cytosolic free Ca^{++} signal (Fig. 1D, arbitrary units) that was completely eliminated by removing Ca^{++} from the Ringer solution. Ca^{++} channels in the CNS, particularly those at presynaptic terminals, are important drug targets and *in situ* bipolar terminals provide an excellent *ex vivo* system for the studies on neuromodulation of presynaptic Ca^{++} channel activity by brimonidine and other neuroactive drugs/drug candidates.

3.2 *In situ* RGCs in the isolated flat-mount retina

Because of the selective vulnerability of RGCs in glaucoma, a detailed characterization of physiological and pharmacological properties of RGCs can help to understand the mechanism of RGC injury in glaucoma and the mechanism of action of neuroprotective drugs, such as brimonidine, as well as to identify novel drug targets for the treatment of glaucoma. *In situ* RGCs in the acutely isolated, superfused retina (Fig. 2) offer a unique opportunity to study the pharmacological properties and intracellular signaling pathways of various neural active drugs and drug candidates on RGCs without significantly altering retinal synaptic connections and RGC gene expression pattern compared to RGC cell lines or even primary cultures.

Fig. 2A shows a bright field image of a piece of live isolated, superfused rabbit retina viewing from the vitreous side. The axon bundles (the main component of the nerve fiber layer) of RGCs are visible. After the inner limiting membrane and nerve fiber layer were carefully poked through and the debris were removed with a cleaning glass micropipette, somas of *in situ* RGCs were revealed and whole-cell recordings could be performed with patch-clamp electrodes (Fig. 2B). We routinely include a membrane-impermeable Ca^{++} dye

in the patch electrodes. Therefore, neurotransmitter agonist (such as NMDA) induced transmembrane currents and cytosolic free Ca^{++} signals can be measured simultaneously. Fig. 2C shows the NMDA (applied with the co-agonist glycine) induced inward current and Ca^{++} signal from an *in situ* RGC. A transient light response from that RGC is also visible immediately after the onset of the excitation light for Ca^{++} imaging. After electrophysiological and optical recordings, the identity of the recorded cell can be confirmed with confocal imaging (Fig. 2D).

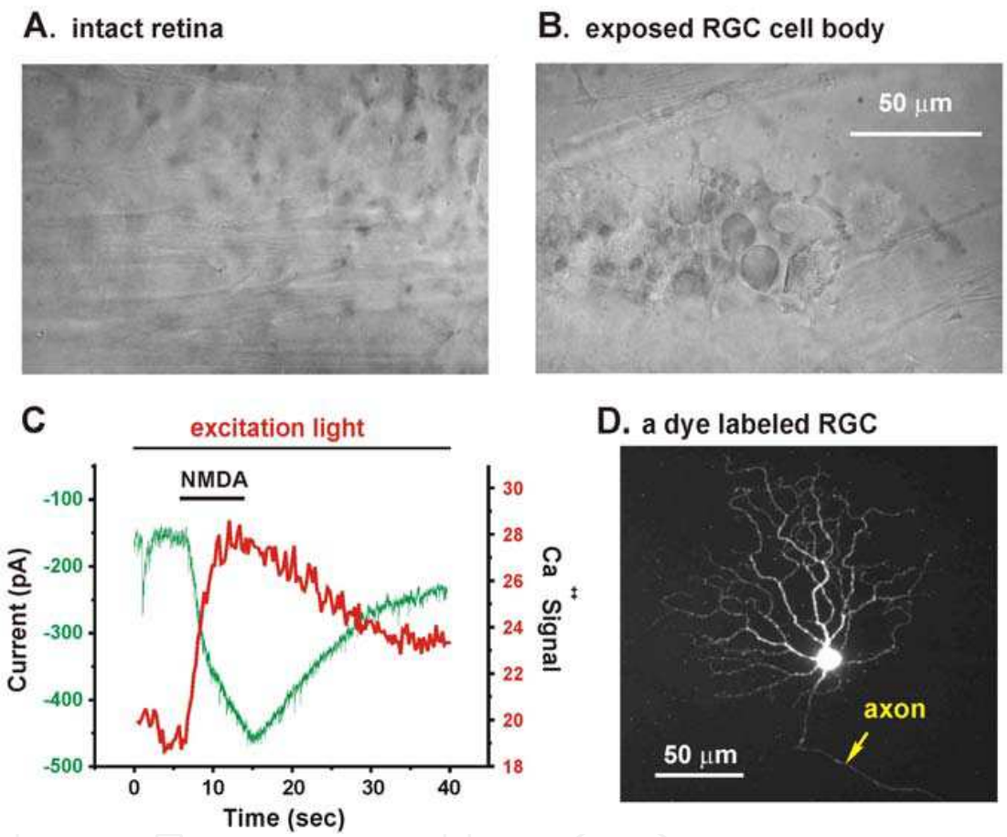

Fig. 2. *In situ* RGCs in the isolated, superfused rabbit retina. See the text for details

3.3 Rabbit retinal excitotoxicity model

Excessive activation of glutamate receptors, particularly the NMDA receptor, has been suggested as a key factor that contributes to high IOP induced RGC loss in experimental glaucoma, regardless how IOP is elevated and what animal species is selected (Dong et al., 2008; Hare et al., 2004; Ju et al., 2009; WoldeMussie et al., 2002).

Intravitreal injection of NMDA, a selective agonist of the NMDA sub-type of ionotropic glutamate receptor, can also induced RGC loss by directly activating the NMDA receptor on RGCs. The rat (Siliprandi et al., 1992) and mouse (Ito et al., 2008) retinal NMDA models have been used in glaucoma research to explore the mechanism of RGC injury and to evaluate efficacy of potential RGC protective drug candidates. However, relatively hard to perform intravitreal injection and lack of macular-like structure are two major disadvantages of the rodent models.

We therefore developed a rabbit retinal NMDA model (Fig. 3, see also Dong et al., 2008). The rabbit has large eyes and it's significantly easier to conduct multiple intravitreal injections in rabbit eyes with low risk of causing accidental retinal or lens damage (both of which can affect RGC survival directly or indirectly). This facilitates *in vivo* studies on the mechanisms of RGC protection by brimonidine or other novel neuroprotective agents.

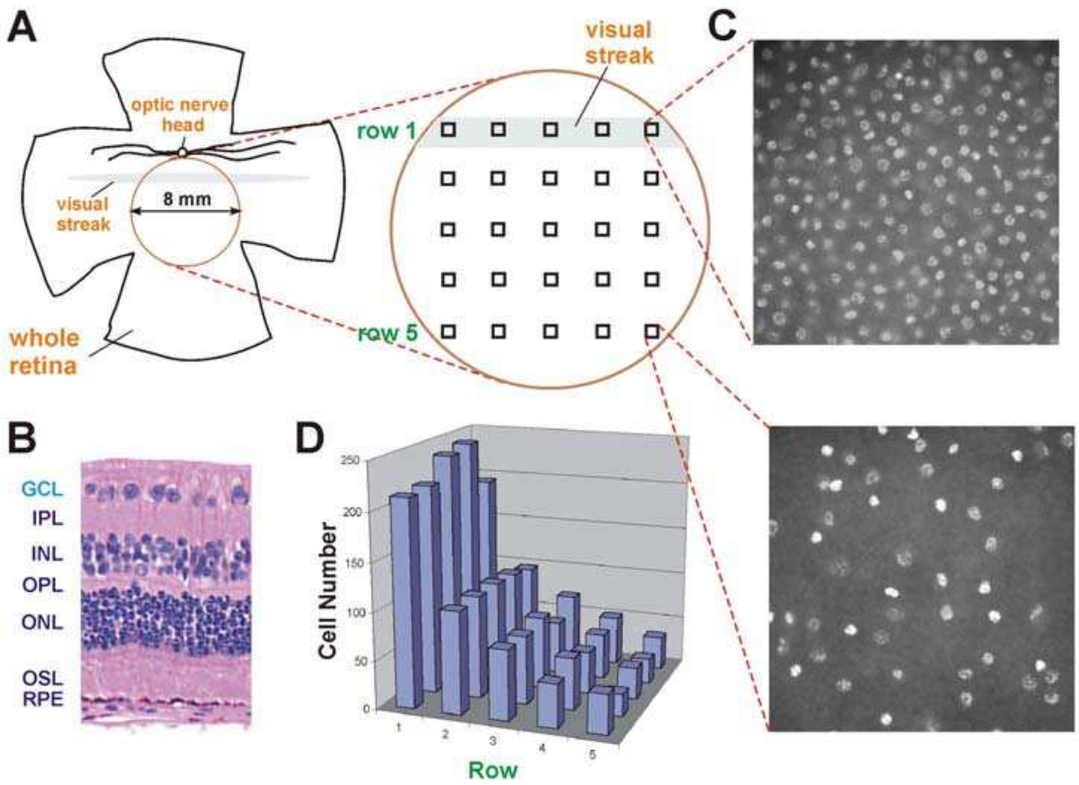

Fig. 3. The rabbit model to evaluate NMDA excitotoxicity. See the text for details

The rabbit retina also has a visual streak that is an elongated macular-like structure that has the highest density of neurons in the retina (Fig. 3C and 3D). This allows us to study the differential vulnerability of RGCs located at the center (visual streak) versus more peripheral retinal locations, which may provide an important clue as to why the central visual fields are more resistant to glaucomatous injury in human glaucoma (Dong et al., 2011). Fig. 3A shows an isolated rabbit retina. We routinely cut out a circular piece (8 mm in diameter) from the central retina using the optic nerve head as a marker to ensure that we compare the same regions of the retina between different experimental groups. We count the neurons in the ganglion cell layer at 25 locations in a 5x5 array using a computer-controlled automated microscope stage (see Dong et al., 2008 for technical details). The circular piece of the retina is aligned carefully so that the first row of 5 sites is located within the visual streak (Fig. 1A). Fig. 1B shows a representative cross section of H&E stained rabbit retina. The individual dots in Fig. 1C are nuclei of RGCs and displaced amacrine cells in the ganglion cell layer labelled with a fluorescent nuclear dye (DAPI). The density of the neurons drops rapidly from the visual streak (row 1) to more peripheral retina (rows 2 to 5). Fig. 1D shows the 3D plot of the cell density profile based on neuron density of these 25 sites.

4. Intracellular Ca^{++} dysregulation and neurodegeneration

There is compelling evidence that intracellular Ca^{++} dysregulation is a key contributor to neurodegeneration in a wide range of CNS degenerative diseases, such as Alzheimer's disease and Parkinson's disease (Bezprozvanny, 2009). In addition to age and genetically related changes in intracellular Ca^{++} handling, Ca^{++} overload as a result of excessive activation of voltage- and ligand-gated Ca^{++} channels on the neuronal cell membrane, particularly the NMDA receptor, plays a central role in intracellular Ca^{++} dysregulation (Choi, 1985; Choi et al., 1988). The NMDA receptor is a type of ionotropic glutamate receptor that is coupled to a cation channel which has a high Ca^{++} permeability (MacDermott et al., 1986; Sattler & Tymianski, 2001). It is therefore sometimes called the ligand-gated Ca^{++} channel. Unlike the other types of ionotropic glutamate receptors (such as AMPA and kainate receptors) that desensitize rapidly and significantly in the continuous presence of the agonist (Hestrin et al., 1990; Lukasiewicz et al., 1995), the NMDA receptor shows significantly less desensitization in the continuous presence of the agonists (MacDermott et al., 1986; Matsui et al., 1998) and is therefore particularly effective in causing intracellular Ca^{++} overload when excessively activated.

Intracellular Ca^{++} overload can trigger neuronal apoptosis via a number of cross-amplifying pathways, including Ca^{++}-activated proteases and their downstream effectors such as calpain and calpain-activated caspases (Das et al., 2005; Sharma & Rohrer, 2004), mitochondrial dysfunction and damage directly produced by Ca^{++} overload (Starkov et al., 2004), and Ca^{++} dependent cytosolic overproduction of free radicals (Brennan et al., 2009; Sattler et al., 1999).

4.1 NMDA receptor, intracellular Ca^{++} dysregulation, and RGC degeneration

Intracellular Ca^{++} dysregulation/overload caused by deregulated retinal glutamatergic transmission is also likely a key mechanism that causes RGC dysfunction (Hare & Wheeler, 2009) and degeneration in disease states, such as acute retinal ischemia (Lagreze et al., 1998) and experimental glaucoma (Dong et al., 2008; Harada et al., 2007; Ju et al., 2009). For example, vulnerability of RGCs in acute retinal ischemia is associated not only with NMDA receptor activity (Lagreze et al., 1998), but also with significantly elevated vitreal glutamate level (Donello et al., 2001; Lagreze et al., 1998). Brimonidine not only protects RGCs in retinal ischemia, but also significantly reduced vitreal glutamate concentration (Donello et al., 2001), suggesting that it either prevents excessive release of glutamate or enhances its uptake, or both.

5. The α2 adrenergic receptor

The α2 adrenergic receptor is a G-protein coupled receptor (GPCRs, Fig. 4) that can signal through both G_{α} and $G_{\beta\gamma}$ subunits (Delaney et al., 2007; Maze, 1991). In the CNS, the major functional role of the α2 receptor is modulation of neurotransmitter release (Starke, 2001). This action is through the classical *presynaptic* inhibition either by inhibiting Ca^{++} channels (Boehm, 1999; Starke, 2001), activating K^{+} channels (Bünemann et al., 2001), or reducing active release sites (Delaney et al., 2007). Presynaptic inhibition by the α2 receptor in the brain is mediated by the $G_{\beta\gamma}$ subunits (Fig. 4B, see also Delaney et al., 2007).

The α2 receptor is coupled to $G_{\alpha i/o}$ (i for inhibition of adenylate cyclase and o for olfactory type). When signaling through the G_{α} subunit, α2 receptor activation leads to inhibition of

adenylate cyclase, resulting in a reduction of cAMP production. cAMP is an important intracellular second messenger that modulates many aspects of cellular function through interacting with various downstream effectors (Maze, 1991), such as protein kinase A. Alpha 2 receptors are known to be expressed in retinal neurons, including retinal bipolar cells (Dong et al., unpublished results) and RGCs (Kalapesi et al., 2005).

5.1 Suppression of NMDA receptor function by brimonidine

As shown in Fig. 2, robust NMDA induced transmembrane currents and cytosolic free Ca^{++} signals can be recorded from *in situ* RGCs in the isolated, superfused retina when they are voltage-clamped close to their resting membrane potential at -70 mV. We reported (Dong et al., 2008) that pretreatment with brimonidine produced a significant suppression of both of these NMDA induced signals in RGCs (Fig. 5B and 5C). NMDA (plus glycine) was applied with a local perfusion micropipette (Fig. 5A) that was connected to a computer-controlled, multi-channel drug delivery system in a 0 Mg^{++} Ringer. This allowed rapid delivery and removal of NMDA as well as effective activation of the NMDA receptor without the voltage-dependent Mg^{++} block. Brimonidine and atipamezole (a selective α2 antagonist) were delivered using both bath (the whole-chamber) and the local perfusion systems. The Ca^{++} dye and some tool compounds, such as GDP-βS, were delivered intracellularly to the RGC using the recording pipette.

The suppressive effect of brimonidine on NMDA responses is mediated by the α2 receptor, because this effect was completely blocked (Fig. 5C) by a highly selective α2 antagonist, atipamezole (Virtanen, 1989). Brimonidine's effect is direct on RGCs since it was also completely blocked (Fig. 5C) by intracellularly applied GDP-βS, a membrane impermeable GPCR inhibitor that blocks exchange of GDP for GTP on the G_{α} subunit of the G-protein (Fig. 4A), an obligate event required for α2 receptor activation. Other intracellularly delivered tool compounds that act at various sites along the α2 receptor signaling pathway also blocked brimonidine's effect on NMDA receptor function (see section 5.2), confirming that brimonidine's effect is direct on RGCs, not an indirect effect through other retinal cells.

5.2 Brimonidine suppresses NMDA receptor function through the $G_{\alpha i}$ pathway

Using tool compounds acting at different sites along the $G_{\alpha i}$ signaling pathway (Fig. 4), we have obtained compelling evidence that brimonidine's effect on NMDA receptor function is mediated almost exclusively by the $G_{\alpha i}$ pathway (Dong et al., 2008). We found that SQ22536, a selective inhibitor of adenylate cyclase (Fabbri et al., 1991), mimicked the effect of brimonidine on NMDA receptor function (Fig. 6A). On the other hand, forskolin (abbreviated as "forsk" in Fig. 6B), an activator of AC (de Souza et al., 1983), blocked the effect of brimonidine (abbreviated as "brimo" in Fig. 6B). This suggests that intracellular cAMP is an important regulator of NMDA receptor function in RGCs (Fig. 6C). A reduction of adenylate cyclase activity, produced either indirectly by brimonidine via α2 receptor activation (Fig. 6D) or directly by SQ222536 (Fig. 6A & 6E), can lead to the same effect: suppression of NMDA receptor function likely through a decrease in intracellular cAMP concentration. On the other hand, forskolin blocks brimonidine's effect likely through neutralizing brimonidine's effect on adenylate cyclase activity and therefore preventing significant alteration of intracellular cAMP concentration (Fig. 6B & 6F).

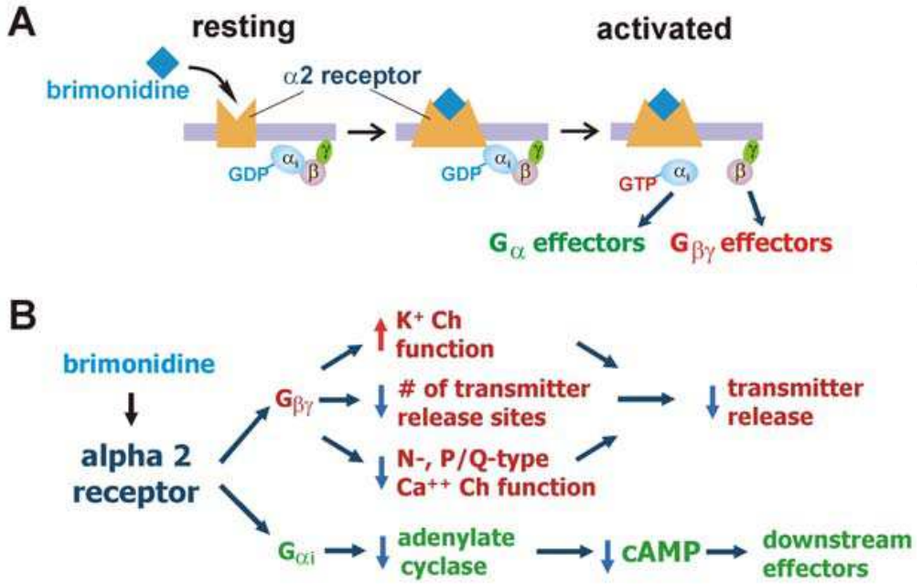

Fig. 4. Activation and intracellular signaling pathways of the α2 receptor

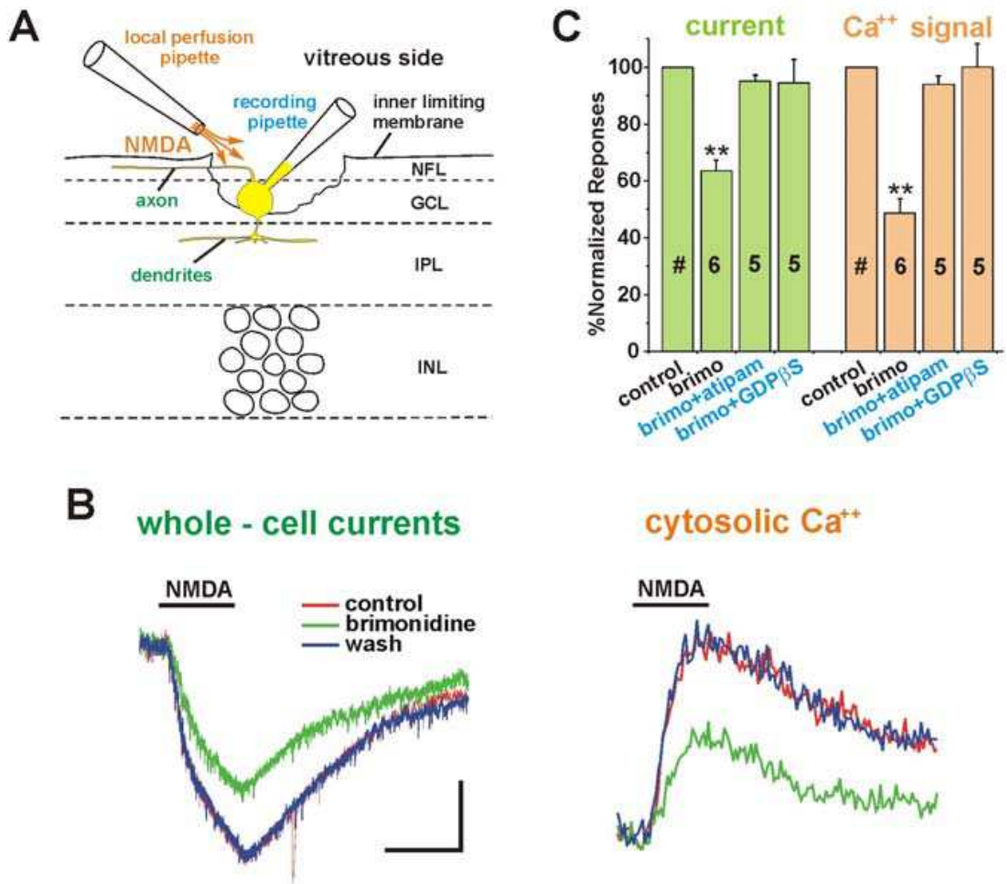

Fig. 5. Brimonidine modulation of NMDA receptor function. See the text for details

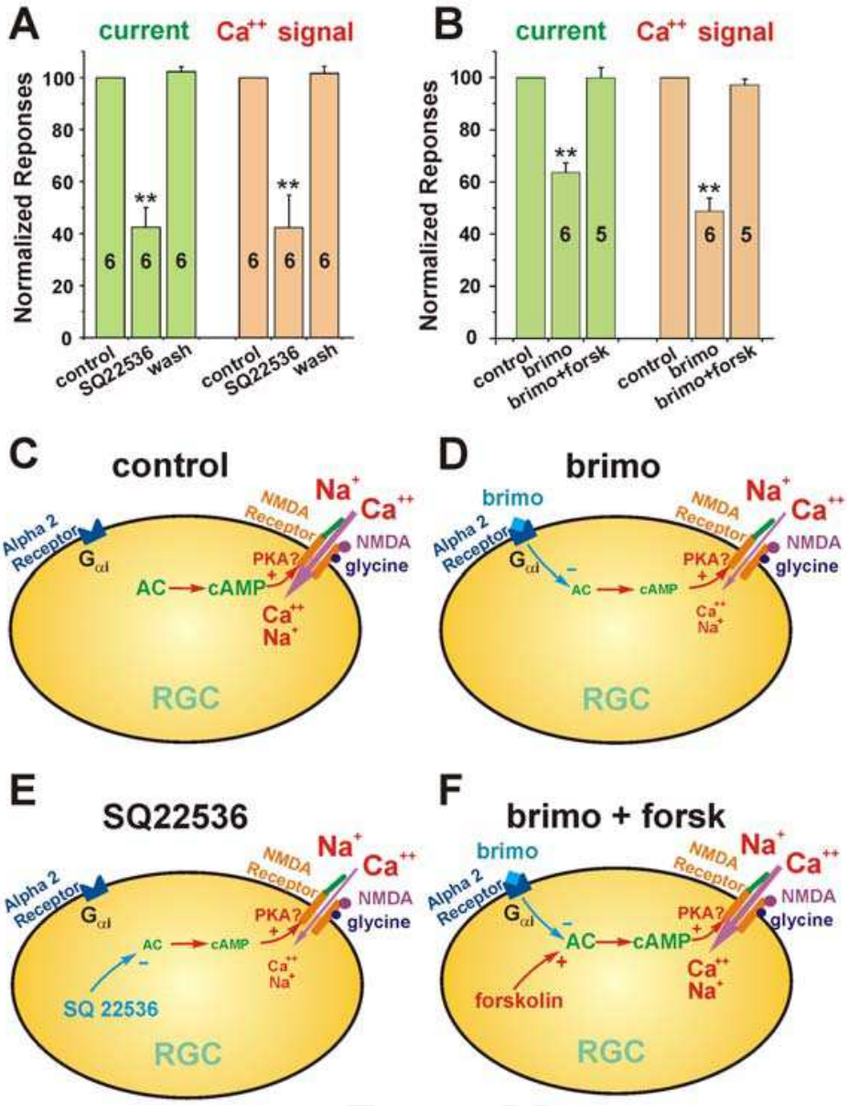

Fig. 6. Effects of an adenylate cyclase (AC) inhibitor and activator. See the text for details

Results with other tool agents provide additional support that brimonidine's effect is mediated by the $G_{\alpha i}$ pathway. To avoid potential global indirect effects of intracellularly acting tool agents on recorded RGCs, we applied those agents intracellularly through the recording pipette. Therefore, the effects are largely limited within the recorded RGCs. Intracellular application of Sp-cAMPS, a synthetic cell-permeable, hydrolysis-resistant cAMP analog, blocks *completely* brimonidine's effect on NMDA receptor function (Fig. 7A). To confirm that the lack of brimonidine's effect is indeed due to the presence of intracellular Sp-cAMPS, but not because of damage caused by patch-clamping, we recorded the same group of RGCs twice: in the presence and absence of Sp-cAMPS. After the first set of recordings were done with the electrode attached (Fig. 7A), we successfully removed the Sp-cAMPS-filled recording electrodes from 5 RGCs without causing significant damage to those cells. After waiting for a few minutes to allow the residual intracellular Sp-cAMPS to diffuse out of the RGCs and wash away, we observed a typical brimonidine-induced

suppression of NMDA induced Ca^{++} signal in the *same* group of RGCs (Fig. 7B, note: the Ca^{++} dye delivered intracellularly by the electrode is a membrane impermeable form of Fluo-4 and therefore stayed inside the RGCs even after the electrode was removed). Thus, "clamping" intracellular cAMP level experimentally with equivalent synthetic cAMP analog is able to eliminate *completely* brimonidine's effect.

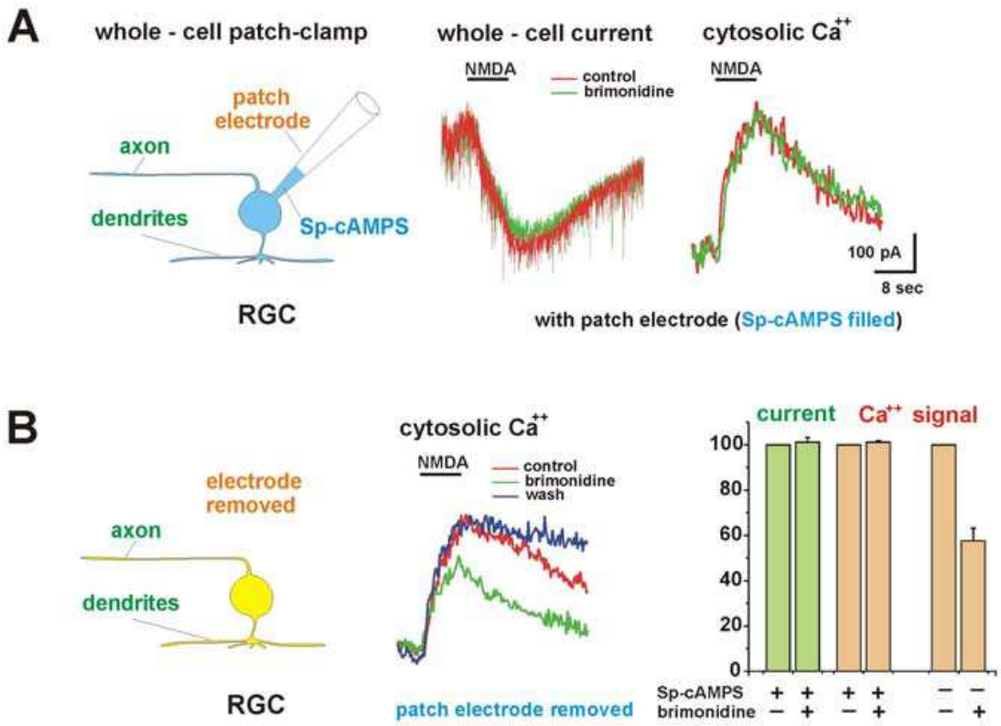

Fig. 7. Brimonidine's effect is blocked by intracellular application of a cAMP analog

In another experiment, we attempted to maintain *endogenous* intracellular cAMP level in RGCs through blocking its active degradation by phosphodiesterases (PDE). When rolipram, a type 4 PDE (a cAMP specific PDE) inhibitor (Teixeira et al., 1997), was intracellularly delivered through the recording electrode into a RGC, brimonidine's suppressive effect on the NMDA receptor mediated transmembrane current and cytosolic free Ca^{++} signal was also completely blocked (Fig. 8A). In this RGC, we succeeded not only in removing the rolipram-filled electrode but also in re-recording the same RGC with a second, rolipram-free electrode. This allowed both electrical and optical recordings being repeated in the absence of the PDE inhibitor and typical brimonidine's effect on NMDA responses was observed (Fig. 8B). These results demonstrate that preserving endogenous cAMP level through blocking its active degradation by PDE is also able to *completely* eliminate brimonidine's effect, confirming that brimonidine's effect is mediated by the $G_{\alpha i}$–AC–cAMP pathway coupled to the α2 receptor.

Fig. 9 summarizes the sites of action of various tool compounds used in our studies to dissect out the intracellular signaling pathway through which brimonidine modulates NMDA receptor function. Among the tool agents, SQ22536 mimics brimonidine's effect by a direct inhibition of the adenylate cyclase. Those in red are all able to block brimonidine's effect at the different sites along the $G_{\alpha i}$ mediated signaling pathway. With these tool agents,

we have demonstrated clearly that brimonidine can down modulate NMDA receptor function in RGCs and this brimonidine's effect is mediated predominantly, if not exclusively, by the $G_{\alpha i}$–AC–cAMP pathway coupled to the α2 receptor.

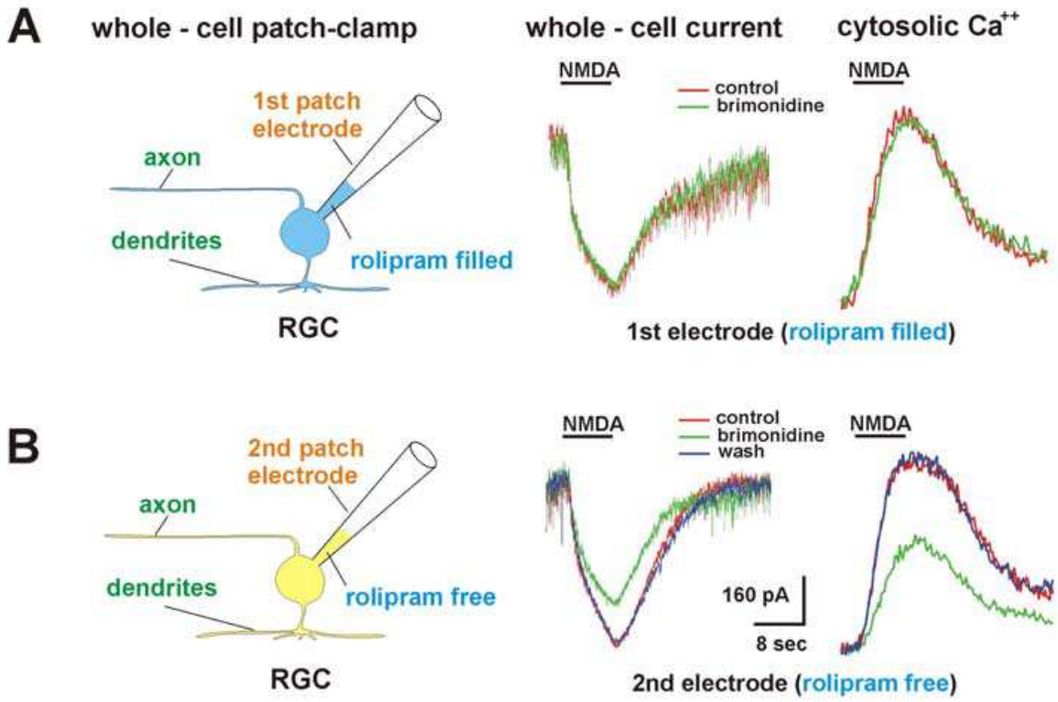

Fig. 8. Brimonidine's effect is blocked by intracellular application of a PDE-4 inhibitor

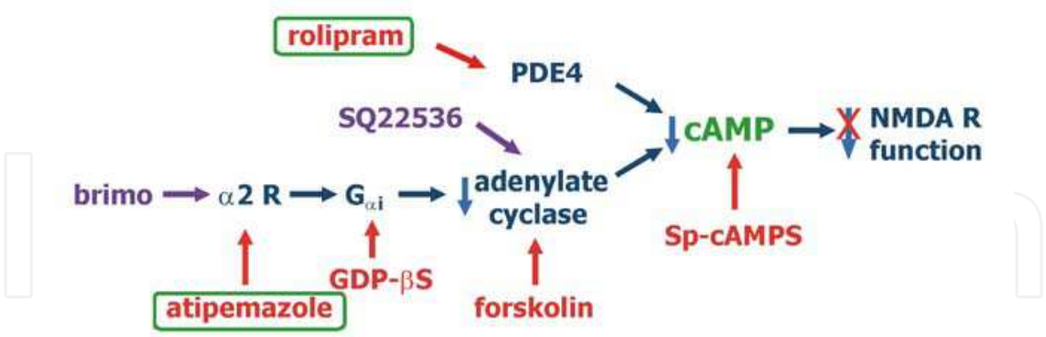

Fig. 9. The sites of action of the tool agents used along the $G_{\alpha i}$–AC–cAMP pathway

6. Validation in *in vivo* models

To test whether α2 receptor mediated modulation of NMDA receptor function in *in situ* RGCs contributes significantly to RGC protection by brimonidine in *in vivo* models, we evaluated in *in vivo* models two of the tool agents, atipamezole and rolipram (Fig. 9), that have a proven CNS bioavailability after systemic application. Only a minority of all tool agents that work well in *in vitro* models can be used in *in vivo* models for target validation or

proof of concept due to practical reasons such as systemic toxicity, bioavailability, and unfavorable pharmacokinetics. Atipamezole and rolipram have already been successfully used in many whole animal studies for other purposes and are therefore chosen for use in our *in vivo* validation experiments.

6.1 Rat glaucoma model

The first *in vivo* model we tested is a rat glaucoma model. In this rat model, elevated IOP (from approximately 15 to 32 mmHg) produced by laser photocoagulation of episclearal and limbal veins leads to approximately 30% RGC loss evaluated at 3 weeks following the laser treatment (WoldeMussie et al., 2001; Dong et al., 2008). We used subcutaneous osmotic pumps to deliver brimonidine alone or in combination with atipamezole or rolipram. Memantine, an NMDA receptor channel blocker, was also used as a positive control to confirm the role of the NMDA receptor in RGC injury in this experimental glaucoma model (WoldeMussie et al, 2002).

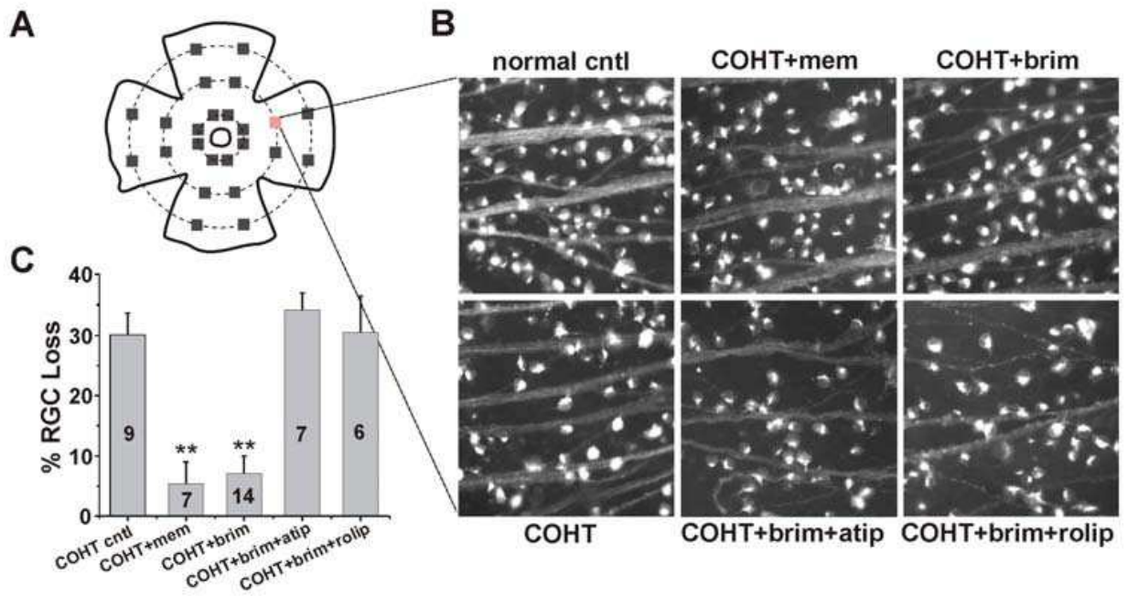

Fig. 10. Brimonidine's protection of RGCs in the rat glaucoma model is mediated by the $G_{\alpha i}$–AC–cAMP signaling pathway coupled to the α2 receptor. See the Dong et al., 2008 for technical details

We used a computer-controlled automated microscope stage and counted dextran tetramethylrhodamine retrogradely labeled RGCs at 24 sites (Fig. 10A). Fig. 10B shows representative images from an intermediate retinal location taken from a normal rat and glaucomatous rats under various treatment conditions: laser treatment alone (COHT for chronic ocular hypertension), COHT treated with memantine (COHT+mem), brimonidine (COHT+brim), brimonidine plus atipamezole (COHT+brim+atip), brimonidine plus rolipram (COHT+brim+rolip). Both memantine and brimonidine provided substantial protection of RGCs in this rat glaucoma model (Fig. 10C), consistent with the observations reported in previous studies (WoldeMussie et al., 2001, 2002). We found that brimonidine's protective effect was completely blocked by co-administration of atipamezole, verifying for

the first time that brimonidine's effect is indeed mediated by the α2 receptor. Furthermore, RGC protection by brimonidine was also *completely* blocked by co-administration of rolipram, a novel finding that identifies PDE-4 as a key endogenous player contributing to brimonidine's protection of RGCs. Together these *in vivo* results suggest strongly that the $G_{\alpha i}$–AC–cAMP signaling pathway coupled to the α2 receptor, described above in *in situ* RGCs, is responsible for RGC protection by brimonidine in this rat glaucoma model.

6.2 Rabbit retinal NMDA model

In animal glaucoma models, ranging from mouse to monkey (including the rat model used here), NMDA receptor mediated excitotoxicity is a major contributor to RGC injury regardless whether IOP elevation is produced biophysically (Dong et al., 2008; Hare et al., 2004; WoldeMussie et al., 2002) or genetically (Ju et al., 2009). We have demonstrated that brimonidine down-modulates NMDA receptor function through the α2 receptor coupled $G_{\alpha i}$–AC–cAMP signaling pathway (see Fig. 4 and Fig. 9 above). We also showed that the same $G_{\alpha i}$ pathway is responsible for brimonidine's protection of RGCs in the rat glaucoma model (Fig. 10). Therefore, it is likely that brimonidine protects RGCs in the glaucoma models, at least in part, through attenuation of NMDA receptor mediated excitotoxicity.

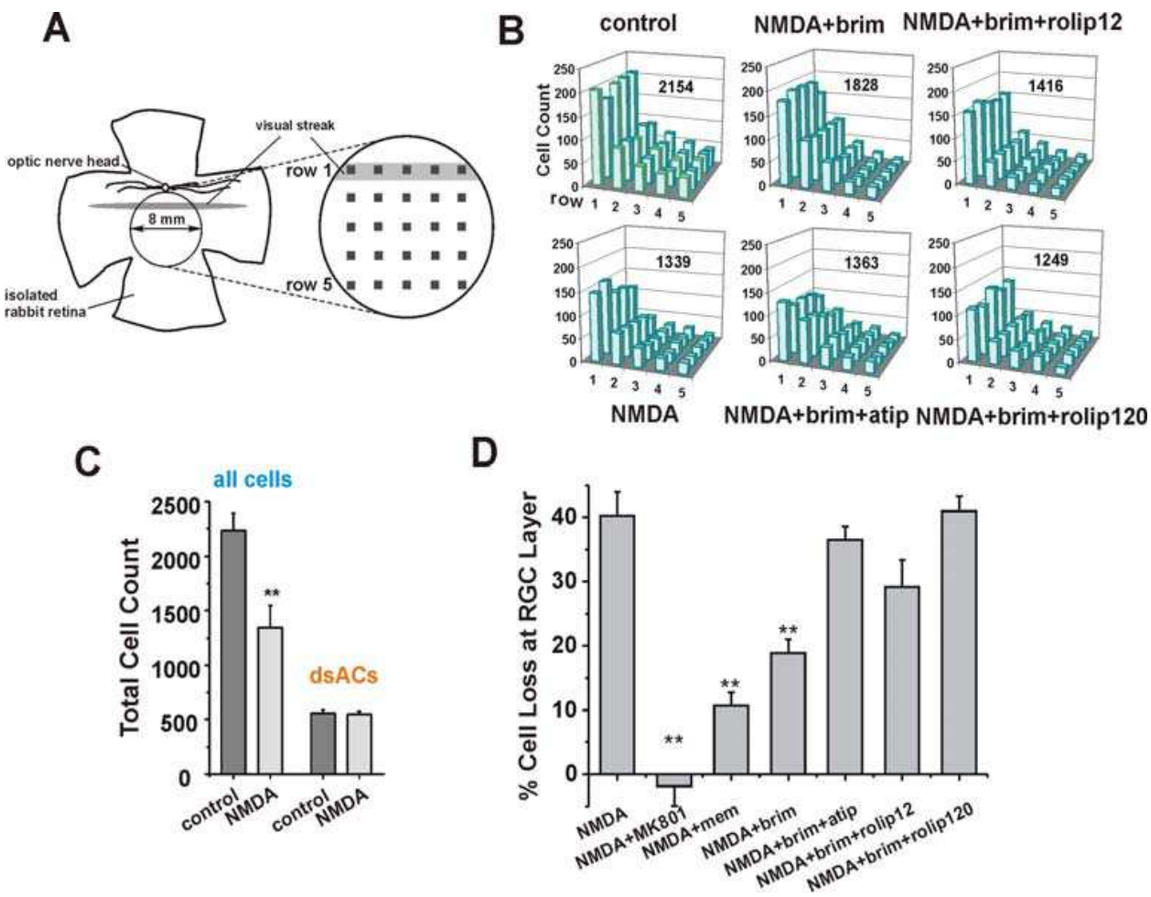

Fig. 11. Brimonidine's protection of RGCs in the rabbit retinal NMDA model is mediated by the $G_{\alpha i}$–AC–cAMP signaling pathway coupled to the α2 receptor. See the text for details

To test this *directly*, we used the rabbit retinal NMDA excitotoxicity model described in section 3 above (Fig. 3). We counted the total number of all neurons in the ganglion cell layer at 25 sites (Fig. 11A) at 2 weeks following intravitreal application of NMDA alone or in combination with other tool agents (Fig. 11D). These tool agents were applied intravitreally 3 times: 1 hour prior to NMDA injection, co-injected with NMDA, and 24 hours following NMDA injection (See Dong et al., 2008 for details). In the rabbit retinal ganglion cell layer, approximately two-thirds of the neurons are RGCs and the remaining one-third are displaced amacrine cells, predominantly displaced starburst amacrine cells (dsACs, 85% of all displace amacrine cells, Vaney, 1984; Vaney et al., 1981). These dsACs can be selectively labeled with a very low dose of DAPI (4',6-diamidino-2-phenylindole, a fluorescent nuclear dye, Vaney et al., 1981; Dong et al., 2010). We found that these dsACs are resistant to NMDA excitotoxicity: the same intravitreal dose of NMDA (3.6 μmol) that produced a substantial cell loss at ganglion cell layer had no effect on dsACs (Fig. 11C), indicating a selective vulnerability of RGCs to NMDA receptor mediated excitotoxicity.

RGC loss produced by intravitreal injection of NMDA is caused by excessive activation of NMDA receptors on RGCs since it can be completely blocked by MK801 (Fig. 11D), a potent and selective NMDA receptor channel blocker (Thompson et al., 1990). RGC loss can also be significantly attenuated by memantine (a less potent, but safer NMDA channel blocker, Fig. 11D). Pretreatment with brimonidine produced a significant protection of RGCs against NMDA excitotoxicity. This protective effect was completely blocked by co-pretreatment with the α2 receptor antagonist atipamezole (Fig. 11D), verifying that brimonidine's protection of RGCs against NMDA excitotoxicity is mediated by the α2 receptor. The PDE-4 inhibitor, rolipram, also blocked brimonidine's effect in a dose-dependent manner at 12 and 120 nmol (Fig. 11D), indicating that this effect is mediated by the $G_{\alpha i}$–AC–cAMP signaling pathway coupled to the α2 receptor (Fig. 4).

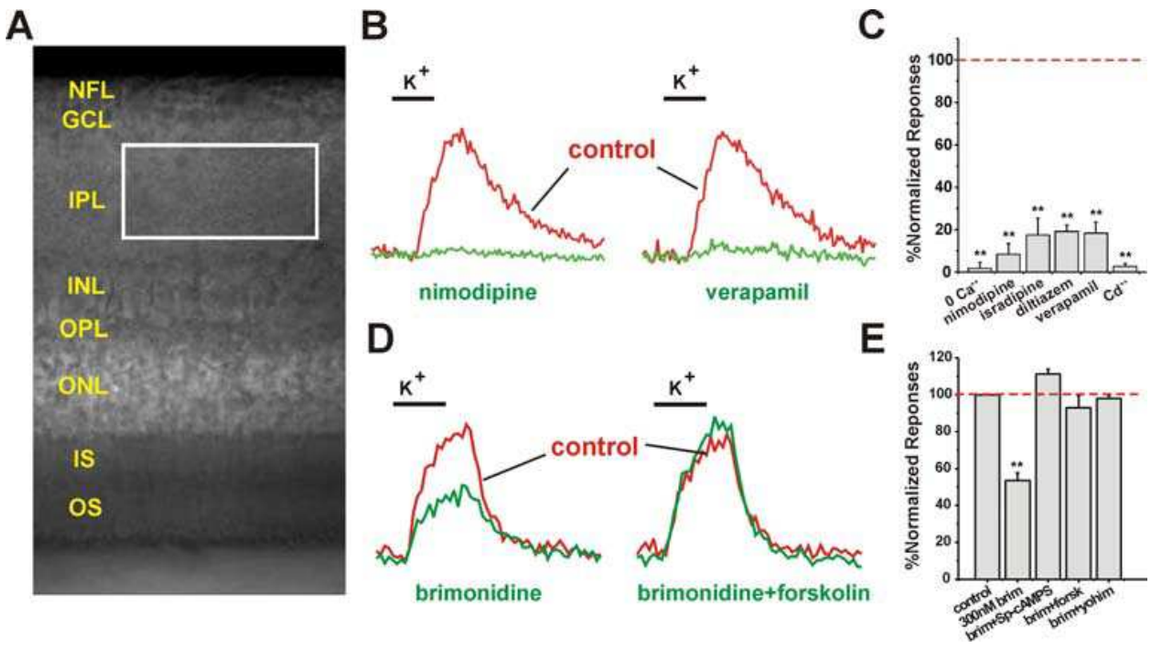

Fig. 12. Brimonidine modulation of L-type Ca^{++} channel function at IPL. See the text for details

We have shown with *in situ* RGCs that brimonidine modulates NMDA receptor function through the α2 receptor coupled $G_{\alpha i}$–AC–cAMP signaling pathway (Dong et al, 2008; see section 5 above). We have also shown that brimonidine protects RGCs through the same $G_{\alpha i}$–AC–cAMP signaling pathway in both experimental glaucoma and retinal NMDA excitotoxicity models (Figs. 10 and 11). Together, our *ex vivo* and *in vivo* data suggest strongly that brimonidine modulation of NMDA receptor function is a major mechanism of RGC protection in experimental glaucoma.

7. Modulation of retinal L-type Ca^{++} channel function by brimonidine at IPL

In addition to modulation of NMDA receptor function postsynaptically in RGCs, brimonidine was also found to modulate the function of voltage-gated Ca^{++} channel at IPL (Dong et al., 2007), a major retinal synaptic layer where communication between RGCs and their presynaptic partners, such as bipolar cells, takes place. In the most regions of CNS, release of neurotransmitters are mediated by voltage-gated N- and P/Q types of Ca^{++} channels (Reid et al., 2003). However, in the retina the L-type Ca^{++} channel plays a dominant role in transmitter release, particularly at photoreceptor and bipolar cell synaptic terminals where glutamate is released (Morgans et al., 2005; Pan, 2000, 2001; Tachibana et al., 1993).

Using several commonly used L-type Ca^{++} channel blockers, we demonstrated that the depolarization (using a high K+ Ringer) induced Ca^{++} signals at IPL were mediated predominantly by the L-type Ca^{++} channel (Dong et al., 2010; see also Fig. 12B & 12C). We also showed that brimonidine down modulated this L-type channel mediated Ca^{++} signal (Dong et al., 2007; see also Fig. 12D). Brimonidine's effect was blocked by Sp-cAMPS, forskolin, and yohimbine (a selective α2 antagonist), indicating that the effect is also mediated by the α2 receptor coupled $G_{\alpha i}$–AC–cAMP signaling pathway (Fig. 12E).

A major contributor to the depolarization induced Ca^{++} signal at IPL is the presynaptic terminals of bipolar cells (Fig. 1C & 1D) where L-type Ca^{++} channels are expressed in high density for glutamate release (Pan, 2000, 2001; Tachibana et al., 1993). We found at the presynaptic terminals from individual *in situ* bipolar cells (Fig. 1C) that the Ca^{++} signal induced by a depolarization voltage step applied through a patch-clamp electrode (Fig. 1D) was also modulated by brimonidine (unpublished observation). Thus, together our results suggest that preventing presynaptic glutamate overrelease by brimonidine is likely an additional neural mechanism contributing to brimonidine's protection of RGCs in experimental glaucoma and acute retinal ischemia. It is also consistent with the observation that brimonidine application reduced vitreal glutamate concentration in acute retinal ischemia (Donello et al., 2001).

8. Other neuroprotective effects by brimonidine

In addition to preventing RGC Ca^{++} overload by modulating activities of both voltage-gated (Fig. 12) and ligand-gated (Fig. 5) Ca^{++} channels that are pre- and post-synaptic to RGCs, brimonidine can also up-regulate survival factors/pathways in the retina. For example, in acute retinal ischemia, brimonidine's neuroprotection is associated with up-regulation of basic fibroblast growth factor, bcl-2, bcl-xl, as well as activation of the PI3 kinase/protein kinase B (Akt) and extracellular-signal-regulated kinase (ERK) pathways (Lai et al., 2002). We believe that some of these beneficial effects of brimonidine may be related to its modulation of NMDA receptor function (Fig. 5). For example, increased expression of bcl-2

and decrease expression of Bax (a proapoptosis member in the bcl-2 family) is associated with blockage of the NMDA receptor by memantine in a mouse glaucoma model (DBA/2J, Ju et al., 2009).

In experimental glaucoma (Lambert et al., 2011) and acute retinal ischemia (López-Herrera et al., 2002), brimonidine also preserves retrograde and anterograde axonal transport in RGCs. This brimonidine's effect may be related to its action on preventing intracellular Ca^{++} overload in RGCs via modulation of NMDA receptor function (Fig. 5), since a healthy soma and unimpaired mitochondrial function are required to provide energy needed for effective transport. It is well established that mitochondria dysfunction caused by NMDA receptor mediated Ca^{++} overload plays a central role in neuronal cell death in disease states (Pivovarova & Andrews, 2010; Stout et al., 1998). Indeed, in experimental glaucoma, it has been shown recently that RGC injury is associated with NMDA receptor mediated mitochondrial dysfunction and can be prevented by NMDA receptor blockade with memantine (Ju et al., 2009).

9. Conclusion

Neuronal Ca^{++} dysregulation, particularly Ca^{++} overload caused by excessive activation of the NMDA receptor and voltage-gated Ca^{++} channels, is an important common final pathway leading to neural dysfunction/death in a wide range of CNS neurodegenerative diseases (Bezprozvanny, 2009).

Our *ex vivo* and *in vivo* findings have provided strong evidence that functional modulation of the NMDA receptor (Fig. 4) and the L-type Ca^{++} channel (Fig. 12) in the retina are two key mechanisms through which brimonidine protects RGCs in animal models of glaucoma and retinal excitotoxicity. Brimonidine also upregulates pro-survival molecules and pathways (Lai et al., 2002). These mechanisms may contribute to brimonidine's IOP-independent preservation of visual function in human glaucoma (also a CNS neurodegenerative disease) observed in a recent randomized, double-masked, multicenter clinical trial (Krupin at al., 2011).

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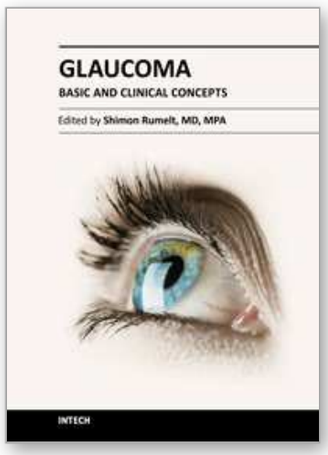

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