

# Structural and functional neuroprotection in glaucoma: role of galantamine-mediated activation of muscarinic acetylcholine receptors

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Glaucoma is the leading cause of irreversible blindness worldwide. Loss of vision due to glaucoma is caused by the selective death of retinal ganglion cells (RGCs). Treatments for glaucoma, limited to drugs or surgery to lower intraocular pressure (IOP), are insufficient. Therefore, a pressing medical need exists for more effective therapies to prevent vision loss in glaucoma patients. In this *in vivo* study, we demonstrate that systemic administration of galantamine, an acetylcholinesterase inhibitor, promotes protection of RGC soma and axons in a rat glaucoma model. Functional deficits caused by high IOP, assessed by recording visual evoked potentials from the superior colliculus, were improved by galantamine. These effects were not related to a reduction in IOP because galantamine did not change the pressure in glaucomatous eyes and it promoted neuronal survival after optic nerve axotomy, a pressure-independent model of RGC death. Importantly, we demonstrate that galantamine-induced ganglion cell survival occurred by activation of types M1 and M4 muscarinic acetylcholine receptors, while nicotinic receptors were not involved. These data provide the first evidence of the clinical potential of galantamine as neuroprotectant for glaucoma and other optic neuropathies, and identify muscarinic receptors as potential therapeutic targets for preventing vision loss in these blinding diseases.

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Glaucoma is the leading cause of irreversible blindness worldwide. It has been estimated that >50 million people around the world are affected by this disease, with >7 million presenting bilateral blindness.<sup>1</sup> Loss of vision due to glaucoma is caused by the selective death of retinal ganglion cells (RGCs), the output neurons that relay visual information from the retina to the brain through their axons in the optic nerve. Although the precise cause of RGC death in glaucoma is unknown, high intraocular pressure (IOP) is a major risk factor for developing this disease. Current treatments for glaucoma are limited to lowering IOP by medication or surgery, but a significant number of patients continue to experience visual loss despite responding well to pressure lowering therapies. Moreover, >50% of patients have normal tension glaucoma characterized by optic nerve degeneration in the absence of high IOP.<sup>2</sup> Therefore, current therapies for glaucoma are insufficient and novel strategies to save RGCs and prevent vision loss would be valuable.

Galantamine is a small molecule acetylcholinesterase (AChE) inhibitor and allosteric ligand of nicotinic ACh

receptors (nAChR) currently used for the symptomatic treatment of Alzheimer's disease. Galantamine was initially thought to ameliorate cognitive deficits in Alzheimer's disease patients only due to its cholinergic boosting activity, but recent studies have shown that it also has neuroprotective effects. *In vitro*, galantamine protects cortical neurons from  $\beta$ -amyloid toxicity and motor neurons from excess glutamate.<sup>3,4</sup> *In vivo*, galantamine improves the survival of hippocampal neurons following transient global ischemia and of dopamine neurons damaged by 6-OHDA.<sup>5,6</sup> Pharmacological antagonists of nAChR have been shown to partially reduce the neuroprotective effect of galantamine against  $\beta$ -amyloid toxicity in culture,<sup>3,7</sup> suggesting that nAChR may be involved in galantamine-induced cell survival. However, the precise mechanisms underlying neuroprotection mediated by galantamine *in vivo* remain poorly defined.

In this study, we examined the role of galantamine in the visual system and investigated whether it stimulates RGC survival in a rat glaucoma model. Our data demonstrate that galantamine leads to structural protection of RGCs from

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**Abbreviations:** RGCs, retinal ganglion cells; IOP, intraocular pressure; ACh, acetylcholine; AChE, acetylcholinesterase; nAChR, nicotinic ACh receptors; mAChR, muscarinic ACh receptors; OHT, ocular hypertension; i.p., intraperitoneal; VEPs, visual evoked potentials; ERG, electroretinogram; PBS, phosphate-buffered saline; Dil, (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; MLA, methyllycaconitine; DH $\beta$ -E, dihydro- $\beta$ -erythroidine hydrobromide; MMA, mecamlamine

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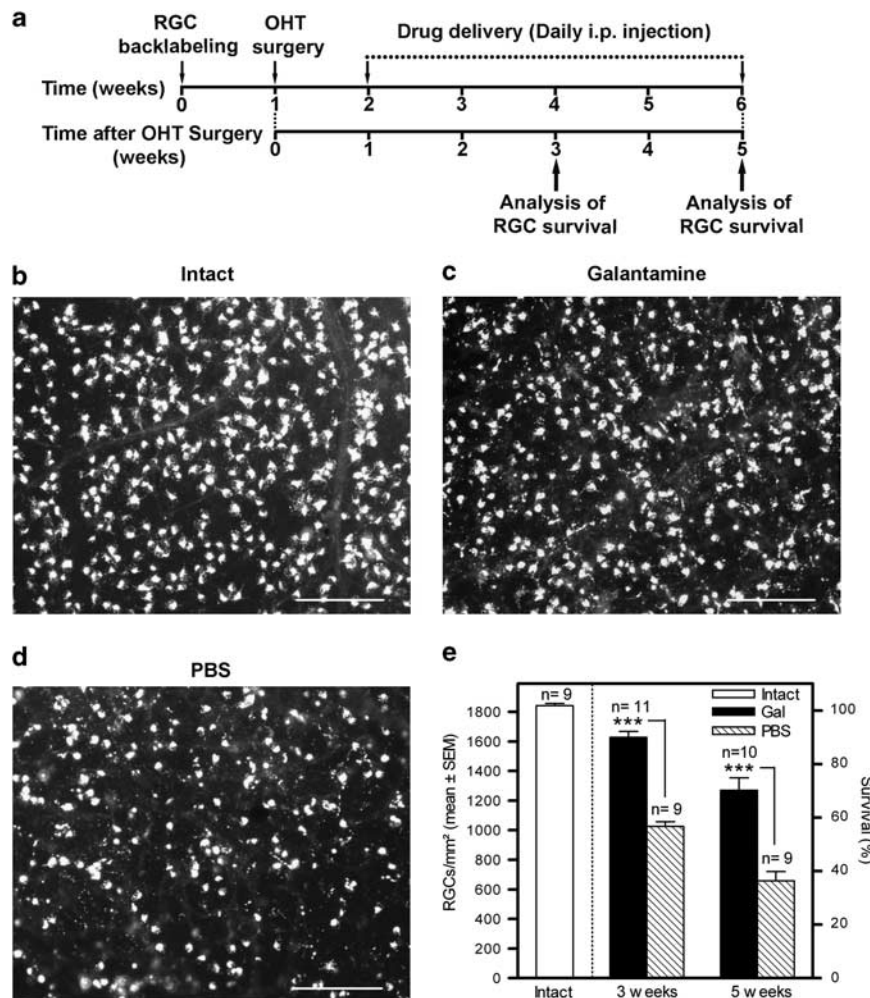
ocular hypertension (OHT) damage. We show that the profound functional impairments caused by high IOP are markedly attenuated in galantamine-treated eyes. Intriguingly, galantamine-induced RGC neuroprotection is mediated through activation of muscarinic ACh receptors (mAChR), and is independent of nAChR. Our study provides the first evidence of the therapeutic potential of galantamine in glaucoma and reveals mAChR as a potential clinical target for this neurodegenerative disease.

## Results

**Galantamine protects RGC soma and axons from hypertension-induced death.** We tested the neuroprotective effect of galantamine *in vivo* in a rat OHT model of glaucoma. Unilateral elevation of IOP was induced after a

single injection of hypertonic solution into an episcleral vein, a procedure named OHT surgery. Gradual increase in eye pressure and progressive death of RGCs was observed in this model, with an excellent linear correlation between IOP increase and degree of RGC loss and optic nerve damage.<sup>8</sup> Inner retinal atrophy, optic nerve degeneration and optic nerve head remodeling in this model are similar to those seen in human glaucoma, making this model one of the best experimental *in vivo* paradigms to study glaucoma.

RGCs were visualized with the fluorescent tracer Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate), which was applied to the superior colliculus at least 1 week before OHT surgery to ensure retrograde labeling before any changes in optic nerve function caused by experimental glaucoma (Figure 1a). Unlike other retrograde markers that leak from the cell body after several weeks, Dil has been shown to persist in RGCs *in vivo* for periods of

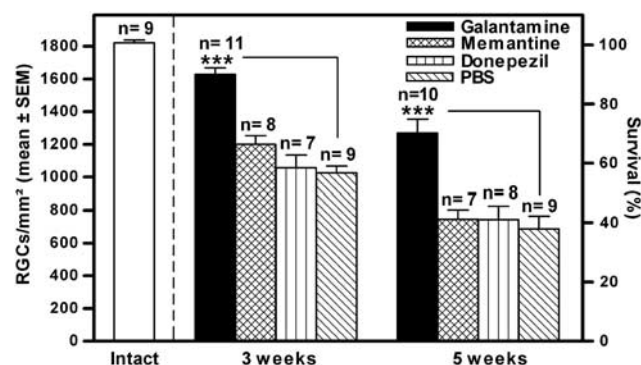


**Figure 1** Galantamine protects RGC soma in glaucoma. (a) Outline of the experimental protocol used to test the effect of galantamine on RGC survival in experimental glaucoma. RGCs were retrogradely labeled with the fluorescent tracer Dil, and ocular hypertension (OHT) surgery was performed a week later. Galantamine treatment (3.5 mg/kg, i.p.) was initiated at ~1 week after OHT surgery, and continued thereafter for the entire duration of the experiment. Retinas and optic nerves were examined at 3 and 5 weeks following OHT surgery. (b) Dil-labeled RGCs in a flat mount preparation from intact, uninjured Brown-Norway rat retina. At 5 weeks after OHT surgery, galantamine treatment (c) led to higher neuronal densities and better preservation of cellular integrity compared with eyes treated with PBS (d). (e) Quantitative analysis of RGC survival in experimental glaucoma after treatment with galantamine (solid bars) or vehicle (PBS, hatched bars) ( $n = 9-11$  rats per group). The density of RGCs in intact, untreated Brown-Norway rat retinas (open bars) is shown as reference. Galantamine markedly increased the number of RGCs that survived at 3 and 5 weeks after OHT surgery (ANOVA,  $***P < 0.001$ ). Data are expressed as the mean  $\pm$  S.E.M. Scale bars (b-d): 100  $\mu$ m

up to 9 months without fading or leakage.<sup>9</sup> Consistent with previous studies, the average total RGC population detected by Dil in intact, non-injured Brown-Norway rat retinas was  $1841 \pm 15$  RGCs/mm<sup>2</sup> (mean  $\pm$  S.E.M.,  $n=9$ ) (Figures 1b and e). Galantamine is a small molecule capable of crossing the blood–brain and blood–retinal barriers; therefore, its neuroprotective effect was evaluated after daily intraperitoneal (i.p.) injection of 3.5 mg per kg of body weight (mg/kg), a dose selected based on previous studies showing efficacy *in vivo*.<sup>5,6</sup> Galantamine treatment was initiated once IOP elevation was detected ( $\sim 1$  week after OHT surgery, Figure 1a) and continued thereafter for the entire duration of the experiment.

Analysis of Dil-positive RGCs in retinal whole mounts showed that galantamine led to higher neuronal densities and better preservation of cellular integrity in glaucomatous eyes compared with control eyes treated with vehicle (phosphate-buffered saline, PBS) (Figure 1b–d). Quantitative analysis confirmed that daily galantamine treatment led to a robust increase in survival of injured RGCs at 3 weeks after OHT surgery (90%:  $1627 \pm 29$  RGCs/mm<sup>2</sup>, mean  $\pm$  S.E.M.,  $n=11$ ) compared with vehicle (55%:  $1025 \pm 22$  RGCs/mm<sup>2</sup>,  $n=9$ ) (one-way analysis of variance (ANOVA),  $P < 0.001$ ). Although neuronal damage was more severe at 5 weeks after OHT surgery, galantamine still protected 70% of RGC soma ( $1270 \pm 74$  RGCs/mm<sup>2</sup>,  $n=10$ ) compared with only 37% with PBS ( $657 \pm 52$  RGCs/mm<sup>2</sup>,  $n=9$ ) (ANOVA,  $P < 0.001$ ) (Figure 1e). We carried out a comparative study on the neuroprotective effect of galantamine with respect to memantine, an *N*-methyl-D-aspartic acid channel blocker, and donepezil, another AChE inhibitor, both currently used in Alzheimer's disease. Our results show that galantamine was more effective than memantine or donepezil at preventing RGC loss in experimental glaucoma (Figure 2).

Glaucoma is characterized by the degeneration of RGC axons in the optic nerve posterior to the lamina cribrosa; hence, we also investigated the effect of galantamine on RGC axon protection. Analysis of optic nerve cross-sections demonstrated a larger number of RGC axon fibers with normal morphology in galantamine-treated eyes compared with PBS-treated control eyes (Figure 3a–c), the latter showing



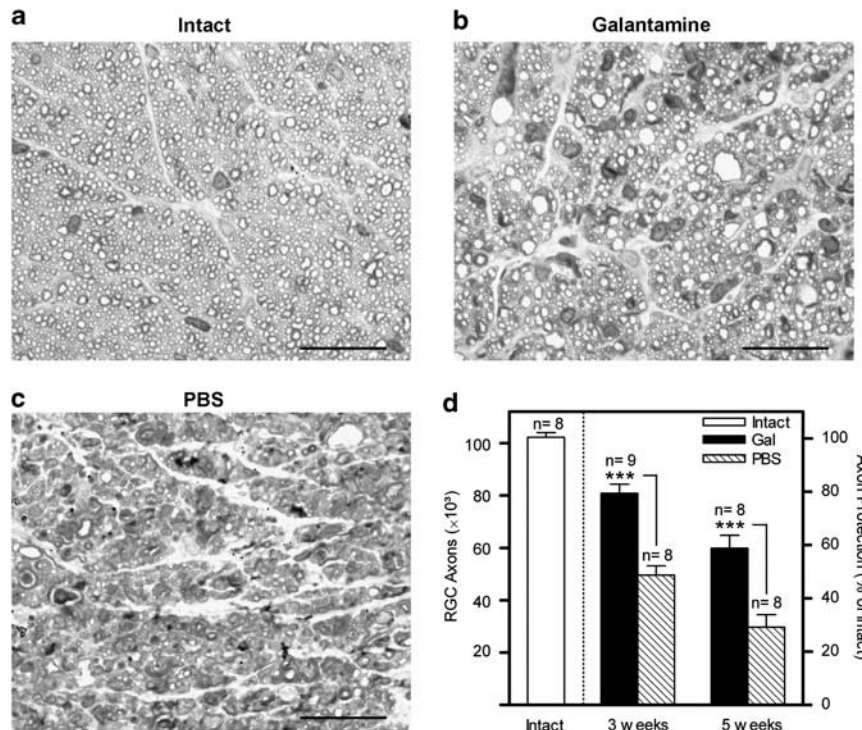
**Figure 2** Comparative analysis of RGC survival in experimental glaucoma mediated by galantamine (solid bars), memantine (crossed hatched bars), donepezil (vertical lines) or vehicle (PBS, hatched bars) at 3 and 5 weeks after OHT ( $n=7-11$  rats per group) (ANOVA, \*\*\* $P < 0.001$ ). Data are expressed as the mean  $\pm$  S.E.M.

extensive axon degeneration including disarray of fascicular organization and degradation of myelin sheaths. Quantitative analysis confirmed that galantamine promoted substantial protection of RGC axons from glaucomatous damage (Figure 3d). Collectively, these results indicate that galantamine effectively protects both RGC soma and axons in experimental glaucoma.

**Galantamine-mediated neuroprotection is not due to decreased IOP.** To investigate if daily treatment with galantamine led to IOP reduction, which could account for the observed neuroprotective effect, we measured eye pressure daily for 2 weeks after OHT surgery and then every other day for the entire duration of the experiment. Our results demonstrate that daily i.p. administration of galantamine did not reduce IOP over a period of several weeks (Figure 4a). The mean sustained pressure elevation among galantamine-treated and PBS-treated groups was similar:  $\sim 34$  mm Hg at 3 weeks after OHT surgery and  $\sim 40$  mm Hg at 5 weeks after OHT surgery (Table 1), well within the range of IOP increases observed in this model.<sup>8</sup> Given that the rate of RGC death is proportional to IOP, the similar elevation in IOP among groups allowed for reliable comparison of the neuroprotective effect of galantamine *versus* vehicle.

To further test whether galantamine-mediated neuroprotection was independent of IOP-induced damage, we examined the effect of galantamine after axotomy of the optic nerve (Figure 4b–e), an acute insult that leads to rapid apoptotic death of RGCs. RGCs were retrogradely labeled with Fluorogold and subjected to optic nerve transection with concomitant intraocular injection of galantamine. The average total RGC population detected with Fluorogold in intact, non-injured Sprague–Dawley rat retinas was  $2223 \pm 24$  RGCs/mm<sup>2</sup> (mean  $\pm$  S.E.M.,  $n=5$ ), a slightly higher density than in Brown-Norway rats, consistent with previous studies.<sup>10</sup> After axotomy, all RGCs survive for 5 days and then die abruptly: the population of RGCs is reduced to  $\sim 50\%$  by 1 week and to  $\sim 10\%$  at 2 weeks after axotomy. In galantamine-treated eyes, 75% of RGCs survived at 1 week after axotomy compared with 50% that survived in the PBS-treated group ( $1611 \pm 47$  RGCs/mm<sup>2</sup>,  $n=6$  and  $920 \pm 32$  RGCs/mm<sup>2</sup>,  $n=5$ , respectively, ANOVA,  $P < 0.001$ ). The effect of galantamine was still marked at 2 weeks after axotomy: 30% of all RGCs remained alive compared with only 10% survival in PBS-treated eyes ( $616 \pm 30$  RGCs/mm<sup>2</sup>,  $n=5$  and  $216 \pm 15$  RGCs/mm<sup>2</sup>,  $n=5$ , respectively, ANOVA,  $P < 0.01$ ) (Figure 4e). Collectively, these results demonstrate that galantamine can delay RGC loss after chronic (glaucoma) or acute (axotomy) optic nerve injury.

**RGC functional deficits in glaucoma are improved by galantamine.** Our results show structural protection of RGCs, both at the level of the cell bodies and axons, but are these neurons functional? To investigate whether galantamine preserved RGC function in glaucoma, we measured visual evoked potentials (VEPs) after flash stimulation. The main target of RGCs in the rat brain is the superior colliculus, thus VEPs recorded from this region provide a faithful representation of surviving RGC function and were used as the primary outcome to assess functional neuroprotection.



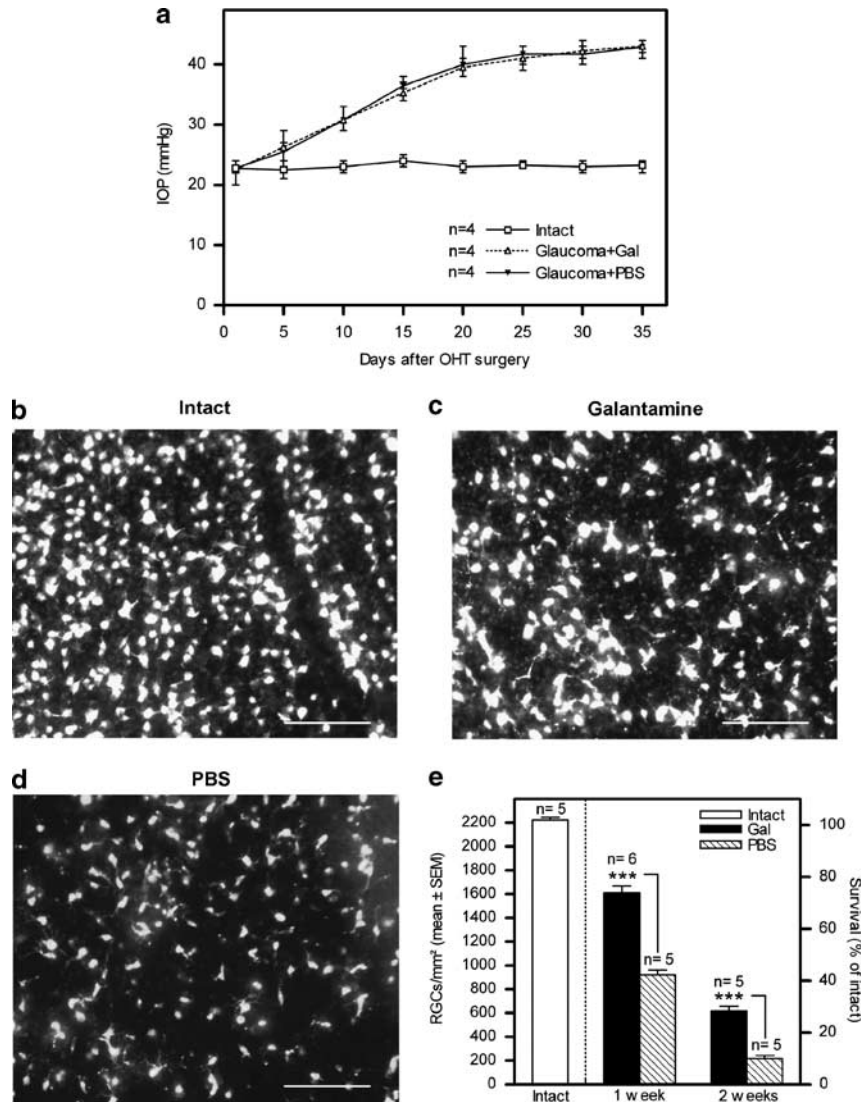
**Figure 3** Galantamine protects RGC axons in glaucoma. Cross-sections of optic nerve segments from intact (a) and glaucomatous eyes treated with galantamine (b) or PBS (c) at 5 weeks after ocular hypertension (OHT) surgery. Galantamine-treated eyes displayed a larger number of axonal fibers with normal morphology compared with PBS-treated control eyes, which showed extensive axon degeneration. (d) Quantitative analysis of RGC axons in the optic nerve following daily i.p. injection of galantamine (solid bar) or PBS (hatched bar) ( $n=8-9$  rats per group) (ANOVA, \*\*\* $P<0.001$ ). The number of axons in the intact, uninjured Brown-Norway rat optic nerve is shown as reference (open bar). Data are expressed as the mean  $\pm$  S.E.M. Scale bars (a-c): 20  $\mu$ m

The flash electroretinogram (ERG) provides information about the outer retina and was not used to assess RGC function. ERGs were routinely performed before VEPs to ensure that the outer retina was functioning properly and that RGCs received adequate input after visual stimulation. All the animals in this study had normal ERGs.

First, we investigated whether galantamine daily treatment had any effect on the VEP response to flash stimulation in normal, non-glaucomatous eyes. Our data demonstrate that VEPs recorded from galantamine-treated normal brains were indistinguishable from those treated with PBS or without treatment (Figure 5a), indicating that galantamine by itself does not alter the response of normal RGCs or target neurons in the superior colliculus. At 3 weeks after OHT surgery, examination of VEP responses to flash stimulation showed substantial reduction in evoked currents recorded from PBS-treated eyes compared with intact, non-glaucomatous controls (Figure 5b). In contrast, galantamine administration led to marked preservation of the VEP. Quantification of peak-to-peak VEP amplitudes demonstrated that galantamine preserved 66% of the intact VEP response compared with only 30% in PBS-treated controls (ANOVA,  $P<0.001$ ).

At 5 weeks after OHT surgery, both PBS-treated and galantamine-treated glaucomatous eyes showed complete obliteration of the VEP (Figure 5c). This lack of response could not be solely attributed to RGC degeneration because galantamine protected almost 70% of RGC soma

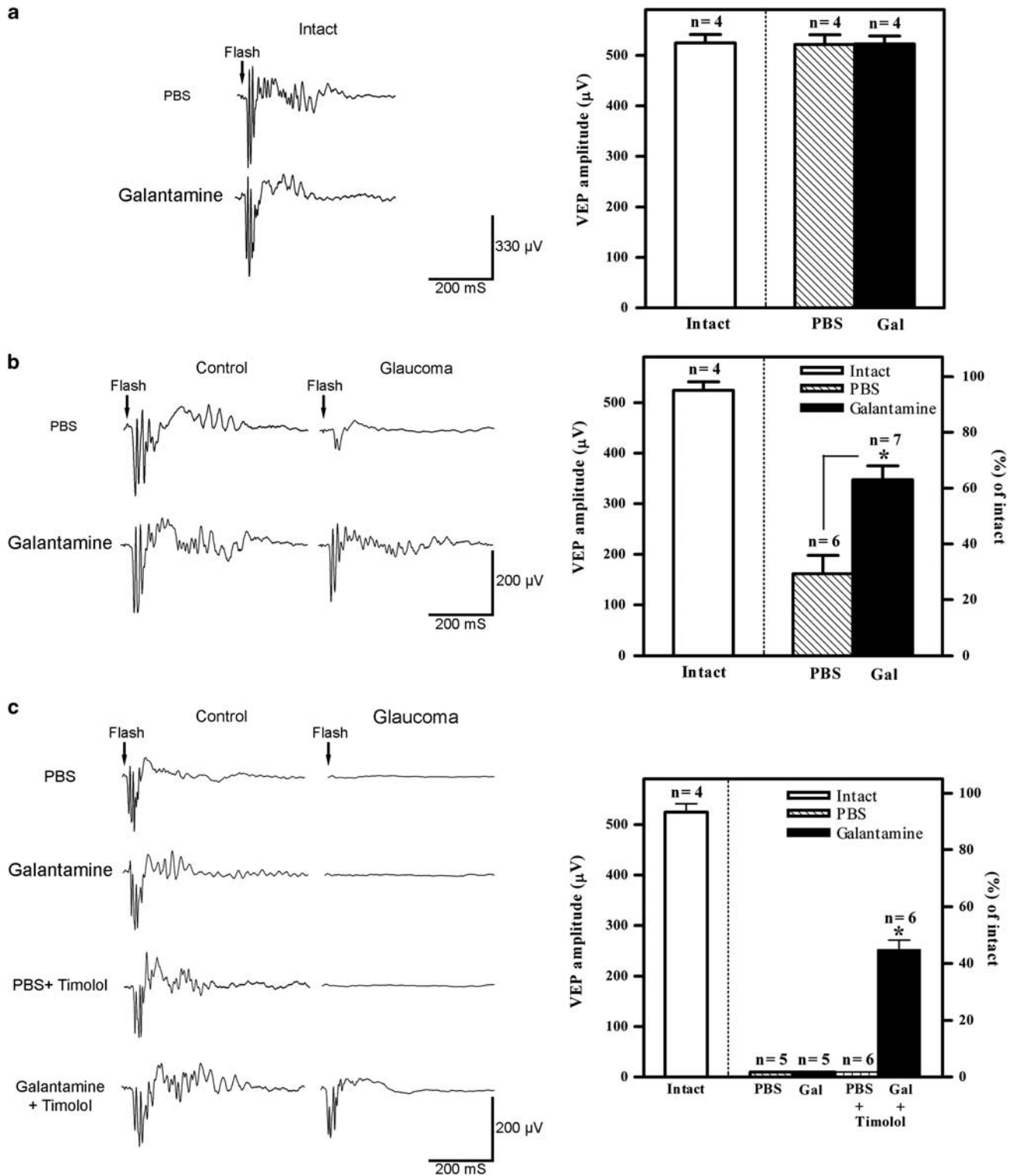
and axons at 5 weeks after OHT (Figures 1 and 3). We then hypothesized that sustained high IOP impairs the visual function of the surviving RGCs. To test this idea, we evaluated the effect of galantamine on the VEP response using a protocol in which IOP in the glaucomatous eye was controlled by topical (corneal) application of timolol, a commonly used  $\beta$ -adrenergic receptor blocker. Treatment with timolol began at 3 weeks after OHT and continued for the entire duration of the experiment. Timolol limited the IOP increase in galantamine-treated glaucomatous eyes to a mean IOP of 35.6 mmHg compared with 42.7 mmHg in eyes without timolol (Table 1). Interestingly, this difference in IOP (7 mmHg) was sufficient to restore 47% of the VEP response in galantamine-treated eyes, but not in PBS-treated eyes (Figure 5c), indicating that IOP reduction only rescued RGC function when combined with galantamine. The recovery of VEP was not due to a neuroprotective effect of timolol because animals treated with PBS and timolol did not show any functional improvement. Furthermore, VEP recovery was not due to increased survival because RGC densities in the presence of timolol and galantamine ( $1387 \pm 50$  RGCs/mm<sup>2</sup>, mean  $\pm$  S.E.M.,  $n=8$ ) were not statistically different from those in eyes treated with galantamine alone ( $1270 \pm 85$  RGCs/mm<sup>2</sup>,  $n=10$ ,  $P>0.05$ ). The absence of VEP responses in eyes treated with PBS also confirmed that topical application of timolol, by itself, was not neuroprotective. Taken together, these results indicate that high IOP leads to dramatic deficits in retinal function that can be markedly attenuated by



**Figure 4** Galantamine-mediated neuroprotection is not due to decreased intraocular pressure. (a) Daily i.p. administration of galantamine did not reduce intraocular pressure (IOP) over a period of several weeks. (b) Fluorogold-labeled RGCs in a flat mount preparation from intact, uninjured Sprague–Dawley rat retina. (c) Galantamine treatment led to marked survival of axotomized RGCs with respect to PBS-treated eyes (d). (e) Quantitative analysis of RGC survival after intraocular injection of galantamine (solid bars) or PBS (hatched bars) ( $n = 5–6$  rats per group) (ANOVA,  $***P < 0.001$ ). The density of RGCs in intact, uninjured Sprague–Dawley rat retinas is shown as reference (open bar). Data are expressed as the mean  $\pm$  S.E.M. Scale bars (a–c): 100  $\mu$ m

**Table 1** Intraocular pressure (IOP) elevation in glaucomatous eyes

Time after OHT surgery	Group	N	Mean IOP (mm Hg)			Peak IOP (mm Hg)	
			Glaucoma	Control	Difference	Glaucoma	Control
3 weeks	Galantamine	27	34.71 $\pm$ 1.8	22.41 $\pm$ 0.2	11.09 $\pm$ 2.4	39.45 $\pm$ 1.4	26.81 $\pm$ 1.3
	Vehicle	23	33.11 $\pm$ 1.6	22.59 $\pm$ 0.3	11.73 $\pm$ 0.3	38.23 $\pm$ 1.2	25.67 $\pm$ 1.6
	No treatment	7	34.11 $\pm$ 0.6	23.09 $\pm$ 0.2	11.02 $\pm$ 0.4	38.41 $\pm$ 0.9	26.25 $\pm$ 1.2
	P-value (ANOVA)		0.6	0.12	—	0.41	0.85
5 weeks	Galantamine	23	42.71 $\pm$ 0.2	23.63 $\pm$ 1.5	17.08 $\pm$ 0.8	49.11 $\pm$ 4.2	25.11 $\pm$ 0.5
	Vehicle	22	39.11 $\pm$ 1.0	23.4 $\pm$ 2.0	16.73 $\pm$ 1.0	48.17 $\pm$ 1.0	26.55 $\pm$ 0.7
	Ga I+ Timolol	6	35.64 $\pm$ 0.8	22.11 $\pm$ 2.2	13.53 $\pm$ 1.4	41.80 $\pm$ 0.8	26.05 $\pm$ 2
	Vehicle+Timolol	6	34.54 $\pm$ 1.6	22.23 $\pm$ 1.3	12.31 $\pm$ 0.5	42.36 $\pm$ 0.5	26.28 $\pm$ 4.2
	No treatment	10	41.35 $\pm$ 0.8	22.80 $\pm$ 1.7	17.55 $\pm$ 1.7	49.65 $\pm$ 0.9	25.08 $\pm$ 0.9
	P-value (ANOVA)		0.45	0.12	—	0.32	0.71



galantamine, and highlight the importance of combining galantamine with IOP-lowering drugs to achieve long-term functional RGC protection.

**ACh muscarinic, but not nicotinic, receptors mediate the neuroprotective effect of galantamine in experimental glaucoma.** In the nervous system, there are two major types of ACh receptors: (i) nicotinic receptors (nAChR) including the  $\alpha 7$  and the  $\alpha 4\beta 2$  nAChR, which are the most abundant subtypes in the brain, and metabotropic mAChRs, which are selectively activated by muscarine-like ligands and include five distinct isoforms (M1–M5) corresponding to the products of five separate genes.<sup>11</sup> To gain mechanistic insight into how galantamine promotes RGC neuroprotection *in vivo*, we asked whether blockade of nAChR or mAChR would compromise galantamine-induced RGC survival. For this purpose, we first assessed the survival of axotomized RGCs after intraocular injection of galantamine in combination with selective pharmacological blockers of nAChR or mAChR. Co-injection of galantamine with scopolamine, an inhibitor of all mAChR types, abrogated the pro-survival effect of galantamine. In contrast, co-administration of galantamine with either methyllycaconitine (MLA), a specific antagonist of  $\alpha 7$  nAChR, dihydro- $\beta$ -erythroidine (DH $\beta$ -E), a specific antagonist of  $\alpha 4\beta 2$  nAChR, or mecamylamine (MMA), a blocker of all neuronal nAChR did not reduce galantamine-induced RGC survival (Figure 6a). A range of concentrations of these nAChR inhibitors was tested (10  $\mu$ M to 10 mM) with similar outcome, indicating that their lack of effect was not the result of suboptimal doses of these drugs. Consistent with these findings, daily i.p. co-injection of galantamine and scopolamine, which readily cross the blood–brain/retinal barrier, completely inhibited RGC neuroprotection in glaucomatous eyes at 5 weeks after OHT (Figure 6b). Administration of MLA, DH $\beta$ -E, MMA or scopolamine, by themselves, did not cause RGC death or adverse effects in non-injured retinas, nor did they promote survival in injured rat retinas at the doses used here (Figure 6c and d).

To establish which mAChRs were involved in RGC survival, the following selective antagonists of mAChR subtypes were co-administered with galantamine: pirenzepine (M1), DX116 (M2), 4-DAMP (M3) or tropicamide (M4). These mAChR antagonists do not cross the blood–brain/retinal barrier or exhibit extremely low-barrier permeability,<sup>12,13</sup> thus their effect was tested on galantamine-induced protection of axotomized RGCs after intraocular injection. Figure 6e shows that while blockade of M2 by DX116 or M3 by 4-DAMP did not have any effect on RGC survival mediated by galantamine, blockade of M1 with pirenzepine completely abrogated RGC neuroprotection. The M4 antagonist tropicamide also reduced RGC survival, albeit to a lesser extent than pirenzepine. Collectively, these data strongly suggest that M1 and M4 mAChR are mediators of galantamine-induced RGC neuroprotection.

## Discussion

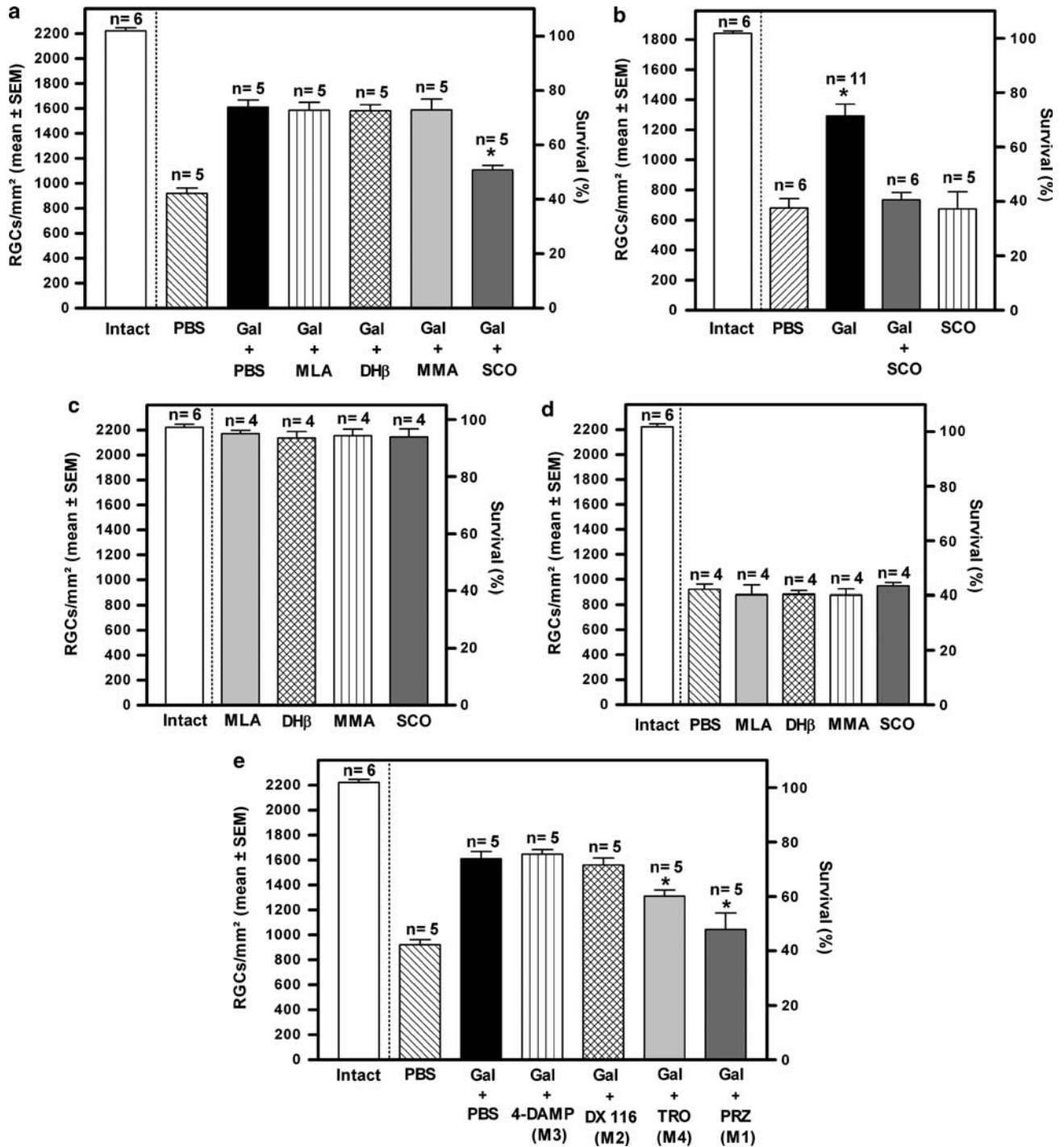
This study supports four major findings. First, galantamine treatment leads to survival of RGC soma and axons in

experimental glaucoma. Second, galantamine-mediated RGC structural protection is independent of IOP-induced damage, as evidenced by the neuroprotective action of this drug after optic nerve axotomy. Third, functional deficits caused by high IOP are markedly improved by galantamine. Fourth, galantamine-mediated neuroprotection occurs primarily through activation of retinal mAChR M1, and is independent of nAChR.

Several recent clinical studies have suggested a correlation between glaucoma and Alzheimer's disease,<sup>14</sup> but the most compelling evidence supporting such link stems from laboratory work. For example, neuronal loss in both glaucoma and Alzheimer's disease occurs by apoptosis,<sup>15</sup> caspases are activated both in Alzheimer's disease and in injured RGCs,<sup>16</sup> and intraocular injection of  $\beta$ -amyloid has been shown to induce RGC degeneration.<sup>17</sup> More recently,  $\beta$ -amyloid deposition was associated with RGC death in experimental glaucoma and blockade of the  $\beta$ -amyloid pathway reduced glaucomatous damage.<sup>18</sup> Although the etiology of glaucoma and Alzheimer's disease may differ, their common features raise the provocative idea that drugs currently used to treat Alzheimer's disease may also have utility in glaucoma. Here, we show that one such drug, galantamine, is a powerful neuroprotectant for injured RGCs. Daily galantamine treatment promoted the survival of RGC soma and axons in glaucoma. Importantly, administration of galantamine by intravitreal injection also led to robust RGC protection after axotomy of the optic nerve. These data highlight several important properties of galantamine: it is effective when administered systemically or by intraocular injection, it promotes structural protection of RGCs in an IOP-independent manner and it delays RGC loss in different models, both acute and chronic, of optic nerve damage.

The neuroprotective effect of galantamine was superior to that conferred by memantine or donepezil. Galantamine has been shown to be a weaker AChE inhibitor than donepezil,<sup>19</sup> therefore other factors likely account for this difference in neuroprotective efficacy. First, donepezil is a non-competitive inhibitor of AChE, which may result in the development of tolerance to donepezil and consequent downregulation of ACh receptors.<sup>20</sup> In contrast, galantamine is a competitive AChE inhibitor and the galantamine–AChE complex follows the typical kinetics of reversible inhibitors, dissociating readily in the presence of excess ACh, with a reduced potential for tolerance.<sup>21</sup> Second, galantamine acts more broadly on other neurotransmitter systems and has been shown to regulate the release of glutamate, serotonin and  $\gamma$ -aminobutyric acid,<sup>22,23</sup> thus potentially modulating neural activity and delaying neurodegeneration. Third, mAChR are amenable to modulation at allosteric sites;<sup>24</sup> hence, it is possible that galantamine may activate mAChR directly, although this possibility presently remains unknown.

Patients with glaucoma experience diminished visual function and poor quality of life; therefore, an ideal neuroprotective drug should preserve the structural viability of RGCs while retaining their ability to respond to visual stimulation. In this study, we aimed at providing a structure–function link based on the neuroprotective effect of galantamine. Our results show that there are major visual deficits in glaucomatous eyes treated with PBS, whereas galantamine treatment



**Figure 6** The neuroprotective effect of galantamine in glaucoma is mediated by activation of ACh muscarinic receptors. (a) Co-injection of galantamine with scopolamine, an inhibitor of all mAChR types, abrogated the prosurvival effect of galantamine. In contrast, the  $\alpha 7$  nAChR antagonist methyllycaconitine (MLA), the  $\alpha 4\beta 2$  nAChR antagonist dihydro- $\beta$ -erythroidine (DH $\beta$ -E) or the antagonist of all nAChR mecamylamine (MMA) did not reduce galantamine-induced survival of axotomized RGCs (ANOVA, \* $P < 0.001$ ). (b) Co-administration of galantamine and scopolamine (SCO) completely inhibited galantamine-induced RGC neuroprotection in glaucomatous eyes at 5 weeks after OHT (ANOVA, \* $P < 0.001$ ). Administration of MLA, DH $\beta$ -E, MMA or SCO, by themselves, did not cause RGC death or adverse effects in non-injured retinas (c), nor did they promote survival in injured rat retinas (d) ( $n = 3-6$ /group). (e) Injection of galantamine in combination with the M1 mAChR blocker pirenzepine (PRZ), the M2 mAChR antagonist DX116, the M3 mAChR blocker 4-DAMP or the M4 mAChR antagonist tropicamide (TRO), showed that galantamine-induced neuroprotection is mediated through activation of M1 and M4 mAChR (ANOVA, \* $P < 0.001$ )

led to substantial preservation of the VEP amplitude at 3 weeks after OHT. Of interest, following longer periods of OHT (5 weeks) galantamine-protected RGCs (70%) did not

respond to light stimulation unless IOP was also reduced. An IOP decrease of just a few mm Hg was sufficient to restore almost 50% of the VEP response in galantamine-treated



eyes, but not in PBS-treated controls. The observation that the majority of RGCs exposed to galantamine remained alive at 5 weeks of OHT, but did not respond to light stimulation, suggests that sustained high IOP has additional deleterious effects on RGC function. We conclude that, in the long-term, structural protection alone is not sufficient to restore visual function unless IOP is also controlled.

Galantamine increases the availability of ACh through its inhibitory action on AChE, the enzyme responsible for ACh breakdown, and it is also an allosteric modulator of nAChR enhancing their sensitivity to ACh.<sup>25</sup> ACh in the retina is released by starburst cholinergic amacrine cells onto RGC dendrites and has a crucial role in visual information processing.<sup>26</sup> Therefore, we postulated that galantamine-induced neuroprotection might result from stimulation of ACh receptors. As galantamine is an allosteric modulator of nAChR, its neuroprotective effect has been compared with that of nicotine. In fact, nicotine has been shown to promote neuronal survival in different models of neurodegeneration through nAChR and downstream activation of survival pathways.<sup>27</sup> Previous *in vitro* studies showed that galantamine promoted the survival of cortical neurons or neuroblastoma cells by  $\alpha 7$  nAChR and stimulation of phosphatidylinositol-3-kinase (PI3K).<sup>3,7</sup> As RGCs express several nAChR subtypes including  $\alpha 7$  nAChR,<sup>28</sup> we initially postulated that nAChR activation would contribute to galantamine-mediated neuroprotection. Surprisingly, our data show that the blockade of nAChR had no effect, whereas inhibition of mAChR completely curtailed the neuroprotective effect of galantamine *in vivo*. The total blockade of galantamine-induced neuroprotection in the presence of mAChR inhibitors indicates that these receptors are the primary locus of the specific action of galantamine in the retina.

Immunocytochemical studies on the localization of mAChR subtypes in primate, rat and chick retinas showed that M2 and M4 are expressed by amacrine cells, and M3 is expressed primarily by bipolar cells.<sup>29–31</sup> In addition, Müller cells, the most abundant glial cell type in the mammalian retina, express M1 and M4 mAChR types.<sup>32</sup> Muscarine was shown to increase intracellular  $Ca^{+2}$  in rabbit RGCs,<sup>33</sup> however, this effect was thought to be indirect because expression of mAChR has not been detected in isolated rat or cat RGCs, and muscarine did not elicit membrane currents measured in whole-cell patch clamp preparations.<sup>34</sup> Our results indicate that galantamine-mediated RGC neuroprotection *in vivo* occurs primarily by activation of M1, a mAChR subtype expressed by Müller cells. The M4 mAChR subtype, expressed by both Müller glia and amacrine cells, also contributes to this effect but to a lesser extent than M1 mAChR. Collectively, these data support a model in which non-cell-autonomous signaling events downstream of mAChR have a major role in galantamine-induced RGC neuroprotection. Activation of M1/M4 mAChR on neighboring Müller glia and amacrine cells may lead to stimulation of signaling pathways and production of prosurvival factors that protect injured RGCs. Other retinal cell types that express these mAChR subtypes, including endothelial cells,<sup>35</sup> may also participate in galantamine-mediated RGC survival.

M1 and M4 mAChR are G-protein-coupled receptors linked to different signal-transduction pathways. M1 mAChR are

preferentially coupled to pertussis toxin (PTX)-insensitive  $G_q/G_{11}$  proteins that stimulate phospholipase C (PLC) and phosphatidylinositol hydrolysis with subsequent  $Ca^{+2}$  mobilization from intracellular stores. M4 mAChR, on the other hand, are preferentially coupled to PTX-sensitive  $G_{i/o}$  proteins that inhibit adenylate cyclase and regulate intracellular cAMP levels. It has become increasingly clear that mAChR downstream signaling pathways converge or intersect with mediators of cell survival. For example, M4 mAChR interacts with the nerve growth factor receptor, through  $G\beta\gamma$  complexes, to enhance PI3K/Akt activation and neuronal survival.<sup>36</sup> Of interest, M1 mAChR through  $G\alpha_q$  and PLC leads to activation of Nrf2, a transcription factor involved in redox homeostasis, which may increase the cellular antioxidant defenses and confer neuroprotection against oxidative stress.<sup>37</sup> Moreover, M1 mAChR activation also regulates the activity of the hypoxia-inducible factor-1, a transcription factor involved in the cellular response to hypoxia.<sup>38</sup> Oxidative stress and ischemia/hypoxia have been proposed to be major contributors to glaucomatous neurodegeneration. An important priority in future studies will be to determine the M1- and M4-coupled signaling pathways underlying galantamine-induced RGC neuroprotection. The precise delineation of these molecular events should be useful for the design of novel therapeutic interventions applicable to glaucoma.

In summary, our study reveals the potent role of galantamine in the protection of RGC structure and function in glaucoma, which could be used in conjunction with standard pressure controlling drugs. Our data also identify retinal mAChR as a novel therapeutic target for prevention of neuronal death and vision loss in optic neuropathies.

## Materials and Methods

**Experimental animals.** All procedures were carried out in accordance with the guidelines of the Society for Neuroscience, the Association for Research in Vision and Ophthalmology, and the Canadian Council on Animal Care for the use of experimental animals. OHT surgery was performed in aging, male Brown-Norway rats (Charles River, Stone Ridge, NY, USA), retired breeders between 10 and 12 months of age (300–400 g). Brown-Norway rats were used because they have a larger eye suitable for the OHT surgical procedure, and this glaucoma model has been well characterized in these animals.<sup>8</sup> The optic nerve axotomy model, which is independent of OHT damage, was used as an acute paradigm of RGC death and was carried out in adult Sprague-Dawley rats (Charles River Canada, Saint-Constant, QC, Canada, 180–200 g). The number of animals used in each experiment is indicated above the bar in the corresponding graph.

**Retrograde labeling of RGCs.** For quantification of neuronal survival, RGCs were retrogradely labeled with Dil (Molecular Probes, Junction City, OR, USA) for the glaucoma model, or with Fluorogold (2%, Fluorochrome, Englewood, CO, USA) for the axotomy model. Dil crystals (3%) or Fluorogold (2%) were dissolved in 0.9% NaCl containing 10% dimethyl sulfoxide and 0.5% Triton X-100. The superior colliculus was exposed and a small piece of gelfoam (Pharmacia and Upjohn, Mississauga, ON, Canada) soaked in tracer was applied to the surface. Seven days after tracer application, the time required for labeling the entire RGC population, animals were subjected to OHT surgery or axotomy.

**Ocular hypertension surgery and optic nerve axotomy.** Surgical procedures were performed under general anesthesia by i.p. injection of 1 ml/kg standard rat cocktail consisting of ketamine (100 mg/ml, Bimeda-MTC Animal Health, Cambridge, ON, Canada), xylazine (20 mg/ml, Bimeda-MTC Animal Health) and acepromazine (10 mg/ml, Ayerst Veterinary Laboratories, Guelph, ON, Canada). Unilateral elevation of IOP was induced as previously described<sup>8</sup> by a single injection of a hypertonic saline solution into an episcleral vein. A plastic ring was applied to the ocular equator to confine the injection to the limbal plexus and a

microneedle was then used to inject 50  $\mu$ l of sterile 1.85 M NaCl solution through an episcleral vein. The plastic ring temporarily blocks off other episcleral veins forcing the saline solution into the Schlemm's canal to create isolated scarring. After injection, the plastic ring was removed and the eyes were examined to assess the extent to which the saline solution traversed the limbal vasculature. Polysporin ophthalmic ointment (Pfizer Canada, Kirkland, QC, Canada) was applied to the operated eye and the animal was allowed to recover from the surgery. Animals were kept in a room with constant low fluorescent light (40–100 lux) to stabilize circadian IOP variations. For optic nerve axotomies, animals were deeply anesthetized (2% isoflurane, 0.8 l/min), the left optic nerve was exposed and carefully transected at 0.5–1 mm from the optic nerve head avoiding injury to the ophthalmic artery. Fundus examination was routinely performed immediately after axotomy and 3–5 days later to check the integrity of the retinal circulation after surgery. Animals showing signs of compromised blood supply were excluded from the study.

**Measurement of IOP.** IOP from glaucomatous and normal eyes was measured in awake animals because general anesthetics reduce IOP.<sup>39</sup> A calibrated tonometer (TonoPen XL, Medtronic Solan, Jacksonville, FL, USA) was used to measure IOP after application of one drop of proparacaine hydrochloride (0.5%, Alcon Laboratories, Fort Worth, TX, USA) per eye. The tonometer was held exactly perpendicular to the corneal surface and ~10 consecutive readings per eye were taken and averaged to obtain an accurate IOP measurement. IOP was measured daily for 2 weeks after OHT surgery, then every other day for the entire duration of the experiment. The mean and peak (maximum) IOP for each eye were calculated and these values were used to estimate the mean and peak IOP for experimental and control groups.

**Drug delivery.** Drug delivery in the glaucoma model was carried out by daily i.p. injection to avoid multiple intraocular injections, which lead to IOP reduction in glaucomatous eyes. For this purpose, the following compounds that cross the blood–brain/retinal barrier were administered alone or in combination: galantamine hydrobromide (3.5 mg/kg, Tocris Bioscience, Ellisville, MO, USA), memantine hydrochloride (4 mg/kg, Sigma-Aldrich, St. Louis, MO, USA), donepezil hydrochloride (4 mg/kg, Jubilant Organosys, Stamford, CT, USA) or scopolamine hydrobromide (1 mg/kg, Tocris Bioscience). Control animals received daily i.p. injections of sterile vehicle (PBS). In some experiments, animals were treated with the  $\beta$ -adrenergic receptor blocker timolol maleate (0.5%, Sabex, Boucherville, QC, Canada) applied daily on the cornea of the glaucomatous eye to control IOP increase.

In the axotomy model, drug delivery was carried out by intravitreal injection of the following compounds in a total volume of 5  $\mu$ l: galantamine hydrobromide (100 mM), MLA citrate (10  $\mu$ M, Sigma-Aldrich), dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ -E, 100  $\mu$ M, Sigma-Aldrich), mecamlamine hydrochloride (MMA, 10 mM, Sigma-Aldrich), scopolamine hydrobromide (10 mM, Tocris Bioscience), pirenzepine dihydrochloride (1 mM, Tocris Bioscience), 11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one (DX116, 1 mM, Tocris Bioscience), Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, 1 mM, Tocris Bioscience) or tropicamide (1 mM, Tocris Bioscience). Control eyes received an intravitreal injection of sterile vehicle (PBS). Drugs were injected into the vitreous chamber using a 10- $\mu$ l Hamilton syringe adapted with a 32-gauge glass microneedle, the tip of which was inserted into the superior hemisphere of the eye, at a ~45° angle, through the sclera into the vitreous body. This route of administration avoided retinal detachment or injury to eye structures, including the iris and lens that release factors that may induce RGC survival. Surgical glue (Indermill, Tyco Health Care, Mansfield, MA, USA) was used to seal the injection site.

**Quantification of RGC soma and axons.** Quantification of RGC bodies and axons was performed in duplicate by an observer masked to the treatment assignments. For RGC density counts, rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer following which both eyes were immediately enucleated. Retinas were dissected and flat-mounted on a glass slide with the ganglion cell layer side up. RGCs were counted within three square areas at distances of 1, 2 and 3 mm from the optic disc in each of the four retinal quadrants (superior, inferior, nasal and temporal) for a total of 12 retinal areas. Macrophages and microglia that may have incorporated fluorescent tracer after phagocytosis of dying RGCs were excluded from our quantitative analysis based on cell-specific markers and morphology.<sup>40</sup> For axon counts, animals received a transcardial injection of heparin (1000  $\mu$ g/kg) and sodium

nitropruside (10 mg/kg) followed by perfusion with 2% PFA and 2.5% glutaraldehyde in 0.1 M phosphate buffer. Optic nerves were dissected, fixed in 2% osmium tetroxide, and embedded in epon resin. Semi-thin sections (0.7- $\mu$ m-thick) were cut on a microtome (Reichert, Vienna, Austria) and stained with 1% toluidine blue. RGC axons were counted at 1 mm from the optic nerve head in five non-overlapping areas of each optic nerve section, encompassing a total area of 5500  $\mu$ m<sup>2</sup> per nerve. The five optic nerve areas analyzed included: one in the center of the nerve, two peripheral dorsal and two peripheral ventral regions. The total area per optic nerve cross-section was measured using Northern Eclipse image analysis software (Empix Imaging, Toronto, ON, Canada), and this value was used to estimate the total number of axons per optic nerve.

**Visual evoked potential and electroretinogram recordings.** For VEP recordings, animals were anesthetized with isoflurane (3% for induction and 1.5% for maintenance) and placed in a stereotaxic head holder. The ERG was continuously monitored and the core body temperature was maintained at 37°C using a feedback controlled heating pad. Atropine sulfate eye drops (1%, Allergan Canada, Markham, ON, Canada) were used to dilate the pupils, and the corneas were protected by application of artificial tears (Allergan Canada). A bilateral craniotomy was performed anterior to the lambda, at bregma coordinates –6.8 and 1.5 mm lateral to the sagittal suture, to expose the cerebral cortex overlying the superior colliculus of each hemisphere. The dura was then incised and a tungsten multiunit recording microelectrode (impedance 0.8  $\Omega$  Microprobe, Gaithersburg, MD, USA) was lowered under microscopic view until the tip touched the surface of the cortex. The skull opening was then filled with agar to protect the tissue from desiccation and, using a micromanipulator (Motorized Microdrive, FHC, Bowdoinham, ME, USA), the microelectrode was advanced vertically to 300  $\mu$ m from the superficial layer of the superior colliculus into the stratum griseum superficiale. Visual stimulation was provided by a diffuse flash ( $f = 1$  Hz, Grass photostimulator, Astro-Med, Brossard, QC, Canada) placed 30 cm away from the contralateral eye. Triggered evoked potentials were averaged over 40 successive presentations. VEP signals were amplified and bandpass filtered between 10 and 1000 Hz and acquired by an analogue/digital interface (CED 1401 plus) to a PC running acquisition software (Signal 2, CED, Cambridge, UK). At the end of the experiment, the final electrode location was marked by passing a direct current of 10 mA for 5 s through the recording electrode. The animals were then perfused with 4% PFA, the brains were removed and processed for serial sectioning. Sections (50  $\mu$ m) were stained with cresyl violet and the electrode location mark was visualized as an iron precipitate following incubation in a 2% K<sub>4</sub>Fe(CN)<sub>6</sub> solution. The depth of recording was confirmed by the position of the mark and the depth reading of the micromanipulator.

For ERG recordings, animals were dark adapted for a 12-h period. Under dim red light illumination, the animals were anesthetized with a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (6 mg/kg), and the pupils were dilated with cyclopentolate hydrochloride 1%. ERGs were recorded with a Dawson, Trick and Litzkow fiber electrode (277X-Static, silver-coated conductive nylon yarn, Sauquoit Industries, Scranton, PA, USA) that was positioned and maintained on the cornea using a drop of 1% methylcellulose. The ERG (bandwidth: 1–1000 Hz;  $\times 10$  000; Grass, P511 amplifier) and oscillatory potentials (bandwidth: 100–1000 Hz;  $\times 50$  000) were recorded simultaneously with the Acknowledge data acquisition system (Biopac MP 100 WS, BIOPAC System, Goleta, CA, USA).

**Statistical analysis.** Data analysis and statistics were performed using the GraphPad InStat software (GraphPad Software, San Diego, CA, USA) by a ANOVA test followed by Bonferroni's multiple comparison post-test.

#### Conflict of interest

The authors declare no conflict of interest.

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