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Critical involvement of extracellular ATP acting on P2RX7 purinergic receptors in photoreceptor cell death

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ABSTRACT

ATP can be released from stressed cells and participate in neurodegenerative processes through the specific ligation of P2RX7 purinergic receptors. Here we demonstrate that extracellular ATP and the more specific P2RX7 agonist, 2′- and 3′-O-(4-benzoylbenzoyl)-ATP (BzATP), both induce photoreceptor cell death when added to primary retinal cell cultures or when injected into the eyes from wild type mice, but not those from P2RX7$^{-/-}$ mice. The photoreceptor cell death was accompanied by the activation of caspase-8 and -9, translocation of apoptosis inducing factor (AIF) from mitochondria to nuclei, and TUNEL-detectable chromatin fragmentation. All hallmarks of photoreceptor apoptosis were prevented by pre-medication or co-application of brilliant blue G (BBG), a selective P2RX7 antagonist that is already approved for the staining of internal limiting membranes during ocular surgery. ATP release is upregulated by nutrient-starvation in primary retinal cell cultures and seems to be an initializing event that triggers primary and/or secondary cell death via the positive feedback loop on P2RX7. Our results encourage the potential application of BBG as a novel neuroprotective agent in retinal diseases, or similar neurodegenerative pathologies linked to excessive extracellular ATP.

Introduction

Photoreceptor degeneration involves the activation of several pathways of regulated cell death that may constitute potential therapeutic targets. Accordingly, attempts have been undertaken to inhibit caspases, which play a central role in the acquisition of the apoptotic morphology,[1, 2] although pharmacological pan-caspase inhibitors have largely failed to preserve the structure and function of photoreceptors.[3, 4] Caspases can be activated as the result of mitochondrial outer membrane permeabilization (MOMP) in thus far that the mitochondrial release of cytochrome *c* results in the Apaf-1 apoptosome-dependent activation of caspase-9. MOMP also results in the mitochondrial release of apoptosis-inducing factor (AIF), which then translocates to the nucleus and participates in caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation,[5] suggesting the existence of redundant cell death mechanisms downstream of MOMP.[4, 6, 7] Pharmacological inhibition of MOMP has indeed been shown to confer some degree of neuroprotection in a model of photoreceptor degeneration induced by retinal detachment.[4]

Upstream of or independently from MOMP, death receptors from the tumor necrosis factor receptor (TNF-R) family proteins including Fas receptor (Apo-1/CD95) or DR4

(death receptor 4) can transmit apoptotic and/or necrotic signals initiated by specific ‘‘death ligands”.[8] Increased TNF-R or CD95 signaling can participate in photoreceptor death induced by retinal detachment (RD),[9, 10] in which case these receptors may either stimulate the initiation of apoptosis (via the activation of caspase-8) or programmed necrosis (via the activation of RIP1 kinase). Indeed, simultaneous inhibition of caspases (with Z-VAD-fmk) and RIP1 kinase (with necrostatin 1) has a more pronounced neuroprotective effect on photoreceptors in RD than either treatment alone.[11] Taken together, these results underscore the existence of multiple cell death mechanisms that have to be inhibited simultaneously to confer optimal neuroprotection.

An alternative to the inhibition of executioner pathways consists in intercepting the initiating events that account for upstream damage signals. Recently, adenosine-5’-triphosphate (ATP) has been discovered as a major extracellular messenger that can contribute to lethal signaling.[12] ATP, which can be released via exocytosis, anion channels or transporters, can act in autocrine and paracrine signaling pathways,[13] for instance by evoking action potentials in brain slices, isolated nerves and glial cells,[14] indicating that ATP acts as a physiological mediator of neurotransmission and neuron-glia communication.[15] Moreover, extracellular ATP

concentrations increase in spinal cord injury,[16] chronic neuropathic pain,[17] and brain ischemia,[18] suggesting a pathological role for extracellular ATP as well. Extracellular ATP can act on purinergic receptors, which are classified into two classes, the ionotropic, ligand-gated P2X receptors and the metabotropic, G protein-coupled P2Y receptors.[19] Among the seven mammalian P2X receptors,[20] the P2X7 receptor (official name: P2RX7) has the highest affinity for ATP. P2RX7 differs from other P2X receptor subtypes by its long cytoplasmic, carboxy-terminal tail (240 amino acids). Brief application of agonists renders P2RX7 permeable to small cations (K^+, Na^+, Ca^{2+}) similar to other P2X receptors,[12] while repeated or prolonged exposure to agonists can lead to the formation of P2RX7-receptor-dependent pores that become permeable to solutes up to 900 daltons, hence triggering cell death.[21] Thus, extracellular ATP can induce apoptotic and/or necrotic cell death by acting on P2RX7.[22] Despite the great interest in the carboxyl-terminal region of P2RX7, the mechanism by which P2RX7 mediates apoptotic signaling is largely unknown. Of note, the residues 436-531 of P2RX7 are similar to a region of TNFR1 that overlaps its death domain,[23] which has led to the proposal of a potential mechanism for P2RX7-induced caspase-8 activity and apoptosis.[24] Thus, the plasma membrane receptor, P2RX7, may cleave and activate caspase-8 during extrinsic apoptotic pathway.

Importantly, P2RX7 is widely expressed in various organs including the immune system (thymus or spleen)[21] and the central nervous system (CNS) (cortex, hippocampus,[25] and spinal cord[26]). P2RX7 is expressed on astrocytes, microglial cells and neurons.[14] In the retina, P2RX7 is expressed on Müller glia,[27] and in both inner and outer retinal neurons, including retinal ganglion cells[28, 29] and photoreceptors.[30] Genetic or functional inactivation of P2RX7 can attenuate the development of several neurodegenerative diseases, including Alzheimer's disease[31] and Huntington's disease.[32] Thus, systemic administration of a pharmacological P2RX7 antagonist, brilliant blue G (BBG) can confer neuroprotective effects in models of Alzheimer's, Parkinson's disease, and spinal cord injury.[31-33]

BBG is a triphenylmethane dye that has been approved for intraoperative use in ocular surgery, in the context of chromovitrectomy, which involves the use of vital dyes to improve the visualization of intraocular tissues during vitrectomy, thereby improving specific procedures such as internal limiting membrane (ILM) peeling.[34] Driven by the recent characterization of BBG as a P2RX7 antagonist,[31-33] we decided to investigate the pathogenic implications of P2RX7 in pathological photoreceptor loss, as well as the therapeutic utility of BBG in this context. As a result of these investigations, we report here that increased extracellular ATP levels contribute to pathological conditions

of photoreceptor loss and that BBG efficiently avoids photoreceptor cell death.

Materials and methods

Animals

All animal experiments were performed according to the guidelines of the Association for Research in Vision and Ophthalmology on adult (8 weeks of age) male C57BL6JJcl mice (CLEA, Tokyo, Japan) and isogenic P2RX7$^{-/-}$ mice (B6.129P2-*P2rx7*^{tm1Gab}/J; stock number 005576, The Jackson Laboratory, Bar Harbor, ME).

Adult mouse primary retinal cell cultures

Adult primary retinal cell cultures were prepared as previously described with minor modifications.[6] Primary retinal cells were cultured in 4-well chamber (NUNC; part of Thermo Fisher Scientific, Rochester, NY) with Neurobasal-A medium (Invitrogen, Carlsbad, CA) containing B27 supplement without antioxidants (NBA/B27AO–; Invitrogen), 1 μg/ml insulin, and 12 μg/ml gentamicin. To determine the number of adherent photoreceptor cells, immunofluorescent staining was performed with a rabbit anti-recoverin antibody (Millipore, Bedford, MA).

For nutrient-starvation, primary retinal cells were cultured for 3 hours (ATP

measurement) or 24 hours (immunocytochemistry and viability assay) with Neurobasal A medium without B27 supplement as 'starvation medium'. P2RX7 agonists (ATP or BzATP; Sigma-Aldrich, Saint Louis, MO) or P2RX7 antagonists (KN-62 or BBG; Sigma-Aldrich) were added to the medium and incubated for 24 hours. To investigate the effect of BBG pre-incubation, cells were cultured in the presence of BBG for 30 min, washed two times with culture medium, and incubated for 24hours. To examine the secondary effects of death ligands, rat anti-mouse TNF-alpha neutralizing antibody (1-10 ng/ml MP6-XT22; R&D systems, Minneapolis, MN)[35] or hamster anti-mouse CD95 ligand neutralizing antibody (1-10μg/ml MFL3; BD biosciences, San Jose, CA)[36] were added to culture medium, while isogenic rat or hamster IgG was added as control.

Viability assay in primary retinal cell cultures

To assess the viability of primary retinal cells, we used calcein AM (2 μM; Invitrogen) or MitoTracker Orange CMTMRos (200 nM, M7510; Invitrogen) that were added for 34 min to primary retinal cell cultures. Then, cultured cells were fixed with 4% paraformaldehyde and photoreceptors were labeled with recoverin. Calcein^{+} or CMTMRos^{+} photoreceptors were counted in 10 random fields by blinded observers

using ImageJ software. Values are given as means ± SD of 10 replicate wells.

Intraocular injections

For evaluation of the toxicity of BzATP and the effect of BBG in vivo, we took advantages of gas compression vitrectomy for uniform diffusion of intravitreally injected solutions. Mouse eyes were vitrectomized using SF6 gas as we and others previously described.[37-40] To investigate the effects of gas compression vitrectomy on the viability of retinal cells, we observed ophthalmic examination every day up to 2 weeks and evaluated histochemical changes in hematoxylin eosin staining and TUNEL positive cells in the retina at 3 and 14 days after gas injections. Two weeks after gas injection, mice were anesthetized with an intraperitoneal injection of pentobarbital, and their pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. Then, 2 µl of liquid (PBS supplemented with 10-20 mM BzATP or BzATP plus 500 µM BBG) was injected into the vitreous cavity with a 32-gauge needle on a Hamilton syringe through posterior to the limbus. Intraocular injections were only performed in the right eye of each animal, and 5 eyes were examined in each group. The mice were sacrificed 24 hours after treatment, and their eyes were harvested, frozen at nitrogen liquid temperature and cryosectioned for histochemical or

ultrastructural examinations.

TUNEL

TUNEL analysis and quantification of TUNEL-positive cells were performed as previously described[4] using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore). Nuclei were counter-stained with propidium iodide or Hoechst 33342. $TUNEL^+$ cells in the ONL were counted by two blinded observers, and results were presented as means ± SD.

Immunohistochemistry

As previously reported,[6] rabbit anti-AIF (R&D Systems, Minneapolis, MN), anti-mouse cleaved caspase-9 (Cell Signaling Technology, Beverly, MA), anti-mouse cleaved caspase-8 (Cell Signaling Technology) and anti-recoverin (Millipore) were used as primary antibodies and incubated at 4°C overnight. Goat anti-rabbit IgG conjugated to Alexa Fluor 546 or 647 (Invitrogen) were used as secondary antibodies and incubated at room temperature for 1 hour.

Electron Microscopy

The posterior segments of enucleated eyes were fixed in PBS containing 1% glutaraldehyde and 1% paraformaldehyde, postfixed in veronal acetate buffer osmium tetroxide (2%), dehydrated in ethanol and water, and embedded in Epon. Primary retinal cell cultures were similarly fixed, dehydrated, and embedded in Epon. Ultrathin sections were cut from blocks and mounted on copper grids. The specimens were observed with H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

Calcium imaging

Primary retinal cells were cultured on 3.5mm coverslip dishes (BD Biosciences, San Jose, CA) in Neurobasal A medium with B27 supplement. Then the calcium indicator, Fluo-4 AM (5μM; Invitrogen) was loaded onto primary cultures. The calcium-dependent fluorescence of Fluo-4 was captured by microscopy during 20 minutes incubation after addition of 1mM BzATP or vehicle PBS. To evaluate the effect of chelation of extracellular Ca^{2+} or P2RX7 antagonist (BBG), the fluorescence of Fluo-4 was measured in culture medium containing 1mM EGTA (Sigma-Aldrich) or 10 μM BBG, respectively.

ATP measurements

For ATP measurements of culture medium, primary retinal cells were starved for 3 hours with Neurobasal A medium without B27 supplement as 'starvation cultures' and cultures with Neurobasal A medium with B27 supplement were prepared as controls. Then 100µl culture medium were collected, centrifuged at 4˚C, and subjected to ATP measurements as follows. The ATP levels of collected medium (100 µl in 96 well microplates; BD Biosciences) were immediately determined by adding luciferin–luciferase reaction buffer (ATP bioluminescent assay kit, FL-AA; Sigma-Aldrich) and a multi-mode microplate reader, Flex Station 3 (Molecular Devices, Sunnyvale, CA). The ATP levels in medium of 'control cultures' were determined by calibration of standard ATP in Neurobasal A medium with/without B27 supplement. To define the detection limit, ecto-nucleotidase (Apyrase; 10 unit /ml**,** Sigma-Aldrich) was added to culture medium. In control experiments, β, γ-methylene-ATP, a potent ecto-ATPase inhibitor (300 µM, Sigma-Aldrich), was added to control or starvation cultures. Values are given as mean ± SD of 10 replicate wells.

Imaging of ATP release

Primary retinal cells were cultured on 3.5 mm coverslip dishes (BD Biosciences) in Neurobasal A medium with B27 supplement. ATP assay mix (a part of the FL-AA kit;

Sigma-Aldrich) was added to culture media (one vial per 1.5 ml culture medium with/without B27 supplement). Light production from the luciferin-luciferase reaction was captured by an electron multiplier charge-coupled camera (ImagEM; Hamamatsu Photonics, Shizuoka, Japan) with a 40 x oil lens (N.A. 1.35; Olympus, Tokyo, Japan) for 3 hours during 5 min exposure periods.

Statistical analysis

Statistical differences between two groups were analyzed by means of the Mann–Whitney U test. Multiple group comparison was performed by ANOVA followed by Tukey–Kramer adjustments. Differences were considered significance at $P < 0.05$ (*) and $P<0.01$ (**). All values were expressed as means ± SD.

Results

P2RX7 mediated photoreceptor death in primary retinal cell cultures

To investigate the possible implication of P2RX7 in photoreceptor degeneration, we took advantage of primary retinal cell cultures.[6, 41] Photoreceptor viability was assessed by means of two fluorescent sensors, calcein AM and MitoTracker CMTMRos, which only label intact and non-apoptotic, metabolically active cells, respectively. Calcein AM, which is non-fluorescent and cell permeable, becomes fluorescent and is trapped in live cells after removal of the AM moiety by cellular esterases. Cells that stain with this dye hence must possess an intact, impermeable plasma membrane. The lipophilic cation MitoTracker CMTMRos labels mitochondria, driven by the mitochondrial transmembrane potential (DY_m), and hence stains cells with intact mitochondrial membranes. Addition of ATP (Figure 1A) or 2'-and 3'-O-(4-benzoyl-benzoyl)-ATP (BzATP) (Figure 1B), a potent, more selective P2RX7 antagonist,[21] resulted in a dose-dependent decline of viable, $calcein^+$ or $CMTMRos^+$ photoreceptors that were identified by immunofluorescence detection of recoverin. Before starting blocking experiments by BBG, we tested whether BBG has potential toxicity. Primary retinal cell cultures were incubated in the presence or absence of 1-10 µM BBG for 24 h. No

significant decline of photoreceptor viability was observed (Figure 2A). BBG showed minimal toxicity at those concentrations *in vitro*. BBG, a potent P2RX7 antagonist[42] as well as another selective P2RX7 inhibitor, 1-[N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), reduced BzATP-induced photoreceptor death (Figure 2B). BBG is a non-competitive and slowly reversible P2RX7 inhibitor that antagonizes P2RX7 by allosteric regulation.[42] Accordingly, a short (30 min) pre-incubation with BBG could reduce BzATP-induced photoreceptor death to some extent, though less efficiently than continuous co-incubation (Figure 2B). Of note, photoreceptors isolated from P2RX7$^{-/-}$ mice readily survived exposure to high dose (1 mM) BzATP, under conditions in which large numbers of wild type photoreceptors died, underscoring that these effects are truly specific (Figure 2C).

These results indicated the specific effect of P2RX7 on photoreceptor death. However, we needed to exclude the secondary effect of death ligands, which could potentially be released by other cell types, on photoreceptor death. To this end, we performed the blocking experiment by neutralizing antibody against TNF-alpha and CD95 ligand. Of note, neither TNF-alpha (1-10 ng/ml MP6-XT22)[35] nor CD95 ligand (1-10 μg/ml MFL3)[36] neutralizing antibody could prevent the photoreceptor death induced by 1mM

BzATP (Figure 2, D and E). These results suggest that P2RX7 stimulation could directly mediate the cell death pathway on photoreceptors, rather than an indirect pathway being mediated via TNF-alpha or CD95 ligands released by other cells.

P2RX7 induced retinal cell death via calcium influx and caspase-8 activation

Transmission electron microscopy revealed the relatively well-preserved organelles in the photoreceptors without BzATP administration, while P2RX7 stimulation by BzATP resulted in apoptotic cell death, characterized by chromatin condensation, as well as in some instances in necrotic cell death (Figure 3A). After culture in the presence of 1 mM BzATP, a significant percentage of photoreceptors exhibited a type of apoptotic DNA fragmentation that could be detected with the TUNEL. To examine caspase-8 activation by P2RX7, TUNEL-positive cells also were stained by immunofluorescence with an antibody that only recognizes active, proteolytically mature caspase-8 (Figure 3B). BBG largely prevented these BzATP-induced signs of the extrinsic cell death pathway (Figure 3, B and C).

As to be expected for an ionotropic receptor, BzATP also stimulated a rapid (20 min) increase in intracellular Ca^{2+} concentrations determined by means of Fluo-4 AM, a Ca^{2+} indicator that was loaded into primary cultures (Figure 3D; top). We performed

the extracellular Ca^{2+} chelating with EGTA to clarify the origin of the Ca^{2+} increase. This increase of intracellular Ca^{2+} was attenuated by addition of 1 mM EGTA into the culture medium (Figure 3D; middle), indicating that BzATP mediated Ca^{2+} influx mainly through the cation channel, not via release from intracellular binding proteins. Furthermore, co-incubation of 10 μM BBG inhibited the Ca^{2+} influx by BzATP (Figure 3D; bottom), suggesting that BBG prevents intracellular calcium signaling by blocking the P2RX7 ion channel.

In conclusion, P2RX7 ligation kills photoreceptors via the influx of Ca^{2+} and caspase-8 activation, suggesting a potential mechanism for the P2RX7-mediated extrinsic pathway in line with previous reports.[23, 24]

Photoreceptor apoptosis induced by intraocular administration of P2RX7 agonists *in vivo*

To determine whether P2RX7 ligation can trigger retinal cell death *in vivo*, we measured retinal cell apoptosis 24 h after intraocular injection of 20 mM BzATP into the eyes of C57BL/6 mice. The BzATP injections were performed 14 days after gas compression vitrectomy to obtain uniform diffusion of the injected solutions. Neither ophthalmic abnormality (i.e. corneal edema) nor signs of retinal cell loss in

hematoxylin eosin staining were observed up to 14 days after gas injection (data not shown), and no TUNEL-positive cells were observed in the retina at 3 and 14 days after injections (Figure 4A). The intraocular injection of BzATP induced $TUNEL^{+}$ apoptotic events in the ganglion cell layer, which is notoriously susceptible to induction of apoptosis by stimulation of P2RX7 receptors,[29, 43, 44] as well as in the inner nuclear layer (INL) and outer nuclear layer (ONL). These TUNEL-detectable DNA degradations accompanied the caspase-8 cleavage (Figure 4B, arrows). *In vivo* apoptosis induction by BzATP was blunted by co-injection of 500 μM BBG (Figure 4, C and D). As in the in vitro system, even high doses of BzATP (10-20 mM) largely failed to induce apoptosis when injected into $P2RX7^{-/-}$ mice (Figure 4, C and D). Transmission electron microscopy confirmed that BzATP induced the death of photoreceptors with hallmarks of apoptosis, such as cell shrinkage and chromatin condensation in the ONL. Moreover, BzATP caused the degeneration of pedicles and spherules in the OPL. The frequency of apoptotic events and degenerative changes in rod spherules and cone pedicles was decreased when BzATP was combined with BBG (Figure 4E).

Taken together, these results confirmed that pharmacological P2RX7 agonists have the potential to induce retinal cell death *in vivo*, under conditions in which P2RX7 antagonists can preserve photoreceptors.

BBG attenuates starvation-induced photoreceptor death in primary retinal cell cultures

Primary retinal cells cultured under starvation conditions (Neurobasal A medium without B27 supplement, a serum substitute) exhibited a significantly elevated level of photoreceptor apoptosis as compared to control cells (cultured in complete, B27-supplemented Neurobasal A medium). AIF was detected in the cytoplasm of control cultures, yet translocated into nuclei containing fragmented DNA upon starvation in agreement with our previous report that starvation induced the mitochondrial cell death pathway in photoreceptors.[6] BBG treatment attenuated the starvation-induced AIF translocation and TUNEL staining (Figure 5, A and C). The proteolytic maturation of caspase-9 was rarely detected in control cells, yet increased upon starvation, an effect that was again reduced by BBG (Figure 5, B and C). Similarly, a 24 h-starvation period led to a decrease in the frequency of viable ($calcein^+$) and metabolically active ($CMTMRos^+$) photoreceptors to approximately half

of that found in control cultures. This decline was again reversed by 30 min pre-incubation with 0.1-10 μM BBG in a dose-dependent manner (Figure 5D). Moreover, $P2RX7^{-/-}$ photoreceptors showed a significantly greater survival rate ($p<0.05$) under starvation conditions (ratio of $calcein^{+}$ cells over controls= 0.73; ratio of $CMTMRos^{+}$ cells over controls=0.64) compared to WT photoreceptors (ratio for $calcein^{+}$ cells=0.52; ratio for $CMTMRos^{+}$ cells=0.50).

Taken together, these results indicate that P2RX7-blockade inhibits the caspase-dependent and caspase-independent arms of the mitochondrial cell death pathway activated by starvation.

Extracellular ATP in stressed retinal cell cultures

Retinal cell cultures spontaneously released ATP (0.22±0.06 nM) under the control conditions. This release increased significantly ($p<0.01$) to 0.38±0.07 nM upon starvation, as quantified by a luciferase-based commercial assay (Figure 6). This difference persisted in the presence of β, γ-methylene-ATP (β, γ-Me-ATP), a potent inhibitor of ecto-ATPases[45] that degrade extracellular ATP, suggesting that the increase in ATP concentrations induced by starvation results from ATP release rather than from reduced ATP degradation. Of note, addition of BBG reduced the starvation-induced

ATP release (Figure 6), suggesting the existence of a positive feedback loop that links activation of P2RX7 receptors to nucleotide release.[46] For a more direct investigation of ATP release, we monitored retinal cell cultures by videomicroscopy to detect ATP-dependent bioluminescence revealed by a luciferin-luciferase assay. The frequency of ATP-releasing cells was low in control cultures, increased upon starvation, and was again decreased when starved cells were pre-incubated with BBG (Figure 7). Thus, BBG inhibits ATP released from starved retinal cells.

Discussion

In the present work, we provide compelling evidence that extracellular ATP and activation of P2RX7 may contribute to the pathological loss of photoreceptors. Thus, addition of P2RX7 agonists to primary retinal cultures or their intraocular injection causes the apoptotic death of photoreceptors in a fashion that is strictly dependent on P2RX7, as demonstrated by the use of $P2RX7^{-/-}$ mice. This death is accompanied by hallmarks of apoptosis, including the activation of caspases, the mitochondrio-nuclear translocation of AIF, and TUNEL-detectable chromatin fragmentation. Finally, we demonstrate that a pharmacological P2RX7 antagonist, BBG, can prevent all stigmata of pathological loss of photoreceptors by inhibiting extracellular ATP acting on P2RX7.

BBG is reported to be a selective antagonist of P2RX7.[42] In the current study, BBG substantially inhibited the effects of the selective P2RX7 agonist BzATP, namely, cell death, caspase-8 activation, and calcium influx. To clarify the specificity of the BBG, we examined another known selective P2RX7 blocker, KN-62. KN-62 showed an effect similar to BBG. Furthermore, the examination with P2RX7 knockout mice

revealed the specificity of BBG *in vitro* and *in vivo*. Thus, we concluded that BBG selectively blocked P2RX7 activation, thereby contributing a neuroprotective effect. Moreover, the blocking experiment with neutralizing antibodies for TNF-alpha or CD95 ligand revealed that P2RX7 could directly induce cell death on photoreceptors rather than indirectly via death ligands released by other cells.

ATP release appears to be (one of) the initializing event(s) that drives retinal cell death by nutrient starvation, a pathological setting *in vitro*. Furthermore, these extracellular ATP released from dying cells may worsen the pathology by promoting the death of neighboring cells, namely, secondary cell death. The following potential mechanisms may account for the increase in extracellular ATP in ocular pathologies. First, acute cell lysis may constitute one source of ATP, in line with the observations that ATP is released by acute stresses such as ischemia,[18] hypotony,[47] or oxygen/glucose deprivation.[48] However, here we observed ATP release from retinal cells in a model of nutrient starvation well before the occurrence of cell death,[6, 41] suggesting that ATP release occurred through a more controlled mechanism than acute cell lysis. Second, massive ATP release may occur from the usurpation of a physiological mechanisms of release (when ATP serves as a neurotransmitter), perhaps as the result of a pathogenic

feed-forward loop. Indeed, it has been shown that extracellular ATP can stimulate further ATP release through the opening of P2RX7 channels,[46] and – as shown here – BBG can reduce ATP release from starved retinal cultures by blocking the positive feedback.

The doses of ATP required to induce photoreceptor cell death are higher than those measured in starved cultures (which are rescued by addition of BBG or knockout of P2RX7). There are three possible explanations for this discrepancy. First, ATP released from cells may be diffused into bulk culture medium and continuously degraded through the action of ecto-ATPases, which are very active both in cultures[45] and *in vivo*[49] in the CNS. Indeed, the local concentration of ATP acting on the plasma membrane exceeds micromolar level, as detected using membrane-bound protein A-luciferase as a spatially localized probe of ATP levels at the extracellular cell surface.[50] Hence, the local increase of ATP in the intercellular space may activate P2RX7 in retinal disorders. Second, the injected solutions diffuse into the intraocular cavity and can be washed out by aqueous humor circulation *in vivo*, thereby lowering the local concentration in the retina. Third, the function of P2RX7 may be modulated not only by extracellular ATP but also by local receptor modifications. Nagasawa et al. reported that P2RX7 channel opening may occur without exogenous ATP

administration in cultured brain astrocytes, suggesting P2RX7 activation by endogenous ATP.[51] Furthermore, P2RX7 may also be sensitized to ATP following ADP ribosylation at the arginine 125 position of P2RX7.[52] These results may provide additional alternative pathways for the activation of P2RX7 under pathological conditions. Further investigations will be needed to elucidate the exact details of P2RX7 activation with respect to fluctuating local ATP concentrations.

In this study, we have shown that BBG may have important neuroprotective effects on the retina. Of note, BBG is already approved for intraocular use in patients, for the surgical procedure of chromovitrectomy. This procedure initially involved the administration of indocyanine green (ICG) as an intraocular surgical adjuvant in 2000.[53, 54] However, upon our initial description that ICG has toxic side effects in 2002,[39] the following clinical and experimental studies reported that ICG induces visual field defects or retinal pigment epithelial (RPE) atrophy.[55-57] Brilliant Blue G (BBG) has been introduced as an alternative staining dye for capsular staining in cataract surgery and chromovitrectomy with satisfactory biocompatibility.[40, 58, 59] Moreover, whereas ICG exposure turned out to trigger apoptotic signaling with caspase activation, BBG has minimal cytotoxicity in retinal cells.[57] In the present study,

we further confirmed the minimal toxicity of BBG by means of primary culture. Recent reports have supported the safety of BBG as a surgical adjuvant,[60, 61, 62] encouraging the ever expanding clinical applications for BBG in vitreo-retinal surgery. It should be noted that a substantial neuroprotective effect of BBG could be obtained at a dose of $\leq$ 10 μM, which is considerably lower than the concentration recommended for use during chromovitrectomy (293 μM). Moreover, the neuroprotective effect of BBG could be obtained through transient exposures of primary cultures, probably due to the characteristics as a slowly reversible antagonist of P2RX7. Considering these results, one might expect BBG to have a broad, long-lasting neuroprotective effect in ocular pathologies. Taken together, our study clarifies (part of) the mechanisms through which BBG exerts its therapeutic effects in clinically relevant models of neurodegenerative diseases,[31-33] and thereby lend support to the potential application of BBG as a novel neuroprotective agent.

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Legends to Figures

Figure 1. P2RX7 agonists induced retinal cell death in primary retinal cell cultures. The viability of retinal cells was assessed by incubation with calcein AM or MitoTracker CMTMRos after 24 h of culture with ATP or BzATP. Photoreceptors were labeled by immunocytochemistry for recoverin (in green). **A:** The frequency of calcein^{+} (**top**; in blue) or CMTMRos^{+} (**bottom**; in red) photoreceptors decreased after incubation with 200 µM to 5 mM ATP in a dose-dependent manner. **B:** BzATP also reduced calcein^{+} (**top**) or CMTMRos^{+} (**bottom**) photoreceptors in a dose-dependent fashion. These quantifications are shown in the **right** panels in **A** and **B**.

Figure 2. BBG attenuated photoreceptor death as a P2RX7 antagonist.

A: The viability of photoreceptors after 24 h of continuous incubation with 1-10 µM BBG. **B:** Photoreceptor death (induced by 1 mM BzATP) was partially reversed by 30 min transient incubation (BBG) or 24 h continuous incubation (co-BBG) of 1-10 µM BBG, or 1-10 µM KN-62. **C:** Photoreceptor viability was preserved in BzATP-treated (1 mM, 24 h) photoreceptors from P2RX7$^{-/-}$ mice (KO) as compared to wild type (WT) controls. **D** and **E:** Neutralizing antibody against TNF-alpha (MP6-XT22) or CD95 ligand (MFL3) failed to prevent BzATP-induced photoreceptor death. *n* = 10 per

group; $^{**}P < 0.01$. Scale bar= 20 μm.

Figure 3. P2RX7 induced caspase-8 cleavage and calcium influx *in vitro*.

A: Electron microscopy revealed the relatively well-preserved organelle of photoreceptors in primary cultures without BzATP administration (**left**). Apoptotic cell death was observed with chromatin condensation (**middle**) after BzATP administration. Necrotic cell death could also be detected after BzATP (**right**). Scale bar= 2 μm.

B: Representative images of immunocytochemistry for cleaved caspase-8 in primary retinal cell cultures (**left**; cleaved caspase-8 in red, **middle**; TUNEL in green, **right**; merged with Hoechst 33342 in blue). Cytoplasmic caspase-8 cleavage was detected in TUNEL-positive cells after 1 mM BzATP administration, and decreased by BBG treatment. **C:** Quantified results of cleaved caspase-8^+ or $TUNEL^+$ cells, $n = 10$ per group; $^{*}P < 0.05$. **D:** The fluorescent images of calcium indicator Fluo-4 in primary retinal cell cultures before and 20 min after BzATP administration. The phase contrast images are shown in the **left** panel. The BzATP-induced increase of Fluo-4 fluorescence (**top**) was attenuated by addition of 1 mM EGTA (**middle**) or 10 μM BBG (**bottom**). Scale bar= 5 μm.

Figure 4. P2RX7 stimulation induced photoreceptor apoptosis/necrosis *in vivo*.

A: Representative TUNEL (in green) and propidium iodide (in red) staining of mouse

retina after 3 (**top**) and 14 (**bottom**) days of gas vitrectomy. **B:** Representative images of cleaved caspase-8 in the mouse retina at 24 h after the intraocular injection of 20 mM BzATP (**top left**; Hoechst 33342 in blue, **top right**; cleaved caspase-8 in red, **bottom left**; TUNEL in green, **bottom right**; merged). In wild type mice, cleaved caspase-8-positive staining was observed in TUNEL-positive apoptotic cells in the inner nuclear layer (INL) and the outer nuclear layer (ONL) after the vitreous injection of BzATP (**white arrows**). **C:** The BzATP injections dose-dependently induced photoreceptor apoptosis in the ONL, as detected by TUNEL (**left**), and this effect was decreased by co-injection of 500 μM BBG (**middle**). Only very few TUNEL-positive photoreceptors were detected upon injection of BzATP into P2RX7$^{-/-}$ mice (**right**). TUNEL-stainings are shown in green and propidium iodide in red. The quantitations are shown in **D**. $n = 6$ per group; $^{**}P < 0.01$. Scale bar= 20 μm. **E:** Transmission electron microscopy revealed BzATP-treated photoreceptors with characteristics of apoptosis, cell shrinkage and chromatin condensation (**white arrow**) in the ONL, and degenerated pedicles and spherules in the OPL (**black arrows**). Apoptotic photoreceptors were decreased, and rod spherules and cone pedicles were well preserved in mice receiving BzATP plus BBG. Scale bar= 2 μm.

Figure 5. Neuroprotective effects of BBG in primary retinal cell cultures. **A** and **B:**

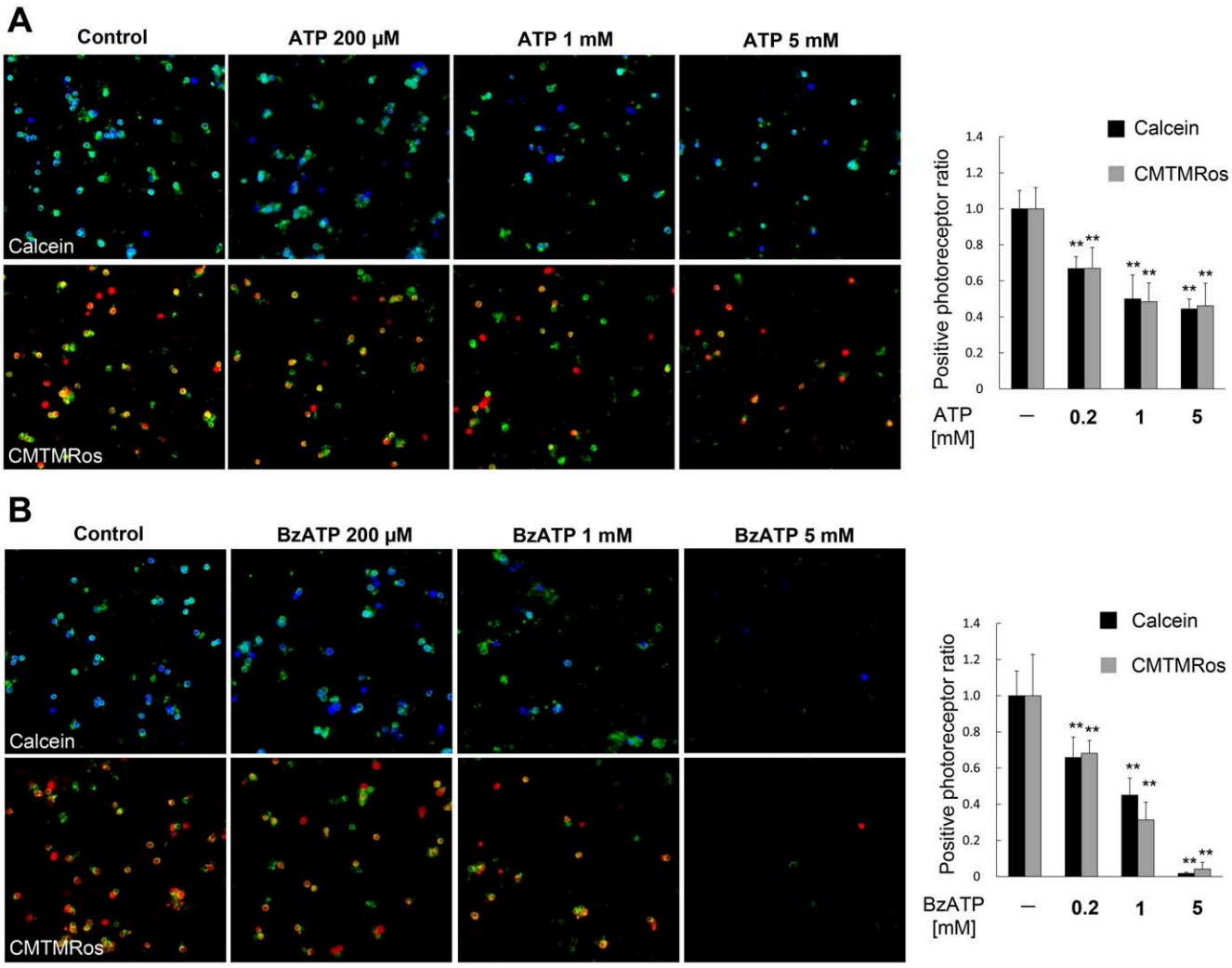
Representative images of immunofluorescence detection of AIF (AIF in red, TUNEL in green, Hoechst33342 in blue) and cleaved caspase-9 (cleaved caspase-9 in red, TUNEL in green, Hoechst33342 in blue) in control cultures and after starvation in the absence or presence of BBG. Scale bar = 5 μm**.** The quantitations are shown in **C**. *n*=10 per group; **P* < 0.05. **D:** The dose-dependent neuroprotective effect of BBG. *n* = 10 per group; ***P* < 0.01.

Figure 6. Nutrient starvation-induced ATP release from primary retinal cells *in vitro*. The ATP levels in primary culture were measured by luciferin-luciferase assay. Total cellular levels of ATP were measured after permeabilization of plasma membranes, while the inferior detection limit of ATP levels was determined by adding apyrase to the culture supernatants. Starvation was induced as in Figure 4 in the presence or absence of BBG or the ecto-ATPase inhibitor, β, γ-Me-ATP (*n* = 10 per group; **P* < 0.05, ***P* < 0.01).

Figure 7. Time-lapse imaging of ATP release induced by starvation. For dynamic imaging of the ATP release, light production from the luciferin-luciferase reaction was captured by an electron multiplier charge-coupled camera for 3 h). Representative images are shown (see Supplemental movies for total time-lapse images). Circles in the **middle** and **bottom** panel indicate events of ATP release induced by starvation and

arrows indicate the same location during the observation periods. Scale bar = 10 μm.

Figure 1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Control
ATP 200 μM
ATP 1 mM
ATP 5 mM
Calcein
CMTMRos
Calcein
CMTMRos
Positive photoreceptor ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
ATP
[mM]
—
0.2
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5
B
Control
BzATP 200 μM
BzATP 1 mM
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Calcein
CMTMRos
Positive photoreceptor ratio
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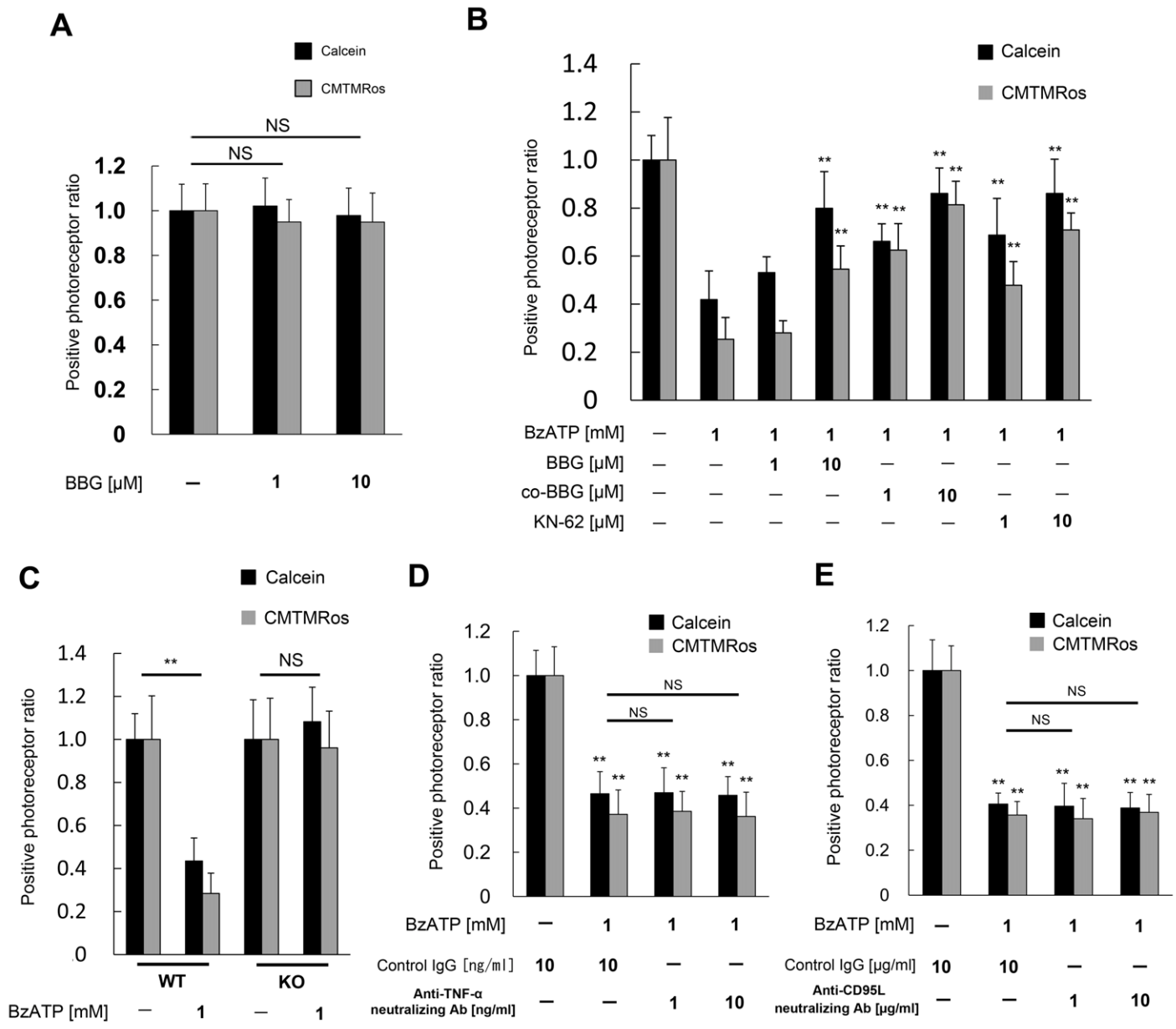
Figure 2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NS
Positive photoreceptor ratio
1.2
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BBG [μM] — 1 10
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Calcein
CMTMRos
Positive photoreceptor ratio
1.4
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0.8
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co-BBG [μM] — — — — 1 10 — —
KN-62 [μM] — — — — — — 1 10
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Calcein
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NS
Positive photoreceptor ratio
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0.4
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BzATP [mM] — 1 — 1
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Calcein
CMTMRos
NS
NS
Positive photoreceptor ratio
1.2
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BzATP [mM] — 1 1 1
Control IgG [ng/ml] 10 10 — —
Anti-TNF-α neutralizing Ab [ng/ml] — — 1 10
E
Calcein
CMTMRos
NS
NS
Positive photoreceptor ratio
1.2
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0.4
0.2
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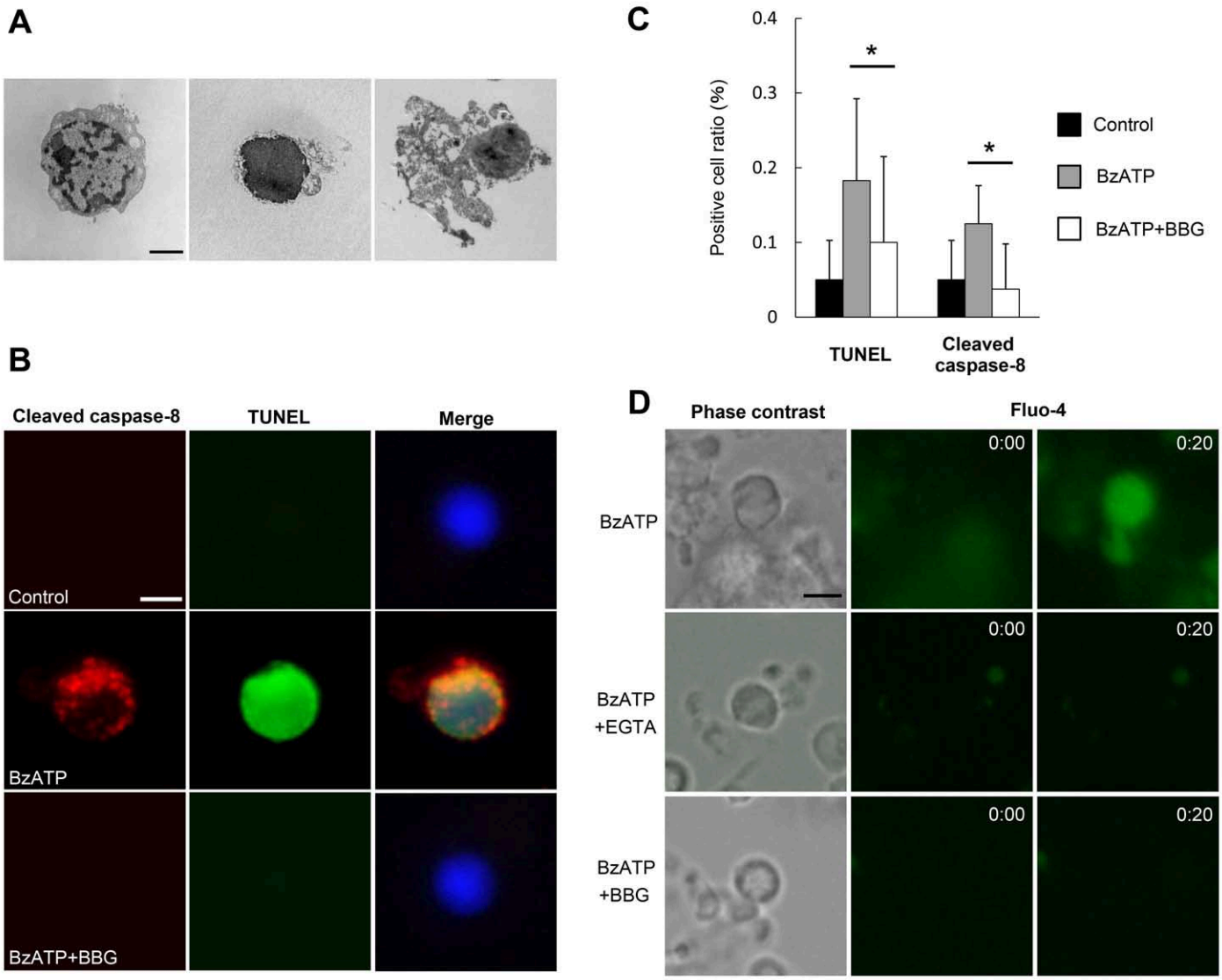
BzATP [mM] — 1 1 1
Control IgG [μg/ml] 10 10 — —
Anti-CD95L neutralizing Ab [μg/ml] — — 1 10

Figure 3


A
B
Cleaved caspase-8
TUNEL
Merge
Control
BzATP
BzATP+BBG
C
Positive cell ratio (%)
0.4
0.3
0.2
0.1
0
*
*
TUNEL
Cleaved caspase-8
Control
BzATP
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D
Phase contrast
Fluo-4
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BzATP +EGTA
BzATP +BBG
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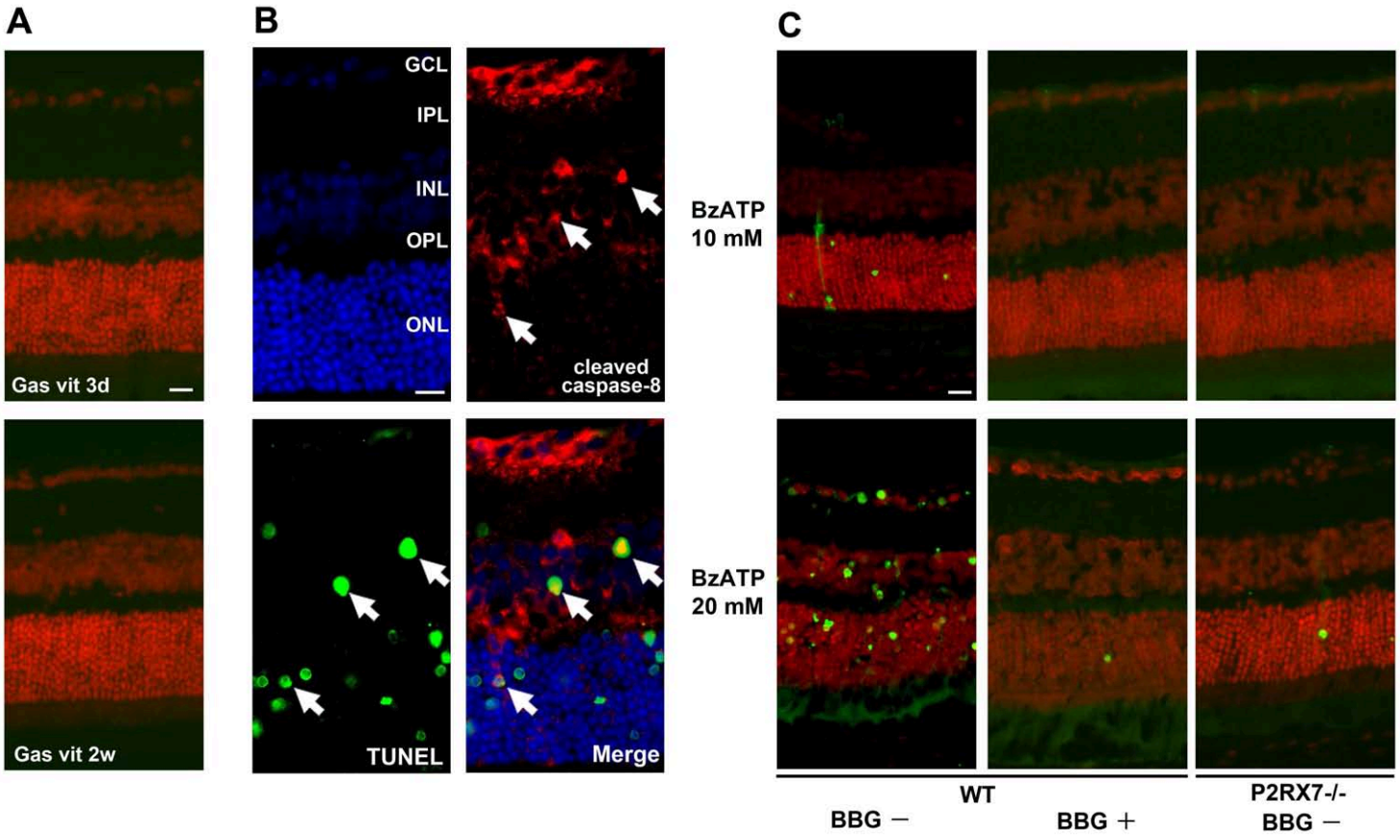
Figure 4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B
C
GCL
IPL
INL
OPL
ONL
Gas vit 3d
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Gas vit 2w
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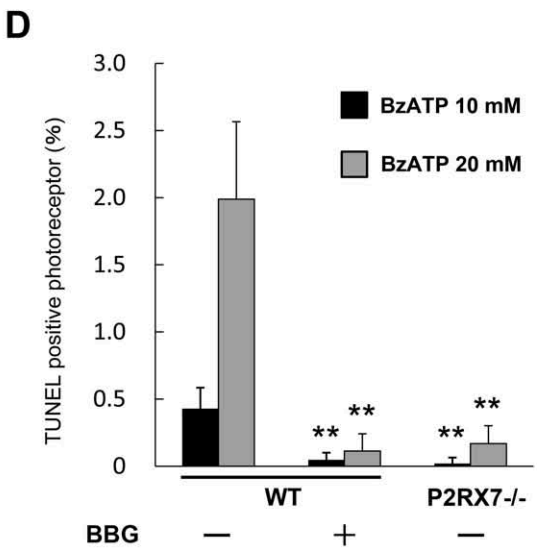
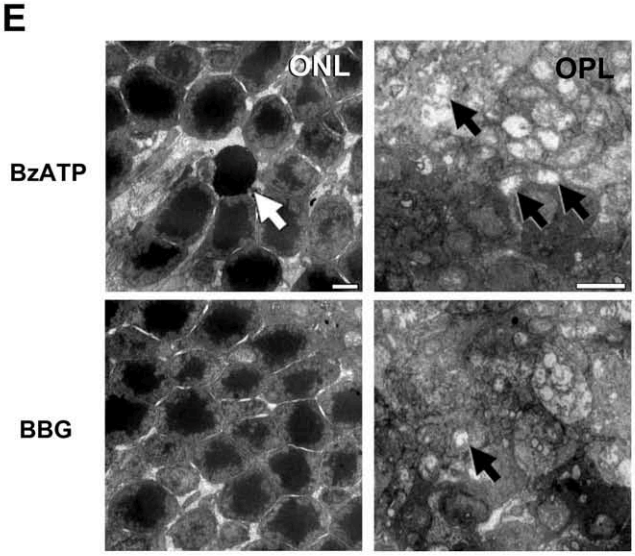
WT
P2RX7-/-
BBG −
BBG +
BBG −


D
TUNEL positive photoreceptor (%)
3.0
2.5
2.0
1.5
1.0
0.5
0
BzATP 10 mM
BzATP 20 mM
**
WT
P2RX7-/-
BBG
—
+
—


E
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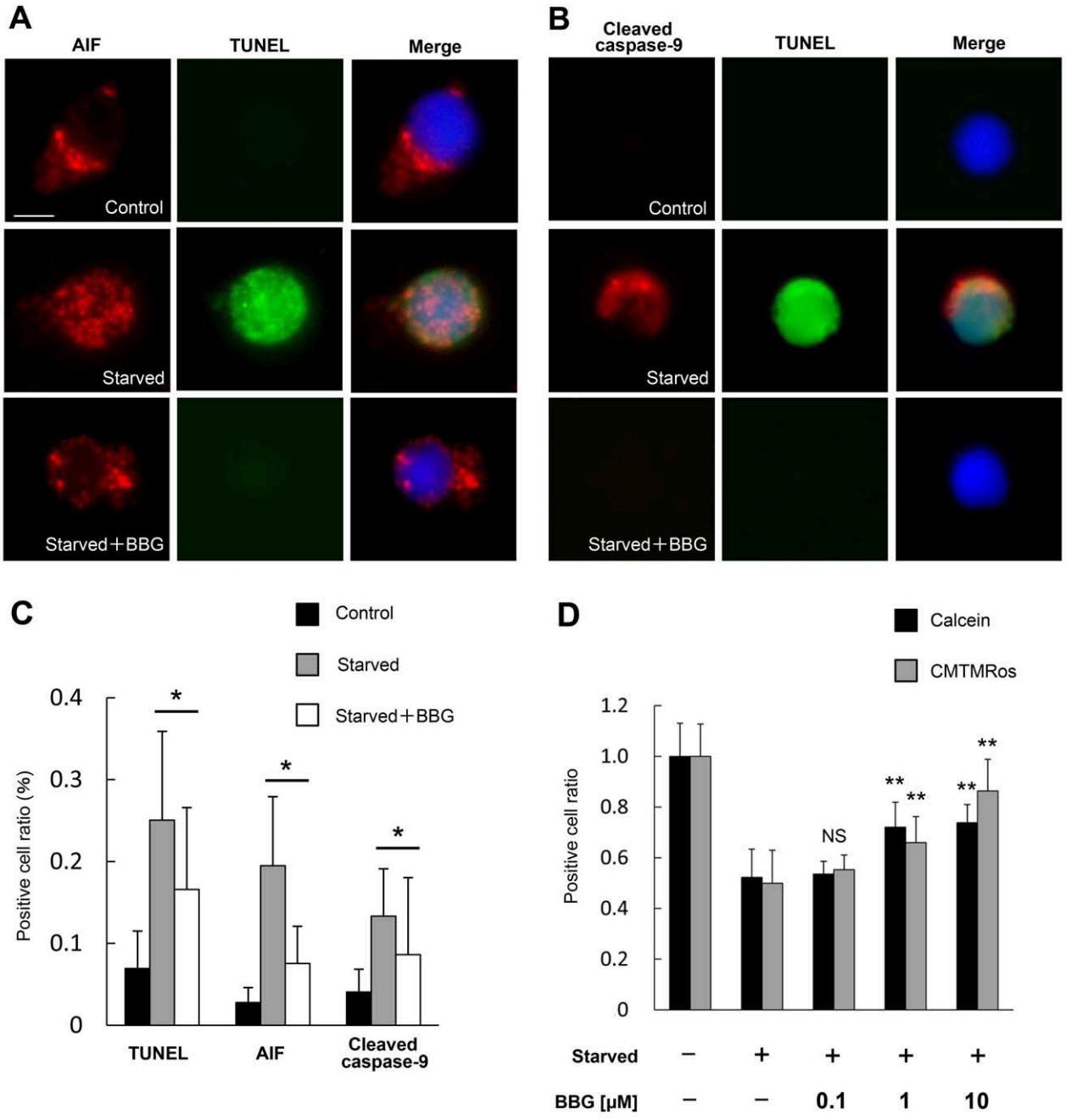
OPL
BzATP
BBG

Figure 5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AIF
TUNEL
Merge
Control
Starved
Starved＋BBG
B
Cleaved caspase-9
TUNEL
Merge
Control
Starved
Starved＋BBG
C
Control
Starved
Starved＋BBG
Positive cell ratio (%)
0.4
0.3
0.2
0.1
0
*
*
*
TUNEL
AIF
Cleaved caspase-9
D
Calcein
CMTMRos
Positive cell ratio
1.2
1.0
0.8
0.6
0.4
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NS
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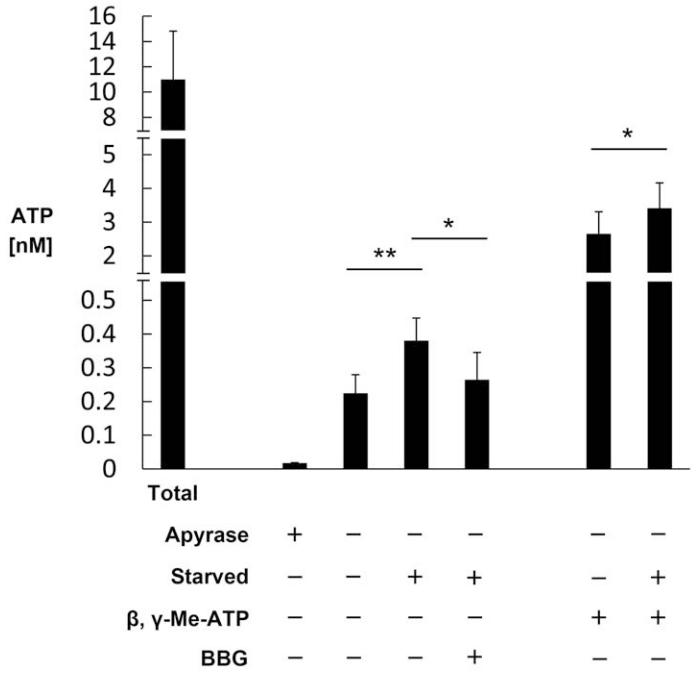
Starved − + + + +
BBG [μM] − − 0.1 1 10

Figure 6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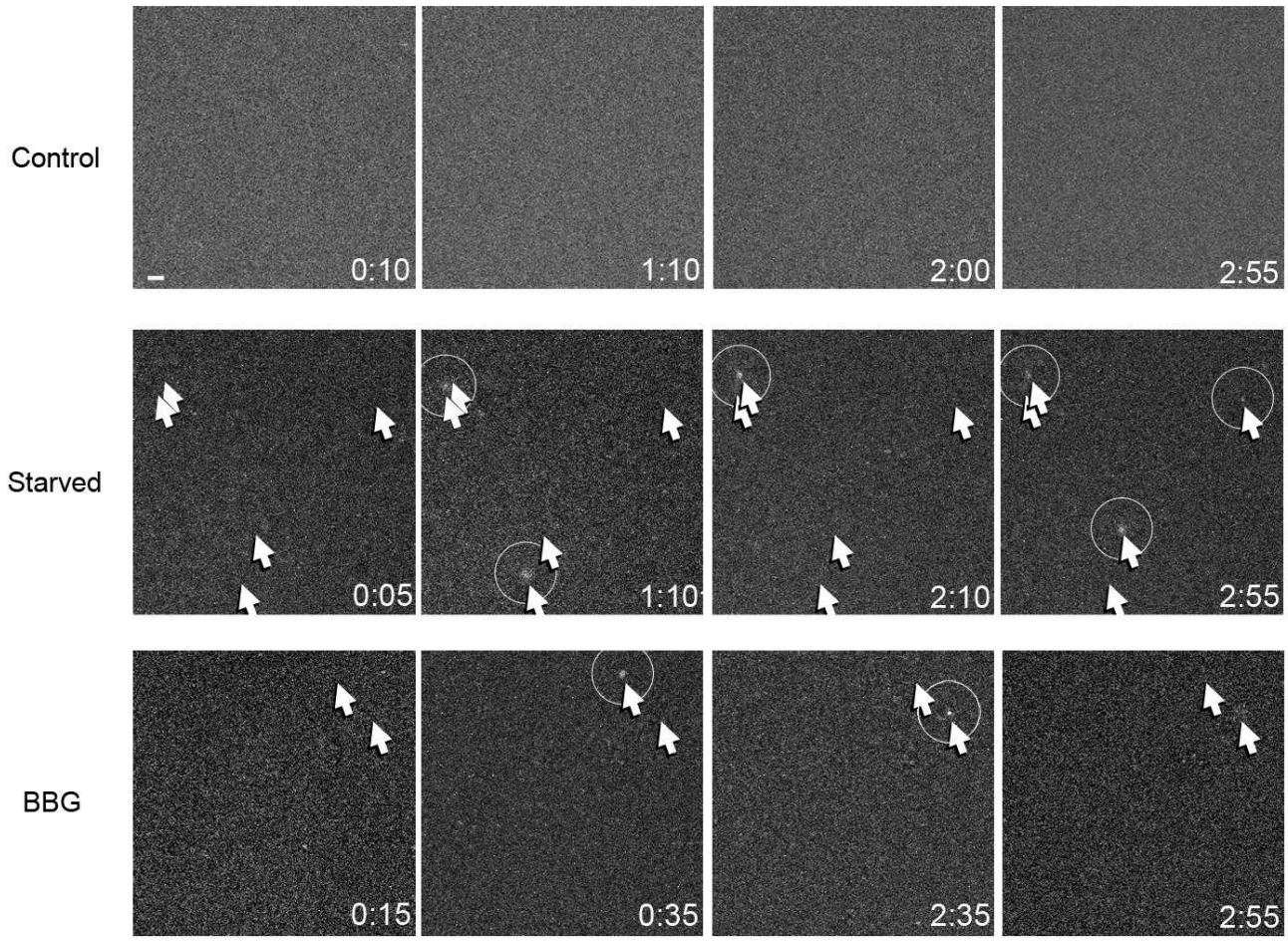
BBG − − − + − −

Figure 7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