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Acute retinal ganglion cell injury caused by intraocular pressure spikes is mediated by endogenous extracellular ATP

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Abstract

Elevated intraocular pressure may lead to retinal ganglion cell injury and consequent visual deficits. Chronic intraocular pressure increase is a major risk factor for glaucoma, a leading blinding disease, and permanent visual deficits can also occur following acute pressure increments due to trauma, acute glaucoma or refractive surgery. How pressure affects retinal neurons is not firmly established. Mechanical damage at the optic nerve head, reduced blood supply, inflammation and cytotoxic factors have all been called into play. Reasoning that the analysis of retinal neurons soon after pressure elevation would provide useful cues, we imaged individual ganglion cells in isolated rat retinas before and after short hydrostatic pressure increments. We found that slowly rising pressure to peaks observed in trauma, acute glaucoma or refractive surgery (50–90 mmHg) did not damage ganglion cells, whereas a rapid 1 min pulse to 50 mmHg injured 30% of these cells within 1 h. The severity of damage and the number of affected cells increased with stronger or repeated insults. Degrading extracellular ATP or blocking the P2X receptors for ATP prevented acute pressure-induced damage in ganglion cells. Similar effects were observed *in vivo*. A short intraocular pressure transient increased extracellular ATP levels in the eye fluids and damaged ganglion cells within 1 h. Reducing extracellular ATP in the eye prevented damage to ganglion cells and accelerated recovery of their response to light. These data show that rapid pressure transients induce acute ganglion cell injury and unveil the causal role of extracellular ATP elevation in such injury.

Introduction

Elevated intraocular pressure (IOP) may cause injury and loss of retinal ganglion cells (RGCs), the neurons that project from the retina to the brain. Anatomical and electrophysiological studies after chronic IOP elevation have shown dysfunction, degeneration and loss of RGCs in various animal species (Weber *et al.*, 1998; Quigley, 1999; Jakobs *et al.*, 2005), and there is evidence that loss of RGCs may begin within hours of IOP elevation, particularly when IOP reaches 50 mmHg or more (e.g. Naskar *et al.*, 2002, Lai *et al.*, 2003). However, the mechanistic link between high IOP and RGC injury is still debated. Mechanical stress at the optic nerve head, where the RGC axons leave the eye, is considered a leading source of injury in cases of chronic or prolonged IOP elevation (for reviews see Quigley, 1999; Burgoyne *et al.*, 2005; Morrison *et al.*, 2005). However, other consequences of pressure elevation, such as the reduction in retinal blood perfusion, release of cytotoxic agents and differential individual susceptibility are also likely to contribute to RGC damage (for reviews see Flammer *et al.*, 2002; Neufeld & Liu, 2003; Libby *et al.*, 2005).

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Taking advantage of the recent advancements in single-cell imaging within tissues (Kettunen *et al.*, 2002) and of a newly developed incubator with hydrostatic pressure control (Previti *et al.*, 2002), we have approached this problem from a new perspective, by monitoring individual RGCs in isolated rat retinas before and after pressure application and searching for signs of cell injury. We focused on short pressure transients reaching values (50 and 90 mmHg) that can be experienced in trauma, acute glaucoma (Saw *et al.*, 2003) and, very briefly (30–90 s), during some ophthalmic surgical procedures such as Lasik (Arbelaez *et al.*, 1997). In humans, this kind of IOP transient causes temporary visual deficits, but vision recovers in the great majority of patients if IOP is reduced to normal levels within hours. However, although rarely, this is not always the case (e.g. Bushley *et al.*, 2000; Varano *et al.*, 2005), suggesting that RGCs may be damaged even by short pressure elevation. Here we found that applying 1 min pressure transients to 50–90 mmHg in isolated rat retinas induces signs of RGC injury within 1 h of pressure application.

Trying to understand which factors mediate such rapid pressure-induced effects on RGCs, we tested the appealing and yet controversial hypothesis that extracellular ATP (eATP) and its cytotoxic $P2X_7$ receptors might have a role in pressure-induced retinal damage. This hypothesis is supported by the observation that mechanical stimulation

of retinal glia induces local eATP release in isolated retinas (Newman, 2001; Fields & Burnstock, 2006), by the presence of immunoreactivity for the cytotoxic $P2X_7$ receptors in the retina (Brandle *et al.*, 1998; Franke *et al.*, 2005; Resta *et al.*, 2005; Zhang *et al.*, 2005) and by the reduced viability of cultured RGCs 24 h after incubation with the $P2X_7$ agonist 2′,3′-benzoyl-4-benzoyl-ATP (Zhang *et al.*, 2005). Other studies, however, apparently conflict with this view, as the specificity of $P2X_7$ immunoreactivity in neurons is debated (Sim *et al.*, 2004; Anderson & Nedergaard, 2006) and short (< 30 min) ATP applications in isolated retinas induce typical signs of $P2X_7$ activation in microglia (Innocenti *et al.*, 2004) and amacrine cells (Resta *et al.*, 2005) but not in RGCs, where $P2X_7$ receptors have not been detected even in single-cell recording soon after ATP application (Taschenberger *et al.*, 1999). The best way to solve this controversy was to directly test the effects of blocking eATP and $P2X_7$ receptors on pressure-induced RGC damage and this was possible in our experimental model. We found that eATP degradation or blockade of the P2X receptors for ATP protected all RGCs from high pressure transients in isolated retinas, indicating a causal link between early pressure-induced RGC damage and eATP. Furthermore, we found that, although exogenous ATP did not affect RGCs within minutes of application as previously reported (Taschenberger *et al.*, 1999; Innocenti *et al.*, 2004; Resta *et al.*, 2005), it did so after 1 h, a finding that might reconcile the apparent conflict in previous observations.

As a final step, we tested the significance of our results *in vivo*, as pressure application to isolated retinas allows the study of individual RGCs with a detail that is still not possible *in vivo* but does not reproduce some of the effects of pressure elevation *in vivo*, such as tangential stress (reviewed in Burgoyne *et al.*, 2005) and blood supply reduction (reviewed in Flammer *et al.*, 2002). Inducing short IOP increments in rats, we detected eATP release in the eye and found acute RGC injury that was prevented by degrading eATP.

Taken together, these findings reveal that rapid pressure transients induce RGC injury within hours, and unveil a mechanistic link between eATP and such acute pressure-induced retinal damage.

Materials and methods

Animal and tissue handling

Experiments were performed on Long Evans hooded rats aged between 15 and 30 days, in accordance with the national and ARVO (Association for Research in Vision and Ophthalmology) regulations on animal experimentation in ophthalmic research. Retinal isolation and cell labelling were performed as already described (Kettunen *et al.*, 2002; Resta *et al.*, 2005). Briefly, Long Evans rats were decapitated, their retinas quickly dissected in oxygenated artificial cerebrospinal fluid (ACSF), flattened onto nitrocellulose filters and labelled by shooting 1.3 μm tungsten particles coated with Oregon Green 488 or Alexa Fluo 564-conjugated 10 000 kDa dextrans (Molecular Probes, Inc.) by means of a gene gun (Bio-Rad). Retinas were then maintained for 2–3 h in oxygenated ACSF within a closed humidified chamber at room temperature (25–28 °C) to allow dextran diffusion. Within this interval RGCs damaged by the shooting started to fragment and/or bleb (V.R. and L.G.-R. unpublished observations; Kettunen *et al.*, 2002), and could thus be excluded from subsequent analysis. In agreement with previous reports (Morgan *et al.*, 2005) we found that gene-gun labelling efficiency decreased with age. In particular, we found optimal labelling between postnatal days (P)15 and 17, with apparently complete dye filling in several RGCs in each retina and a low rate (5–10%) of labelled cells dying 2–3 h after gene-gun shooting ($N = 30$ retinas). In comparison, retinas from animals between P24 and P30 had a lower number of apparently complete dye filling of RGCs per retina and a higher rate (12–20%) of labelled cells dying 2–3 h after gene-gun shooting ($N = 15$ retinas). Older retinas (2 months, $N = 10$), at least in our hands, displayed an even higher (20–35%) rate of labelled cells dying 2–3 h after gene-gun shooting. For this reason we constrained most of our experiments on isolated retinas to the P15–P17 age range.

Imaging living retinal ganglion cells

Imaging was performed by placing retinas in a 4 mL custom-made chamber (containing oxygenated ACSF) positioned on the stage of either a Zeiss Axioplan or a Zeiss Axioskop 2 FS Plus epifluorescence microscope equipped with a black and white CCD camera (Chroma 1600, DTA, Pisa, Italy), using the proprietary software. Observations were performed at room temperature (25–28 °C) using a 40× Olympus water immersion objective (numerical aperture 0.80). Retinas were rapidly screened for labelled RGCs, starting from random locations. RGCs were identified by the presence of the axon, which is clearly visible after dextran labelling and can be easily followed in its centripetal course from the cell body towards the optic nerve head. Following visual inspection at different focal depths, three images were taken for each RGC, focusing on the dendritic tree (3 s exposure), soma (0.5 s exposure) and axon (2 s exposure). To minimize light exposure and reduce the risk of photo-damage, excitation light was kept at about 2% of its maximum intensity using an Attoarc (Zeiss) and a neutral density filter (ND 1.0; Omega filter). Furthermore, no more than five RGCs were studied per retina, imaging each cell only at two time points (before and after treatment), unless otherwise specified. Reference points within the observation chamber allowed each recorded cell to be retrieved in subsequent observations by means of its coordinates. Cells showing signs of damage (such as fragmentation or membrane blebbing) in the screening preceding the pressure stimuli were excluded from the analysis, as were brightly labelled RGCs because of their high risk of photodamage. Control cells were imaged as for the treated cells. The contrast and brightness of the micrographs shown here were adjusted using Adobe Photoshop (San Jose, CA, USA). In some instances, in order to enhance the visibility of the dendritic trees, images have been inverted to display cells as dark profiles against a lighter background and partially (50%) superimposed to their rendering with the emboss filter.

Pressure-controlled incubation

A bioreactor chamber was used to incubate retinas in 100 mL ACSF under controlled temperature (33 ± 0.5 °C), pH (7.4 ± 0.05) and gas composition (air, prefiltered at 0.2 μm, with 5% CO_2). The hydrostatic pressure in the incubator chamber could be regulated at will in the range 0–110 mmHg by electronically controlled gas admission (Previti *et al.*, 2002). Within the incubator retinas were allowed to stabilize at resting pressure (12–14 mmHg) for at least 5 min, then a pressure stimulus was applied, followed by 1 h at resting pressure before subsequent analysis. Each pressure test was normally performed on at least two different sets of retinas. Each retina underwent only one test unless otherwise specified. Loss of membrane integrity was assessed by adding propidium iodide (PI) (final concentration 1.5 μM) for 1 min to the ACSF. Oxidized ATP (oATP) was preincubated for 2 h, Brilliant Blue G (BBG) for 30 min and apyrase for 5–10 min. All chemicals were from Sigma unless otherwise specified.

In a typical experiment four to six retinas were isolated from littermates and labelled. After 2–3 h RGCs were imaged in a set of randomly selected retinas, which were then transferred to the incubator for treatment. Meanwhile, RGCs were imaged from the remaining retinas. Once the first set of retinas finished treatment, the second set underwent the appropriate control (e.g. pressure stimulus in the presence of a pharmacological agent). The sequential order of treatment and control was randomized from experiment to experiment. This experimental design allowed control and treated retinas from littermates, and a treatment and corresponding control to be performed on the same day.

Pressure transients in vivo

Long Evans rats were deeply anaesthetized with Avertine (10 mL/kg of body weight i.p., 3.3% tri-bromo-ethanol, 2% tertiary amyl alcohol in saline) and their right eye cannulated with a 30 gauge needle inserted in the vitreal chamber and connected to a column of ACSF. The IOP was increased to the desired value by positioning the column at the appropriate height (760 mmHg = 10 m H_2O), and opening the connection between the needle and the column. Cannulation and IOP increments were performed while monitoring the animal under a dissecting microscope to carefully avoid eye damage. Control eyes were cannulated but no pressure transient was applied. To monitor the time course of IOP elevation, in six animals the eyes were connected with one needle to the ACSF column and through a second needle to a piezo-resistive pressure sensor (RS Electronics) whose output was fed continuously to a computer. To analyse the effects of degrading eATP *in vivo*, 2 µl apyrase (estimated intraocular concentration 30 U/mL) was injected intraocularly 5 min before pressure application. Control animals received vehicle (phosphate-buffered saline) injection. To evaluate cell damage, PI-labelled cells were sampled across the retinal area in 10 evenly spaced (370×250 µm^2) sample fields per retina. Total cell density in the ganglion cell layer was also computed in some of the sampled fields and the average value used to determine the percentage of affected cells in the ganglion cell layer. Considering the limited amount of affected cells (12%, see Results), we did not attempt any histological analysis of the RGC population in fixed retinas as this percentage would be below the counting error (see Perry *et al.*, 1983).

Measurement of ATP levels

At 90 s after a 2 min IOP pulse to 50 mmHg, 5 µl fluid samples were collected from the vitreal chamber in 14 anaesthetized P30 rats as described previously (Resta *et al.*, 2005) and eATP levels measured by the luciferin/luciferase assay with a luminometer (Wallac Victor 31420, Perkin Elmer).

Electrophysiological recordings

These experimental procedures were performed as described previously (Caleo *et al.*, 2003). Briefly, rats were anaesthetized with urethane (20% solution in saline; 0.7 mL/100 g of body weight, i.p.; Sigma) and placed in a stereotaxic frame. Both eyes were fixed by means of adjustable metal rings surrounding the external portion of the eye bulb. Body temperature was continuously monitored and maintained at 37 °C by a thermostat-controlled electric blanket. After exposure of the cerebral surface, a glass micropipette (tip resistance 2 MΩ) filled with 3 M NaCl was inserted into the brain at the appropriate stereotaxic coordinates (2.1 mm anterior and 3.3 mm lateral to lambda) and lowered to reach the optic tract, which is composed of RGC axons [mostly from the contralateral eye in rats (Lund *et al.*, 1980)]. The first evoked visual activity was usually encountered at a depth of 3.6 mm from the pial surface and had an audible 'swish', characteristic of discharges from fibres of the optic tract. The optic tract location was confirmed by histological reconstruction of the electrode track (see Results) in three animals as described by Caleo *et al.* (2003). The visual stimuli were light flashes of 1.25 s generated by a VSG2/5 card (Cambridge Research Systems, Rochester, UK) on a display (Sony Multiscan G500) positioned 20–30 cm in front of the rat's eyes. The stimulus frequency was 0.2 Hz, flash contrast 80% and mean luminance 15 cd/m^2. Signals were amplified 25 000-fold, bandpass filtered (500–5000 Hz) and conveyed to a computer for storage and analysis with a custom-made program (based on a National Instrument Card). Multi-unit spikes were discriminated from background by a voltage threshold that was set between 3.5 and 4.5 times the SD of noise, as described by Caleo *et al.* (2003). Responses were averaged over 10 consecutive stimulations. The recovery time was defined as the first time after pressure stimulation when the variable under consideration reached 85% of its value before pressure application.

Statistical analysis

To compare the effects of the different treatments on RGCs in isolated retinas, we rated each RGC as injured when displaying at least one sign of damage (e.g. blebbing or PI permeability) and as non-injured otherwise. Pairwise comparisons between different treatments were performed by generating 2×2 contingency tables with raw data (treatment A/treatment B; injured/non-injured RGCs) and using the Fisher 2×2 two-tailed test, which allows the exact computation of probability. Bonferroni's procedure was used to correct for multiple tests on the same datasets (Krauth, 1988). In the analysis of *in vivo* data, the average density of injured cells observed after IOP elevation was assessed in each retina and the significance of treatments vs. controls was assayed using the non-parametric Kruskal–Wallis one-way ANOVA on ranks with 0.05 significance for pairwise comparisons (Siegel & Castellan, 1988). Average data are reported as mean and SE unless otherwise specified. Statistical analysis was performed with Sigmastat (Systat Software).

Results

Brief pressure pulses cause rapid retinal ganglion cell injury in isolated retinas

Individual RGCs were labelled by biolistic delivery of fluorescent dextrans in isolated retinas, and analysed before and after pressure transients to detect the appearance of signs of cell damage, such as membrane blebbing, loss of membrane integrity (as revealed by permeability to PI), soma swelling or shrinkage, fragmentation and/or loss of processes. We mostly focused on retinas from P15–P17 rats, as at these ages we obtained optimal RGC labelling and observed a very limited proportion (5–10%) of cells damaged by the gene-gun shooting (see Materials and methods).

We first tested whether light exposure or incubation at resting pressure affected RGCs. We found no sign of damage in any of the 45 RGCs analysed 5 h after the first imaging (0/45 RGCs; $N = 23$ retinas) or in the 20 RGCs analysed after 18 h ($N = 10$). Similarly, we observed no sign of damage after 1 h (0/48 RGCs; $N = 14$) or 3 h (0/27 RGCs; $N = 10$) of incubation at 12–14 mmHg, corresponding to the mean IOP that we measured in our rat strain.

We next explored the effects of short pressure increments above resting level, reaching pressure peaks that may be experienced in acute glaucoma, trauma and, very briefly (i.e. 60–90 s), in the course of some ophthalmic surgery. In a first set of experiments, the pressure was rapidly (8 ± 0.5 mmHg/s) raised to 50 mmHg and returned to resting level after 1 min (1 × 50). 1 h later, we observed small blebs in the cell soma of 12 out of 41 RGCs analysed (Fig. 1a–c; $N = 7$) and loss of plasma membrane integrity, as revealed by PI uptake in the cytoplasm, in six of these cells (Fig. 1b). The percentage of affected RGCs was small (29%) but statistically significant (see Table 1). No sign of damage was detected in either the dendrites or the axon.

To explore whether pressure-induced effects are cumulative, we exposed a set of retinas to a sequence of seven 1 min spikes at 50 mmHg (7 × 50), separating sequential spikes by 1 min at resting pressure, and found that 29 out of the 39 RGCs tested displayed PI permeability within 1 h ($N = 12$). Most of these cells also displayed blebbing in the soma (22/39 RGCs; $N = 12$), which was commonly more conspicuous than after a single 50 mmHg transient (Fig. 1d). Furthermore, many RGCs had blebs throughout the dendrites (20/39 RGCs; $N = 12$).

To explore whether it is the time spent at the pressure peak or the occurrence of pressure transients that mostly affects cells, we exposed retinas to a single insult of 7 min at 50 mmHg (slow-50), raising and decreasing the pressure very slowly (3 ± 0.5 mmHg/min). This stimulus exposed retinas to 50 mmHg for 7 min, as did the 7 × 50 sequence, but had no rapid transient. We found that none of the 26 labelled RGCs analysed after the slow-50 stimulus showed signs of damage (0/26 RGC, $N = 9$ retinas) at 1 and 2 h after the end of stimulus (data not shown).

Finally, we found that a sequence of seven 1 min spikes reaching 90 mmHg (7 × 90) affected all RGCs. At 1 h after this stimulus we observed severe soma (Fig. 1e) and dendritic blebbing (Fig. 1f and g) as well as loss of membrane integrity in all RGCs analysed (31/31 RGCs; $N = 12$) except the α-RGCs. Notably, this stimulus also caused blebbing in some axons (19/54 axons; $N = 25$; not shown). At 2 h after the 7 × 90 insult, the α-RGCs also displayed soma and dendritic blebbing as well as PI incorporation (7/7 α-RGCs), suggesting that these neurons take longer to respond to pressure but do not qualitatively differ from the smaller non-α-RGCs (data not shown). For this reason, and considering that α-RGCs represent a minority of RGCs, we have excluded them from subsequent analysis.

A quantitative summary of these data is shown in Fig. 1h. As shown in Table 1, all of the stimuli except the slow-50 affected a statistically significant number of RGCs compared with control (Fisher 2 × 2 one-tailed test and Bonferroni's procedure for multiple comparisons).

TABLE 1. *P*-values for pairwise comparisons between different pressure stimuli

	Pressure stimuli			
	1 × 50	7 × 50	Slow-50	7 × 90
Compared with:				
Control	3×10^{-5}	9×10^{-15}	1	$< 10^{-15}$
1 × 50	–	6.2×10^{-5}	–	–
7 × 50	–	–	3.3×10^{-10}	–

Pairwise comparisons were performed to evaluate the significance of the differences in the proportion of injured retinal ganglion cells (RGCs) observed in postnatal day 15–17 retinas at 1 h after the various pressure stimuli. Each RGC was scored as injured when displaying blebbing or propidium iodide permeability and as uninjured otherwise. Comparisons were made using the Fisher 2 × 2 exact two-tailed test. Each table entry indicates the *P*-value obtained for the comparison between the stimulus indicated in the column header and that indicated in the row header. As multiple comparisons were based on the same data sets, we used Bonferroni's procedure for multiple tests, finding that all comparisons made were significant at the 0.01 level except for the single insult of 7 min at 50 mmHg (Slow-50) vs. control. Pressure stimuli: 1 × 50, pulse of 1 min at 50 mmHg; 7 × 50, seven 1 min 50 mmHg insults; 7 × 90, seven 1 min 90 mmHg insults.

Retinal ganglion cell damage is not due to stirring artefacts

Pressure changes were produced by modulating the rate of air supply to the incubator, which may alter stirring of the incubation medium (Macdonald & Fraser, 1999). To investigate whether stirring *per se* contributes to cell damage, we performed experiments modulating the air supply as for the 7 × 90 stimulus but leaving the incubator exit valve open. This caused medium stirring without a pressure increase. 1 h later we found no blebbing or PI permeability (0/15 non-α-RGCs; $N = 4$ retinas, $P < 0.001$, Fisher 2 × 2 test, data not shown).

Extracellular ATP mediates rapid pressure-induced retinal ganglion cell damage in isolated retinas

Taken together, the previous results show that fast pressure transients rapidly damage RGCs in isolated retinas. Searching for a mechanism for this effect, we focused on cytotoxic factors, reasoning that ischaemic effects are unlikely to play a role in isolated retinas kept under controlled incubation. Recent studies have suggested the hypothesis that eATP might contribute to pressure-induced retinal damage (Newman, 2001; Zhang *et al.*, 2005). We tested the protective effects of eATP blockade using the 7 × 90 stimulus, which, by affecting all RGCs, provided a solid statistical comparison. When retinas were incubated in ACSF containing 30 U/mL apyrase, an enzyme that degrades eATP (North, 2002), no blebbing or PI permeability was detected 1 h (0/41 RGCs; $N = 14$), 2 h (0/18 RGCs; $N = 8$) or 3 h (0/7 RGCs; $N = 5$) after the 7 × 90 stimulus. Examples of this protective effect are illustrated in Figs 2a–d and 3a–d. We also found that 2 h preincubation with oATP (300 μM), an irreversible $P2X_7$ receptor blocker (Murgia *et al.*, 1993; North, 2002), prevented RGC blebbing and PI permeability at 1 and 2 h after the 7 × 90 stimulus (0/11 RGCs; $N = 10$), as shown in Fig. 3e. Finally, incubation with 0.5 μM BBG, considered a selective reversible inhibitor of rat $P2X_7$ receptors at this concentration (North, 2002), protected RGCs from pressure-induced blebbing and PI permeability at 1 and 2 h after the 7 × 90 stimulus (0/27 RGCs; $N = 10$), as illustrated in Figs 3f and 4a and b. The protective effect of BBG was reversible; RGCs that were protected from a 7 × 90 stimulus applied in the presence of BBG became sensitive to this same pressure stimulus applied 20 min after BBG removal by washing (11/11 RGCs; $N = 5$), as illustrated in Fig. 4a–c. All of these protective effects were statistically significant (pairwise comparisons with 7 × 90 using the Fisher 2 × 2 tests: apyrase, $P < 10^{-15}$; oATP, $P < 10^{-9}$; BBG, $P < 10^{-15}$; Bonferroni's procedure for multiple comparisons, significance level 0.01).

Finally, to investigate whether rapid pressure-induced eATP-mediated damage was also observed in more mature retinas, we analysed P24–P29 rats, an age when rat RGCs appear to have attained maturity both morphologically (Yamasaki & Ramoa, 1993) and electrophysiologically (Wang *et al.*, 1997). As the efficiency of RGC labelling per retina decreases with age (see Materials and methods), in these retinas we focused on the 7 × 90 stimulus, obtaining the same results as for the younger retinas. A 7 × 90 stimulus affected all tested RGCs (14/14; $N = 7$) and damage was not observed when the 7 × 90 stimulus was applied following a 2 h preincubation with oATP (300 μM) (0/11; $N = 5$; $P < 10^{-11}$, Fisher 2 × 2 test).

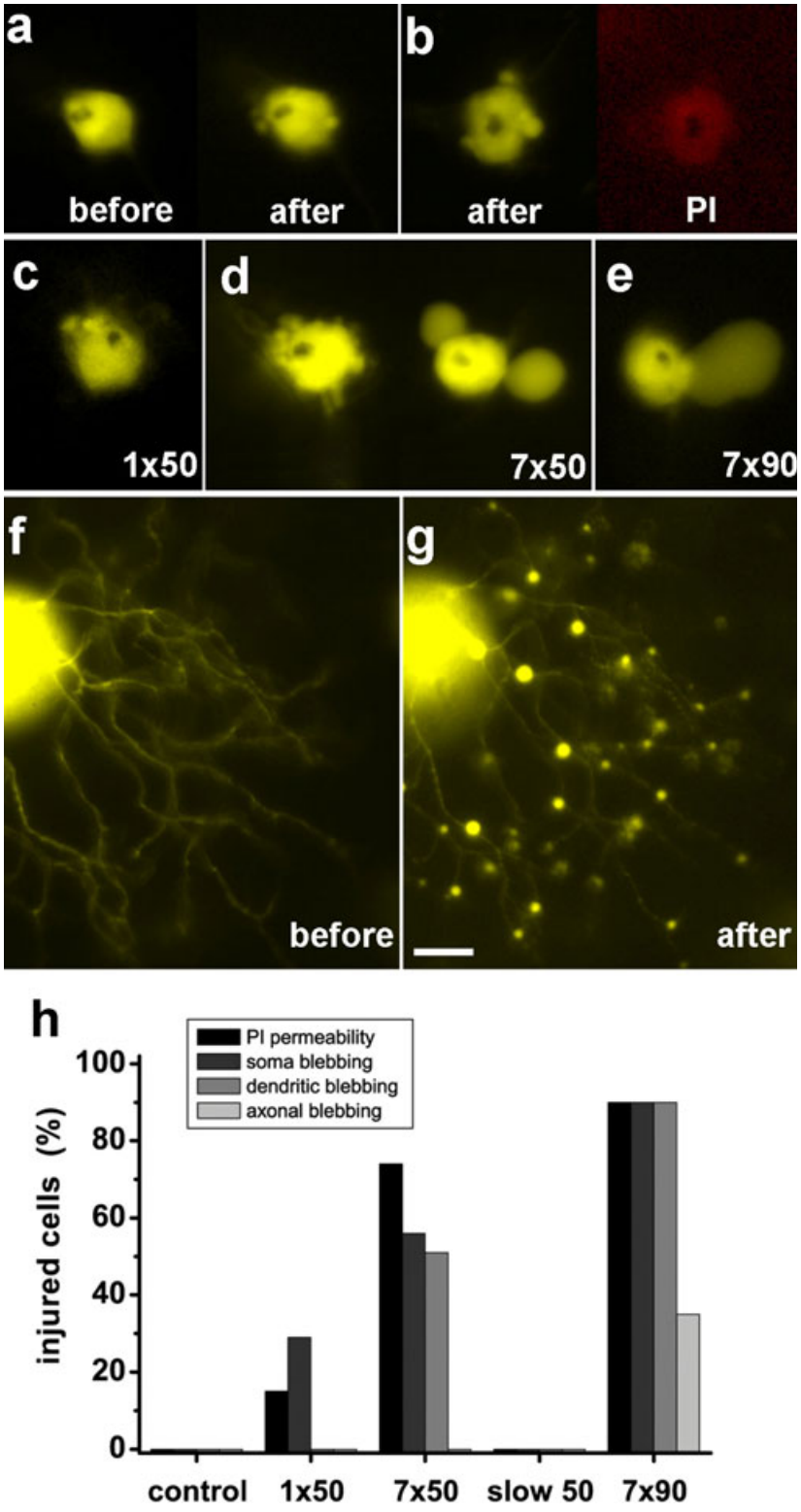


FIG. 1. Short transients of hydrostatic pressure cause rapid retinal ganglion cell (RGC) damage in isolated rat retinas. (a) An affected RGC soma before and 1 h after a pulse of 1 min at 50 mmHg (1 × 50). Note the appearance of small blebs after pressure. The black spot in the soma is the gene-gun bullet. (b) Example of a cell displaying soma blebbing (left) and loss of membrane integrity, as revealed by propidium iodide (PI) permeability (right) at 1 h after a 1 × 50 insult. (c–e) Typical soma blebbing was small after a 1 × 50 insult (c), increased with seven 1 min 50 mmHg insults (7 × 50) (d) and more so after seven 1 min 90 mmHg insults (7 × 90) (e). (f and g) An example of dendritic blebbing observed 1 h after a 7 × 90 insult. The cell is shown before (f) and after (g) the stimulus. Calibration bar: 10 μm (a–e); 20 μm (f and g). (h) Quantification of RGCs affected 1 h after pressure transient as a function of the different pressure stimuli. The percentages of cells displaying PI permeability, soma, dendritic and axonal blebbing are separately quantified. As specified in the text, α-RGCs are not considered in this quantification. Slow 50, single insult of 7 min at 50 mmHg.

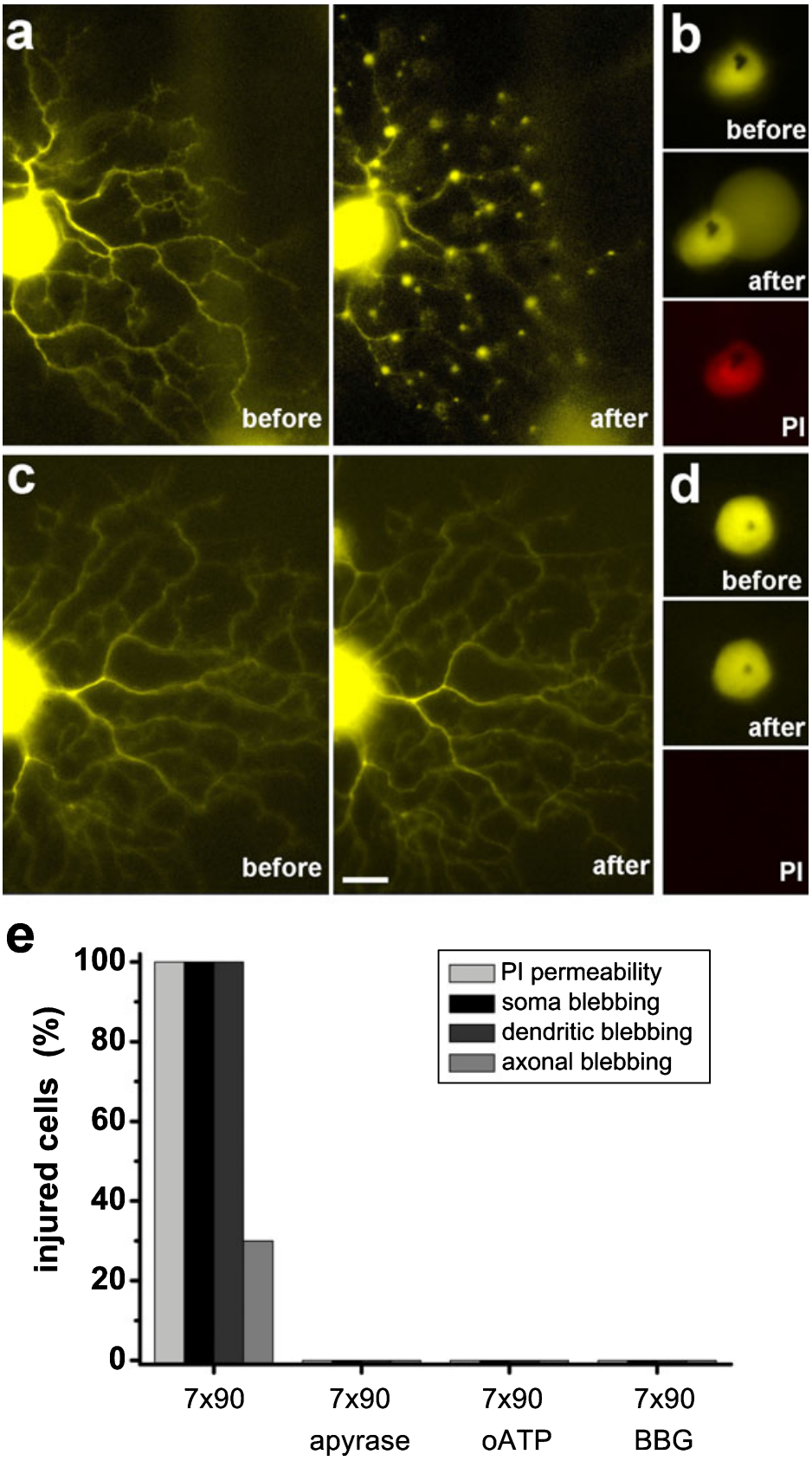


FIG. 2. Retinal ganglion cell (RGC) damage induced by rapid pressure transients in isolated retinas is prevented by degrading extracellular ATP (eATP). Example of an RGC dendritic tree (a) and a soma (b) before and 1 h after seven 1 min 90 mmHg insults (7 × 90), which induced blebbing and propidium iodide (PI) permeability (b, bottom). Preincubation with apyrase (30 U/mL) totally prevented dendritic (c) and soma (d) blebbing as well as PI permeability (d, bottom) after a 7 × 90 pressure stimulus. Calibration bar: 20 μm (a and c); 10 μm (b and d). (e) Quantification of injured non-α-RGCs after eATP degradation with apyrase of P2X receptor blockade with oxidized ATP (oATP) or Brilliant Blue G (BBG).

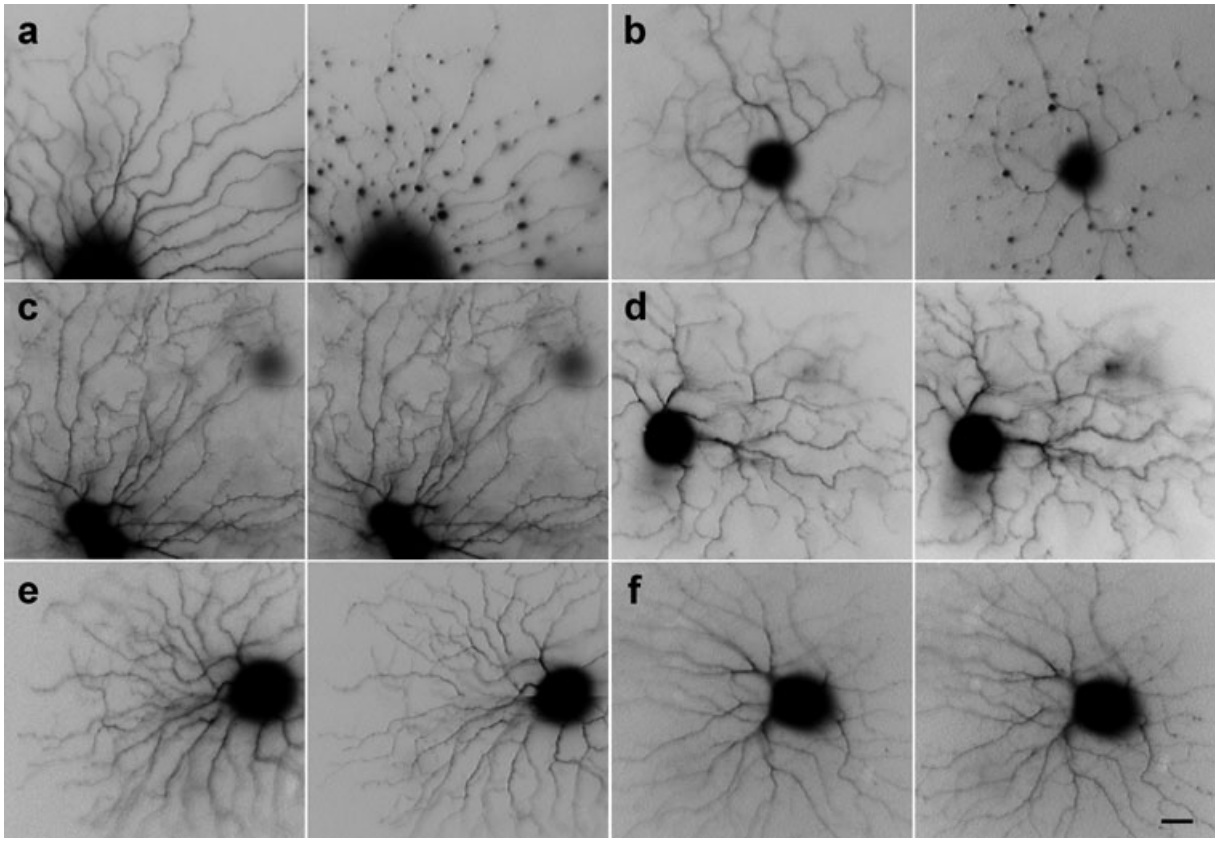


FIG. 3. Retinal ganglion cell (RGC) damage induced by rapid pressure transients in isolated retinas is prevented by degrading extracellular ATP or by blocking P2X receptors. (a and b) Examples of RGC dendritic trees before (right panel) and 1 h after (left panel) seven 1 min 90 mmHg insults (7 × 90). Notice conspicuous dendritic blebbing, which is induced in all RGCs by this pressure insult. (c–f) Examples of RGC dendrites before (right panel) and 1 h after (left panel) a 7 × 90 pressure stimulus applied in the presence of 30 U/mL apyrase (c and d), after 2 h preincubation with 300 μM oxidized ATP (e) or in the presence of 0.5 μM Brilliant Blue G (f). Calibration bar, 20 μm.

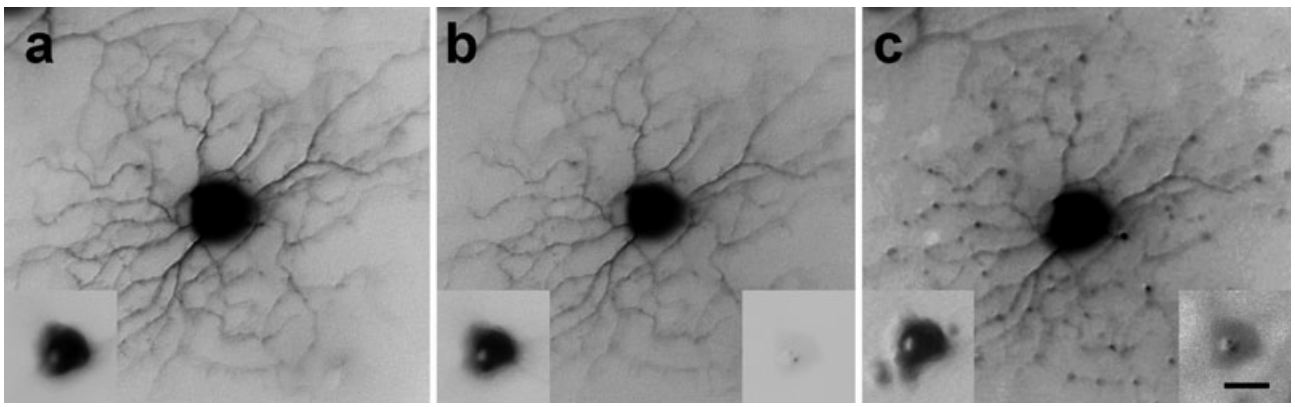


FIG. 4. Reversible protective effects of the $P2X_7$ blocker Brilliant Blue G (BBG). Example of a retinal ganglion cell (RGC) dendritic tree and soma (insets) before (a) and 1 h after (b) seven 1 min 90 mmHg insults (7 × 90) delivered in the presence of 0.5 μM BBG. No blebbing is observed in the dendritic tree or in the soma (left inset) and propidium iodide (PI) permeability is not detected (right inset). (c) The same RGC is injured by a second 7 × 90 stimulus, delivered after BBG removal by washing. Blebbing is observed in the dendrites and soma (left inset), together with PI permeability (right inset). Calibration bar: 20 μm (a–c), 10 μm (insets).

Exogenous ATP application mimics pressure-induced retinal ganglion cell damage

The previous findings provide a strong indication that eATP mediates acute pressure-induced RGC damage, possibly via the cytotoxic $P2X_7$ receptors, which are blocked by oATP and BBG (Murgia *et al.*, 1993; North, 2002). However, we and others have failed to detect RGC responses to exogenous ATP that might be attributable to $P2X_7$ receptor activation, at least within up to 30 min of ATP application (Taschenberger *et al.*, 1999; Innocenti *et al.*, 2004; Resta *et al.*, 2005). With this in mind, we tested the effects of incubating isolated retinas in ATP for more than 30 min. In agreement with previous studies, we found no blebbing or PI permeability when RGCs were analysed in

P15 retinas within 30 min of incubation in ATP (0.1 or 1 mM). However, after 1 h of ATP incubation we observed a dose-dependent effect. Half of the RGCs that we tested (9/16, $N = 8$) displayed soma and dendritic blebbing as well as PI uptake in 0.1 mM ATP, and all RGCs except the α-RGCs displayed blebbing and PI permeability (35/35, $N = 12$) after 1 h in 1 mM ATP (0/15, $N = 12$). Similarly, all tested cells were injured (10/10 non-α-RGCs, $N = 4$) at 1 h after the application of 0.1 mM 2′,3′-benzoyl-4-benzoyl-ATP, a potent, albeit not selective, $P2X_7$ receptor agonist (North, 2002). The RGC damage induced by ATP (1 mM) was totally prevented by 2 h preincubation in ACSF containing either the irreversible P2X receptor blocker oATP (300 μM; 0/20 non-α-RGCs, $N = 9$) or the selective inhibitor of rat $P2X_7$ receptors BBG (0.5 μM; 0/15 non-α-RGCs, $N = 6$). The effects of 1 h incubation in ATP (1 mM) and the protective action of P2X blockade are illustrated by the examples in Fig. 5.

Brief intraocular pressure increments injure retinal ganglion cells in vivo

We next tested whether eATP-mediated pressure-induced RGC damage was also observed *in vivo*. IOP cannot be manipulated with the same degree of freedom as hydrostatic pressure *in vitro* but we can still address the qualitative question of whether rapid RGC damage occurs after short IOP transients and test the role of eATP in this process. Connecting the vitreal chamber of anaesthetized rats with a column of ACSF held at 68 cm above the eye to raise IOP to 50 mmHg, we first measured the time course of IOP elevation with a piezo-resistive

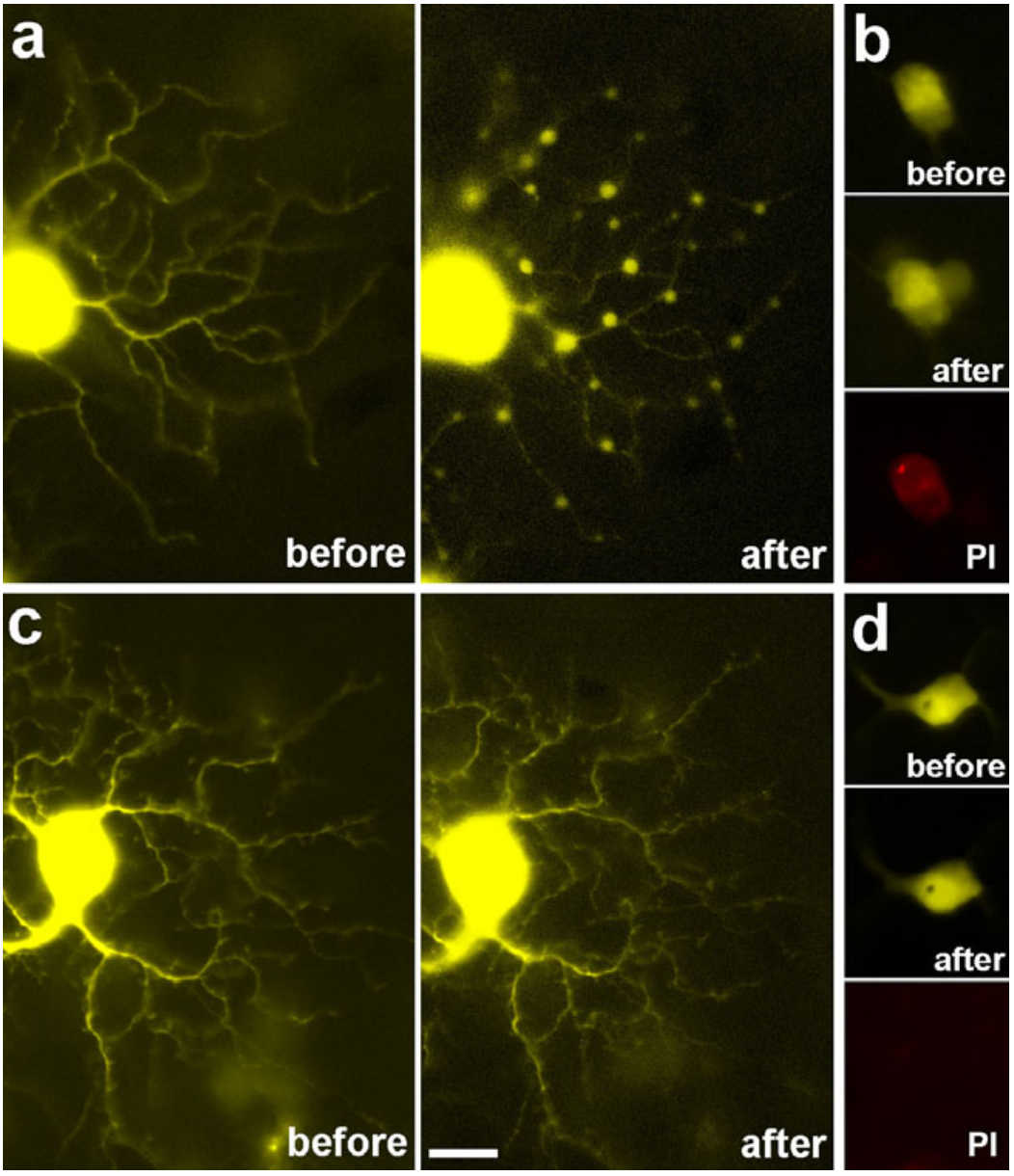


FIG. 5. Incubation in the presence of ATP injures retinal ganglion cells (RGCs) within 1 h. The dendritic tree (a) and soma (b) of a dextran-labelled RGC before (a, left; b, top) and 1 h after retinal incubation in oxygenated artificial cerebrospinal fluid containing 1 mM ATP. ATP induced extensive dendritic (a, right) and soma (b, middle) blebbing as well as propidium iodide (PI) permeability (b, bottom). Example of an RGC dendritic tree (c) and a soma (d) before (c, left; d, top) and 1 h after application of 1 mM ATP in the presence of the $P2X_7$ blocker Brilliant Blue G (BBG) (0.5 μM). BBG prevented ATP-induced blebbing (c, right; b, middle) and cell permeability to PI (d, bottom). Calibration bar: 20 μm (a and c); 10 μm (b and d).

pressure sensor (see Materials and methods). In P25–P30 rats we found a repeatable increase rate of 1.6 ± 0.3 mmHg/s ($N = 6$), probably reflecting the physical properties of the eye, and a similar decrease rate. Younger animals (P15–P17, $N = 4$) gave less reproducible results and were therefore excluded from further *in vivo* analysis.

Next we applied either a 2 min IOP transient (treated) or no pressure changes (control) to anaesthetized P25–P29 rats and 1 h later isolated the retinas and tested them for PI permeability in the ganglion cell layer. In the ganglion cell layer of pressure-treated animals we found an average of 160 ± 30 PI-permeable cells/mm^2 ($N = 8$ retinas; Fig. 6a), whereas an average of 35 ± 8 PI-permeable cells/mm^2 were observed in sham-treated animals ($N = 8$ retinas). No notable difference in the density of PI-labelled cells was observed across the retina. On the basis of their soma size (Sun *et al.*, 2002), most of these cells appeared to be RGCs (data not shown). We also analysed the effects of raising IOP to 90 mmHg for 1 min, finding after 1 h a percentage of PI-labelled cells in the ganglion cell layer very similar to that observed at 1 h after 2 min at 50 mmHg (control, 20 ± 10 PI-permeable cells/mm^2; 90 mmHg, 150 ± 50 PI-permeable cells/mm^2). Repeated pressure spikes were not studied *in vivo* because IOP measurements showed the lack of a reproducible pattern of IOP variation ($N = 4$, data not shown).

In-vivo *intraocular pressure spikes increase extracellular ATP levels in the eye*

To study the potential involvement of eATP in mediating RGC damage induced by transient IOP increase *in vivo*, we first determined whether IOP elevation increases eATP levels in the eye. We therefore measured eATP levels in fluid samples taken from the vitreal eye chamber after a single IOP spike (2 min at 50 mmHg). Knowing that eATP can be rapidly degraded by endogenous ectoATPases in the eye (Newman, 2001), we performed this measure at 90 s after the IOP spike and found a five-fold increase in ATP levels when comparing rats subjected to IOP elevation with control rats that only received eye cannulation but no IOP increment (control, 0.37 ± 0.15 μM, $N = 8$ animals; treated, 2.1 ± 0.6 μM, $N = 6$; $P < 0.01$, Student's *t*-test).

Degrading extracellular ATP prevents retinal ganglion cell damage induced by intraocular pressure pulses in vivo

Next we tested whether eATP blockade prevented RGC PI permeability induced by a single IOP spike *in vivo*. We performed intraocular injection of either apyrase (30 U/mL intraocularly; $N = 8$) or vehicle ($N = 4$) 5 min before a 2 min IOP elevation to 50 mmHg. Although vehicle did not significantly change the density of PI-permeable RGCs at 1 h after the IOP increment, apyrase greatly reduced this density, as illustrated qualitatively in Fig. 6b and quantitatively in Fig. 6c (no parametric Kruskal–Wallis one-way ANOVA on ranks, pairwise comparison with the Holm-Sidak method at 0.05 significance level; all comparisons significant except apyrase + pressure vs. control and vehicle + pressure vs. pressure). Similarly, a protective effect of apyrase was observed at 1 h after increasing IOP to 90 mmHg for 1 min (data not shown).

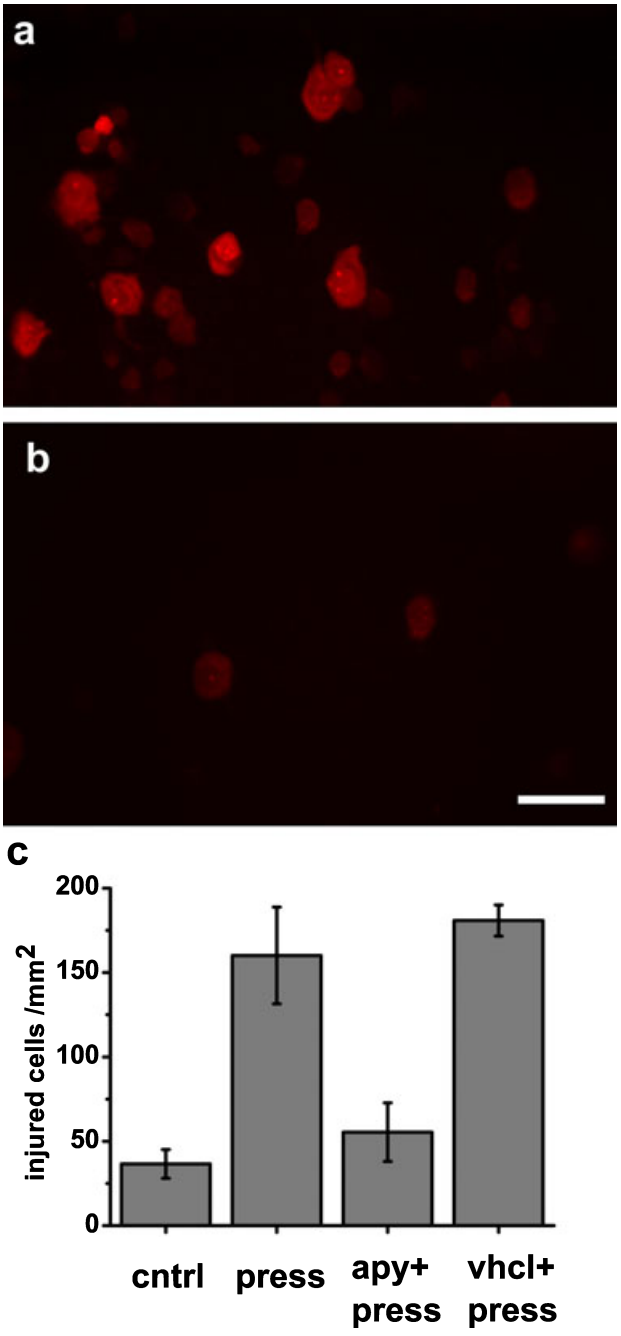
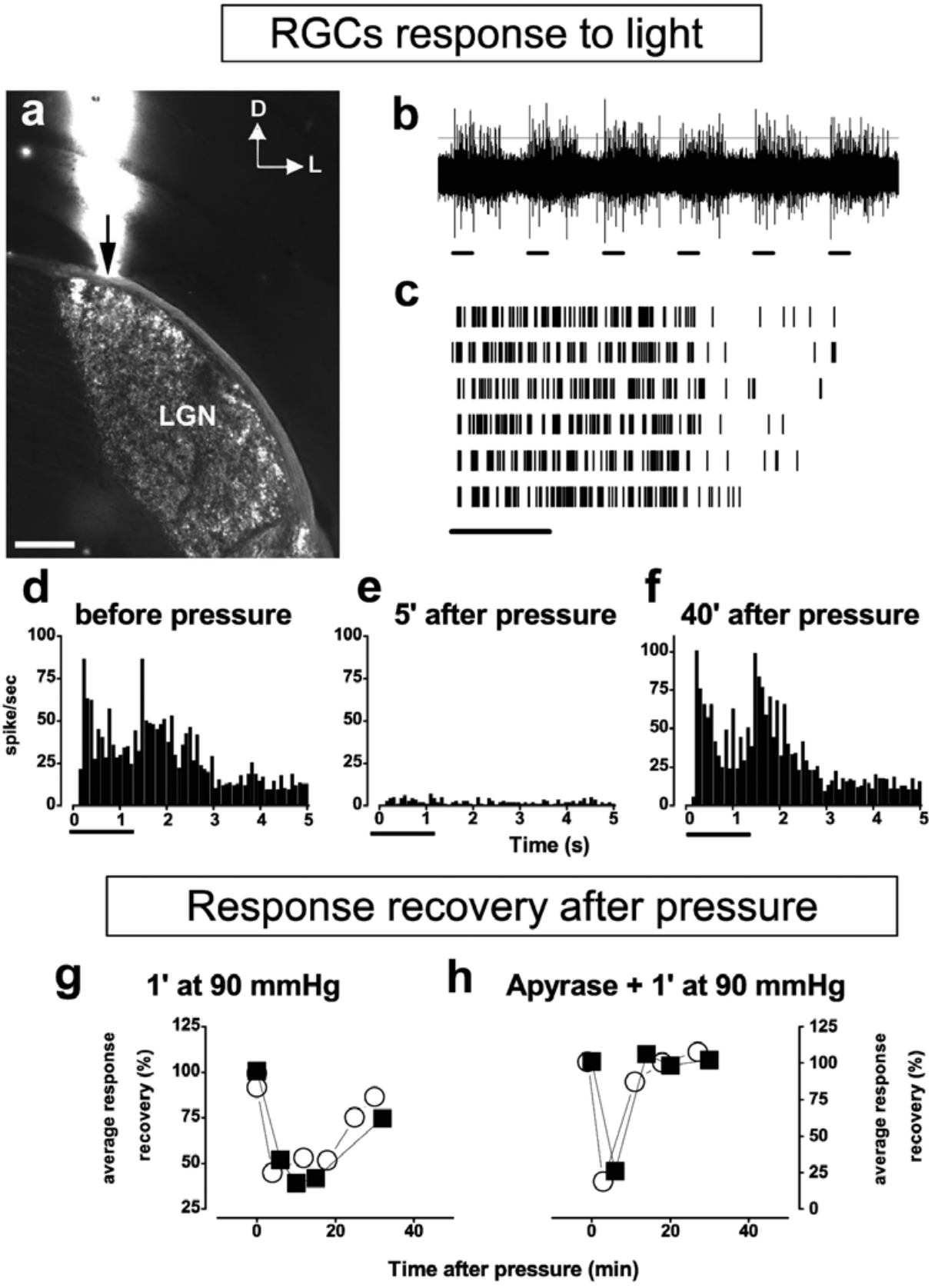


FIG. 6. Brief intraocular pressure (IOP) transients rapidly damage retinal ganglion cells *in vivo* but extracellular ATP degradation prevents such damage. (a) Increasing IOP to 50 mmHg for 2 min damages neurons in the ganglion cell layer within 1 h, as revealed by propidium iodide (PI) permeability. (b) Such pressure-induced PI permeability is largely prevented by intraocular injection of apyrase (30 U/mL intraocular) 5 min before the IOP increment. Calibration bar: 20 μm (a and b). (c) Density of PI-permeable cells in the ganglion cell layer in control animals (cntrl), in animals subjected to an IOP transient (press; 2 min, 50 mmHg) and in animals that received intraocular injection of either apyrase (apy + press) or vehicle (vhcl + press) 5 min before IOP elevation. Retinas were analysed 1 h after the IOP transient.

Degrading extracellular ATP speeds up recovery of the retinal ganglion cell light response after intraocular pressure pulses in vivo

To further explore the effects of eATP degradation on RGCs following IOP increments, we recorded RGC light responses before and after a single IOP transient. It has long been known that short pressure transients can temporarily impair the RGC light response (reviewed in Troy & Shou, 2002). This deficit is normally reversible, and we wondered whether and how recovery might be affected by degrading

eATP in the eye. For this purpose, multiunit spike activity evoked by a light flash was recorded in the optic tract (Fig. 7a–d) at 5 min intervals before and after an IOP pulse. Typically, in multiunit recordings, both the onset and offset of a light flash evoke significant increments in firing rate, as shown in Fig. 7b–d. After a single IOP spike at 50 mmHg this response to light is temporarily reduced, and even more



so after a 1 min spike at 90 mmHg (Fig. 7e), and then gradually it recovers (Fig. 7f), in line with that observed in single RGC recording (Troy & Shou, 2002). Quantifying response as the average firing rate in the interval 0–2 s after flash onset, the average maximal response decrement caused by a 1 min 90 mmHg IOP spike was 80 ± 16% ($N = 15$; data expressed as mean ± SD) and the average time needed to recover at least 85% of the initial response was 32 ± 4 min after the IOP spike. Two typical time courses for response recovery in normal animals are illustrated in Fig. 7g. Apyrase treatment (30 U/mL intraocularly) at 1–3 h before IOP elevation shortened the average response recovery time to 11.2 ± 2.6 min ($N = 8$), while not significantly altering spike activity before pressure application (data not shown). Two examples of response recovery after IOP spike in apyrase-treated animals are illustrated in Fig. 7h. The difference in recovery time between normal and apyrase-treated animals was statistically significant ($P < 0.001$, Student's *t*-test). Thus, reducing eATP levels in the eye accelerates the recovery of RGC light responses.

Discussion

Here we have investigated the acute effects of pressure transients on RGCs. Our results show that rapid pressure transients at or above 50 mmHg injure RGCs *in vitro* and *in vivo*. This damage is prevented by eATP degradation and by pharmacological blockers of the P2X purinergic receptors for eATP. In the following discussion we will briefly consider the characteristics of this rapid pressure-induced RGC damage, the mechanisms by which eATP could act to mediate it and the potential relevance of these findings to pathology.

Rapid retinal ganglion cell damage induced by short pressure transients

Rapid pressure-induced RGC damage was observed both *in vitro* and *in vivo* following short (minutes) pressure transients. The *in-vitro* and *in-vivo* approaches used complemented each other to provide information about this phenomenon. *In-vitro* analysis revealed features of pressure-induced damage at the single cell level, allowed us to analyse qualitatively the relevance of different pressure parameters (duration, rate of increase and peak value) to cell damage and made it possible to perform controlled pharmacological tests. Finally, an *in-vitro* analysis allowed the problem of potential ischaemic conditions associated with IOP changes *in vivo* to be bypassed. The main limitations of the *in-vitro* approach were that pressure was not as complex a stimulus as are *in-vivo* IOP elevations, which have both hydrostatic and stress-related components, and that most experiments were limited to young retinas (P15–P17) in order to optimize cell labelling efficiency. *In-vivo* experiments were limited by a reduced degree of freedom in pressure manipulations, which restricted the age range tested and the detail at which single cell damage could be investigated, but still allowed testing of whether rapid RGC damage occurred after IOP elevation *in vivo* and whether it was mediated by eATP.

In our opinion, three features of acute pressure-induced RGC damage appear most worthy of consideration: its existence, its additive nature and its association with rapid pressure transients. The occurrence of a temporary visual deficit associated with high IOP transients has long been known. However, electrophysiological recordings from RGCs during this phenomenon and the complete functional recovery that normally follows have focused attention mostly on transient alterations in light responses (Troy & Shou, 2002). Here we show that, following a rapid (1–2 min) pressure transient at 50 mmHg, there is also a small (29%) but significant percentage of RGCs exhibiting loss of membrane integrity within 1 h of pressure elevation both *in vitro* and *in vivo*. Interestingly, similar proportions of RGCs are lost within a few days after permanent IOP elevation in rats (Naskar *et al.*, 2002; Ben Simon *et al.*, 2006). The analysis of individual RGCs in isolated retinas has also shown that damage induced by pressure transients can be cumulative, as we observed using a sequence of pressure spikes (7 × 50). When compared with the effects of a single pressure transient (1 × 50), these sequential stimuli considerably increased both the percentage of affected cells and the extent of individual cell damage, increasing the frequency and extent of soma blebbing, the frequency of PI labelling and causing the appearance of dendritic blebbing. The use of sequential pressure insults on isolated retinas has also shown that all RGC types are affected, although α-RGCs take longer to display signs of damage, and the soma, dendrites and axon differ in their vulnerability to pressure transients, with the highest vulnerability in the soma and the lowest in the axon. Interestingly, dendritic and soma alterations in RGCs with still apparently viable axons and the lack of a cell-type-specific vulnerability to damage have also been observed in retinas from glaucoma animal models exposed to prolonged (months to years) periods of elevated IOP (Weber *et al.*, 1998; Jakobs *et al.*, 2005). The finding of the highest pressure resistance in the axons is also of interest, as this higher resistance might be an important feature considering that axons enter the optic nerve head, the region of the eye where, for mechanical and geometrical reasons, IOP-induced mechanical stress is maximal (Burgoyne *et al.*, 2005).

Most of our analysis *in vitro* was focused on P15–P17 retinas, in which we obtained optimal RGC labelling, leaving open the possibility that more mature RGCs might be differently affected by

FIG. 7. Extracellular ATP degradation accelerates recovery of retinal ganglion cell (RGC) light response after an intraocular pressure (IOP) pulse. (a) Coronal section through the thalamus of a postnatal day 25 rat that received an intravitreal injection of Alexa 488-conjugated cholera toxin B into the contralateral eye to label retinogeniculate projections (white grainy staining) and was recorded 1 day later. The track of the recording electrode is marked with 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; white labelling, arrow) and shows that the tip of the recording micropipette was located within the optic tract overlying the medial portion of the dorsal lateral geniculate nucleus (LGN). D, dorsal; L, lateral. Calibration bar, 400 μm. (b) Example of extracellular activity recorded in the optic tract. The trace shows multiunit responses to a series of light flashes. Horizontal bars under the trace represent light ON periods (duration of each flash 1.25 s). The continuous line placed on the trace exemplifies qualitatively the thresholding procedure for spike discrimination. (c) Raster display showing an additional example of spike recordings in response to six consecutive light flashes. Each vertical bar on the raster plot represents an extracellularly recorded action potential. Each row is the response to a single flash. The horizontal bar under the trace indicates flash duration (1.25 s). The top row corresponds to the first flash of the series. This raster display is derived from a different animal than the trace in b. Spike responses are typically plotted as peristimulus time histograms (PSTHs), which were obtained by computing the firing rate every 78 ms and averaging over 10 consecutive stimulations. (d–f) Example of a PSTH from multiunit spike activity recorded in the optic tract in response to a 1.25 s flash of light right before (d), 5 min (e) and 40 min after (f) an IOP transient of 1 min to 90 mmHg. The bars underneath each graph represent flash duration. Notice that RGC activity was elicited by both light onset and offset, giving rise to a double peak in the PSTH. The example illustrates a case of strong reduction of RGC activity soon after IOP. (g) Typical time-course of response recovery after 1 min IOP transient to 90 mmHg in control animals. Symbol size is larger than error bars. Average response was quantified as the average spike activity in the 0–2 s time interval of each PSTH. (h) Intraocular injection of apyrase prior to IOP increments greatly reduced the time of RGC light response impairment after a 1 min IOP transient to 90 mmHg.

pressure than these younger RGCs. In this latter respect, however, a limited set of experiments on P24–P29 retinas, an age range when rat RGCs appear indistinguishable from adult RGCs both morphologically (Yamasaki & Ramoa, 1993) and electrophysiologically (Wang *et al.*, 1997), showed that, at least for the 7 × 90 stimulus, the RGCs appear to behave just like the younger RGCs. We cannot exclude, however, that the degree of pressure-induced damage caused by milder stimuli might change with age.

We have observed that abrupt pressure transients injure RGCs (1 × 50 and 7 × 50), whereas slowly elevating pressure to the same peak level (slow-50) did not. It is worth considering that the 7 × 50 sequence and the slow-50 stimulus expose retinas to 50 mmHg for the same amount of time (7 min) but the first damages most RGCs and the second damages none. This strongly indicates that it is rapid pressure variation that mostly affects RGCs. From a cell biological point of view this finding is not totally unexpected. Cells have a notorious capability to adapt within large ranges of external physical variables; culture studies show that neuronal and non-neuronal cells from the eye can withstand prolonged steady-state hyperbaric conditions at or above 50 mmHg without loss in cell viability (Wax *et al.*, 2000), as do many other cell types (reviewed in Macdonald & Fraser, 1999). However, abrupt variations in external conditions challenge the cellular capability to adapt and might induce injurious effects, as we have observed here for rapid pressure variations. In future, it will be important to investigate how slowly pressure has to change in order to avoid RGC damage.

Mechanisms of extracellular ATP-mediated pressure-induced damage

Recent studies have shown that localized mechanical stimulation induces focal eATP release in isolated retinas (Newman, 2001). Furthermore, exogenous application of 2′,3′-benzoyl-4-benzoyl-ATP to primary cultures of RGCs reduced cell survival within 24 h (Zhang *et al.*, 2005). These findings lend support to the hypothesis that endogenous eATP might have a role in rapid pressure-induced RGC injury. Exposing isolated retinas to our most severe pressure insult (7 × 90), which normally affects all RGCs, we found that degrading eATP with apyrase or using the P2X receptor blockers oATP or BBG totally prevented RGC damage. *In vivo*, we found that eATP levels are increased in the eye after IOP increments and that eATP degradation by apyrase prevents RGC damage and promotes light response recovery after transient IOP elevation *in vivo*. These results strongly suggest that eATP mediates the acute RGC damage observed after abrupt pressure transients *in vitro* and *in vivo*. These findings raise a number of questions, as we will briefly discuss.

By measuring the average eATP concentrations in eye fluid samples, we showed that eATP release is associated with IOP changes but these bulk measures cannot provide a quantitative indication of the peak eATP levels that cells might experience. The use of soluble luciferase has provided estimates of local eATP peaks around 100 μM after mechanical stimulation in isolated retinas (Newman, 2001) but even these measures might underestimate the eATP concentration close to the release site, as suggested by studies using cellular detectors expressing luciferase on their plasma membrane (Pellegatti *et al.*, 2005).

Elucidating this issue is of particular interest considering that eATP concentrations of above 100 μM are normally required to activate the $P2X_7$ cytotoxic receptors (North, 2002), whose involvement is strongly supported by the inhibition of pressure-induced RGC damage obtained with either oATP or BBG. However, as neither blocker is strictly selective for $P2X_7$ (Murgia *et al.*, 1993; North, 2002), we cannot exclude that other P2X receptors might be involved. The analysis of pressure effects in the retina of $P2X_7$ knockout mice will provide an important contribution towards the elucidation of this issue.

The mechanisms of eATP-induced RGC damage are also still unclear. $P2X_7$ expression in RGCs has been shown by immunostaining (Brandle *et al.*, 1998; Franke *et al.*, 2005; Resta *et al.*, 2005; Zhang *et al.*, 2005) but the specificity of $P2X_7$ immunoreactivity in the nervous system is still debated (Sim *et al.*, 2004; Anderson & Nedergaard, 2006). Furthermore, studies attempting to detect $P2X_7$-mediated effects in RGCs have yielded apparently contrasting results. ATP application to RGCs isolated from immature retinas does not elicit membrane potential alterations attributable to rapid $P2X_7$ pore opening (Taschenberger *et al.*, 1999) and neither immature nor mature isolated retinas display loss of RGC membrane integrity within minutes of ATP application (Innocenti *et al.*, 2004; present results) or membrane blebbing (present data). However, when longer time intervals are considered, cytotoxic effects are observed that can be attributable to the $P2X_7$ receptors; 24 h incubation in 50 μM 2′, 3′-benzoyl-4-benzoyl-ATP reduces RGC survival in culture unless the $P2X_7$ receptors are pharmacologically inhibited (Zhang *et al.*, 2005) and we found here that 1 h retina incubation in the presence of ATP induces RGC blebbing and PI permeability, unless oATP or BBG is present. These findings are not irreconcilable with a direct cytotoxic action of eATP on RGCs. A delayed effect of eATP could be explained by considering that $P2X_7$ pore opening requires sustained activation (Di Virgilio *et al.*, 1998; North, 2002), and that this may be contrasted by local eATP degradation, as suggested by the strong expression of the CD39 ecto-ATPase observed around RGCs (Zimmermann, 1996; Braun *et al.*, 2004; V.R. and L.G.-R. unpublished results). However, the possibility that eATP affects RGCs indirectly, e.g. via eATP-activated bystander cells such as microglia (Ferrari *et al.*, 1996; Innocenti *et al.*, 2004) or macroglia (Fields & Burnstock, 2006), should also be considered in future studies.

Potential relevance to pathology: a neuroprotective action of extracellular ATP blockade

Elevated IOP is a major risk factor for glaucoma (Leske *et al.*, 2003). Hypertensive glaucoma is typically associated with chronic (months to years) IOP elevation, a condition that cannot be tested satisfactorily in isolated retinas whose viability is normally limited to a few days. However, IOP fluctuations on a daily basis are becoming increasingly investigated as an independent risk factor for glaucoma progression (Asrani *et al.*, 2000; Hughes *et al.*, 2003). Given the rate of outflow through the trabecular meshwork and aqueous production it is unlikely that these fluctuations will resemble in duration or abruptness those used here but it will be important in the future to understand how brisk a pressure insult needs to be to injure RGCs.

Intraocular pressure elevation reaching 50 mmHg or more can be experienced in trauma, acute glaucoma (Saw *et al.*, 2003) and during refractive surgery such as Lasik (Arbelaez *et al.*, 1997), where the IOP elevation is very brief but also rather fast. Typically, a temporary visual deficit occurs in acute high IOP conditions but no permanent damage ensues if IOP is rapidly reduced to normal levels. However, although very rare, this is not always the case (e.g. Bushley *et al.*, 2000; Varano *et al.*, 2005). The results of the present study may provide a cellular explanation for prolonged or even permanent visual deficits following short IOP elevation at or above 50 mmHg. We found that short pressure transients injure some RGCs and increase the vulnerability of many more, as shown by the effects of multiple pressure insults. RGC injury induced by a single 50 mmHg pressure transient is limited and probably reversible, as suggested for example by light response recovery, but, given the additive nature of

such damage, it is not difficult to speculate that, in conditions of particular individual vulnerability and/or of a pre-existing pathology, pressure transients might contribute to irreversible RGC injury in a way similar to the cumulative damaging effects that we observed in isolated retinas after multiple pressure insults. Indeed, *in vivo*, increased RGC vulnerability after IOP increments might cumulate with or even contribute to the secondary injury observed in the days following reperfusion after pressure-induced retinal ischaemia (reviewed in Osborne *et al.*, 2004). Taken together, these considerations suggest that eATP blockade, which prevents rapid RGC pressure-induced damage, may represent a useful novel neuroprotective tool in conditions associated with rapid high IOP increments.

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Conflict of interests

V.R., L.G.-R. and F.D.V. have applied for a US Patent based on the present results.

Abbreviations

ACSF, artificial cerebrospinal fluid; BBG, Brilliant Blue G; eATP, extracellular ATP; IOP, intraocular pressure; oATP, oxidized ATP; P, postnatal day; PI, propidium iodide; RGC, retinal ganglion cell.

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