$P2X_7$ Receptor in Microglia Cell Death in Relation to Glaucoma

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Aims

The scope of this project is to investigate mechanisms involved in neurodegeneration in glaucoma, identifying potential novel drug targets for neuroprotection.

The initial aims of the research were to:

1. Investigate whether an increase in permeability to large molecules through the microglial cell membrane could be measured in response to ATP stimulation.
2. Determine the involvement of the P2X7 receptor in this process.
3. Investigate the link between increased membrane permeability and mechanisms of cell death.
4. Further work aims to determine the role of gasdermins in this process and whether these may mediate the increased permeability and be Involved in microglia and neuronal death in relation to glaucoma.

Background

Glaucoma is a group of optic neuropathies in which there is progressive degeneration of retinal ganglion cells (RGCs); if left untreated total blindness will occur. There is no cure and all the treatments for glaucoma attempt to slow the progression of the disease. Receptors known as purinergic receptors, which get their name as they are activated by purines, have been shown to be of interest in relation to RGC death in glaucoma[1,2]. One variant, P2X7, is a trimeric ATP-gated cation channel that allows the influx of sodium and calcium and the efflux of potassium. This receptor is found in the retina. The processes involving P2X7 include mast cell degranulation, inflammation and cell death. Cell death is the ending of a cell performing biological functions and can follow several different and unique pathways. Pathways include: necrosis which involves reactive oxygen species, increased calcium and activated proteins such as calpains and cathepsins; necroptosis which involves TRADD/TRAF, RIP1 and RIP3; apoptosis which involves various caspases (Caspases 3, 6, 7, 8, 9 and 10) and pyroptosis, an inflammatory cell death pathway involving the inflammasome and the gasdermin D pore.

Materials and Methods

The BV2 cell line, which is a mouse microglial cell line, was used for all experiments and a P2X7 CRISPR knockout variant of the BV2 cells was also used.

The main techniques used were:

YO-PRO dye assay (measuring membrane permeability to large molecules)

0.2% YO-PRO solution made up in phenol red free DMEM/F12 with L-Glutamine and buffered with 15mM HEPES medium was added to BV2 cells. At time zero, fluorescence was measured. To record YO-PRO fluorescence an excitation wavelength (490nm) and emission wavelength (520nm) was measured using a Flexstation 3 multi mode microplate reader(*Molecular Devices*). ATP concentrations of 50µM, 100µM, 300µM, 500µM, 1mM, 3mM and 5mM were added to initiate the experiment. The plate was read every 5 minutes for 210 minutes using Softmax Pro v5.4.6 software. This procedure was performed with both the BV2 and the P2X7 CRISPR knockout variant which only used the 300µM, 1mM, 3mM and 5mM concentrations of ATP.

Antagonist Studies

In experiments using antagonists the 0.2% YO-PRO HEPES media was mixed with the antagonist of choice and all conditions made up to a 0.1% DMSO concentration. The plate was then left to incubate for 120 minutes.

LDH assay (measuring cell lysis)

PD150606 and ATP LDH Assay

BV2 cells were either incubated with or without 50µM PD150606 for 90 minutes and to initiate the experiment the cells were either stimulated with 3mM ATP or 0.1% DMSO media (vehicle control). Samples were taken at time point 0, 1, 2, 3, 4, 5, 6, 7 and 8 hour. At the end of the 8 hours 2% triton X was added to lyse the cells and give a reading of total LDH.

$P2X7^{K/O}$ variant of BV2 cells and BV2 LDH Assay

BV2 cells and the P2X7 CRISPR knockout variant had ATP concentrations of 3mM or 0mM added to initiate the experiment. Samples were taken at time point 0, 1, 2, 4, and 8 hour. At the end of the 8 hours 2% triton X was added to lyse the cells and give a reading of total LDH.

Data analysis was conducted with Softmax Pro and Graphpad Prism.

Results

ATP-induced Membrane Permeability Changes (Yo-Pro uptake).

Increased YO-PRO uptake was observed when BV2 cells were stimulated with high concentrations of ATP, 1mM, 3mM and 5mM (Figure 1), however this did not occur in BV2 cells treated with P2X7 inhibitor AZ10606120 or a P2X7 CRISPR knockout variant of the BV2 cells (Figure 2 and 3)

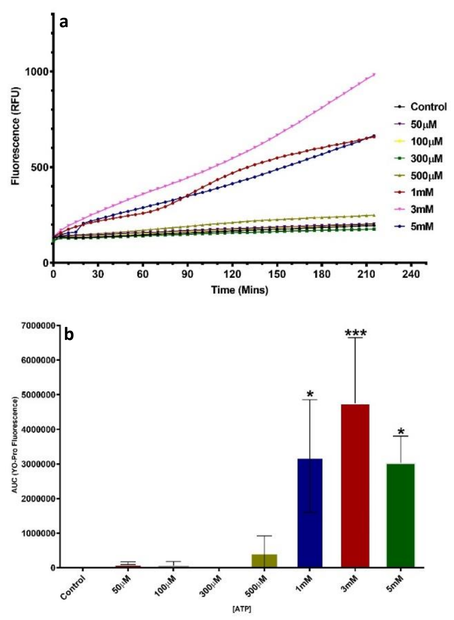

Figure 1 (a) Mean values for YO-PRO uptake of BV2 microglial cells in response to increasing concentrations of ATP (n=4) (b) Mean values +S.E.M of AUC for data in Figure 1a * Indicates significance compared to control (P<0.05) using one-way ANOVA with Dunnett's post hoc test.

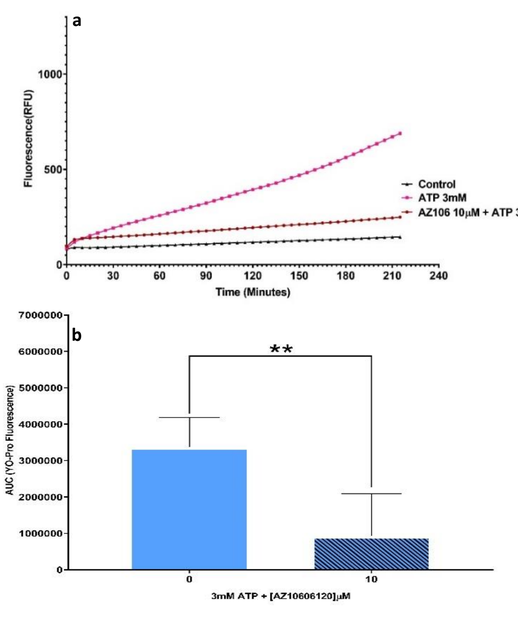

Figure 2 (a) Mean values for YO-PRO uptake of BV2 microglial cells in response to 3mM ATP and either 0µM or 10µM AZ10606120 (b) Mean values +S.E.M of AUC for data in Figure 2a (n=5) * Indicates significance (P<0.05) using students T-test

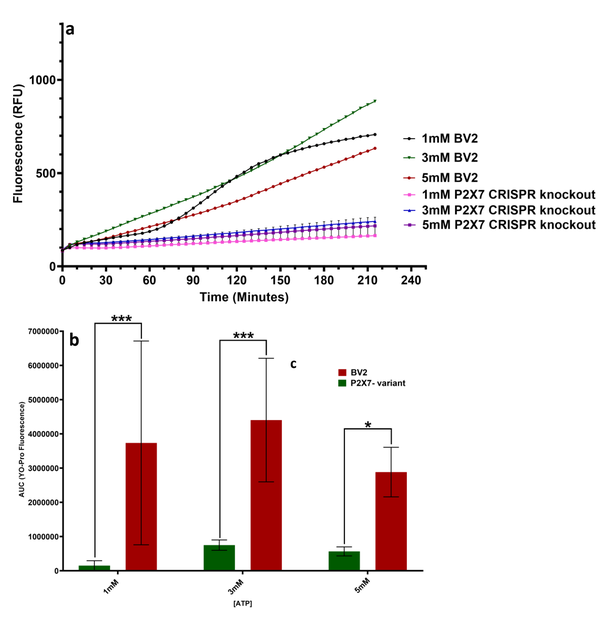

Figure 3 (a) Mean values for YO-PRO uptake of BV2 microglial cells and P2X7 CRISPR knockout variant of the BV2 microglial cells in response to increasing concentrations of ATP (n=4) (b) Mean values +S.E.M of AUC for data in Figure 3a * Indicates significance (P<0.05) using one-way ANOVA with Dunnett's post hoc test.

Effect of Inhibitors of Enzymes associated with Cell Death

Antagonists for enzymes involved in pyroptosis, necroptosis and apoptosis were seen to have no effect on the YO-PRO uptake when treated BV2 cells stimulated with 3mM ATP (Data not Shown, Table 1). However an inhibitor of the calcium-activated protease (calpain), PD150606, did lower YO-PRO uptake (Figure 4).

Antagonist	Proposed Protein Target	Proposed Cell death Mechanism Involvement	Effect on YO-PRO Uptake
PD150606	Calpain		Decreased
Calpeptin	Calpain I		None
Calpain Inhibitor 3	Calpain I + II		None
EST	Calpain-I activation		None
MCC950	NALP3	Pyroptosis	None
Necrostatin	RIPK1	Necroptosis	None
Necrosulphonamide	MLKL, possibly Gasdermin D	Necroptosis, possibly pyroptosis	None
Ac-YVAD-cmk	Caspase 1, Some Caspase 4	Pyroptosis	None
Z-VAD-FMK	Caspase 1, 3, 7 and 8	Pyroptosis and Apoptosis	None
Z-DEVD-FMK	Caspase 3 Inhibitor	Apoptosis	None
Ac-DEVD-CHO	Caspase 3/7	Apoptosis	None
Z-IETD-FMK	Caspase 8	Apoptosis	None

Table 1: Overview of antagonists used

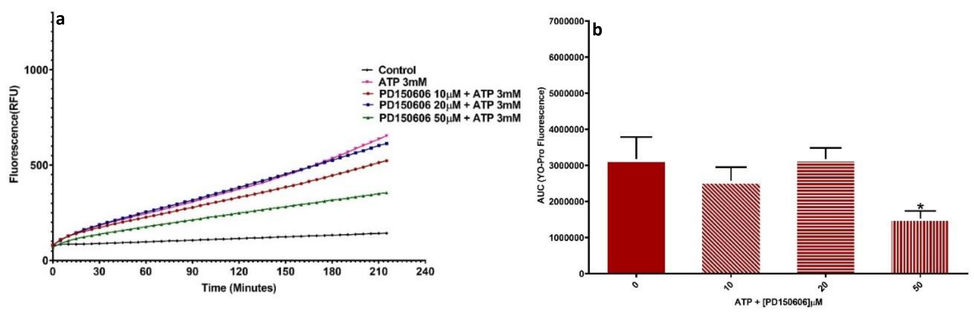

Figure 4 (a) Mean values for YO-PRO uptake of BV2 microglial cells in response to 3mM ATP and either 0µM, 10µM, 20µM or 50µM PD150606 (n=3) (b) Mean values +S.E.M of AUC for data in Figure 4a * Indicates significance (P<0.05) using one-way ANOVA with Dunnett's post hoc test

ATP-induced Lytic Cell Death

BV2 cells stimulated with 3mM ATP saw a significant steady increase in LDH release. This was not observed in the P2X7 CRISPR knockout variant of the BV2 cells or the control (Figure 5). BV2 cells incubated with PD150606 and stimulated with 3mM ATP saw a significant decrease in LDH release compared to ATP alone (Figure 6). Images of the cells taken over the course of the 8 hours (Figure 7) show that both groups of cells treated with 3mM ATP have had morphological changes when compared to the control group, however, these morphological changes differed. For the 3mM ATP population the cells appear swollen and rounded, whereas for the PD150606 + 3mM ATP cells appear less swollen and seem more shrunken.

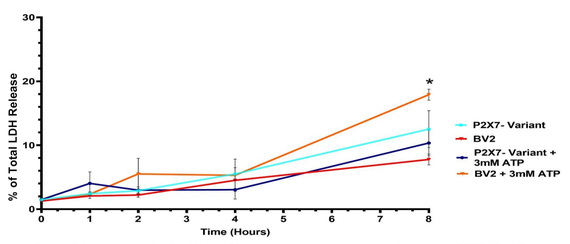

Figure 5 Mean values for % of total LDH release over a time course of 8 hours in P2X7 CRISPR knockout variant of the BV2 microglial cells and wild type BV2 microglia cells (n=3) * Indicates significance (P<0.05) using two-way ANOVA with Tukey post hoc test by comparison of BV2 + 3mM ATP to either P2X7 CRISPR knockout variant of the BV2 microglial cells, P2X7 CRISPR knockout variant of the BV2 microglial cells + 3mM ATP or BV2 cells

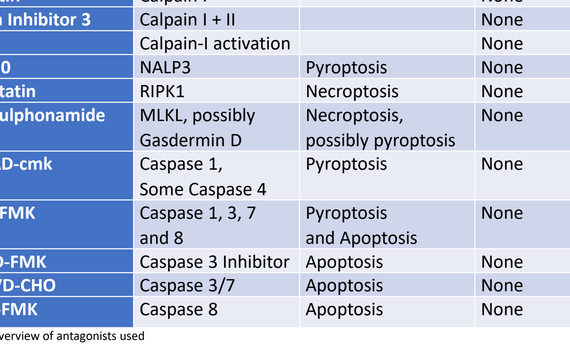

Figure 6 Mean values for % of total LDH release over a time course of 8 hours in BV2 microglia cells (n=4) * Indicates significance (P<0.05) using two-way ANOVA with Tukey post hoc test by comparison of 3mM ATP to either Control or 50µM PD150606 + 3mM ATP

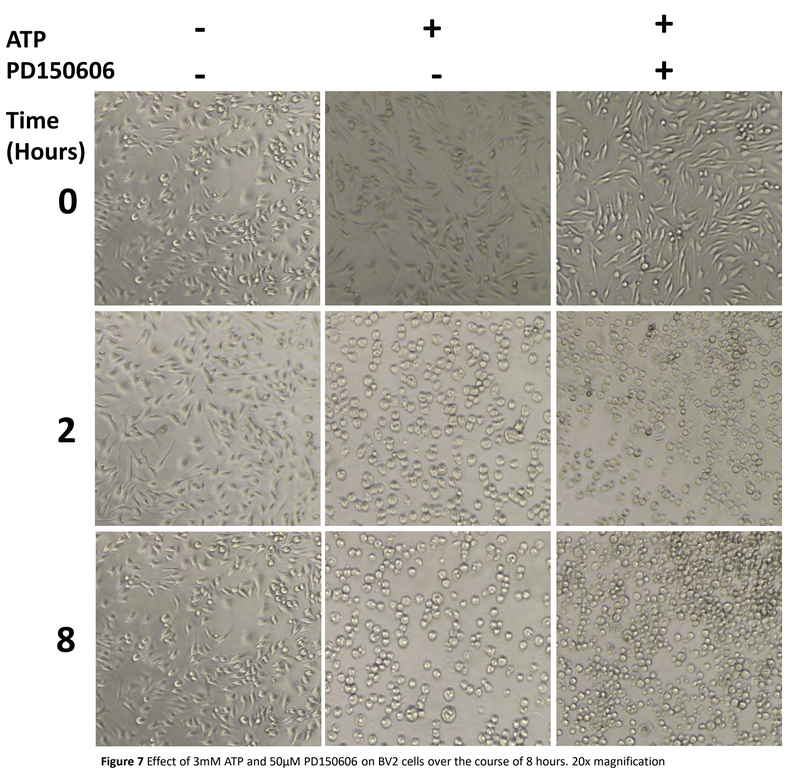

Figure 7 Effect of 3mM ATP and 50µM PD150606 on BV2 cells over the course of 8 hours. 20x magnification

References

[1] Reichenbach, A and Bringmann, A. (2016). Purinergic signaling in retinal degeneration and regeneration. *Neuropharmacology.*. 104, 194-211.

[2] Sanderson, J, Dartt, D, Trinkaus-Randall, V, Pintor, J, Civan, M, Delamere, N, Fletcher, E, Salt, T, Grosche, A and Mitchell, C. (2014). Purines in the eye: recent evidence for the physiological and pathological role of purines in the RPE, retinal neurons, astrocytes, Müller cells, lens, trabecular meshwork, cornea and lacrimal gland. *Experimental Eye Research*. 127: 270-279.

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Conclusions and Summary

1. ATP was found to increase microglial membrane permeability to large molecules.
2. This was found to be P2X7 dependent.
3. Although none of the apoptotic, necroptotic or pyroptotic antagonists were able to block this increase, the calcium-activated protease (calpain) inhibitor, PD150606, did inhibit the P2X7 mediated increase in YO-PRO uptake.
4. ATP-induced LDH release was also inhibited by PD150606 suggesting an effect on lytic cell death although morphological changes which may suggest a switching of cell death pathways indicate that further investigation is required.

Future work would involve western blotting and qPCR gene analysis to look at the presence of certain proteins during ATP stimulation such as gasdermins, caspases, calpains and their substrates. Other assays, such as the MTS assay, could also be carried out to assess the cells viability under certain ATP and inhibitor conditions.