Title: Adenosine tri-phosphate induced photoreceptor death and retinal remodelling in rats

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Abstract:

Many common causes of blindness involve the death of retinal photoreceptors followed by progressive inner retinal cell remodelling. For an inducible model of retinal degeneration to be useful, it must recapitulate these changes. Intravitreal administration of adenosine tri-phosphate (ATP) has recently been found to induce acute photoreceptor death. The aim of this study was to characterise the chronic effects of ATP on retinal integrity. Five week old, dark agouti rats were administered 50mM ATP into the vitreous of one eye and saline into the other. Vision was assessed using the electroretinogram and optokinetic response and retinal morphology investigated via histology. ATP caused significant loss of visual function within one day and loss of 50% of the photoreceptors within 1 week. At three months, 80% of photoreceptor nuclei were lost, while total photoreceptor loss occurred by six months. The degeneration and remodelling was similar to that found in heritable retinal dystrophies and age-related macular degeneration and included inner retinal neuronal loss, migration and formation of new synapses; Müller cell gliosis, migration and scarring; blood vessel loss and; retinal pigment epithelium migration. In addition, extreme degeneration and remodelling events such as neuronal and glial migration outside the neural retina and proliferative changes in glial cells were observed. These extreme changes were also observed in the two year old P23H Rhodopsin transgenic rat model of retinitis pigmentosa. This ATP-induced model of retinal degeneration may provide a valuable tool for the development of pharmaceutical therapies or for the testing of electronic implants aimed at restoring vision.
Introduction:

Retinal degeneration due to photoreceptor death, as occurs in age related macular degeneration (AMD) and in inherited disorders such as retinitis pigmentosa, accounts for fifty percent of blindness in the Western world (Taylor et al., 2005). These diseases are characterised by loss of photoreceptors, followed by inner retinal neuronal cell death and progressive remodelling (Fletcher and Kalloniatis, 1996; Gargini et al., 2007; Jones et al., 2012; Jones et al., 2003; Marc and Jones, 2003; Marc et al., 2003; Marc et al., 2008; Pignatelli et al., 2004; Strettoi et al., 2002). There are few treatment strategies for these diseases, but those that exist involve therapies to slow photoreceptor death using pharmacological agents and more recently electronic implants (O'Brien et al., 2012; Stingl et al., 2013a; Stingl et al., 2013b; Trifunović et al., 2012). In particular, investigation into the efficacy of retinal implants has fuelled the need for the development of inducible animal models of retinal degeneration that mimic the human retinal disease phenotype and are experimentally tractable. Thus, while there are many transgenic or natural genetic mutations in small animals, including genetic models of retinitis pigmentosa (Fletcher et al., 2011), few inducible models of retinal degeneration are available where the timing can be regulated.

Of the available inducible models, acute light damage has recently become popular and extensively researched (Hunter et al., 2012; Marc et al., 2008; Wenzel et al., 2005). This model is effective for inducing photoreceptor death and recapitulates many of aspects of retinal degenerations. However, this method is limited in application because, while it is very effective in albino animals, pigmented animals are relatively resistant to light induced damage (LaVail and Gorin, 1987). Also, other aspects of the genetic background, even between albino strains can moderate the effectiveness of this method (Danciger et al., 2007; LaVail et al., 1987a; LaVail et al., 1987b). Pharmacological treatments to induce retinal degeneration have also been developed, but many of these, like light damage, have their drawbacks. Systemic administration of N-methyl-N-nitrosourea (MNU) has been found to induce tumour formation in addition to photoreceptor death (Tsubura et al., 2011), which makes it a poor choice for long term studies of retinal implants. Similarly, methanol administration has detrimental systemic effects on the central nervous system and affects both inner and outer retinal neurons to induce blindness in rodents and
humans (Eells et al., 1996; Murray et al., 1991). Systemic administration of iodoacetate has also been used as a model of retinal degeneration in rodents but induces bilateral vision loss (Noell, 1951; Scott et al., 2011; Wang et al., 2011). Other novel inducible models of retinal degeneration, which can be administered to one eye only without systemic side effects, are desirable.

Recently, intravitreal administration of adenosine tri-phosphate (ATP) has been found to be effective for inducing photoreceptor death and loss of retinal function in the injected eye, suggesting it is a potential candidate for an inducible model of retinal degeneration (Notomi et al., 2011; Notomi et al., 2013; Puthussery and Fletcher, 2009). ATP is a naturally occurring molecule traditionally associated with cellular energy metabolism and with purinergic neurotransmission outside the cell (Burnstock, 2012). Extracellular ATP mediates fast, excitatory neurotransmission through activation of two classes of purine receptor, the ionotropic, ligand-gated P2X receptors and the metabotropic, G protein-coupled P2Y receptors. In the rat eye, injection of ATP (45mM) into the vitreous was found to induce photoreceptor-specific death within 18 hours in the rat via a P2X-receptor mediated mechanism (Puthussery and Fletcher, 2009). The photoreceptors were the only neurons affected, while the RPE and inner retinal neurons were spared. Of the purine receptor classes expressed in the eye, the P2X7-receptor (P2X7-R) has been implicated in the mechanisms underlying photoreceptor death in response to high concentrations of ATP (Notomi et al., 2011; Notomi et al., 2013; Puthussery and Fletcher, 2009). Previous work from our laboratory has demonstrated the expression of P2X7-Rs on the synaptic terminals of rod and cone photoreceptors in the rat, whereas many of the other P2 subtypes investigated were not (review: (Ward et al., 2010), P2X7: (Puthussery and Fletcher, 2004); P2X2: (Puthussery and Fletcher, 2006); P2X3: (Puthussery and Fletcher, 2007); P2X7: (Puthussery et al., 2006); P2Y1: (Ward and Fletcher, 2009)). In addition, while the P2X7-R has been shown to mediate a physiological function as a neurotransmitter receptor in the rodent retina (Vessey and Fletcher, 2012), recent work shows that if ATP concentrations exceed that which can be adequately degraded by ectonucleotidases, excessive stimulation of P2X7-Rs on photoreceptors causes apoptotic cell death (Notomi et al., 2011; Notomi et al., 2013; Puthussery and Fletcher, 2009). This appears to proceed via a P2X7-R specific mechanism, as high doses of a P2X7-R ligand, BzATP, largely fail
to induce photoreceptor apoptosis when injected into the eye of P2X7-R knockout mice (Notomi et al., 2011). Together this work suggests that the mechanism of ATP-induced photoreceptor death is specifically via a P2X7-R mediated neurotoxic response akin to NMDA mediated neurotoxicity in the inner retina (Olney et al., 1987).

Development of inducible models of retinal degeneration is important for trialling vision restorative therapies. The aim of this study was to characterise the effects of ATP on the integrity of retinal neurons and glia in the rat over six months and determine whether this may be a useful model system. In addition, we compare some unusual and “extreme” aspects of remodelling characterised in the ATP-model with a heritable model of retinal degeneration, the P23H rhodopsin transgenic rat. Our findings indicate that ATP-induced photoreceptor death recapitulates many of the anatomical changes that occur in retinal degenerations, providing a viable model for the investigation of the effects of photoreceptor death on retinal function and second order, neuronal remodelling.
Methods:

Animals: Five week old, Dark agouti rats (n=45) were obtained from the Animal Resource Center (ARC), WA, Australia. Heterozygous Pro23His-line 3 transgenic rats (P23H Rats) were obtained by crossing homozygous Pro23His rats with Sprague-Dawley rats. The Pro23His rat were originally produced by Xerogen Biosciences (Formerly Chrysalis DNX transgenic Sciences, Princeton NJ) and developed and supplied with support of the National Eye Institute by Professor Matthew LaVail, Beckman Vision Centre, University of California San Francisco School of Medicine. The breeding pairs for the work described in this study were kindly donated by Dr Krisztina Valter-Kocsi and Professor Jonathan Stone at The Australian National University, Canberra, and bred at the University of Melbourne. Rats were housed under < 30 lux cage illumination at the University of Melbourne animal facility on a 12 hour light/dark cycle. Food and water was available ad libitum. All experiments adhered to the Association of Vision Research and Ophthalmology (ARVO) standards for the ethical treatment of animals and complied with the requirements of the Animal Ethics Committee of the University of Melbourne (Ethics number #1011840).

Intraocular injections of ATP in Dark Agouti rats: At five weeks of age, dark agouti rats were anaesthetised by an intramuscular administration of a mixture of ketamine (60 mg/kg, Provet Pty. Ltd., VIC, Australia) and xylazine (5 mg/kg, Provet). In addition, the corneal reflex was anaesthetised with topical administration of Alcaine (0.5%, Alcon laboratories, VIC, Australia). Using a 30G needle, a single injection of 2 µL of 1 M ATP prepared in sterile saline vehicle (0.9%) was injected into the vitreous of one eye using a Hamilton syringe. The contralateral control eye received 2 µL of saline vehicle (Dureau et al., 2001). Based on an estimated vitreal volume of 40 µL, the concentration of ATP at the retina was 50 mM.

Visual acuity: At one, three and six days after administration of ATP, awake behaving rats (n=6) were placed in an OptoMotry system ® (CerebralMechanics Inc., Lethbridge, AB, Canada; (Douglas et al., 2005; Prusky et al., 2004)) and tested for visual responsiveness under photopic conditions using a virtual optokinetic drum. Spatial frequency thresholds were assessed by monitoring the head tracking response to a range of spatially modulated, sinusoidal gratings (contrast, 100%;
mean photopic luminance, 100 cd/m²) presented using a simple staircase protocol, rotating at a constant velocity (12 deg/s). As motion detected in the clockwise direction represents the left eye response and motion detected in the anti-clockwise direction represents the right eye response, separate spatial frequency thresholds could be determined for ATP- and saline-injected eyes independently (Douglas et al., 2005). All measurements were made by an observer, who was blinded to the stimulus condition. The spatial frequency threshold (cycles/degree) was defined as the highest spatial frequency that elicited a reliable head tracking response.

**Electroretinogram (ERG):** To assess retinal function after ATP administration, ERGs were recorded from saline- and ATP-treated eyes of (n=9) rats at 1 and 6 days after ATP, as previously described (Vessey et al., 2011). Animals were dark adapted overnight. The next day, the rats were anaesthetised using a mixture of ketamine (60mg/kg) and xylazine (5 mg/kg). The cornea was anaesthetised (Alcaine; Proparacaine Hydrochloride 0.5% from Alcon), the pupil dilated (Midriacyl; Tropicamide, 0.5% from Alcon) and to maintain body temperature, rats were placed on a heating pad. Using an Ag/AgCl electrode that was placed gently on the center of the cornea and a reference electrode placed in the mouth, ERGs were recorded. Responses were amplified (gain×5000; −3 dB at 1 Hz and 1 kHz, ADInstruments, Castle Hill, New South Wales, Australia) and digitized at 10 kHz. To elicit the ERG response, a full field flash of 2.1 log cd.s/m² was generated by a Nikon photography flash (Nikon SB900, NSW, Australia) and delivered via a custom made Ganzfeld. Two consecutive flashes (0.8 sec inter stimulus interval) were used to assess the rod and cone responses independently (Jobling et al., 2013; Lyubarsky et al., 1996; Lyubarsky and Pugh, 1996). The first flash elicited responses from both the rod and cone pathways (mixed response). The second flash elicited responses only from the cones. The rod responses were isolated by digital subtraction of the cone response from the mixed response.

**ERG component analysis:** Due to the paucity of response from the ATP-treated eyes, it was not possible to model the ERG responses. Instead, the rod photoreceptor response of the ERG, the a-wave, was determined by measuring the amplitude (µV) from the pre-stimulus baseline to the trough of the waveform. The amplitude of the rod post-photoreceptor response, the b-wave, was measured from the a-wave trough to the peak of the waveform or if no a-wave was present, from the
prestimulus baseline. The implicit times of the a- and b-waves were measured from the time of flash presentation to the trough of the a-wave or the peak of the b-wave, respectively. The cone post photoreceptor response, b-wave, was analysed by measuring the amplitude, from the prestimulus baseline to the peak of the waveform, and the implicit time until the maximum response was reached.

**Gross histology:** The gross retinal morphology of ATP- and saline-injected eyes was ascertained using haematoxylin and eosin stained paraffin sections and toluidine blue stained resin sections (Ho et al., 2012). Rats were anaesthetised with intramuscular administration of a mixture of ketamine and xylazine (60:5 mg/kg, Provet Pty. Ltd.) and euthanized using sodium pentobarbital (120 mg/kg, Provet Pty. Ltd.). The eyes were isolated and the anterior portions of the eye and lens were removed by dissection. For paraffin sectioning, whole eyes were fixed overnight in 4% paraformaldehyde containing 3% sucrose, 5% acetic acid and 60% ethanol. Eyes were then dehydrated in graded alcohols before being embedded in paraffin wax. Retinae were then sectioned at 4 µm, placed on poly-L-lysine® coated slides and incubated overnight at 37 °C. Sections were deparaffinised, stained with Mayer's haematoxylin and eosin and coverslipped. For resin sections, eyecups were fixed overnight in 1% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, 0.01% calcium chloride in 0.1 M phosphate buffer pH 7.4 (PB). Eyecups were washed in PB, and then dehydrated in a graded series of methanol (75%, 85%, 95%, and 100%) and acetone (100%). Tissues were embedded in an epon resin (ProSciTech Pty. Ltd., QLD, Australia) and polymerised overnight at 60°C. Retinae were sectioned (1 µm) on an ultramicrotome (Reichert-Jung Ultracut S; Reichert, Depew, NY, USA) and stained using 1% toluidine blue.

A microscope (Axioplan; Carl Zeiss, Göttingen, Germany) was used to view retinal sections with a X40 oil objective and images were captured using a digital camera and computer software (SPOT, version 3.5.2; Diagnostic Instruments, WA, Australia). Images were converted to grayscale and adjusted for white levels, brightness, and contrast with graphics editing software (Adobe Photoshop CS4; Adobe Systems, San Jose, CA, USA). Retinal cellular layers (multiple sections n > 2) in saline- and ATP-treated eyes were imaged within 150 µm of the optic nerve at one week (n = 7, paraffin), three months (n = 6, resin) and six months (n = 6, resin). The number of rows of photoreceptor and inner nuclear layer cells were quantified.
manually at 5 regions per image using a cell counter application (ImageJ 1.43; National Institutes of Health [NIH], Bethesda, MD). The number of ganglion cells across the retina were quantified and expressed per mm of retinal length. In addition, to determine if there was an eccentricity dependent effect of ATP on photoreceptor number, full tile scans of toluidine blue stained retinæ from optic nerve to ciliary body were collected using an LSM confocal microscope (Carl Zeiss, Göttingen, Germany). These images were used to generate “spider plot” analyses (Mittag et al., 1999), in which retinæ were divided into ten locations from optic nerve (ON) to the ciliary body and the number of rows of photoreceptors counted and averaged. An Abercrombie correction was used to normalise cell counts and account for differences in section thickness between paraffin and resin sections (Abercrombie and Johnson, 1946).

Fluorescence Immunohistochemistry: Fluorescence immunohistochemistry was used to assess morphology of retinal cell classes using previously described techniques (Vessey and Fletcher, 2012). The posterior eye cups of saline- and ATP-treated eyes at three (n=6) and six (n=6) months post injection were fixed for 30 minutes in 4% paraformaldehyde in PB, washed three times in PB, and cryoprotected in a series of graded sucrose solutions (10%, 20%, and 30% in PB). Saline- and ATP-treated eyes were then embedded together in optimal cutting temperature compound (OCT; Tissue-Tek, Sakura, Torrance, CA), frozen and sectioned transversely at 14 µm on a cryostat at −20°C (Microm, Walldorf, Germany). Sections were collected on Poly-L-lysine coated slides (Menzel-Gläser, Braunschweig, Germany) and stored at −20°C.

For immunofluorescent labelling, slides were defrosted, washed in PB and sections were incubated in a blocking solution (10% normal goat serum [NGS], 1% Bovine Serum Albumin [BSA], 0.5% Triton-X in PB) for 1 hour before incubation overnight, at room temperature, in primary antibody (Table 1) diluted in antibody buffer (3% NGS, 1% BSA, 0.5% Triton-X in PB). After washing in PB, sections were incubated with appropriate secondary antibody: goat anti-guinea pig, goat anti-mouse, or goat anti-rabbit conjugated to fluorescent dyes (AlexaFluor 488, AlexaFluor 594, or AlexaFluor 643; diluted 1:500; Life Sciences, VIC, Australia) and a nuclear dye, 4',6-diamidino-2-phenylindole (DAPI; diluted 1:300; Life Sciences, VIC, Australia) for 90 minutes. The sections were washed in PB, coated in a glycerol/Mowiol-based
mounting media, and covered with a glass coverslip. All antibodies listed were used as tissue markers and were found to label cellular morphology and distribution as carefully demonstrated in previous publications (Table 1).

Fluorescein-labeled peanut agglutinin (PNA) was used to visualize cone photoreceptors. PNA binds preferentially to a commonly occurring structure, galactosyl (b-1,3) N-acetylgalactosamine, and has previously been found to label cone photoreceptors of rodents (Vessey et al., 2011) and many other species (Hageman and Johnson, 1986).

Guinea pig anti-Vesicular Glutamate Transporter (VGLUT1) was used to label Photoreceptor and bipolar cell terminals as has been shown previously for rodent retinae (Johnson et al., 2003; Vessey et al., 2012). It is affinity purified using the immunogenic peptide and recognizes one band of 60 kDa on immunoblot of synaptic membrane fraction from rat cerebral cortex (Melone et al., 2005).

Mouse anti-Calbindin D28k was used to label horizontal cells, as has been previously shown in rodent retina (Ho et al., 2012; Vessey and Fletcher, 2012). It recognizes one band of 28 kDa on immunoblot of synaptic membrane fraction from rat cerebral cortex and combinant calbindin D-28k (Manufacturer’s product sheet).

Mouse anti-Protein Kinase Cα (PKC) was used to label rod bipolar cells as has been previously shown in rodent retina (Greferath et al., 1990; Ho et al., 2012; Vessey et al., 2012). The antibody reacts with the 80 kD polypeptide of PKC on immunoblot of bovine brain PKC, extracts of rat glioma and murine NIH 3T3 cell lines and rat brain (Manufacturer’s product sheet).

Mouse anti-Calretinin was used to label amacrine and ganglion cells as has been previously shown in rodent retina (Ho et al., 2012; Vessey et al., 2012). In the rodent retina, calretinin is present in ChAT amacrine cells as well as other GABAergic wide-field amacrine cells and ganglion cells (Araki and Hamassaki-Britto, 2000; Gábrisel and Witkovsky, 1998). It recognizes a band of 29 kDa on immunoblot of synaptic membrane fraction from rat cerebral cortex. In mouse cerebral cortex it labels a subpopulation of neurons but does not label cerebral cortex of calretinin knockout mice (Manufacturer’s product sheet).
Lectin from *Bandeiraea simplicifolia* BS-I isolecitin B4 FITC was used to label blood vessels. This marker has been previously established to label the retinal microvessels via histochemistry (Tyler and Burns, 1991) and has been used in a number of studies as an identifier for rodent retinal blood vessels (van Wijngaarden et al., 2007; Vessey et al., 2011).

Rabbit polyclonal anti-ionized calcium binding adaptor molecule 1 (Iba1) was used to label microglia (Vessey et al., 2011). The specificity of this antiserum has been previously demonstrated by testing in immunoblots of rodent cortex proteins, where it was shown to react with a unique band of the expected molecular size, 17 kDa (Ito et al., 1998).

Mouse anti-glutamine Synthetase (GS) was used to label Müller cells as has been previously shown for rodent retina (Vessey et al., 2012; Vessey et al., 2011). The glutamine synthetase antibody produces a single expected band of 45 kDa on immunoblots from rat brain (see manufacturer's datasheet) and in mouse retina (Chen and Weber, 2002; Nasonkin et al., 2011).

Rabbit polyclonal anti-glial fibrillary acid protein (GFAP) was used to label astrocytes and gliotic Müller cells (Vessey et al., 2011). The specificity of this antiserum has been previously demonstrated by testing in immunoblots of rodent retinal proteins, where it was shown to react with a unique band of the expected molecular size, 51 kDa (Chen and Weber, 2002).

Rabbit monoclonal anti-cyclin-D1 was used to label cells in G1-S transition, as has been shown previously in retina (Albarracin and Valter, 2012; Bienvenu et al., 2010). The anti-cyclin-D1 antibody produces an expected band of around 33 kDa on immunoblots from mouse testes (see manufacturer's datasheet and (McIver et al., 2012)) and regenerating mouse skeletal muscle (Galatioto et al., 2010).

Rabbit monoclonal anti-1098 bp Ki-67 motif-containing cDNA fragment (Ki-67) was used to label all cycling cells, those in G1, S, G2 and M, as has been shown previously in retina (Glaschke et al., 2011) and in the mouse small intestine (Bergner et al., 2014). The gene encodes 15 exons with a large exon 13 containing 16 homologous, highly conserved 22 amino acid sequence elements called the “Ki67 motif”. Nine of the Ki67 motif regions include a highly immunogenic amino acid
sequence (amino acid 2319-2323, FKELF; (Kubbutat et al., 1994)) that forms the epitope for many Ki67 monoclonal antibodies including the SP6 clone (Pathmanathan and Balleine, 2013). According to the manufacturer, the SP6 clone recognizes a band of 356 kDa on western blots of SKBR3 cell lysates, matching reports using other Ki-67 clones (Key et al., 1993).

Images were taken using the LSM 5 Meta confocal laser scanning microscope (Zeiss, Oberkochen, Germany) using a 20x air or 40x/1.3 oil immersion objective at a resolution of 1024 x 1024 pixels. Gain settings were at the same level when taking images for saline- and ATP-treated tissue sections. Scale bars were digitally added to the images by Zeiss LSM Image Browser software (v4.2.0.121, Zeiss, Oberkochen, Germany). Images were adjusted for black levels, contrast and brightness in Adobe Photoshop CSE version 4 (Adobe Systems, CA, USA) using the same settings for consistency between samples. In all cases, two separate representative images of ATP treated retina are presented in order to show variation in the degenerative process across the retina and in particular to highlight differences between regions where photoreceptor nuclei remained or were absent.

**Statistical analysis:** Results are expressed as the mean ± Standard Error of the Mean (SEM). Electroretinogram responses and spatial frequency thresholds across time (one, three and six days) and treatment (saline vs. ATP) were analysed by two-way, analysis of variance (2-way ANOVA). Similarly, a two-way ANOVA was used to determine changes in cell counts over time (one week, three months, and six months) and treatment (saline vs. ATP). A Bonferroni post-hoc test was used to analyse the effect of ATP treatment at individual ages (Graphpad Prism v.4, San Diego, CA, USA). In all figures, statistical significance is expressed as *P <0.05.

**Results:**

*Intravitreal administration of ATP induces loss of photoreceptors*

The time course of the effects of 50mM ATP on the structure and function of the retina was assessed in rats over one week, three months and six months. Retinal function was assessed using the electroretinogram at one and six days after ATP administration (Fig.1). A twin-flash paradigm was used to isolate rod and cone
pathway function separately and representative waveforms are shown (Fig.1A and D, rod and cone respectively). Rod photoreceptor (a-wave) function and postphotoreceptor (b-wave) function was significantly reduced, but still detectable at one day after ATP treatment (Fig.1B and C). By six days after ATP injection, the rod pathway response was indistinguishable from baseline recording noise (for both a- and b-wave; two way ANOVA for time p<0.001 and treatment p<0.0001, Bonferroni post-hoc for saline vs ATP, *p<0.05 at 1 and 6 days). Cone pathway function was negligible within one day and was still undetectable at six days after ATP (Fig.1E; cone b-wave; two way ANOVA for time p=0.25 and treatment p<0.0001, Bonferroni post-hoc for saline vs ATP, *p<0.05 at 1 and 6 days). Similarly photopic visual function, as measured by optokinetic spatial frequency threshold, was negligible and significantly reduced compared to the saline-treated fellow eye at one, three and six days after ATP-injection (Fig.1F, Two way ANOVA for time p=0.98, treatment p<0.0001, Bonferroni post-hoc for saline vs ATP, *p<0.05 at 1, 3 and 6 days). In the saline-treated fellow eye, spatial frequency threshold was ~0.53 ± 0.5 cycles per degree and although slightly lower, perhaps due to contributions from ipsilateral (ATP-treated) visual centres, this response was not significantly different to the response from un-injected rats (0.58 ± 0.01, p>0.05). This suggests that ATP induces loss of cone derived retinal responses and loss of functional photopic vision within 24 hours and complete loss of both rod and cone derived retinal function within one week.

In line with this loss of visual function, the structure of the retina after intravitreal administration of ATP was found to be aberrant (Fig.2). In all cases, two separate representative images of ATP treated retina are presented in order to show variation in the degenerative process across the retina. One week after ATP-treatment, there was specific loss of the photoreceptors across most regions of the retina, while the inner retina was relatively preserved (Fig.2B, C). At three months after ATP treatment, the photoreceptor layer was further reduced with only a few rows of nuclei and inner/outer segments detected in some regions (Fig.2E) while in other regions photoreceptors were completely absent (Fig.2F). At six months, the photoreceptor layer was completely degenerate (Fig.2H) and in some regions the retinal pigment epithelium (RPE) had migrated into the retina (Fig.2H, arrowhead). The inner retinal neurons also appeared affected (Fig.2I). In contrast, the morphology of saline-
treated eyes (Fig.2A, D, G) was similar to un-injected control rat eyes at each age (data not shown).

To quantify the gross retinal changes induced by ATP, the number of rows of photoreceptor nuclei and inner nuclear layer nuclei were determined and the numbers of nuclei across the ganglion cell layer were counted also (Fig.3). ATP treatment induced a specific and significant loss of photoreceptor nuclei, approximately 50% at one week, ~80% at three months and by six months there was, on average, less than one layer of cells (Fig.3A, Two way ANOVA, time p=0.0003, treatment p<0.0001, Bonferroni post-hoc for saline vs ATP, *p<0.05 at 1 week, 3 and 6 months). When quantified across the retina as a function of distance from the optic nerve, ATP was found to alter photoreceptor row number differentially across eccentricity. At three months, on average there were no photoreceptors centrally and some residual photoreceptors in the far periphery (Fig.3B). At six months, the number of photoreceptors in the periphery was reduced to less than one layer (Fig.3C). In contrast, although the number of rows of nuclei in the inner nuclear layer decreased with age in ATP- and saline-treated eyes, there was no significant effect of the treatment at any time (Fig.3D, Two way ANOVA, time p<0.0001, treatment p=0.15). ATP treatment had no effect on the number of cells in the ganglion cell layer at one week and three months after the initial insult, however, after six months, cell numbers were significantly reduced in the ATP treated eye (Fig.3E, Two way ANOVA, time p=0.0016, treatment p=0.0352, Bonferroni post-hoc for saline vs ATP, *p<0.05 at 6 months only). This suggests that 50mM ATP provides a single insult that induces photoreceptor dysfunction and death within one week. Furthermore, over time almost all photoreceptors and also some amacrine and/or ganglion cells in the ganglion cell layer are lost as part of an ongoing degenerative process.

**ATP induces photoreceptor degeneration and remodelling of inner retinal neurons**

To further investigate the time course of changes following ATP treatment, various retinal cell markers were used to probe the neurodegenerative process. Retinae from saline- and ATP-treated eyes were labelled for cone photoreceptors (PNA, green), photoreceptor and bipolar cell terminals (VGLUT1, red) and cell nuclei (DAPI, blue) at three (Fig.4A-C) and six months post-injection (Fig.4D-E). In the saline-treated
eyes, PNA-labelled cone photoreceptor inner/outer segments and terminals were apparent across the outer retina and outer plexiform layer respectively at three- (Fig.4A) and six-months (Fig.4D). Cone terminals were found to co-localise with VGLUT1-labelled synapses in the outer plexiform layer in a manner consistent with healthy retinal tissue (Sherry et al., 2003). In ATP-treated eyes, although there were still regions of photoreceptor nuclei at three months (Fig.4B), PNA labelled cone inner/outer segments were absent and cone photoreceptor terminals were displaced or absent from the outer plexiform layer. In addition, VGLUT1-labelled photoreceptor terminals were displaced into the outer nuclear layer (arrowhead, Fig.4B). In other regions, where photoreceptor nuclei were absent, a few sparse VGLUT1 positive photoreceptor terminals remained (Fig.4C). By six months after ATP administration, evidence of PNA-labelled cones and VGLUT1 positive photoreceptor terminals was negligible (Fig.4E&F). Also in some regions, the inner retina had begun to remodel significantly, with large holes representing either pigmented cells from the RPE and/or fluid filled vacuoles located in the inner retina (Fig.4E, asterisk) as has been described previously (Marc and Jones, 2003) and loss of VGLUT1 positive bipolar cell terminals in the inner plexiform layer (Fig.4F).

To investigate the response of inner retinal neurons, retinæ were labelled for rod bipolar cells with an antibody against protein kinase C α (PKCa; Fig.5). At three months post-ATP injection, rod bipolar cell morphology was relatively well maintained in most regions of the retina (Fig.5B) and, in general, similar to saline-injected (Fig.5A) or healthy retinae (Uesugi et al., 1992). In regions where photoreceptors remained, rod bipolar cell nuclei were located in the outer regions of the inner nuclear layer, their dendrites contacted remnant VGLUT1 positive photoreceptor terminals in the outer plexiform layer and PKCa/VGLUT1-positive terminals laminated in the inner most region of the inner nuclear layer (Fig.5B). However, in regions devoid of photoreceptors, rod bipolar cell morphology was more irregular (Fig.5C). By six months after ATP-injection, PKCa-positive rod bipolar cells were still present although they appeared reduced in number, their overall morphology was abnormal and they laminated irregularly in the inner plexiform layer (Fig.5E-F). In addition, there were instances of extreme remodelling events, with rod bipolar cell dendrites exiting the neural retina and forming VGLUT1-positive contacts in the choroid (Fig.5F, arrowhead). These instances of neural retina emigration into
the choroid were also observed at the light microscopy level (Fig.5G, arrowheads). Given the unusual nature of this neuronal remodelling, it was of interest to determine if this change was representative of heritable retinal degenerations. Two year old P23H rat retinas were compared (Fig.5H). The P23H rat had no outer nuclear layer, regions of reduced and remodelled inner nuclear layer and an abnormal distribution of VGLUT1 positive synapses. In particular, there were distinct regions of PKCα-positive/VGLUT1 positive synapses in the choroid (Fig.5H, arrow), similar to that seen in the ATP-treated retina at 6 months.

Next, horizontal cells were investigated in the ATP-treated eyes. Horizontal cells, labelled with an antibody against calbindin, were evident at both three and six months (Fig.6). At three months after ATP injection, calbindin positive cell bodies were present in the inner nuclear layer adjacent to the outer plexiform layer (Fig.6B - C) much like those observed in the saline-treated eye (Fig.6A). However, their processes were altered compared to saline-treated eyes and had remodelled to contact the few remaining VGLUT1-positive photoreceptor terminals (Fig.6B - C). By six months after ATP administration, calbindin positive cells had begun to migrate within the inner nuclear layer (Fig.6E) and were found to extend processes into the inner plexiform layer to form contacts with VGLUT1 positive bipolar cell terminals (Fig.6E). In other areas, as was seen with the PKC positive rod bipolar cells, horizontal cell processes exited the neural retina and formed connections with VGLUT1 positive synapses in the choroid (Fig.6F, arrowheads).

Amacrine and ganglion cells were identified with an antibody against calretinin (Fig.7). At three months following ATP, calretinin positive amacrine cells in the inner nuclear layer and ganglion cells / amacrine cells in the ganglion cell layer were generally well preserved (Fig.7B - C) and of similar morphology to saline-injected retinae (Fig.7A). Furthermore, in regions of ATP-injected eyes with remnant photoreceptors, the processes of these cells were found to laminate in three layers within the inner plexiform layer (Fig.7B), similar to control retinae (Fig.7A) and consistent with healthy rodent retinae (Sherry et al., 2003). In regions where photoreceptor nuclei were absent, calretinin positive processes appeared irregular, yet still formed three distinct laminae (Fig.7C). By six months after ATP-injection, calretinin positive cells were less regular in number and spacing and their processes...
were frequently disorganised (Fig. 7E). In some regions, severe remodelling had occurred with processes found to extend into the choroid (Fig. 7F, arrowheads). Overall, the effects of ATP treatment on neuronal remodelling correlate well with events common to retinal degenerations (Marc et al., 2003). However, there were instances of neurons and process connections outside the neural retina, an extreme and unusual remodelling event that is not commonly described for retinal degenerations, but was also found to be apparent in the two year old P23H rat retina.

**ATP induces loss and remodelling of intraretinal blood vessel profiles**

To determine if ATP-induced retinal degeneration had effects on blood vessels, sections were labelled with IB4-lectin (Fig. 8). In saline treated control retinae, blood vessel profiles labelled with IB4-lectin (green) were apparent in the ganglion cell layer / nerve fibre layer (superficial plexus), at the border of the inner plexiform and inner nuclear layer (inner plexus) and also in the outer plexiform layer (deep plexus, Fig. 8A, D). This lamination of the intraretinal blood vessels was consistent with healthy rodent retinae (Vessey et al., 2011). Three months after ATP administration, in regions where photoreceptors were still present, blood vessel profiles were similar to that seen in saline treated eyes (Fig. 8B). In regions where photoreceptors were absent, the vasculature appeared abnormal and the number of blood vessel profiles appeared to be reduced (Fig. 8C). At six months after ATP, the number of blood vessel profiles was reduced and morphology was abnormal, although vessels in the superficial plexus were still apparent in all regions (Fig. 8E-F).

**ATP induces Müller cell gliosis, scarring and extra-retinal migration**

The main macroglia of the retina, Müller cells, were labelled with an antibody against glutamine synthetase (GS, green), and the astrocytes and gliotic Müller cells were labelled with an antibody against glial fibrillary acidic protein (GFAP, red; Fig. 9). In the saline treated eyes, GS positive, Müller cell bodies were present in the middle of the inner nuclear layer and their processes extended across the transverse retina to form the inner and outer-limiting membrane (Fig. 9A, D). In these control eyes, GFAP-labelled astrocytes were found in the nerve fibre layer at the base of the ganglion cell layer. There was no GFAP immunoreactivity present in the Müller cell processes, which indicates the saline injected rat retinae were healthy. Three
months after ATP injection, in regions where photoreceptor nuclei were still present in a defined outer nuclear layer, the structure of the GS labelled, Müller glia was consistent with that seen in saline injected eyes however, all Müller cells were also positive for GFAP, ie. gliotic (Fig.9B). In regions devoid of photoreceptors, the Müller glia were not only GFAP-positive but were also remodelled, with nuclei at both the top and bottom of the inner nuclear layer and processes that ran diagonally across the retina (Fig.9C). There was also evidence of Müller cell hypertrophy and glial scar formation at the margins of the retina (Fig.9C). By six months after ATP, there appeared to be less GS positive, Müller cells and the remaining cells were significantly remodelled. In addition, large holes, likely fluid filled vacuoles or pigmented displaced RPE cells, encapsulated by the Müller glia, were apparent in the inner nuclear layer (Fig.9E, asterisk), and in other regions, GFAP/GS positive, Müller cells were found to leave the neural retina and enter the choroid (Fig.9F, arrowhead).

**ATP induces Müller cell expression of cell cycling markers**

Another aspect of the late stage retinal degenerative process recently highlighted in the literature is that Müller glial cells begin to express markers of cell cycling and proliferation (Albarracin and Valter, 2012; Wan et al., 2008). However, as yet this has not been described for heritable retinal degenerations. To investigate this, retinae from two year old P23H rats and three and six month ATP-treated rat eyes were labelled for Müller cells (GS, green) and the cell cycling marker, cyclin D1, a marker of G1/S phase (cyclin D1, red; Fig.10). In P23H rats, there were regions of retina where Müller cell expression of glutamine synthetase was reduced suggesting a change in glutamate/glutamine recycling in these cells. In these regions also the Müller cells labelled for cyclinD1 (Fig.10A). This response was apparent in the ATP treated eyes at three (Fig.10C - F) and six months (Fig.10G - J) but never in saline treated, contralateral control eyes (Fig.10B). In particular, at six months after ATP, there was evidence of RPE cell expression of cyclin D1 (Fig.10G, arrowhead) and strong labelling of Müller cells. In addition, cyclin D1 expression was also apparent in Müller cells that had migrated outside the neural retina into the choroid (Fig.10I, arrow).
To further probe whether Müller cell were indeed expressing markers of re-entering the cell cycle, three and six month ATP treated retinae were labelled with an antibody against Ki-67, a protein that is present during all active phases of the cell cycle (G₁, S, G₂, and mitosis) (Abdouh and Bernier, 2006). Ki67 was not present in saline-treated control eyes (Fig. 11A, D) or in regions of three month, ATP-treated retina in which photoreceptor nuclei were still present (Fig. 11B). Ki67 labelling was apparent in GS-positive Müller cells and also other cells in regions of ATP-treated retina in which the photoreceptor layer was absent at both three and six months (Fig. 11C and E, F). Taken together these results suggest that ATP-treatment induces retinal degeneration and neuronal and Müller glial remodelling consistent with that seen in retinal degenerations. Furthermore, this model recapitulates the more extreme instances of remodelling, neuronal migration into the choroid and Müller cell expression of cell cycle markers seen in the aged P23H rat retina.

Discussion
In this study we characterised the effects of a single, intravitreal injection of 50mM ATP on the integrity of rat retinal neurons and glia over six months. ATP caused complete loss of visual acuity within one day, loss of rod and cone pathway retinal function and 50% of the photoreceptors within 1 week, and ongoing retinal degeneration. At three months, 80% of the photoreceptor nuclei were lost, while inner retinal cells and the general integrity of the inner plexiform layer and ganglion cell layer remained intact. At six months, photoreceptors were absent and extreme degenerative and remodelling events such as neuronal and glial migration outside the neural retina and phenotypic changes in glial cell markers were observed. These extreme changes were very similar to that observed in the two year old P23H rat model of retinitis pigmentosa. Overall, the use of intravitreal ATP administration to induce photoreceptor death and retinal remodelling recapitulates many of the changes that occur in retinal degenerations. This model may provide a valuable tool for the development of pharmaceutical therapies aimed at slowing vision loss or for the testing of electronic implants aimed at restoring vision in retinal degenerations.

**ATP induces photoreceptor death, visual dysfunction and retinal remodelling consistent with previous reports of retinal degenerations**
Many common causes of blindness involve the death of retinal photoreceptors. This is followed by progressive inner retinal cell remodelling (Fletcher and Kalloniatis, 1996; 1997; Gargini et al., 2007; Jones et al., 2012; Jones et al., 2003; Marc and Jones, 2003; Marc et al., 2003; Marc et al., 2008; Pignatelli et al., 2004; Strettoi et al., 2002; Zhu et al., 2013). For an inducible model to be useful, it must recapitulate these changes. In the present study, ATP was found to induce complete loss of photopic visual responsiveness within one day as measured by the cone electroretinogram and the optokinetic response. By six days after ATP administration, both rod and cone pathway electroretinogram responses were negligible. In contrast, vision in the saline-injected, contralateral control eye was normal suggesting no systemic side effects. This finding is in line with work by Puthussery et al, 2009, which showed that the function of the retina, as measured by the electroretinogram, was absent five days after ATP administration (Puthussery and Fletcher, 2009). In addition to loss of functional vision, ATP induced photoreceptor specific death and an ongoing degenerative process. These changes occurred despite the likelihood that the ATP would be broken down by endogenous ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) in the eye within the first day after injection (Nedeljkovic et al., 2005; Ricatti et al., 2009). Previous work has shown that ATP induces retinal degeneration via a direct neurotoxic action on the photoreceptors, rather than the RPE or microglia or the inner retinal neurons, likely via a P2X7-R mediated action (Notomi et al., 2011; Notomi et al., 2013; Puthussery and Fletcher, 2009). Our data support this and furthermore show that ATP induces an initial insult, which causes photoreceptor failure and subsequent loss, followed by ongoing retinal degeneration and remodelling.

In retinal degenerations, both heritable and acquired, the remodelling process has been suggested to occur in three stages, documented and reviewed thoroughly by Marc et. al., 2003 (Marc et al., 2003). Broadly, Phase 1 involves a primary insult to the photoreceptors and photoreceptor death, Phase 2 is characterised by extensive photoreceptor death and changes in the inner retinal neurons and glia, while Phase 3 occurs in response to the loss of the last of the photoreceptors and involves global remodelling of the remnant retina. With regards to ATP induced photoreceptor death, Phase 1 was found to occur within the first week after injection, during which the initial ATP insult induced rapid photoreceptor dysfunction and death, affecting both
rods and cones. At three months after ATP administration, Phase 2 degeneration was found to occur in regions where the photoreceptors remained and early Phase 3 was found to occur in regions devoid of photoreceptor nuclei and terminals. By six months after ATP administration, evidence of cones and photoreceptor terminals was negligible and many examples of Phase 3 remodelling (early, mid and late) were apparent (Marc et al., 2003).

It has been suggested that as long as photoreceptors remain, retinal remodelling is subtle and abstruse, however when all photoreceptors are lost significant remodelling occurs and it is similar in most retinal degenerations (Jones et al., 2012; Jones et al., 2003). In general, our findings relating to neuronal, glial and blood vessel remodelling at three months after ATP induced photoreceptor death seem concordant with this hypothesis. At three months, evidence of Phase 2 degeneration and remodelling (Marc et al., 2003) in regions where photoreceptor nuclei remained included: displaced photoreceptor terminals into the remaining outer nuclear layer; rod bipolar and horizontal cell dendrite retraction and extension to contact remnant photoreceptor terminals; relative preservation of the inner retina, specifically bipolar cell terminals and, amacrine/ganglion cells and their processes; Müller glia were structurally sound but were gliotic; and blood vessel profiles were similar to that seen in saline treated eyes. Evidence of early Phase 3 remodelling in regions devoid of photoreceptors included: Müller glia hypertrophy, scar/seal formation and remodelling, with Müller cell nuclei at both the top and bottom of the inner nuclear layer and aberrant processes; abnormal vasculature, with the number of blood vessel profiles reduced. Taken together the retinal changes in neurons, glia and blood vessels, observed at three months after ATP-induced photoreceptor death best represent Phase 2 and early Phase 3 stages of retinal degeneration and remodelling (Marc et al., 2003).

By six months after ATP administration, Phase 3 remodelling was apparent (Marc et al., 2003). Evidence of Phase 3 remodelling events affecting neurons included: a reduction in number of amacrine and/or ganglion cells in the ganglion cell layer; migration of horizontal and bipolar cell towards the inner plexiform layer; migration of amacrine and ganglion cells towards the glial seal; and development of multicellular fascicles and microneuromas, for example, horizontal cells had begun to migrate
within the inner nuclear layer and were found to extend processes into the inner plexiform layer to make contact with bipolar cell terminals. Evidence of Phase 3 remodelling events affecting Müller glia included: hypertrophy and gliosis; consolidation of the glial seal at the margins of the transverse retina; and Müller cell processes surrounding invading RPE cells and new neurites. In addition, there was evidence of late Phase 3 remodelling events affecting the blood vessel and RPE including: abnormal morphology; vessel and RPE cell loss; and invasion of RPE into the neural retina, consistent with changes seen in genetic rat models of retinal degeneration with age (Villegas-Pérez et al., 1998; Wang et al., 2003). Ten major types of restructuring of the retina have been suggested to occur in Phase 3, that are consistent across heritable retinal degenerations in humans and mice (Jones et al., 2003) and we see many examples of these changes in the ATP treated eyes at three months and all of these changes at six months (Table 2).

**ATP induces extreme neuronal and glial remodelling consistent with the P23H rat model of retinitis pigmentosa**

In addition to the retinal remodelling changes detailed above, two types of extreme remodelling event were also observed: 1) glial and neural cell exit from the neural retina into the choroid and formation of new extra-retinal neuronal connections and 2) alterations in Müller glia phenotype, including loss of glutamine synthetase and expression of cell cycling markers. These changes were not specific to ATP-induced retinal degeneration, but were also observed in the P23H rat model of retinitis pigmentosa at two years. This suggests that these extreme remodelling events are a late stage consequence of photoreceptor death and the degenerative process.

With regards to glial and neural cell exit from the neural retina into the choroid, this type of remodelling event has been described in the light damage induced model of retinal degeneration in the rat, in which emigration of Müller cells and neurons of all classes from the retina into the choroid was observed (Marc et al., 2008). Marc et al (2008) suggest, in an unpublished observation, that this event has been observed in human retinitis pigmentosa. In addition, it may occur in human AMD as well (Pow and Sullivan, 2007; Sullivan et al., 2007). We suggest that neuronal and glial emigration and new neuronal synapse formation in the choroid may be an extreme remodelling event that is consistent across retinal degenerations in both retinitis
pigmentosa and AMD in humans and animal models. We hypothesise that this occurs in response to failure and loss of the RPE as part of an ongoing degenerative process. The RPE and Bruch’s membrane, a layered structure consisting mainly of collagen, provide a structural barrier between the choroid and the retina (Guymer et al., 1999). In regions where the RPE dies or remodels and invades the retina, the barrier between the retina and choroid would be weakened. This would allow migrating glia and neurons to exit the neural retina and enter the choroid however more research is required to confirm if failure of RPE integrity precedes neuronal/glial migration into the choroid.

In addition, here we show that in both the P23H rat and also in the ATP-induced model, Müller glia undergo extreme changes in phenotype including loss of glutamine synthetase and concomitant increase in expression of cell cycling markers, cyclinD1 and Ki67. Glutamine synthetase is a key enzyme expressed by Müller cells that is important in the degradation of glutamate released in neurotransmission to glutamine for recycling (Bringmann et al., 2009). The loss of glutamine synthetase suggests that in some regions Müller glia are no longer able to perform one of their key neuroregulatory functions. A loss of glutamine synthetase and/or increase in glutamate levels in Müller glia has been reported previously in retinal degenerations such as light damage (Albarracin and Valter, 2012; Marc et al., 2008), retinal detachment (Lewis et al., 1994; Marc et al., 1998) and retinitis pigmentosa (Fletcher and Kalloniatis, 1997). However, in addition to this, we see Müller cell expression of cell cycling markers in these regions of depleted glutamine synthetase. Cyclin D1 in Müller cells has been reported following light damage (Albarracin and Valter, 2012) and NMU models of retinal degeneration (Wan et al., 2008). Albarracin et al, suggest that Müller cells are undergoing proliferative changes in order to restore retinal structure, similar to the role of fibroblasts in skin injuries and scarring. However, an alternative hypothesis is that Müller glia are de-differentiating to a progenitor like state as occurs in fish (Moshiri et al., 2004; Raymond et al., 2006) and birds (Fischer, 2005; Fischer and Omar, 2005). In these species, Müller cells are able to de-differentiate and re-enter the mitotic cell cycle to regenerate retinal neurons, restoring some level of functional vision. In mammals it is believed this process is halted by lack of appropriate differentiation factor expression. However, it has been reported that in rodents these proliferating glia are
able, with the correct stimulus, to form inner retinal neurons such as bipolar and amacrine cells (Karl et al., 2008), as well as rhodopsin expressing photoreceptors (Wan et al., 2008). The present model of ATP induced retinal degeneration may prove useful for testing which factors support glial de-differentiation.

**Other applications and limitations of ATP-induced retinal degeneration**

The heterogeneity of retinal degenerations and the variability in the progression of these diseases means that investigating therapeutic strategies to ameliorate these disorders can be problematic. The advantage of an inducible model of retinal degeneration is that the start and process of the disease can be controlled and in this case the agent, ATP, can be administered to one eye only, so systemic side effects are negligible and viable vision remains in the other eye. In these respects the ATP-induced retinal degeneration model has advantages over many of the other models such as: NMU, which affects both eyes and induces cancer with time (Tsubura et al., 2011); iodoacetate, which affects both eyes (Noell, 1951; Scott et al., 2011; Wang et al., 2011); and light damage, which has limited applicability in pigmented animals (Hunter et al., 2012; Marc et al., 2008).

The ATP-induced retinal degeneration model does have potential limitations. One of these is that both the rod and cone photoreceptors appear to undergo simultaneous degeneration. This is unlike many heritable retinal degenerations such as retinitis pigmentosa in which rods degenerate before cones (Fletcher et al., 2011), but similar to AMD in which both rods and cones are affected (Curcio, 2001). In addition, as purinergic receptors are located throughout the rodent retina (Ward et al., 2010), there is the potential for ATP to induce significant damage to neurons other than photoreceptors (Hu et al., 2010). Inner retinal neurons, such as calretinin positive amacrine and ganglion cells express P2X7-Rs (Vessey and Fletcher, 2012). Thus although these inner retinal neurons remain morphologically intact up to three months after the ATP induced insult, their function may be altered. Despite these potential limitations, our preliminary evidence suggests that ATP-induced photoreceptor degeneration is a viable model in larger vertebrates such as the cat providing a useful model for testing of electronic devices aimed at restoring vision in humans (unpublished observation). Our data in the rat suggest that the optimal time to test such devices would be three months after ATP administration, as this is a
time when most photoreceptors are lost, but retinal remodelling is not rampant and ganglion cell numbers have not been significantly reduced. However, testing of retinal implants at later stages may also be beneficial for comparison with late stage, retinal degenerations in humans.

**Conclusion**

In the present study we show intravitreal ATP administration induces photoreceptor death and retinal remodelling. We find it replicates many of the changes that occur in retinal degenerations and that it induces some extreme remodelling events that are also common to heritable retinal degenerations and AMD. This model provides a valuable tool for investigating the degenerative process, glial cell de-differentiation and proliferation, as well as for investigating therapies aimed at slowing vision loss or electronically restoring vision.

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**Conflict of interest statement:** None

**Role of authors:** All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: ELF, KAV; Acquisition of data: KAV, ELF, FA, UG; Analysis and interpretation of data: KAV, ELF, FA, UG; Drafting of the manuscript: KAV, UG, FA, AIJ, JAP, TH, RUI, ANB, ELF; Critical revision of the manuscript for important intellectual content: KAV, UG, FA, AIJ, JAP, TH, RUI, ANB, ELF; Statistical analysis: KAV, FA; Obtained funding: ELF, ANB; Administrative, technical, and material support: ELF; Study supervision: KAV, ELF
**Literature cited**


Figure Legends

Figure 1. The time course of the effects of 50mM ATP on retinal function at one and six days. Twin, full field 2.1 log cd.s/m² intensity flashes were used to isolate the rod and cone pathway electroretinogram responses of saline- and ATP-treated eyes. (A) Representative rod pathway waveforms from saline- (grey) and ATP-treated (black) eyes. (B) Rod a-wave amplitude and (C) rod b-wave amplitude were assessed for saline- (grey bar, n=9) and ATP-treated (black, n=9) eyes at 1 and 6 days after intravitreal injection. (D) Representative cone pathway waveforms from saline- (grey) and ATP-treated (black) eyes. (E) Cone b-wave amplitudes were assessed for saline- (grey bar, n=9) and ATP-treated (black, n=9) eyes at 1 and 6 days after intravitreal injection. (F) Spatial frequency threshold in saline-treated and ATP-treated eyes was assessed under photopic conditions, using the optokinetic response at 1, 3 and 6 days after injection. Significance of p<0.05 between saline and ATP treated eyes indicated by *.

Figure 2. The time course of the effects of 50mM ATP on the gross structure of the retina at one week, three months and six months. Gross retinal structure was assessed in saline-treated and ATP-treated regions of retina at one week (A, saline; B-C, ATP; paraffin), three months (D, saline; E-F, ATP; resin) and six months (G, saline; H-I, ATP; resin). At each time point, two example regions of ATP-treated retinae are presented. Arrowheads in H indicate aberrant pigmented (RPE) cells within the neural retina. Scale bar, 20 µm. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Figure 3. Quantification of the effects of 50mM ATP on the gross structure of the retina at one week, three months and six months. (A) The average number of rows of photoreceptor nuclei were quantified for saline- (grey bars) and ATP-treated (black bars) eyes at one week, three months and six months. (B-C) To determine if there was an effect across eccentricity, rows of photoreceptor nuclei were also quantified at 10 regions across the retina from the optic nerve (ON) to the peripheral ciliary body at (B) three and (C) six months. (D) The average number of rows of inner nuclear layer cells were quantified in saline (grey bars) and ATP treated (black bars) eyes at one week, three and six months. (E) The average number of cells across the
ganglion cell layer were also quantified. Significance of p<0.05 between saline (n=6) and ATP treated eyes (n=6) indicated by *.

**Figure 4. Cone and rod photoreceptors degenerate following ATP treatment.** Sections of retinae from saline- and ATP-treated eyes were labelled for cone photoreceptors (PNA, green), photoreceptor and bipolar cell terminals (VGLUT1, red) and cell nuclei (DAPI, blue) at three months (A, saline; B-C, ATP) and six months post-injection (D, saline; E-F, ATP). At each time point, two example regions of ATP-treated retinae are presented. Arrowhead in (B) indicates an abnormal, clump of VGLUT1-positive photoreceptor terminals displaced in the ONL. Asterisks (*) in (E) indicate RPE cell migration and/or vacuole structures in the retina. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

**Figure 5. Rod bipolar cell morphology following ATP-induced photoreceptor degeneration and in P23H rat retinae.** Sections of retinae from saline- and ATP-treated rat eyes, and a two year old P23H rat retina for comparison, were labelled for rod bipolar cells (Protein kinase C α, green), photoreceptor and bipolar cell terminals (VGLUT1, red) and cell nuclei (DAPI, blue). (A - C) ATP-treated rat retinae at three months (A, saline; B - C, ATP). (D - F) ATP-treated rat retinae at six months (D, saline; E - F, ATP). At each time point for ATP-treated retinae, two example regions are presented. (G) Light microscope image of ATP-treated rat retina at six months. (H) Two year old P23H rat retina. Arrowheads in (F & H) indicate extreme remodelling event of VGLUT1/PKCα-positive synapses displaced into the choroid in 6 month ATP retina (F) and two year old P23H rat retina (H). Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

**Figure 6. Horizontal cell morphology following ATP-induced photoreceptor degeneration.** Sections of retinae from saline- and ATP-treated eyes were labelled for horizontal cells (Calbindin D28k, green), photoreceptor and bipolar cell terminals (VGLUT1, red) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinae are presented. Arrowheads in (F) indicate
extreme remodelling event of horizontal cell processes associating with VGLUT1-positive synapses displaced into the choroid. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Figure 7. Amacrine and ganglion cell morphology following ATP-induced photoreceptor degeneration. Sections of retinas from saline- and ATP-treated eyes were labelled for a subset of amacrine and ganglion cells (Calretinin, green) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinas are presented. Arrowheads in (F) indicate extreme remodelling event of amacrine cell processes displaced into the choroid. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Figure 8. Blood vessel morphology following ATP-induced photoreceptor degeneration. Sections of retinas from saline- and ATP-treated eyes were labelled for blood vessels (Isolectin B4, green) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinas are presented. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

Figure 9. Glial cell morphology following ATP-induced photoreceptor degeneration. Sections of retinas from saline- and ATP-treated eyes were labelled for Müller glial cells (GS, green), astrocytes and Müller cell gliosis (GFAP, red) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinas are presented. Asterisk (*) in (E) indicates holes representing fluid filled vacuoles and/or RPE cell migration into the retina. Arrowheads in (F) indicate extreme remodelling event of GFAP/GS positive, Müller cell processes displaced into the choroid. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
**Figure 10. Müller cells express the cell cycling marker, cyclin D1, in P23H rat retinae and ATP-induced photoreceptor degeneration.** Sections of retinae from two year old P23H rat model of retinitis pigmentosa (A), saline-treated rat eyes at six months (B), ATP-treated rat eyes at three months (C, tile scan; D - F magnification of three areas from C), and ATP-treated rat eyes at six months post-injection (G, tile scan; H - J magnification of three areas from G), were labelled for Müller glial cells (GS, green), cell cycling (cyclin D1, red) and cell nuclei (DAPI, blue). Arrow in (G) indicates RPE cyclin D1 expression. Arrow in (I) indicates extreme remodelling event of cyclinD1 positive Müller cells displaced into the choroid. Scale bar in all, 100 μm. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

**Figure 11. Müller cells express the cell cycling marker, Ki67, following ATP-induced photoreceptor degeneration.** Sections of retinae from saline- and ATP-treated eyes were labelled for Müller glial cells (GS, green), cell cycling (Ki67, red) and cell nuclei (DAPI, blue) at three months (A, saline; B-C, ATP) and 6 months post-injection (D, saline; E-F, ATP). Scale bar, 20 μm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Table 1. Primary antibodies used for immunohistochemical analysis.

All antibodies listed were used as tissue markers and were found to label cellular morphology and distribution as previously demonstrated in previous publications.

<table>
<thead>
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<th>Cell type/Target</th>
<th>Antibody/Lectin</th>
<th>Dilution</th>
<th>Source</th>
<th>Immunogen</th>
</tr>
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<td>Cones</td>
<td>Fluorescein labelled Peanut Agglutinin (PNA) from <em>Arachis hypogaea</em> (peanuts)</td>
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<td>Cat# FL-1071; Vector Laboratories, Burlingame, CA</td>
<td>NA</td>
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<td>Photoreceptor and bipolar cell terminals</td>
<td>Guinea pig anti-Vesicular Glutamate Transporter (VGLUT1), polyclonal</td>
<td>1:1000</td>
<td>Cat# AB 5905; Millipore, Merck, VIC, Australia</td>
<td>C-terminal peptide of rat VGLUT1, GATHSTVQPRPPPPVREY</td>
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<tr>
<td>Horizontal cells</td>
<td>Mouse anti-Calbindin D28k, monoclonal</td>
<td>1:4000</td>
<td>Cat# AB 300; Swant, Bellinzona, Switzerland</td>
<td>Purified bovine kidney calbindin-D-28K</td>
</tr>
<tr>
<td>Rod bipolar cells</td>
<td>Mouse anti-Protein Kinase Cα (PKC), monoclonal (MC5)</td>
<td>1:400</td>
<td>Cat# P5704; Sigma-Aldrich, NSW, Australia</td>
<td>Peptide Amino acids 296-317 of PKC</td>
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<tr>
<td>Amacrine and ganglion cells</td>
<td>Mouse anti-Calretinin, monoclonal</td>
<td>1:1000</td>
<td>Cat# 63B; Swant, Bellinzona, Switzerland</td>
<td>Recombinant human calretinin - 22k</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>Lectin from <em>Bandeiraea simplicifolia</em> BS-I isolectin B4 FITC</td>
<td>1:75</td>
<td>Cat# L2895; Sigma-Aldrich, NSW, Australia</td>
<td>N/A</td>
</tr>
<tr>
<td>Microglia</td>
<td>Rabbit anti-Ionized calcium binding adaptor molecule 1 (Iba1), polyclonal</td>
<td>1:1500</td>
<td>Cat# 019-19741; Wako Pure Chemical Industries, Richmond, VA, USA</td>
<td>Synthetic peptide corresponding to the C-terminus of Iba1: PTGPPAKKAISELP</td>
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<tr>
<td>Müller cells</td>
<td>Mouse anti-glutamine Synthetase (GS), monoclonal (GS-6)</td>
<td>1:1000</td>
<td>Cat# MAB302; Millipore, Merck, VIC, Australia</td>
<td>Glutamine synthetase purified from sheep brain</td>
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<tr>
<td>Astrocytes and gliotic Müller cells</td>
<td>Rabbit anti-glia fibrillary acid protein (GFAP), polyclonal</td>
<td>1:20,000</td>
<td>Cat# Z0334; Dako, Carpinteria, CA, USA</td>
<td>Bovine spinal cord GFAP</td>
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<tr>
<td>Cells in G1-S transition</td>
<td>Rabbit anti-cyclin-D1, monoclonal (SP4)</td>
<td>1:2</td>
<td>Cat# AB21699; Abcam, Sapphire Bioscience, NSW, Australia</td>
<td>A synthetic peptide from the C-terminus of human cyclin D1 (amino acids 250-295, Uniprot seq: P24385)</td>
</tr>
<tr>
<td>All cycling cells, those in G1, S, G2 and M</td>
<td>Rabbit anti-1098 bp KI67 motif-containing cDNA fragment, monoclonal (SP6)</td>
<td>1:1000</td>
<td>Cat# AB16667; Abcam, Sapphire Bioscience, NSW, Australia</td>
<td>A synthetic peptide from the C-terminus of human Ki-67 (amino acids 2319-2323, FKELF, within amino acids 2300-2400, Uniprot seq: P46013)</td>
</tr>
</tbody>
</table>
Table 2. In the ATP treated eyes there are remodelling events consistent with the 10 major types of restructuring of the retina seen in retinal degenerations (Jones et al., 2003). Evidence of the remodelling event is indicated by a plus (+) sign, absence by a minus (-).

<table>
<thead>
<tr>
<th>Remodelling Event</th>
<th>3 mon</th>
<th>6 mon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell death including ganglion cell loss</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Relocation of all types of surviving neurons</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fragmentation of the inner plexiform layer lamination</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Evolution of new neurites in complex fascicles surrounded by Müller cell processes</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Formation of new synapses throughout the retina</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Müller glia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migration</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypertrophic columns formation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glial seal at the distal margin of the retina</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Blood vessel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrophy and remodelling</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RPE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasion of RPE into the retina</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 1. The time course of the effects of 50mM ATP on retinal function at one and six days. Twin, full field 2.1 log cd.s/m² intensity flashes were used to isolate the rod and cone pathway electroretinogram responses of saline- and ATP-treated eyes. (A) Representative rod pathway waveforms from saline- (grey) and ATP-treated (black) eyes. (B) Rod a-wave amplitude and (C) rod b-wave amplitude were assessed for saline- (grey bar, n=9) and ATP-treated (black, n=9) eyes at 1 and 6 days after intravitreal injection. (D) Representative cone pathway waveforms from saline- (grey) and ATP-treated (black) eyes. (E) Cone b-wave amplitudes were assessed for saline- (grey bar, n=9) and ATP-treated (black, n=9) eyes at 1 and 6 days after intravitreal injection. (F) Spatial frequency threshold in saline-treated and ATP-treated eyes was assessed under photopic conditions, using the optokinetic response at 1, 3 and 6 days after injection. Significance of p<0.05 between saline and ATP treated eyes indicated by *.

129x63mm (300 x 300 DPI)
Figure 2. The time course of the effects of 50mM ATP on the gross structure of the retina at one week, three months and six months. Gross retinal structure was assessed in saline-treated and ATP-treated regions of retina at one week (A, saline; B-C, ATP; paraffin), three months (D, saline; E-F, ATP; resin) and six months (G, saline; H-I, ATP; resin). At each time point, two example regions of ATP-treated retinas are presented. Arrowheads in H indicate aberrant pigmented (RPE) cells within the neural retina. Scale bar, 20 µm. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Figure 3. Quantification of the effects of 50mM ATP on the gross structure of the retina at one week, three months and six months. (A) The average number of rows of photoreceptor nuclei were quantified for saline- (grey bars) and ATP-treated (black bars) eyes at one week, three months and six months. (B-C) To determine if there was an effect across eccentricity, rows of photoreceptor nuclei were also quantified at 10 regions across the retina from the optic nerve (ON) to the peripheral ciliary body at (B) three and (C) six months. (D) The average number of rows of inner nuclear layer cells were quantified in saline (grey bars) and ATP treated (black bars) eyes at one week, three and six months. (E) The average number of cells across the ganglion cell layer were also quantified. Significance of p<0.05 between saline (n=6) and ATP treated eyes (n=6) indicated by *. 
129x63mm (300 x 300 DPI)
Figure 4. Cone and rod photoreceptors degenerate following ATP treatment. Sections of retinae from saline- and ATP-treated eyes were labelled for cone photoreceptors (PNA, green), photoreceptor and bipolar cell terminals (VGLUT1, red) and cell nuclei (DAPI, blue) at three months (A, saline; B-C, ATP) and six months post-injection (D, saline; E-F, ATP). At each time point, two example regions of ATP-treated retinae are presented. Arrowhead in (B) indicates an abnormal, clump of VGLUT1-positive photoreceptor terminals displaced in the ONL. Asterisks (*) in (E) indicate RPE cell migration and/or vacuole structures in the retina. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

297x196mm (300 x 300 DPI)
Figure 5. Rod bipolar cell morphology following ATP-induced photoreceptor degeneration and in P23H rat retinae. Sections of retinae from saline- and ATP-treated rat eyes, and a two year old P23H rat retina for comparison, were labelled for rod bipolar cells (Protein kinase C α, green), photoreceptor and bipolar cell terminals (VGLUT1, red) and cell nuclei (DAPI, blue). (A - C) ATP-treated rat retinae at three months (A, saline; B - C, ATP). (D - F) ATP-treated rat retinae at six months (D, saline; E - F, ATP). At each time point for ATP-treated retinae, two example regions are presented. (G) Light microscope image of ATP-treated rat retina at six months. (H) Two year old P23H rat retina. Arrowheads in (F & H) indicate extreme remodelling event of VGLUT1-/PKCα-positive synapses displaced into the choroid in 6 month ATP retina (F) and two year old P23H rat retina (H). Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Figure 6. Horizontal cell morphology following ATP-induced photoreceptor degeneration. Sections of retinae from saline- and ATP-treated eyes were labelled for horizontal cells (Calbindin D28k, green), photoreceptor and bipolar cell terminals (VGLUT1, red) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinae are presented. Arrowheads in (F) indicate extreme remodelling event of horizontal cell process associating with VGLUT1–positive synapses displaced into the choroid. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

297x198mm (300 x 300 DPI)
Figure 7. Amacrine and ganglion cell morphology following ATP-induced photoreceptor degeneration. Sections of retinae from saline- and ATP-treated eyes were labelled for a subset of amacrine and ganglion cells (Calretinin, green) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinas are presented. Arrowheads in (F) indicate extreme remodelling event of amacrine cell processes displaced into the choroid. Scale bar, 20 μm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Figure 8. Blood vessel morphology following ATP-induced photoreceptor degeneration. Sections of retinae from saline- and ATP-treated eyes were labelled for blood vessels (Isolectin B4, green) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinae are presented. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Figure 9. Glial cell morphology following ATP-induced photoreceptor degeneration. Sections of retinas from saline- and ATP-treated eyes were labelled for Müller glial cells (GS, green), astrocytes and Müller cell gliosis (GFAP, red) and cell nuclei (DAPI, blue) at three months (A, saline; B - C, ATP) and six months post-injection (D, saline; E - F, ATP). At each time point, two example regions of ATP-treated retinas are presented. Asterisk (*) in (E) indicates holes representing fluid filled vacuoles and/or RPE cell migration into the retina. Arrowheads in (F) indicates extreme remodelling event of GFAP/GS positive, Müller cell processes displaced into the choroid. Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
Figure 10. Müller cells express the cell cycling marker, cyclin D1, in P23H rat retinas and ATP-induced photoreceptor degeneration. Sections of retinas from two year old P23H rat model of retinitis pigmentosa (A), saline-treated rat eyes at six months (B), ATP-treated rat eyes at three months (C, tile scan; D - F magnification of three areas from C), and ATP-treated rat eyes at six months post-injection (G, tile scan; H - J magnification of three areas from G), were labelled for Müller glial cells (GS, green), cell cycling (cyclin D1, red) and cell nuclei (DAPI, blue). Arrow in (G) indicates RPE cyclin D1 expression. Arrow in (I) indicates extreme remodelling event of cyclinD1 positive Müller cells displaced into the choroid. Scale bar in all, 100 µm. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

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Figure 11. Müller cells express the cell cycling marker, Ki67, following ATP-induced photoreceptor degeneration. Sections of retinae from saline- and ATP-treated eyes were labelled for Müller glial cells (GS, green), cell cycling (Ki67, red) and cell nuclei (DAPI, blue) at three months (A, saline; B-C, ATP) and 6 months post-injection (D, saline; E-F, ATP). Scale bar, 20 µm. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

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This study characterised the chronic effects of ATP on retinal integrity. ATP caused loss of visual function within one day, loss of fifty percent of the photoreceptors within one week and total photoreceptor loss by six months. This inducible model provides a valuable tool for investigating the retinal degenerative process.
Graphical abstract: ATP induces retinal degeneration and remodelling of neurons.