Purinergic Signalling
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ORIGINAL ARTICLE

Increased Ap_4A levels and ecto-nucleotidase activity in glaucomatous mice retina

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Abstract

The pathogenesis of glaucoma involves numerous intracellular mechanisms including the purinergic system contribution. Furthermore, the presence and release of nucleotides and dinucleotides during the glaucomatous damage and the maintenance of degradation machinery through ecto-nucleotidase activity are participating in the modulation of the suitable extracellular complex balance. The aim of this study was to investigate the levels of diadenosine tetraphosphate (Ap_4A) and the pattern of ecto-nucleotidase activity expression in glaucomatous retinas during the progress the pathology. Ap_4A levels were analyzed by HPLC in glaucomatous retinas from the DBA/2J mice at 3, 9, 15, and 23 months of age. For that, retinas were dissected as flattened whole-mounts and stimulated in Ringer buffer with or without 59 mM KCl. NPP1 expression was analyzed by RT-PCR and western blot and its distribution was assessed by immunohistochemistry studies examined under confocal microscopy. Glaucomatous mice exhibited Ap_4A values, which changed in stimulated retinas as long as the pathology progressed varying from 0.73 ± 0.04 (3 months) to 0.170 ± 0.05 pmol/mg retina (23 months). Concomitantly, NPP1 expression was significantly increased (82.15%) in the DBA/2J mice at 15 months. Furthermore, immunohistochemical studies showed that NPP1 labeling was stronger in OPL and IPL labeling tangentially in the vitreal part of the retina and was upregulated at 15 months of age. Our findings demonstrate that Ap_4A decreased levels may be related with exacerbated activity of NPP1 protein in glaucomatous degeneration and in this way contributing to elucidate different mechanisms involved in retinal impairment in glaucomatous degeneration.

Keywords DBA/2J · Nucleotides · Ecto-nucleotidases · NPP1 · Ap_4A · Retina · Glaucoma

Introduction

Nucleotides are becoming relevant chemical messengers in the physiology of the eye [1]. Apart from the more relevant representative of nucleotides, ATP, others such as diadenosine polyphosphates are becoming also significant since it participates in processes such as tear secretion, regulation of IOP, or protection of the ciliary body from neurodegeneration [2, 3].

Diadenosine polyphosphates are structurally formed by two adenosines bridged by a variable phosphate chain (from 2 to 7). The main representative of this family is diadenosine tetraphosphate, abbreviated Ap_4A [4, 5]. Diadenosine polyphosphates are stored in synaptic vesicles, mainly stored with monoamine and with acetylcholine and therefore their exocytotic release can be achieved either by using agents such as veratrine or 4-aminopyridine or simply by stimulating nerve terminals with 59 mM KCl in the presence of extracellular Ca^{2+} [6, 7]. Once at the extracellular milieu, diadenosine polyphosphates interact with P2 purinergic receptors [6, 8], and their actions are terminated by the action of ecto-nucleotidases [9]. Most of these Ap_nA-hydrolyzing enzymes display biochemical characteristics typical of members of the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family. This group of ecto-enzymes is formed by seven members termed NPP1-NPP7. All E-NPPs ecto-enzymes, but NPP2, are type-II or type-I single-span transmembrane proteins [9–11]. Among all, NPP1, NPP2, and NPP3 are capable

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of hydrolyzing diadenosine polyphosphates. In this sense, they cleave these dinucleotides providing as products AMP and adenosine 5′(n–1) phosphate, these three NPPs presenting values of Km in the low micromolar range [12]. According to studies carried out in neural models, NPP1 seems to be the main ecto-nucleotidase cleaving diadenosine polyphosphates [13, 14].

The presence and identification of nucleotides and dinucleotides in ocular secretions [15–17] in the neural retina [18, 19] and their regulation by ecto-nucleotidases are indicating their relevance in ocular physiology processes as previously commented [2].

In the retina, the purinergic signaling system performs different roles in neuronal information processing [1]. The neural retina, the retinal pigmented epithelium (RPE) and glial cells express P1 (adenosine) and P2 (nucleotide) receptors [20] and ecto-enzymes including NPP1 and NTPD2 in Müller cells and ecto-ATPase located on photoreceptors, RPE, and Müller cells [21–24]. Furthermore, purinergic signaling is activated in the retina under pathophysiological conditions, which are mediating visual transduction and intracellular pathway cascades leading to neurodegenerative processes in retinal cells [25–29].

In this sense, the present work describes the changes in Ap_4A concentration and the increase in the expression of NPP1 with the development of the glaucomatous pathology, suggesting the possible role of these molecules in the physiopathology of this disease.

Materials and methods

Animals

The experiments were performed with female DBA/2J and C57BL/6J mice for each stage of age, obtained from the European distributor of Jackson Laboratories mice (Charles Rivers Laboratories). C57BL/6J and DBA/2J mice were divided in groups corresponding to different ages: 3, 9, 15, and 23 months of age. The total number of animals used for NPP expression and Ap_4A quantification was 15 animals per age.

All animal maintenance and experimental procedures were in compliance with institutional, Spanish and European guidelines of animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed and handled with the authorization and supervision of the Institutional Animal Care and Use Committee from the Complutense University of Madrid. Four mice were housed per cage in temperature and light-controlled rooms maintained on a 12-h light/dark cycle, and all animals had access to food and water ad libitum.

For immunohistochemical studies and sample collection of mice retinas, animals were perfused with an i.p. injection of a lethal dose of pentobarbital (Dolethal Vetoquinol®, Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain).

IOP and electroretinogram measurements

IOP and electroretinogram (ERG) measurements were carried out by anesthetizing the mice with an intraperitoneal (i.p.) injection of ketamine (95 mg/kg, Imalgene 1000®, Merial, Barcelona, Spain) and xylazine (5 mg/kg, Rompún®, Bayer, S.A., Barcelona, Spain).

For IOP measurements, control and glaucomatous mice were anesthetized and maintained on a warmed pad. The intraocular pressure was measured using a non-invasive rebound tonometer (Tonolab®; iCare Finland Oy, Helsinki, Finland). Both eyes of each animal were measured immediately after anesthesia at 3, 9, 15, and 23 months, and IOP was measured at the same time to avoid changes due to the circadian rhythm.

For electroretinogram recordings the mice were dark-adapted overnight before the ERG recordings. Pupil mydriasis was induced by instilling 1% tropicamide (Colircusí Tropicamida 1% ®; AlconCusí, S.A., El Masnou, Barcelona, Spain) in the right eye of each mouse. Scotopic threshold responses were recorded in response to light stimuli produced signals generated in the retina were amplified (1000×) and band filtered between 0.3 and 1000 Hz with a Grass amplifier (CP511 AC amplifier, Grass Instruments, Quincy, MA).

Electrical signals were digitized at 20 kHz using a Power Lab data acquisition board (AD Instruments, Chalgrove, UK). Light stimuli were calibrated periodically with a photometer (Mavo Monitor USB, Gossen, Nürenberg, Germany). The recordings were processed with the criteria established by the International Society for Clinical Electrophysiology of Vision (ISCEV).

The STR was analyzed for each stimulus, positive STR (pSTR) was measured from the baseline to the peak of the positive deflection, approximately 110–120 ms from the flash onset, and the negative STR (nSRT) was measured from the baseline to the peak of the negative deflection after the pSTR, approximately 220 ms from the onset of the flash. ERG wave amplitudes were analyzed for each animal group, and the percentage difference between the DBA/2J eyes and the control eyes was obtained for each stimulus and was further averaged (mean ± standard error of the mean, SEM).

HPLC measurements

Control and glaucomatous retinas at 3, 9, 15, and 23 months of age ($n = 3$ each group) were isolated under a stereo microscope. Control samples of C57BL/6J and DBA/2J mice were

maintained in Ringer solution (non-stimulated retinas) and the stimulated retinas of DBA/2J mice and age-matched controls were treated with 59 mM potassium chloride (KCl) in Ringer solution for 1 min. The supernatants (1000 μl) were collected in 1.5-ml tubes and maintained in ice during 5 min. The tubes were heated in a 98 °C bath for 2 min and transferred to ice 10 min, to precipitate the proteins [30]. Then samples were centrifuged at 22,000×*g* for 10 min at 4 °C to pellet the proteins and maintained at freeze until high-pressure liquid chromatography (HPLC) analysis.

NPP1 activity was analyzed in glaucomatous mice model. DBA/2J retinas at 3 and 15 months of age ($n = 4$ for each age) were incubated in Locke's solution (composition in mM: NaCl, 140; KCl, 4.5; $CaCl_2$, 2.5-, KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 5.5; HEPES, 10; pH 7.4). Then, 10 μM (250 pmol) Ap_4A was added to the wells and retinas were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. To assess Ap_4A cleavage, samples of the incubated medium were taken at different time points ($t = 0$ min, 15 min, 30 min, 1 h, 2 h) and the level of Ap_4A was measured by HPLC detection (see the next paragraph). Ap_4A was incubated in parallel but in the absence of retinas as a negative control.

After thawing, levels of Ap_4A were analyzed in 1000 μl of the supernatant using a reversed-phase HPLC equipped with an isocratic pump (model 1515; Waters). The chromatographic conditions consisted of a C_{18} reverse-phase column, 250 mm length and 4.6 mm in diameter (Hyperchrome, Scharlab). It was equilibrated with a mobile phase containing 10 mM KH_2PO_4, 2 mM tetrabutylammonium bisulfate (TBA), and 20% acetonitrile adjusted to pH 7.5 with 5 M KOH. The flow rate throughout chromatographic runs was 2 ml/min. Ap_4A was measured at 260 nm wavelength.

RT-PCR and quantitative real-time PCR

Total RNA from isolated retinas was extracted using RNeasy® plus mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. After digestion with TURBO DNase (Ambion, Austin, TX, USA), total RNA was quantified and reverse transcribed using M-MLV reverse transcriptase, 6 μg of random primers and 350 μM dNTPs (Invitrogen, San Francisco, CA, USA). Quantitative real-time PCR (Q-PCR) reactions were carried out using LuminoCt® qPCR readymix (Sigma-Aldrich), 5 μl of the RT product, and specific oligonucleotide primers (all from Sigma-Aldrich) for ecto-nucleotide pyrophosphatases/phosphodiesterases 1 and 3 (NPP1 and NPP3, respectively) and for tissue non-specific alkaline phosphatase (TNAP), in combination with specific Taqman MGB probes (Roche, Basel, Switzerland). For NPP1, the primers used were forward 5′-cggacgctatgattccttaga-3′ and reverse 5′-agcacaatgaagaagtgagtcg-3′. For NPP3, the primers used were: forward 5′-gatgcacaggacgaggagac-3′ and reverse 5′-tgcacgtccatatttgagttg-3′. For TNAP, the primers used were forward 5′-aatgaggtcacatccatcctg-3′ and reverse 5′-cacccgagtggtagtcacaa-3′. The probes designed were FAM-5′-gccaggaa-3′-MGB for NPP1, FAM-5′-ctgctggg-3′-MGB for NPP3, and FAM-5′-ggatgctg-3′-MGB for TNAP. Fast thermal cycling was performed using a StepOnePlus® Real-Time System (Applied Biosystems, Foster City, CA, USA) as follows: denaturation, one cycle of 95 °C for 20 s, followed by 40 cycles each of 95 °C for 1 s and 60 °C for 20 s. The results were normalized as indicated by parallel amplification of GAPDH housekeeping gene. For GAPDH, commercial primers and TaqMan MGB probe were supplied by Applied Biosystems.

Western blot

C57BL/6J and DBA/2J retinas ($n = 5$ mice for each stage of age) were homogenized with a lysis buffer (50 mM HEPES, pH 8, 150 mM NaCl, 1% NP-40 (*w*/*v*), 0.5% sodium deoxicolate, 0.1% SDS and inhibitor proteases (1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM sodium fluoride, and 2 mM sodium orthovanadate) and were loaded, were subjected to 10% SDS-PAGE, and were transferred to polyvinyldene fluoride membranes using the Mini-PROTEAN Electrophoresis System and the Mini Trans-Blot Electrophoretic Transfer System (Bio Rad). Then were incubated overnight at 4 °C in TBS1× 0.1% Tween 2-20 containing 5% non-fat milk (blocking buffer) and monoclonal anti-NPP1 primary antibody (1:100; sc-166649, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Finally, immunoreactive bands were detected by chemiluminiscence using corresponding HRP secondary antibody (1:10,000, Jackson ImmunoResearch) and were studied to analyze the alterations in the expression of NPP1 levels in the glaucomatous mice model during the progress of the pathology and with respect to the aged matched controls (3, 15, and 23 months). The densitometric analysis was performed by using Kodak Molecular Imaging Software (Kodak). The densitometry values of each sample were normalized to the respective densitometric GAPDH values.

Immunohistochemistry

DBA/2J mice at 3, 15, and 23 months ($n = 3$ mice at each time point) were anesthetized and perfused transcardially with phosphate-buffered saline (PBS) followed by a fixative solution of 4% paraformaldehyde in 0.1 M PBS at pH 7.4 at 4 °C. The eyes were enucleated and dissected to avoid damaging retina and were immersed in this solution for 1 h at 4 °C and then washed in PBS before being cryoprotected in 10% sucrose for 1 h and 30% overnight at 4 °C. Finally, the eyecups were subsequently embedded in in tissue freezing medium (Tissue-Tek© OCT) and 10-μm-thick retinal sections were cut on a cryostat (Microm, Walldorf, Germany) in a horizontal

plane and were collected on poly-L-lysine-coated slides and stored at − 20 °C until use.

For immunofluorescence studies, sections were permeabilized with PBS-0.25% Tx-100 for 30 min, followed by pre-incubation with the blocking solution, containing 10% normal donkey serum (NDS; Jackson ImmunoResearch, West Grove, PA, USA) and 0.1% Triton X-100 in PBS for 1 h at room temperature. Then, the slices were incubated with goat polyclonal anti-NPP1 primary antibody (1:250; ab-40003, Abcam, MA, USA) diluted in PBS-0.1% Tx-100 and 1% normal donkey serum overnight at 4 °C. Subsequently, sections were washed in PBS-0.1% Tx-100 and incubated with FITC-conjugated (green) donkey anti-goat IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:200 dilution for 1 h, in darkness at RT. Finally, the nuclei were stained with propidium iodide (red, Sigma-Aldrich, St. Louis, MO) diluted 1:500 in PBS for 10 min and sections were rinsed in PBS and mounted in fluoromount Vectashield (Vector Laboratories, Palex Medical, Barcelona) and coverslipped.

Microscope images were obtained with a confocal microscope (Zeiss LSM 5 Zeiss Mikroskopie, Jena, Germany) equipped with an argon laser (458/488/514 nm) and a helium-neon laser (543 nm). Images were collected as 512–512 pixels and projections of four frames using the single or the multi-track scanning module. Images acquired were processed using the Adobe Photoshop 8.0 software (Adobe Systems, Inc., San Jose, CA, USA).

Statistical analysis

Data were plotted as the mean ± standard error of the mean (SEM). The results were analyzed using Graph Pad Instat®3 for Windows® (GraphPad Instat Software, San Diego, CA, USA). Data were analyzed using unpaired *t* test for two-group comparisons. A value of $p \leq 0.05$ was considered statistically significant.

Results

Extracellular Ap_4A levels in stimulated and non-stimulated retinas

In order to verify that the glaucomatous DBA/2J presented the pathological changes due to the development of the glaucoma, both IOP and ERG measurements were performed along the whole study. In Fig. 1a, the changes in IOP in both control (C57BL/6J mice) and the glaucomatous (DBA/2J) animals are shown. The IOP values remained stable in the control animals along their life. On the contrary, in the DBA/2J there was an elevation in IOP, which was maximal at 15 months of age

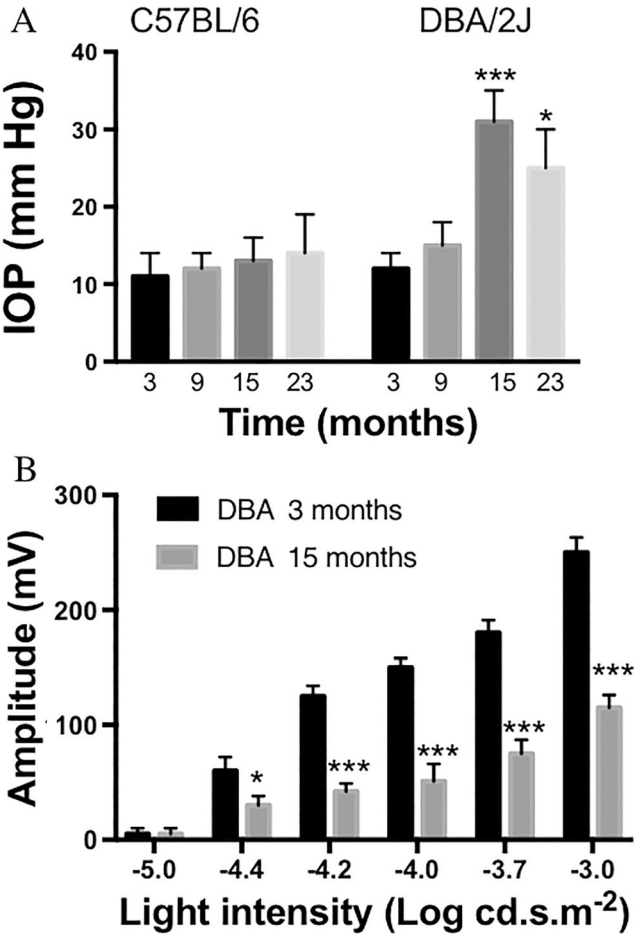

Fig. 1 Variations in the intraocular pressure (IOP) and electroretinogram recordings in control and glaucomatous mice. **a** Measurements of intraocular pressure in the control (C57) and glaucomatous (DBA/2J) mice as a function of age. Significant differences were observed in DBA/2J mice at 15 and 23 months of age when compared with the 3 months mice, which corresponds to the moment before the pathology starts (***$p < 0.001$, *$p < 0.05$). **b** Scotopic threshold responses (STR) from DBA/2J glaucomatous mice as a function of age. Between 3 months (before the pathology starts) and 15 months (when it is fully established), it was possible to observe a statistically significant reduction in the STR amplitudes when the animals presented the pathology (*$p < 0.05$. ***$p < 0.001$)

(11.5 ± 3.1 mmHg at 3 months vs. 31.3 ± 4.2 mmHg at 15 months, ***$p < 0.001$).

In the same way, the ERG patter along the pathology in the DBA/2J mice was tracked. As presented in Fig. 1b, the pSTR amplitudes showed significantly decreases at all tested light stimuli, being maximal at − 3.0 log Cd.s.m^{-2} (46%). Altogether, both IOP and ERG studies indicated that the DBA/2J mice presented the expected features of the glaucomatous pathology in this animal model.

The study on the presence of the dinucleotide Ap_4A in the retinas of both normal and glaucomatous mice depicted interesting results. Extracellular Ap_4A levels in control mice (C57BL/6J) decreased with age between 3 and 23 months in basal and stimulated conditions (Fig. 2a).

The same stages of age were studied in DBA/2J mice to assess Ap_4A concentrations during the progression of the pathology. The release of Ap_4A from the retinas of glaucomatous mice demonstrated a significant increase measurable from 15 months (1.25 ± 0.30 pmol/mg retina) compared to 23 months (2.98 ± 0.18 pmol/mg retina) in basal conditions (*$p < 0.05$).

There were no significant changes in the stimulated Ap_4A levels at 3 and 15 months (1.57 ± 0.17 pmol/mg retina), in contrast to that observed in Ap_4A levels with age (3.15 ± 0.23 pmol/mg retina). Furthermore, Ap_4A levels in glaucomatous mice were significantly increased in stimulated retinas compared to non-stimulated retinas (87%, *$p < 0.05$) at 3 months (Fig. 2b).

In addition, the net released Ap_4A was measured in control mice and the levels remained constant during animal aging (Fig. 3). Interestingly, in DBA/2J mice, the dinucleotide level was strongly decreased between 3 and 23 months of age respectively (from 0.73 ± 0.04 pmol/mg retina to 0.17 ± 0.05 pmol/mg retina).

Expression of NPP1 transcript is enhanced in glaucomatous retinas at different stages of age

In a previous study, we reported the presence of ecto-nucleotide pyrophosphatase/phosphodiesterase (NPPs) activity able to hydrolyze extracellular diadenosine polyphosphates in neural cells. The expression of both NPP1 and NPP3 isozymes was confirmed in neural cells, although diadenosine polyphosphates hydrolysis was mainly mediated by NPP1 [14]. In order to analyze the presence of NPP1 and NPP3 able to hydrolyze diadenosine polyphosphates in mouse retina, both normal (C57BL/6J) and glaucomatous (DBA/2J) mice were sacrificed at 3, 9, or 15 months of age and total RNA from their isolated retinas was purified, retro-transcribed, and quantified by Q-PCR. Moreover, the expression of a tissue-

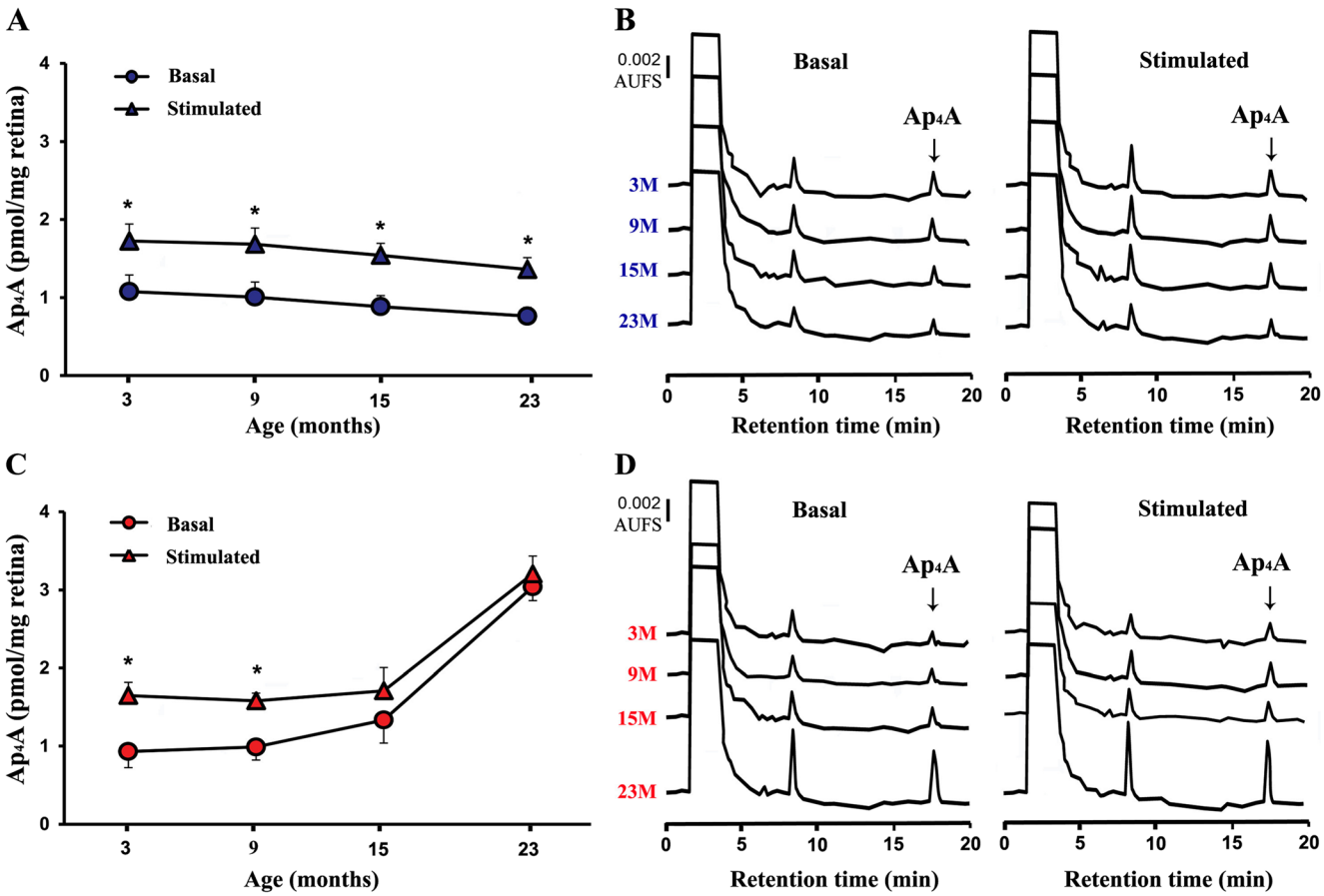

Fig. 2 Ap_4A release in control (C57) and glaucomatous (DBA) mice. **a** Basal and KCl stimulated Ap_4A release from whole mount retinas obtained from C57BL/6J mice. Panel represents the mean of Ap_4A amounts at the given mice ages either under basal (blue circles) or stimulated conditions (blue triangles). **b** Representative HPLC elution profiles of basal (left panel) and stimulated (right panel) C57BL/6J retinas. **c** Basal and KCl stimulated Ap_4A release from whole mount retinas obtained from glaucomatous DBA/2J mice. The panel represents the mean of Ap_4A amounts at the given mice ages either under basal (red circles) or stimulated conditions (red triangles). **d** Representative HPLC elution profiles of basal (left panel) and stimulated (right panel) DBA/2J retinas. Results are the mean ± SD of three independent experiments (*$p < 0.05$)

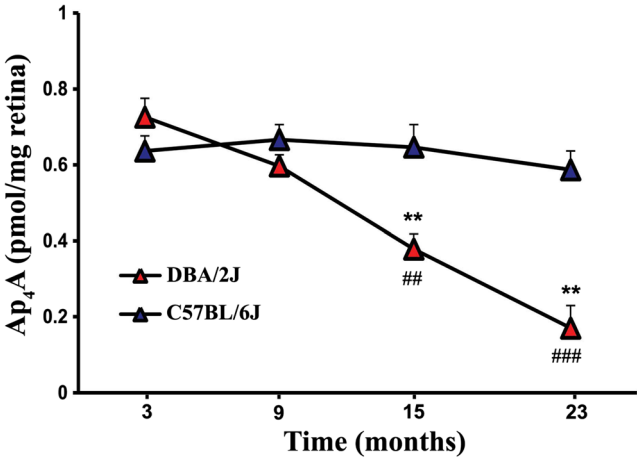

Fig. 3 Net stimulated Ap_4A release in both control (blue triangles) and glaucomatous (red triangles) mice followed from 3 and 23 months. The net stimulated release of Ap_4A was obtained in each stage of age by subtracting basal release from 59 mM KCl stimulated release. Values are the mean ± SD of eight experiments ($^{**}p < 0.01$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$)

non-specific alkaline phosphatase, able to hydrolyze a wide spectrum of extracellular nucleotides but not dinucleotides, was also analyzed [31]. As shown in Fig. 4, the expression of NPP1, NPP3, and TNAP was detected in retina from both normal and glaucomatous mice, TNAP transcript being the most abundant one, followed by NPP1 and to a lesser extent NPP3 (comparison of normalized ratios versus GAPDH housekeeping transcript). Interestingly, the expression of NPP1 was significantly increased in mice glaucomatous retinas compared to control ones at all ages analyzed (Fig. 4a). On the contrary, no differences were observed in the expression on either NPP3 or TNAP ecto-enzymes (Fig. 4b, c, respectively). Moreover, whereas NPP1 expression was maintained invariable for 3 to 15 months old in control retinas, an upregulation of NPP1 was observed in glaucomatous retinas from older mice, suggesting that NPP1 and, consequently, extracellular dinucleotide levels could be specifically involved in the development of glaucomatous phenotype.

Alterations of NPP1 protein levels in glaucomatous mice model

In order to quantify possible ecto-nucleotide pyrophosphatase changes, NPP1 expression was analyzed by western blot (Fig. 5a, b). NPP1 is weakly expressed in DBA retinas at 3 months and its expression was increased at 15 months (82.15%, $^{**}p < 0.01$). In older glaucomatous mice, it was possible to see a reduction when comparing with DBA/2J at 15 months. To further determine the differences with respect to the matched controls, NPP1 was evaluated and the results were significantly increased at 3 months (92.45%, $^{*}p < 0.05$) and 15 months (94.65%, $^{*}p < 0.05$).

The activity of this enzyme cleaving Ap_4A in animals before (3 months of age) and after glaucoma appears (15 months of age) was measured during 120 min. As it can be seen in Fig. 5c, the cleavage of Ap_4A by NPP1 in the 3-month-old retinas only permitted the detection of 123.1 ± 20.3 pmol of this dinucleotide after 2 h of incubation with the retinas ($n = 4$). Interestingly, when the same experiment was performed with the glaucomatous retinas (15 months old), the remaining Ap_4A was 26.6 ± 15.4 pmol ($n = 4$). Ap_4A in the absence of any tissue (control) was hydrolyzed very slowly (Fig. 5c).

Furthermore, immunohistochemistry studies were analyzed to identify possible alterations in the distribution of ecto-nucleotidase activity during the progress of glaucomatous impairment. NPP1 immunoreactivity was detected in the outer plexiform layer (OPL) and inner plexiform layer (IPL) in control and pathological retinas. At 15 months, when the retinal dysfunction was observed in a previous study [32], NPP1 immunoreactivity was increased in DBA/2J mice. On the contrary, as shown in Fig. 6, the expression of de ecto-nucleotidase protein was reduced in the pathological mice at 23 months compared to 15 months. This localized retinal immunolabeling with NPP1 suggesting the relevant role of these proteins in the synaptic connectivity through the plexiform layers.

Discussion

Diadenosine polyphosphates (Ap_nA) are molecules that act via P2 receptors to serve as neuromodulators [6, 33, 34] and contribute to several physiological and physiopathological mechanisms in the nervous system [35–38]. Besides, these signaling molecules are involved in numerous ocular physiological processes including tear secretion, corneal wound healing, retinal detachment, or intraocular pressure regulation [39–41]. Dinucleoside polyphosphates are in the modulation of retinal processing via P2 receptors after being released from nerve endings [19, 42, 43]. For this reason, the assessment of extracellular concentrations reached by Ap_4A and other nucleotides could be a relevant feature since these compounds could play an important function during the visual impairment in glaucomatous damage. In this sense, it is very interesting to indicate that Ap_4A basal concentration at 23 months of age was statistically higher than the measured in the previous stages (see Fig. 1). The reason for such an increase is not clear, although some ideas can be suggested. The dinucleotide Ap_4A has been claimed as an "alarmone." An alarmone is a molecule whose concentrations increase as a consequence of environmental changes [44]. It could be the case that the variations in the retinal environment due to glaucoma pathology may trigger the production of this dinucleotide. This makes sense since Ap_4A has also been described as a molecule that protects the eye's neural structures [33] and in the same way it could be

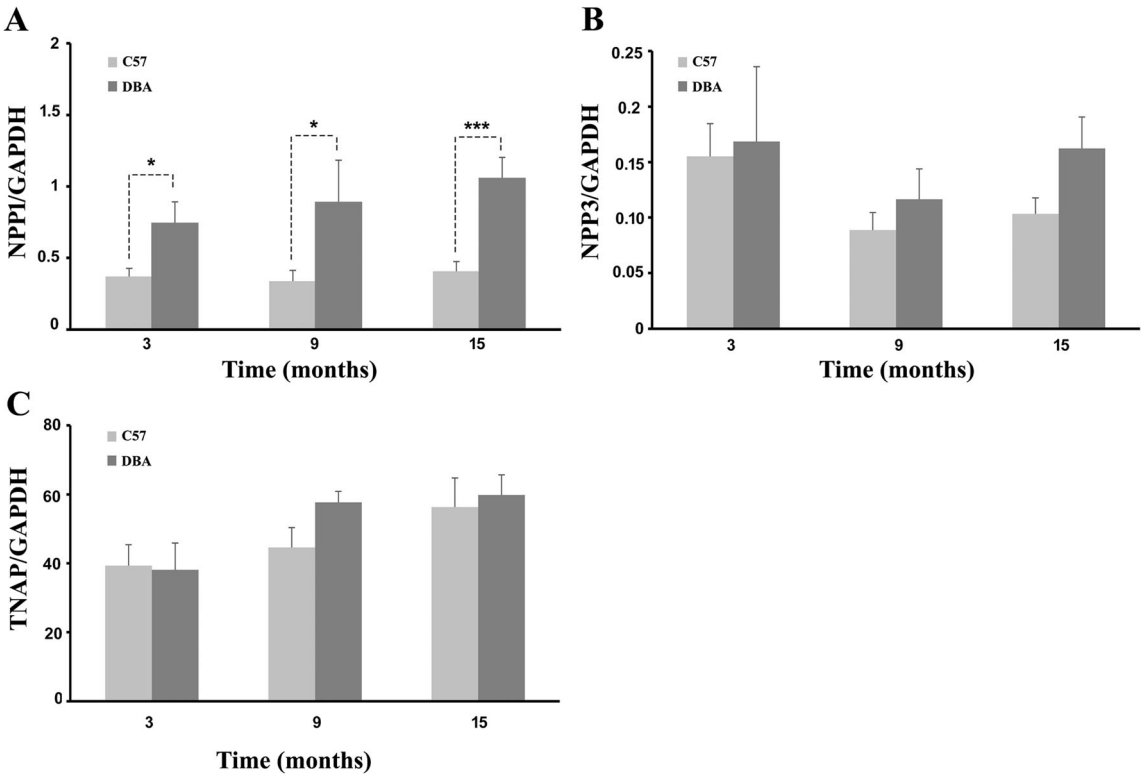

Fig. 4 Normalized expression of ecto-nucleotidases NPP1, NPP3, and TNAP transcripts in glaucomatous versus control retinas at different ages. Retinas from C57BL/6J and DBA/2J mice at 3, 9, and 15 months of age were isolated and total RNA was extracted and retro-transcribed. NPP1 (**a**), NPP3 (**b**), and TNAP (**c**) mRNAs were quantified by Q-PCR, using GAPDH as housekeeping gene. Results are mean ± s.e.m. of three independent experiments in triplicate. $*p \leq 0.05$, $***p \leq 0.001$ (unpaired *t* test)

also protecting the retina. Nevertheless, these ideas deserve further research to be confirmed.

Considering these findings, extracellular dinucleotide levels and their suitable regulation by degradation mechanisms, involving ecto-nucleotidase activity, suggest an important point to consider in the maintenance of the extracellular medium. Ecto-nucleotidases comprise a relevant family of proteins involved in the regulation of nucleotide-dependent signaling in the nervous system as previously noticed [9–13, 45, 46].

Previous studies demonstrated increased levels of these nucleotides in aqueous humor and other anterior ocular structures in glaucomatous patients [40, 47, 48]. In accordance with these reports, ATP and Ap_4A concentrations were increased in the aqueous humor (J. Pintor, personal communication) of DBA/2J mice, a well-characterized model of glaucoma [18]. Furthermore, relevant changes in nucleotide levels were observed in murine retinas during glaucomatous degeneration [19, 49] and in DBA/2J mice.

In this study, stimulated Ap_4A concentration was dramatically reduced in the glaucomatous mice from 3 to 23 months of age whereas dinucleotide levels in the control mice remain constant, suggesting a potential implication of ecto-nucleotidase activity in the pathology development. Indeed, at 23 months of age both, basal and stimulated Ap_4A levels, were very close each other. This is probably indicating that the cell damage that occurs at this stage of the pathology is producing a non-controlled release of this dinucleotide. Also, it is important to bear in mind that the reduction in the released Ap_4A may be a consequence of the increased expression of other enzymes such as TNAP which could partially eliminate it from the extracellular milieu (Fig. 4). It is clear that the reason for such lack of release of Ap_4A is a complex subject that demands further studies. Ap_4A net release levels observed in glaucomatous retinas could be indicating the loss of the potential neuroprotective effect of this compound with aging in relation to the observed results in ciliary processes [33] and central nervous system [50]. Furthermore, although biological properties of dinucleotides remain unknown, some authors have described the potential therapeutical role of Ap_4A in retinal impairments [51, 52] and brain injury mediated by inhibition of apoptotic process [50, 53].

Due to the presence of changes in Ap_4A concentrations during retinal degeneration, the activity of ecto-nucleotidases was evaluated at different ages to analyze their involvement in the regulation of Ap_4A levels in the extracellular milieu. Our data suggest that a reduction of extracellular Ap_4A may contribute to protection against retinal injury through exacerbated ecto-nucleotidase activity leading to the concomitant ATP

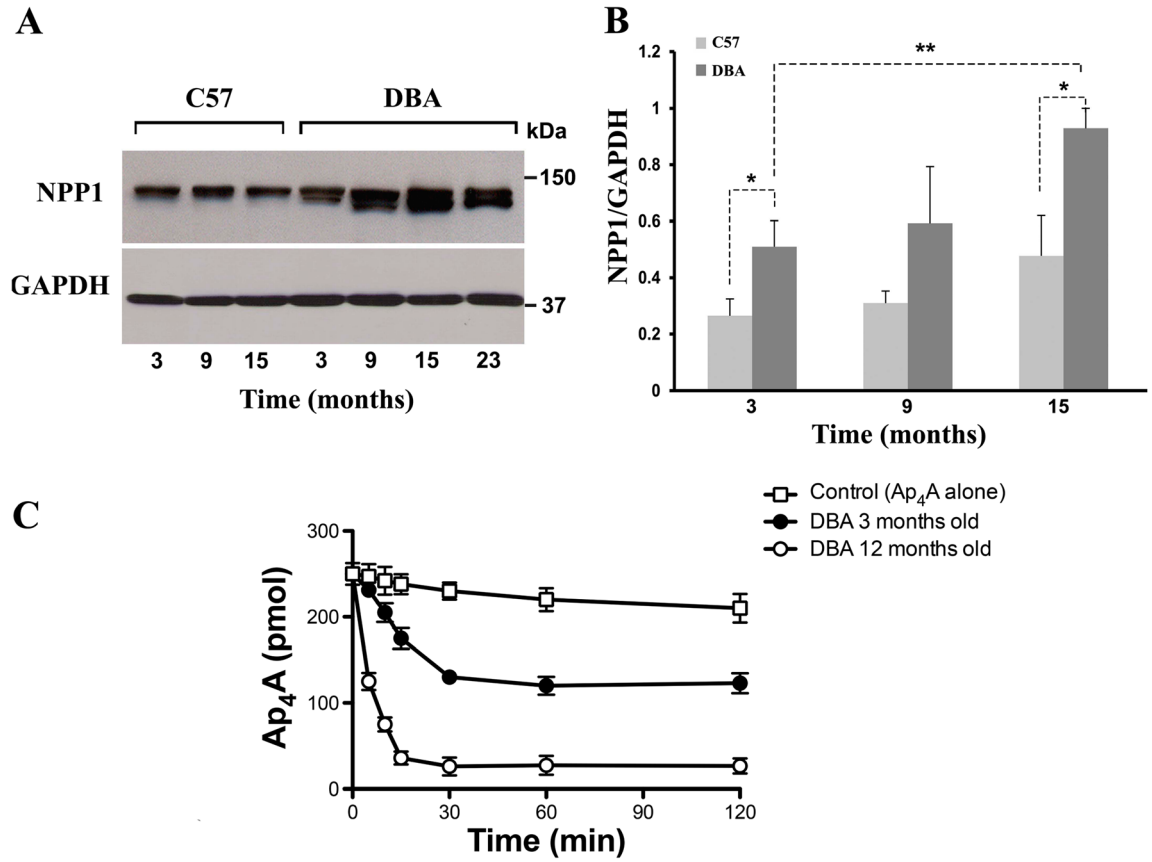

Fig. 5 NPP1 expression and activity in the glaucomatous and control retinas with aging. **a** A representative western blot of NPP1 (≈ 105 kDa) levels in C57BL/6J and DBA/2J mice at 3, 9, 15, and 23 months. GAPDH (37 kDa) levels are shown as a loading control. **b** Western blot analysis of NPP1 at 3, 9, 15, and 23 months of age. Values are the mean ± SD of five independent experiments (**$p < 0.01$). **c** Study on the degradation of Ap_4A for 2 h assayed on 3- and 15-month-old retinas as described in the "Materials and methods" section. Control trace corresponds to Ap_4A incubated in the absence of any retina. Values are the mean ± SD of four independent experiments

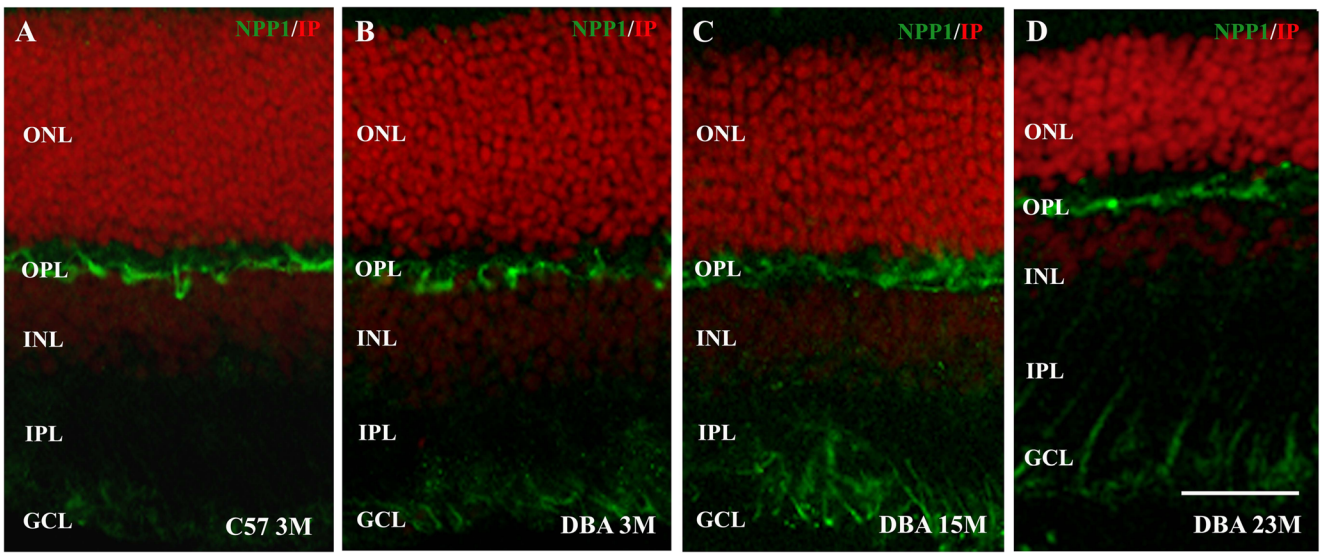

Fig. 6 Immunohistochemical localization of NPP1 in glaucomatous retinas. Vertical sections at 3 months in the control C57BL/6 mice (**a**) and 3, 15 and 23 months of central retinas from DBA/2J mice (**b**–**d**). NPP1 staining (green) showed a significant increment in DBA/2J mice retinas at 15 months (**c**) with respect to 3 months (**b**) and 23 months (**d**). Nuclei were stained with a nuclear marker (propidium iodide; red). All images were collected from the central area of the retina. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 20 μm

release. This is an important aspect to take into account since the generated ATP consequent to the Ap_4A cleavage by NPP1 and NPP3 can activate P2X receptors. This triggers different physiological processes (Fig. 7), including apoptotic signalways through P2X7 receptors stimulation, mainly in the retinal ganglion cells [31, 54, 55].

The second aim of the study was to analyze the expression of the main ecto-enzymes involved in the degradation of dinucleonucleotides: the ecto-nucleotide pyrophosphatase (E-NPP) [12] family and tissue-non-specific alkaline phosphatase (TNAP), the latter is unable to cleave Ap_4A since it can only act on nucleotides with 5′ phosphate ends [56]. NPP1, NPP3, and TNAP transcripts have been quantified in glaucomatous and control mice retinas by quantitative real-time PCR in other neuronal populations [14]. Interestingly, the current results showed a significant upregulation of NPP1 in mice glaucomatous retinas and are in agreement with immunoblot, histochemical, and hydrolysis studies. In this sense, it was relevant to see that the presence of NPP1 at 3 months of age was able to cleave 50.8% of the incubated Ap_4A in 2 h, while in the case of the 15-month-old glaucomatous retinas, the percentage of Ap_4A degradation was 89.4%. These two values were consistent with the NPP1 expression observed in the western blots where the bands intensity at 15 months of age doubled the intensity of the 3 months old.

It is interesting to see that although the glaucomatous mice develop the pathology from the sixth month of age, it was possible to see significant differences with the control animal from 3 months old. These differences became more robust when the animals developed the pathology. This may suggest that the C7BL/6J mice might not be the best control animal for some studies and in this sense, several functional studies suggest significant differences in the C57BL/6J strain with respect to the glaucomatous mouse [57]. This is one of the reasons why age-dependent studies in DBA/2J mice were imperative.

The neural retina expresses the activity of various extracellular nucleotide-degradative enzymes showing specific immunolabeling for the ecto-nucleotide pyrophosphatase 1 (NPP1) along the retina. Vertical section analysis revealed that NPP1 distribution is observed in retinal plexiform layers, suggesting their implication in visual information modulation.

Both extracellular Ap_4A degradation and the increase of NPP1 expression could contribute to retinal impairment in glaucomatous damage. Exacerbated NPP1 activity during the progression of the pathology may explain the diminished dinucleotide extracellular levels. It is worthy to note that the activity of the enzyme NPP3 needs to be taken into consideration. This enzyme hydrolyzes Ap_4A at the same rate as NPP1 does [11]; nevertheless, its expression is far lower that NPP1 (see Fig. 4). We estimate that more than 90% of the dinucleotide cleavage is due to mainly to NPP1.

Further investigations are necessary to confirm the involvement of extracellular nucleotide and NPP1 expression in the main neuronal populations responsible for the modulation of synaptic transmission and in the major glial component of the retina, the Müller cells [21, 24]. Double immunolabeling experiments should be also necessary to co-localize NPP1 expression with different markers could reveal roles in the axon synaptic terminals and optic nerve fiber layer of the retinal circuitry.

Considering the results presented and recent studies in our group, nucleotide and dinucleotide levels and purinergic signaling are contributing to the modulation of the visual function in DBA/2J mice [58]. ATP levels and the vesicular nucleotide transporter (VNUT) were increased in glaucomatous mice when the retinal degeneration was developed (at 15 months), and this suggests that the exacerbated degradation of this signaling molecule in other nucleotides diminishes Ap_4A levels during the progression of the pathology through hydrolytic activity of ecto-nucleotidases [18]. The NPP1 increase may be in part responsible for the reduced Ap_4A concentrations in the retina of aged DBA/2J mice. Furthermore, dinucleotide alterations in concentration were accompanied by an increase in intraocular pressure and a loss of retinal function in this model [32, 59, 60]. The present manuscript supports a previous work showing altered purinergic signaling in chronic glaucoma [49] by adding the involvement of the dinucleotide Ap_4A to that of the ATP.

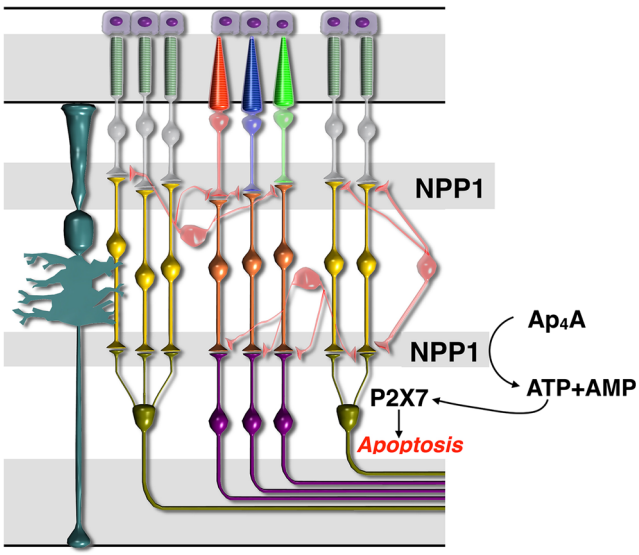

Fig. 7 The possible connection between Ap_4A, NPP1, and glaucoma. The increase in expression in both plexiform layers permits the action of the enzyme cleaving the dinucleotide to produce ATP. This ATP generated in the inner plexiform layer can stimulate the P2X7 receptors present in the retinal ganglion cells triggering an apoptotic process that will conduct cell death

In summary, we have demonstrated that Ap_4A levels and NPP1 ecto-enzyme were altered during the progress of the pathology. Increased levels of NPP1 in DBA/2J has been detected at 15 months of age concomitant with decreased Ap_4A release that could be associated with retinal impairment, suggesting a novel pharmacological approach.

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Compliance with ethical standards

Conflicts of interest María J. Pérez de Laraa declares that she has no conflict of interest.

Ana Guzmán-Aranguez declares that she has no conflict of interest.

Rosa Gómez-Villafuertes declares that she has no conflict of interest.

Javier Gualix declares that he has no conflict of interest.

María Teresa Miras-Portugal declares that she has no conflict of interest.

Jesús Pintor declares that he has no conflict of interest.

Ethical approval All animal maintenance and experimental procedures were in compliance with institutional, Spanish and European guide-lines of animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed and handled with the authorization and supervision of the Institutional Animal Care and Use Committee from the Complutense University of Madrid.

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