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Steroid induced neuroprotection of damaged photoreceptor cells



A thesis submitted to the National University of Ireland, Cork,

in fulfilment of the requirements for the degree of

Doctor of Philosophy

by

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i. Abstract

Retinitis Pigmentosa (RP) is the name given to a group of hereditary diseases causing progressive and degenerative blindness. RP affects over 1 in 4000 individuals, making it the most prevalent inherited retinal disease worldwide, yet currently there is no cure. In 2011, our group released a paper detailing the protective effects of the synthetic progestin 'Norgestrel'. A common component of the female oral contraceptive pill, Norgestrel was shown to protect against retinal cell death in two distinct mouse models of retinal degeneration: in the Balb/c light damage model and the Pde6b^{rd10} (rd10) model. Little was known of the molecular workings of this compound however and thus this study aimed to elucidate the protective manner in which Norgestrel worked.

To this aim, the 661W cone photoreceptor-like cell line and *ex vivo* retinal explanting was utilised. We found that Norgestrel induces a increase in neuroprotective basic fibroblast growth factor (bFGF) with subsequent downstream actions on the inhibition of glycogen synthase kinase 3β. Progesterone receptor expression was subsequently characterised in the C57 and rd10 retinas and in the 661W cell line. Norgestrel caused nuclear trafficking of progesterone receptor membrane complex one (PGRMC1) in 661W cells and thus Norgestrel was hypothesised to work primarily through the actions of PGRMC1. This trafficking was shown to be responsible for the critical upregulation of bFGF and PGRMC1-Norgestrel binding was proven to cause a neuroprotective bFGF-mediated increase in intracellular calcium.

The protective properties of Norgestrel were further studied in the rd10 mouse model of retinitis pigmentosa. Using non-invasive diet supplementation (80mg/kg), we showed that Norgestrel gave significant retinal protection out to postnatal day 40 (P40). Overactive microglia have previously been shown to potentiate photoreceptor cell loss in the degenerating rd10 retina and thus we focussed on Norgestrel-mediated changes in photoreceptor-microglial crosstalk. Norgestrel acted to dampen pro-inflammatory microglial cell reactivity, decreasing chemokine (MCP1, MCP3, MIP-1α, MIP-1β) and subsequent damaging cytokine (TNFα, Il-1β) production. Critically, Norgestrel up-regulated photoreceptor-microglial, fractalkine-CX3CR1 signalling 1000-fold in the P20 rd10 mouse. Known to prevent microglial activation, we hypothesise that Norgestrel acts as a vital anti-inflammatory in the diseased retina, driving fractalkine-CX3CR1 signalling to delay retinal degeneration.

This study stands to highlight some of the neuroprotective mechanisms utilised by Norgestrel in the prevention of photoreceptor cell death. We identify for the first time, not only a pro-survival pathway activated directly in photoreceptor cells, but also a Norgestrel-driven mediation of an otherwise damaging microglial cell response. All taken, these results form the beginning of a case to bring Norgestrel to clinical trials, as a potential therapeutic for the treatment of RP.

ii. Author's Declaration

This thesis has not been submitted in whole or part to University College Cork or any other university for any degree. This thesis is, unless stated, the original work of the author.

Signed: _____

Alice Wyse Jackson

iii. Acknowledgements

Knowing that the acknowledgements section is the only section that anyone (outside of my examiners!) will ever look at, I find myself panicking slightly that I can't thank everyone and that I'm going to miss someone out (I won't go down the thanking my Grandmother's hairdresser's son route). If I do, please forgive me and know that each and every person that I have had the pleasure of meeting and interacting with throughout these past three years, has been invaluable in helping me keep my sanity. So thank you.

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vi. Abbreviations

661W: Cone photoreceptor-like cell line

βE2: 17β-estradiol

AC: Adenylate cyclase

AKT: Protein kinase B

AMD: Age-related macular degeneration

ANOVA: Analysis of variance

AP-1: Activator protein-1

ATP: Adenosine triphosphate

BAX: BCL2-associated X protein

BCL-X_L: B-cell lymphoma-extra large

bFGF: Basic fibroblast growth factor (FGF2)

BSU: Biological services unit

C57: C57BL/6 Mice

$[Ca^{2+}]_i$: Intracellular calcium concentration

cAMP: 3',5'-cyclic adenosine monophosphate

CD206: Mannose receptor (MRC1)

CD68: Cluster of differentiation 68

CHX10: CEH10 homeodomain-containing homolog

CM: Conditioned media

CNS: Central nervous system

CREB: cAMP response element-binding protein

CX3CL1: Fractalkine/Chemokine ligand 1

CX3CR1: CX3C chemokine receptor 1

DAMP: Damage-associated molecular pattern

DEX: Dexamethasone

DME: Diabetic macular edema

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ERα: Oestrogen receptor α

ERβ: Oestrogen receptor β

ERG: Electroretinogram

ERK: Extracellular signal-related kinase

FA: Fluocinolone acetonide

FBS: Foetal bovine serum

FDA: Food and Drug Administration (Federal Agency)

FGF2: Basic fibroblast growth factor (bFGF)

GCL: Ganglion cell layer

GFAP: Glial fibrillary acidic protein

GPCR: G-protein coupled receptors

GPR30: G protein-coupled receptor 30 (G protein-coupled estrogen receptor 1; GPER)

GR: Glucocorticoid receptor

GRα: Glucocorticoid receptor α

GS: Glutamate synthase

GSK3β: Glycogen synthase kinase 3β

H2AX: H2A histone family, member X

HMGB1: High mobility group box 1 protein

HMW: High molecular weight

Iba1: Ionized calcium-binding adaptor molecule 1

IL-1α: Interleukin-1α

IL-1β: Interleukin-1β

IL-6: Interleukin-6

INL: Inner nuclear layer

iNOS: Inducible nitric oxide synthase

IOP: Intraocular pressure

IP: Intraperitoneal

IPL: Inner plexiform layer

IRβ: Insulin receptor β

IS: Inner segment of photoreceptors

LC3B: Light chain 3B

LMW: Low molecular weight

M1: Pro-inflammatory microglial activation state

M2: Anti-inflammatory microglial activation state

MAPK: Mitogen activated protein kinase

MCAO: Middle cerebral artery occlusion

MCP1: Monocyte chemotactic protein-1 (Chemokine (C-C Motif) Ligand 2; CCL2)

MCP3: Monocyte chemotactic protein-3 (Chemokine (C-C Motif) Ligand 7; CCL7)

ME: Macular Edema

MIP-1α: Macrophage inflammatory protein-1α (Chemokine (C-C Motif) Ligand 3; CCL3)

MIP-1β: Macrophage inflammatory protein-1β (Chemokine (C-C Motif) Ligand 4; CCL4)

mPRα: Membrane progesterone receptor α (PAQR7)

mPRβ: Membrane progesterone receptor β (PAQR8)

mPRγ: Membrane progesterone receptor γ (PAQR5)

MRC1: Mannose receptor (CD206)

mRNA: Messenger ribonucleic acid

MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

N=: Number of biological replicates

n=: Number of technical replicates per N number

NF-κB: Nuclear factor-κB

NFM: Neurofilament M

NORG: Norgestrel

OCT: Optical coherence tomography

ONL: Outer nuclear layer

OS: Outer segment of photoreceptors

p: Phosphorylation

P: Post-natal day

P2Y1: P2Y purinoceptor 1

PAQR: Progestin and AdipoQ

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate-buffered saline

***Pde6b*:** Phosphodiesterase-6b

PEDF: Pigment epithelium derived factor

Peripherin 2: Peripherin/RDS (retinal degeneration slow)

PGRMC1: Progesterone receptor membrane component 1

PGRMC2: Progesterone receptor membrane component 2

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PMH: Postmenopausal hormone

PNA:	Peanut agglutinin
PR A/B:	Progesterone receptor A/B
PS:	Pregnenolone sulphate
rAION:	Rodent anterior ischemic optic neuropathy
RCS:	Royal College of Surgeons rat model
Rd:	Retinal degeneration
Rd10:	rd10/rd10 Mice (B6.CXBI-Pde6b^{rd10}/J)
RDS:	Retinal degeneration slow
RGC:	Retinal ganglion cell
Rhod:	Rhodopsin
ROM1:	Rod outer segment protein 1
ROS:	Reactive oxygen species
RP:	Retinitis pigmentosa
RPE:	Retinal pigment epithelium
RT-qPCR:	Real time quantitative polymerase chain reaction
SC:	Subcutaneous
SDS-PAGE:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFI:	Science foundation Ireland
siRNA:	Small interfering ribonucleic acid
TA:	Trimacinolone acetonide
TBI:	Traumatic brain injury
TNFα:	Tumour necrosis factor α
TUNEL:	Terminal dUTP nick-end labelling
TUNEL+:	Terminal dUTP nick-end labelled positive
VEGF:	Vascular endothelial growth factor

vii. Publications and Presentations

Publications

Wyse-Jackson, A.C. & Cotter, T.G. (2016) The synthetic progesterone "Norgestrel" is neuroprotective in stressed photoreceptor-like cells and retinal explants, mediating its effects via basic fibroblast growth factor, protein kinase A and glycogen synthase kinase 3β signalling. *Eur. J. Neurosci.*, **43**, 899–911.

Wyse-Jackson, A.C., Roche, S.L., Byrne, A.M., Ruiz-Lopez, A.M., & Cotter, T.G. (2016) Progesterone Receptor Signalling in Retinal Photoreceptor Neuroprotection. *J. Neurochem.*, **136**, 63–77.

Wyse-Jackson, A.C., Roche, S.L., Moloney, J.N., Ruiz-Lopez, A.M., Byrne, A.M., & Cotter, T.G. Progesterone analogue protects stressed photoreceptors via bFGF-mediated calcium influx. *Eur. J. Neurosci.*, in press.

Wyse-Jackson, A.C.*, Roche, S.L.*, Gomez-Vicente, V., Lax, P., Ruiz-Lopez, A.M., Byrne, A.M., Cuenca, N. & Cotter, T.G. Progesterone attenuates microglial-driven retinal degeneration and stimulates protective fractalkine-CX3CR1 signaling. *PLoS One,* in press.

Roche, S.L., **Wyse-Jackson, A.C.**, Byrne, A.M., Ruiz-Lopez, A.M., & Cotter, T.G. (2016) Alterations to retinal architecture prior to photoreceptor loss in a mouse model of retinitis pigmentosa. *Int. J. Dev. Biol.*, **60**, 127–139.

Byrne, A.M., Roche, S.L., Ruiz-Lopez, A.M., **Wyse-Jackson, A.C.**, & Cotter, T.G. (2016) The synthetic progestin norgestrel acts to increase LIF levels in the rd10 mouse model of retinitis pigmentosa. *Mol. Vis.*, **22**, 264–274.

Wyse-Jackson A.C. A Closer look at Science Series: How a contraceptive pill could help the blind see again. *Cork's Evening Echo,* September 16 2015

Publications in preparation

Wyse-Jackson, A.C., Groeger, G., & Cotter, T.G. (2016) Beyond anti-inflammation: steroid induced neuroprotection in the retina.

Presentations

May 2014: Doctoral Showcase, UCC, Cork, Ireland. *Oral Presentation.* ***Finalist.***

Nov 2014: Retina Conference, Dublin, Ireland. *Poster presentation.* ***2nd place prize.***

March 2015: Science for all, UCC, Cork, Ireland. *Oral Presentation.* ***Finalist.***

Oct 2015: European Retina Meeting, Brighton, England. *Oral Presentation.*

Nov 2015: Retina Conference, Dublin, Ireland. *Poster Presentation.*

May 2016: ARVO Conference, Seattle, WA, USA. *Poster Presentation.*

June 2016: International Cell Death Society Conference, Cork, Ireland. *Oral and Poster Presentation.* ***1st place prize, Oral Presentation.***

Chapter 1. Introduction: Steroid induced neuroprotection of the diseased and damaged retina

1. 1. Retinal degenerations

Many eye diseases are classified as retinal degenerations. This blanket term refers to a number of retinal dystrophies including retinitis pigmentosa (RP), age-related macular degeneration (AMD) and diabetic macular edema (DME), all of which result in a progressive loss of photoreceptor cells. Retinal degeneration can be triggered by a number of different genetic aetiologies (Daiger *et al.*, 2013), though common disease features include night-blindness or nyctalopia, visual field loss and subsequent deterioration of visual acuity – shown in the case of retinitis pigmentosa in figure 1.1.1.

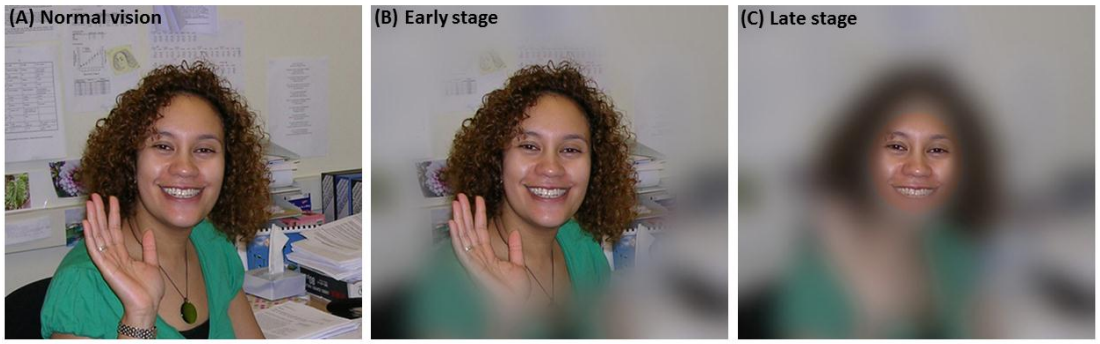


Figure 1.1.1. Images to show the progression of visual field loss in retinitis pigmentosa. (A) *Normal Vision.* ***(B)*** *Early retinitis pigmentosa: mild peripheral visual field loss with good central vision.* ***(C)*** *Late retinitis pigmentosa: marked peripheral visual field loss resulting in "tunnel vision" (Images taken from Carter, 2016).*

1.1.1. Phenotype of retinitis pigmentosa

Retinitis pigmentosa encompasses a set of hereditary diseases causing degenerative blindness. Initial symptoms occur independent of age and thus RP diagnosis may occur anywhere from early infancy to late adulthood (Koenekoop *et al.*, 2003). Caused by several different genomic mutations (McLaughlin *et al.*, 1993), the same mutation can manifest itself very differently across any number of different individuals. In general, however, RP is characterised by the early loss of rod photoreceptors followed by a slow, progressive

deterioration of the cone photoreceptor cells. Pigmentary changes typically arise following photoreceptor degeneration in RP and are a result of the release of pigment by cells of the adjacent retinal pigment epithelium (RPE). These pigmented deposits are opthalmoscopically visible in advanced stages of the disease and are referred to as 'bone-spicule formations' due to their branching patterns (Figure 1.1.2).

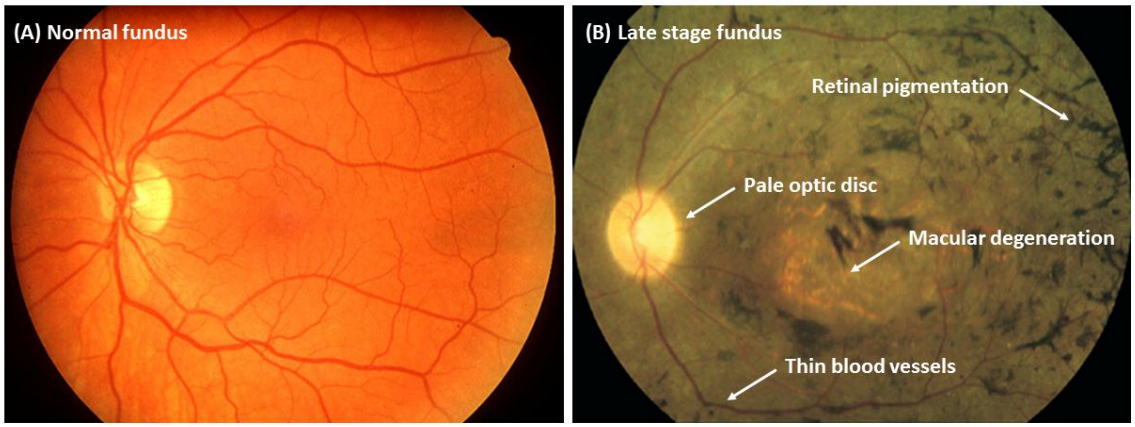


Figure 1.1.2. Images to show the fundus of a patient with retinitis pigmentosa vs. a healthy individual. (A) *Normal retina: No retinal pigmentation, a bright optic disc and plump retinal blood vessels are visible. No evidence of macular degeneration.* ***(B)*** *Late retinitis pigmentosa: widespread marked retinal pigmentation, a pale optic disc and thin retinal blood vessels can be seen. Macular thinning/degeneration occurs in the late stages of RP and results in reduced central vision.*

1.1.2. Genetic basis of retinitis pigmentosa

Mutations in a large variety of genes have been linked to autosomal-dominant (adRP), autosomal-recessive (arRP), X-linked RP (xlRP) (Table 1.1.1) and digenically inherited RP. AdRP and arRP comprise approximately 20% of RP cases whilst xlRP is responsible for approximately 10% (Hartong *et al.*, 2006). Although rare, digenic inheritance of the disease also occurs, as shown in the study of three familial cases of RP (Kajiwara *et al.*, 1994). In this study, mutations were discovered in the unlinked photoreceptor-specific genes rod outer segment protein 1 (ROM1) and peripherin/RDS (retinal degeneration slow; peripherin 2). Interestingly, in these families only double heterozygotes developed RP and thus the first report of digenic inheritance in a human disease was discovered (Kajiwara *et al.*, 1994).

Whilst many cases of RP are inherited, isolated instances also occur in about 40% of patients, mostly due to spontaneous mutation.

Form of retinitis pigmentosa	Number of identified genes	Mapped and identified genes
Autosomal-dominant (adRP)	27	ARL3, BEST1, CA4, CRX, FSCN2, GUCA1B, HK1, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF4, PRPF6, PRPF8, PRPF31, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRNP200, SPP2, TOPORS
Autosomal-recessive (arRP)	57	ABCA4, AGBL5, ARL6, ARL2BP, BBS1, BBS2, BEST1, C2orf71, C8orf37, CERKL, CLRN1, CNGA1, CNGB1, CRB1, CYP4V2, DHDDS, DHX38, EMC1, EYS, FAM161A, GPR125, HGSNAT, IDH3B, IFT140, IFT172, IMPG2, KIAA1549, KIZ, LRAT, MAK, MERTK, MVK, NEK2, NEUROD1, NR2E3, NRL, PDE6A, PDE6B, PDE6G, POMGNT1, PRCD, PROM1, RBP3, RGR, RHO, RLBP1, RP1, RP1L1, RPE65, SAG, SLC7A14, SPATA7, TTC8, TULP1, USH2A, ZNF408, ZNF513
X-linked (xlRP)	3	OFD1, RP2, RPGR

Table 1.1.1. List of all genes associated with inherited forms of autosomal-dominant (adRP), autosomal-recessive (arRP) and X-linked retinitis pigmentosa (xlRP) ("RetNet: Summaries," 2016)

1.1.3. Retinitis pigmentosa-induced changes in visual processing

There are two types of photoreceptor: rods and cones. These cells are responsible for converting light energy in to information to be processed by the visual system. Rod cells contain long cylindrical outer segments, have many disks and have a high concentration of pigment, all features enabling rods to be extremely light sensitive. In low light, visual processing occurs solely due to rod signalling, for these cells can be triggered by a single photon. Rods cannot mediate colour vision and thus colours cannot be seen at low light levels: only one type of photoreceptor cell is active. This is also why nyctalopia is one of the initial symptoms of RP. Rods are lost first in RP and so few rods are left to process visual signals, leading to night-blindness. Alternately, cones require significantly brighter light (i.e. a larger numbers of photons) in order to produce a signal and are primarily responsible for spatial acuity and for colour vision. Central vision is lost as cones of the central retina degenerate, thus central vision is lost secondarily to peripheral vision (Wong, 1993). The human retina contains about 120 million rod cells and 6 million cone cells - the cellular structure of the retina being illustrated in figure 1.1.3.

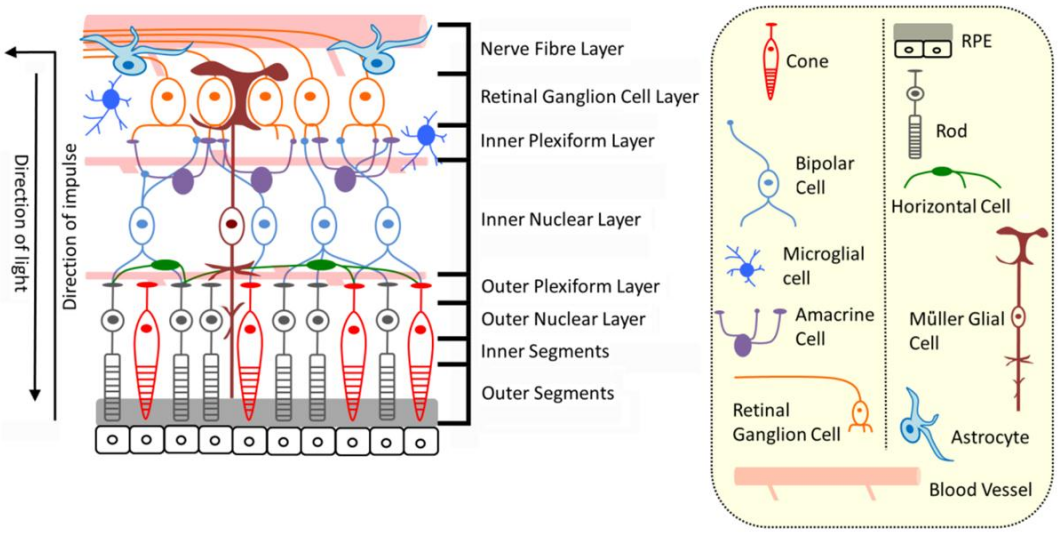


Figure 1.1.3. Simplified schematic of the retina. *Illustrates the variety of cell types found in the retina along with their connections. (Taken from Roche et al., 2016)*

Visual processing occurs *via* a tightly controlled photo-transduction cascade. Packets of light energy in the form of photons are absorbed and processed by rhodopsin molecules in the membrane discs of rod outer segments (Figure 1.1.4). Once activated, rhodopsin makes repeated contact with the G-protein transducin to further activate a cGMP phosphodiesterase (cGMP PDE). The resultant hydrolysis of cGMP - the second messenger in the photo-transduction cascade, closes cGMP-gated channels leading to hyper-polarisation of the cell and subsequent signalling. Rhodopsin kinase will phosphorylate rhodopsin, inducing a termination of the photo-transduction cascade (Leskov *et al.*, 2000).

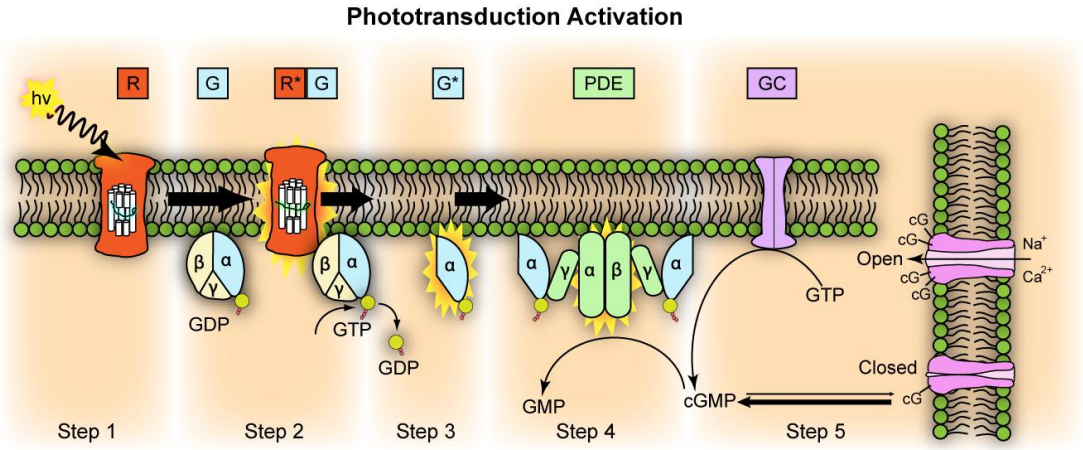


Figure 1.1.4. Representation of molecular steps in photoactivation *(taken from Corneveaux, 2016. Modified from Leskov et al., 2000). Shown is the outer membrane disk found in a rod cell. Step 1: Incident photon (hv) is absorbed and activates rhodopsin via a conformational change to R*. Step 2: R* contacts with G-protein transducin molecules. This contact activates the molecule to G*, catalyzing its activation to by the release of bound GDP in exchange for cytoplasmic GTP. Step 3: This release expels the G-protein β and γ subunits leaving the α subunit. Step 4: G* α subunit binds inhibitory γ subunits of phosphodiesterase (PDE) activating its α and β subunits. Activated PDE hydrolyzes cGMP. Step 5: Guanylyl cyclase (GC) synthesizes cGMP. Reduced levels of cytosolic cGMP cause cyclic nucleotide gated channels to close preventing further influx of Na^+ and Ca^{2+}.*

Rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit beta is the beta subunit of the protein complex PDE6 that is encoded by the *Pde6b* gene. This gene is included in the array of genes that cause RP (Table 1.1.1), mutations of which account for 4-5% of autosomal-recessive cases. cGMP is an essential component in the rod signal transduction pathway (Cote, 2004; Kennan *et al.*, 2005) and as such, PDE6 is crucial in transmission and amplification of visual signal. Specifically, the beta subunit is essential for normal

functioning of this protein and mutations in this subunit have been shown to cause retinal degeneration.

1.1.4. Genetic mouse models of retinitis pigmentosa

Clearly, our understanding of the genetic abnormalities leading to RP is increasing. However, the mechanism by which the many mutations described lead to cell death has yet to be elucidated. Unfortunately, the direct study of human RP is hindered by the difficulty in obtaining relevant human tissue samples. Consequently, a number of animal models now exist harbouring the same mutations found in humans, thereby representing the human disease both genetically and phenotypically.

Perhaps the best characterized mouse model of retinal degeneration is the naturally occurring rd1 (retinal degeneration) mouse. These mice carry a defect in the beta subunit of cGMP PDE, resulting in photoreceptor degeneration. Long recognised, the rd1 mouse has proved to be an essential tool in furthering our understanding of the disease (Pittler & Baehr, 1991). Unfortunately, this model experiences a rapid loss of photoreceptors, overlapping with postnatal development of the retina. As such, this has resulted in limitations to its use (Sanyal & Bal, 1973; Carter-Dawson *et al.*, 1978). The development of potential treatments is difficult using this mouse model; there is only a narrow time frame before onset of disease, which coincides with retinal development.

Alternately, the rd10 mouse model of RP, which possesses a mutation in *pde6b* (Chang *et al.*, 2002, 2007; Han *et al.*, 2013), has proven to be a suitable model for studying the course

of degeneration and developing treatments. This is because unlike in the rd1 mouse, photoreceptors are lost in the rd10 at a rate that closely resembles human disease progression. A loss of rods is observed from postnatal day (P)18 in the rd10 and peaks ~P21 (Gargini *et al.*, 2007; Barhoum *et al.*, 2008; Samardzija *et al.*, 2012; Roche *et al.*, 2016). This is accompanied by morphological changes in rod and cone outer segments as well as a loss of photoreceptor axon terminals at P20 (Barhoum *et al.*, 2008; Roche *et al.*, 2016). Therefore, the nuclear and plexiform layers in the retina have established their mature state (Caley *et al.*, 1972) before the onset of photoreceptor loss. Degeneration of photoreceptors follows a clear gradient from the central to the peripheral regions of the retina, with a delay of 2-3 days observed between these regions (Gargini *et al.*, 2007; Roche *et al.*, 2016). (Barhoum *et al.*, 2008; Roche *et al.*, 2016). Degeneration also occurs more rapidly in the central retina in comparison to the periphery (Barhoum *et al.*, 2008). Similar to human RP, rods are lost first with cones persisting into adulthood (Gargini *et al.*, 2007; Barhoum *et al.*, 2008; Roche *et al.*, 2016).

In the rd10 retina, cells other than photoreceptors are also affected at time-points coinciding with photoreceptor loss. A shortening of horizontal cell processes, reduced bipolar cell connectivity with both the outer nuclear layer (ONL) and retinal ganglion cell (RGC) layer, as well as a reduction in the size of amacrine cell appendages were observed in the central retina at P20 (Barhoum *et al.*, 2008; Puthussery *et al.*, 2009; Roche *et al.*, 2016). Therefore, although the primary insult is present in rods, other retinal cell types are also affected by the mutation during the course of photoreceptor degeneration.

1.2. Steroid-induced neuroprotection

Although recent advances in molecular tools have facilitated the successful implementation of gene therapy for some forms of retinal degenerations (Cashman *et al.*, 2015; Guo *et al.*, 2015; Wu *et al.*, 2015; Zhang *et al.*, 2015); retinal diseases can be highly heterogeneous. For example, mutations in more than 50 genes are known to cause non-syndromic retinitis pigmentosa and nearly 3100 mutations have been reported in these genes (Daiger *et al.*, 2013). Therefore, a neuroprotective, mutation-independent treatment strategy is highly desirable in the fight against degenerative blindness. As such, the concept of 'neuroprotection' is one therapy currently under investigation for the treatment of retinal disease (Doonan & Cotter, 2012; Trifunović *et al.*, 2012). Quite simply, neuroprotective strategies in the retina aim to conserve the photoreceptor layer, leading to preservation of photoreceptor cell function. These strategies involve the administration of differing molecules ranging from growth factors (O'Driscoll *et al.*, 2008), to antioxidants (Payne *et al.*, 2014), to steroids (Ishikawa *et al.*, 2014), the focus of this study.

Steroids are a family of organic compounds containing a four cycloalkane ring core (Figure 1.2.1). There are hundreds of different types of steroids present in nature and all have varied function. In the human body however, the majority of steroids are derived from the membrane lipid 'cholesterol', which can form a number of different steroids derivatives through a series of enzymatic modifications. In this review of the current literature, we will focus on two such steroid groups associated with retinal cell neuroprotection: glucocorticoids and sex steroids.

Glucocorticoids are a class of corticosteroid. Binding to the glucocorticoid receptor (Edelman, 2010), the name glucocorticoid comes from the role that they play in glucose metabolism regulation, their synthesis in the adrenal cortex and the steroid structure: **Glu**cose + **cor**tex + ster**oid**. Three glucocorticoids used therapeutically in the retina are dexamethasone (DEX), trimacinolone acetonide (TA) and fluocinolone acetonide (FA) (Figure 1.2.1). Similar to other corticosteroids, these glucocorticoids are renowned for their anti-inflammatory properties and it was this characteristic that first led to their use in the retina several decades ago (Edelman, 2010). More recently, increased clinical use has shown these glucocorticoids to have neuroprotective properties in retinal cells, though far less is known of the molecular mechanism leading to this protection.

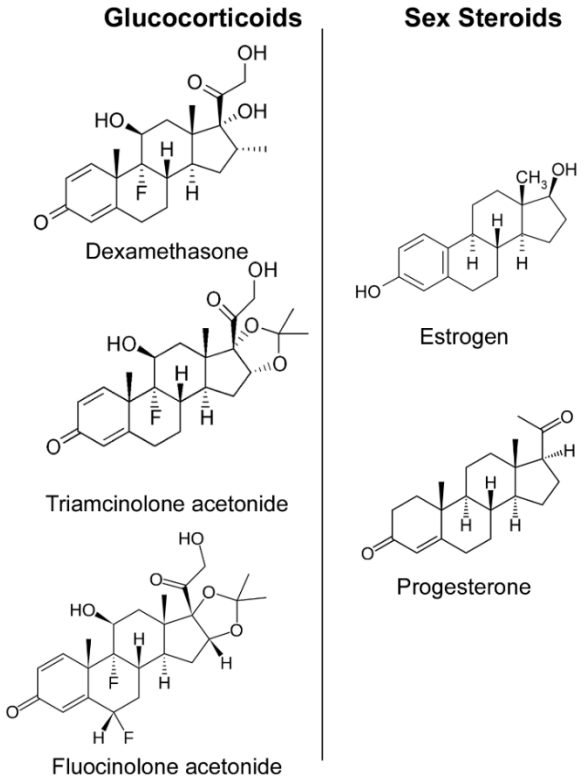


Figure 1.2.1. Chemical structures of steroids with recognised neuroprotective potential in the retina.

Alternately, the sex steroids can be divided into three functional groups: oestrogens, progestogens and androgens. The late 1970's saw the emergence of this area of research

(Toran-Allerand, 1976, 1980; Toran-Allerand *et al.*, 1983) and oestrogen and progestins have since been shown to promote neuronal survival and growth (Garay *et al.*, 2008; Mannella *et al.*, 2009). Whilst androgens were investigated in relation to eye diseases in the 1950's (Gurling, 1955), these studies were not continued and so will not be discussed in this review. Instead, a comparison of the two sex steroid groups: oestrogen and progestogen, will be focussed upon.

The glucocorticoids and sex steroids share a very similar structure (Figure 1.2.1). As such, it is likely that there are observations and subsequent advances to be made from studying the two steroid groups in parallel. Through emphasizing similarities and differences between glucocorticoids: dexamethasone (DEX), trimacinolone acetonide (TA) and fluocinolone acetonide (FA) and sex steroids: oestrogen, progesterone; insights gained from one group will be highlighted with the aim of aiding future studies of the other. Through this, we hope to facilitate the development of the sex steroids as neuroprotective therapies for retinal diseases.

1.3. Glucocorticoids as retinal neuroprotectants

1.3.1. Glucocorticoids: Evidence for protection

In certain retinal degeneration disease models, glucocorticoids have been shown to be neuroprotective to photoreceptors. For example, in the rodent light damage model, dexamethasone (DEX) prevents photoreceptor cell death (Fu *et al.*, 1992; Wenzel *et al.*, 2001, 2003; Hao *et al.*, 2002; She *et al.*, 2008). Trimacinolone acetonide (TA) is protective to

photoreceptors in a rabbit model of subretinal haemorrhage (Bhisitkul *et al.*, 2008) and preserves the inner segment/outer segment photoreceptor interface in patients with diabetic macular edema (DME) (Shin *et al.*, 2012). Glybina *et al.* also demonstrated specific protection of photoreceptors using retinal implants of fluocinolone acetonide (FA) in two inherited retinal degeneration models, the Royal College of Surgeons (RCS) rat model (2009) and the s-334ter-4 rat model (2010). These studies (Table 1.3.1) were completed over the same time period as several different human clinical trials of varying degrees of success and all looked at the potential for glucocorticoids to act as anti-inflammatories in the eye. These studies have been nicely reviewed by Cebeci & Kir (2015).

Clinical trials used various formulations of these steroids, demonstrating protection of vision in humans. DEX is five and twenty times more potent an anti-inflammatory agent than TA and FA respectively (Jermak *et al.*, 2007; Edelman, 2010), however its short half-life and high solubility means that it is not retained in the eye (Kwak & D'Amico, 1992). To combat this, Ozurdex (Allergan, Irvine, CA, USA), a slow-release DEX intravitreal implant, was developed. At 0.46mm in diameter and 6mm in length, this rod-like implant is used to treat patients with uveitis and to protect vision in patients with diabetic macular edema (DME) and macular edema (ME) related to branch or central retinal vein occlusion (Haller *et al.*, 2010, 2011). Approved by the FDA in 2009 and the European Commission in 2010, long term evaluation of patients receiving multiple implants are now available and seem promising (Moisseiev *et al.*, 2013).

TA, due its more lipophilic nature, is better retained post-injection within the eye (Beer *et al.*, 2003). Abraldes *et al.* (2009) reviewed all the clinical trials of TA in the treatment of

DME and found that TA can increase visual acuity in certain cases. For example, 'I-vation' (SurModics, Eden Prairie, MN, USA), a helical sustained-release implant containing 0.925µg, demonstrated the effectiveness of TA in treating DME. Major complications involving increased intraocular pressure (IOP) and cataract development (Dugel *et al.*, 2009) however, meant that phase 2b trials were terminated ("Clinicaltrials.gov. A Study of MK0140 in Diabetic Patients with Macular Edema.," 2016). Finally, similar to Ozurdex and I-vation, there has been a slow-release implant of FA developed, 'Retisert' (Bausch & Lomb, Rochester, NY, USA). This implant had some success in clinical trials for uveitis, however as with TA's 'I-vation', patients showed increased cataract formation and raised IOP which has consequently restricted its use (Schwartz & Flynn, 2011).

In general, advanced research in this area is directed towards the development of slow-release systems (Glybina *et al.*, 2009, 2010; Schwartz & Flynn, 2011). These enable steroids to be retained better within the eye, thereby reducing the number of intravitreal injections required and consequently decreases associated complications. In addition to this, intravitreal implants systems negate the need for a vehicle; i.e. the solution in which the steroid suspended before injection. These vehicles can often cause complications: a case well illustrated by the first studies of TA. When TA was first injected intravitreously, it was suspended in benzyl-alcohol using a product originally designed for intramuscular injection to treat dermatological conditions. These studies not only found the vehicle to be toxic to the retina (Macky *et al.*, 2007), but also found TA itself to be toxic at high doses (4 - 20mg of TA in 0.1mL injection). TA is only safe and neuroprotective in humans at the lower dose of 0.5 - 1mg (Yu *et al.*, 2006). Difficulties associated with administration of individual systems, serve to highlight the need for specific testing of various drug delivery routes – rather than simply trialling the compound itself.

In all of the aforementioned trials, visual acuity was improved through corticosteroid administration. Accordingly, there must have been some degree of photoreceptor protection. The trouble with this deduction however, is that many of these trials were completed in patients suffering from Macular Edema (ME) (Beer *et al.*, 2003; Haller *et al.*, 2010). ME occurs when fluid and protein deposits collect on or under the macula of the eye, resulting in poor communication between the photoreceptors and other retinal neurons. This means that photoreceptors lose out on vital signalling and thus loss of vision occurs thereafter (National Eye Institute, 2015). Since ME is not associated with photoreceptor death in its early stages, it is difficult to determine if glucocorticoids affect photoreceptor cells directly. Glucocorticoids promote a healthy retina through excess fluid reduction. Therefore, the photoreceptors are not as stressed and so are less likely to die, as normally occurs in the later stages of this disease. Both human studies into ME and animal trials into other retinal diseases, agree that glucocorticoids can protect vision. However, the mechanism of action by which this occurs, be it directly or indirectly on the photoreceptors, has not yet been elucidated.

Steroid	Model Used	Dose + Route of Admin.	Degree of Protection; Variable	Ref.
DEX	Mouse light damage	52mg/kg in single IP injection	Complete; ONL Complete; ERG	(Wenzel *et al.*, 2001)
	Mouse light damage	52mg/kg in single IP injection	Qualitative; retinal neuronal layer integrity	(Hao *et al.*, 2002)
	Dog light damage	3 x SC injections (2 @ 0.18mg/kg, 1 @ 1.8mg/kg)	None found in retinal sections	(Gu *et al.*, 2009)
TA	Rabbit subretinal haemorrhage	2mg in single intravitreal injection	Quantitative; ONL	(Bhisitkul *et al.*, 2008)
	Human-Diabetic macular edema	4mg in single intravitreal injection	Quantitative; IS/OS as measured by OCT	(Shin *et al.*, 2012)
FA	Royal College of Surgeons Rat	0.2µg/day equivalent release from intravitreal implant	Quantitative; ONL Quantitative; ERG	(Glybina *et al.*, 2009)
	S334ter-4 rat	0.2µg/day equivalent release from intravitreal implant	Quantitative; ONL Quantitative; ERG	(Glybina *et al.*, 2010)
Oestrogen and derivatives	Rat light damage	2.5mg	Qualitative; retina	(O'Steen, 1977)
	S334ter-3 rats	2µL of 10mM in single intravitreal injection	Quantitative; ONL	(Dykens *et al.*, 2004)
	Rat light damage	500µg/kg in single IP injection	Qualitative; ONL Qualitative; ERG	(Yu *et al.*, 2004)
Progestins	RD10 mouse	100mg/kg IP injection on alternate days	Quantitative; ONL Qualitative; ERG	(Doonan *et al.*, 2011)
	Mouse light damage	100mg/kg IP injection every 3 days	Qualitative; ONL	(Doonan *et al.*, 2011)
	Rat retinal ischemia reperfusion	4mg/kg in 2 x IP injections	Qualitative; INL	(Lu *et al.*, 2008)

Table 1.3.1. Summary of studies which used the steroids under review as therapeutics in models of retinal degeneration. *(ERG: electroretinogram; INL: inner nuclear layer; IP: intraperitoneal; IS/OS: inner segment/outer segment of photoreceptors; OCT: optical coherence tomography; ONL: outer nuclear layer; SC: subcutaneous; Qualitative: study reports images only; Quantitative: study undertook statistics which proved to be significant)*

1.3.2. Glucocorticoids: Mechanism of action

Retinal cells have a bi-modal response to glucocorticoids, due to signalling through differing glucocorticoid receptor (GR) isoforms (Chrousos & Kino, 2005; Oakley & Cidlowski, 2011). Alternative splicing of the GR gene transcript generates two C-terminal glucocorticoid receptor isoforms: GRα and the classic GR. The GR gene also has several initiation sites, which can give rise to isoforms of differing lengths: GRβ, GRγ, GR-A and GR-P (Oakley & Cidlowski, 2011). The classical GR and GRα initiate a response through genomic signalling (Edelman, 2010). This mechanism allows for ligand-receptor complex binding and sequential internalisation to the nucleus. Here, they will bind to glucocorticoid response elements, directly regulating the transcription of various genes. For example, GRα may inhibit key transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Zanchi *et al.*, 2010). As with many steroid hormones however, glucocorticoids may also activate a non-genomic signalling system. This means that a membrane-bound form of GR may directly activate an intracellular signalling cascade, thus transmitting signalling information at a much faster speed than the slower classical GR signalling (Groeneweg *et al.*, 2012). Precisely how these two signalling mechanisms result in increased cell survival is still ill-defined, though what is known will be discussed below.

With regard to the direct action of glucocorticoids on photoreceptors, Wenzel *et al.* attempted to explicate their mechanism through use of the light damaged mouse model of retinal degeneration. This is a method whereby adult, albino Balb/c mice are exposed to a bright a damaging light over a length of time and their photoreceptors degenerate as a result (Doonan *et al.*, 2011). Wenzel *et al.* found that intraperitoneal injection of DEX activated the GR, which in turn reduced the levels of the transcription factor, activator

protein-1 (AP-1) within the retina (Wenzel *et al.*, 2001) (Figure 1.3.1A). Interestingly however, AP-1 has conversely been shown under certain conditions to induce photoreceptor death (Wenzel *et al.*, 2005). Hao *et al.* further investigated this mechanism in mice. They found that DEX only protected against bright light death, where AP-1 was strongly implicated. In dim light induction however, where AP-1 did not play a role, DEX had no effect (2002).

In contrast to these mouse investigations, a study completed in dogs using bright light showed no protection of photoreceptors with dexamethasone, though the concentration of DEX used was far less than in the aforementioned mouse trials (Gu *et al.*, 2009). This dog trial found again that despite a DEX-induced decrease in AP-1 activation, and an inhibited phosphorylation of c-Fos and extracellular signal-regulated kinases 1/2 (ERK1/2) (Figure 1.3.1B); neither intraocular or systemic injection of DEX could protect against light-induced photoreceptor degeneration. Gu *et al.* hypothesised that the discrepancy in DEX efficacy between the mouse and dog studies had to do with the differing role that AP-1 may play in photoreceptor cell death. Certainly since these studies were completed, AP-1 has been

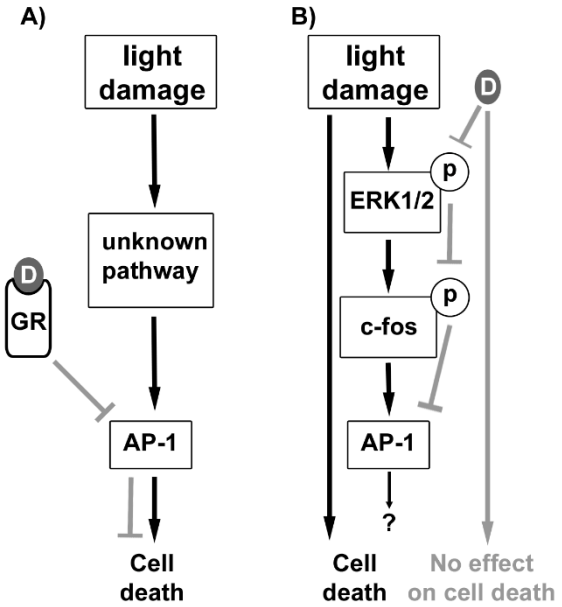


Figure 1.3.1. Known signalling pathways of dexamethasone in the retina. (A) *Dexamethasone (D) activates the glucocorticoid receptor (GR) and inhibits activator protein-1 (AP-1), which prevents cell death (Wenzel et al., 2001). This was determined in a mouse light damage model.* ***(B)*** *Light damage causes cell death in a dog model and this is concurrent with an increase in the phosphorylation (p) of ERK1/2 and c-fos and an increase in AP-1 activity. Whilst dexamethasone inhibits these three events, it has no effect on cell death (Gu et al., 2009).*

recognised to promote cell death or survival, depending on its dimer composition (Meng & Xia, 2011). Therefore, it is too simplistic to say that AP-1 activation will always lead to cell death. Thus DEX-induced reduction of AP-1 may not always be neuroprotective to photoreceptor cells (Figure 1.3.1).

In a recent study, it was shown that DEX administration to light-damaged Balb/c mice significantly increased levels of protective retinal BCL-X_L in photoreceptor inner segments (Cubilla *et al.*, 2013) – as is the case in other tissues whereby DEX has anti-apoptotic actions (Kfir-Erenfeld *et al.*, 2010). In comparison, high levels of cleaved caspase-3 and Bax, both drivers of apoptosis (Peng *et al.*, 2014); and phosphorylated histone H2AX, a marker of DNA breakage and repair (van Attikum & Gasser, 2009) were found in mifepristone GR antagonist-treated controls (Cubilla *et al.*, 2013). This study is one of the first to elucidate the signalling pathways associated with DEX-induced photoreceptor cell survival *in vivo*.

Whilst it is evident that some progress is being made in understanding the actions of DEX in promoting neuronal survival, very little is known about the intracellular signalling mechanisms of TA and FA in neurons. Using microarrays (Smit-McBride *et al.*, 2011) and through analysis of selected genes (Rehak *et al.*, 2011), both have been shown to regulate genes in a characteristic anti-inflammatory manner. However, these studies did not manage to determine the pathway leading to transcription changes. FA can bind to the GR (Ayalasomayajula *et al.*, 2009), but it is not known if this promotes retinal cell survival. What is known however, is that when TA has cytotoxic effects (i.e. at high doses), they are independent of classical GR signalling (Chung *et al.*, 2007), suggesting a toxic non-GR

mechanism. Unfortunately, due to the lack of published studies in this area, it is impossible to conclude how TA and FA might signal to protect photoreceptor cells directly.

As mentioned previously, it is believed that many of the effects that glucocorticoids have on photoreceptors, may occur indirectly. This is through their actions on other retinal cell types, with downstream consequences on the photoreceptors. Glucocorticoids have been widely used to inhibit inflammation, a finding recently validated in the retina (Edelman, 2010). For example, Glybina *et al.* found that a retinal implant releasing the equivalent of 0.2µg/day FA was effective in protecting photoreceptors in the RCS and the transgenic S334ter-4 rat models (2009, 2010). They demonstrated that this protection results from a decrease in activation and recruitment of microglia to damaged photoreceptors – a process now commonly accepted to potentiate photoreceptor cell degeneration (Zhao *et al.*, 2015; Roche *et al.*, 2016). This diminished microglial response leads to reduced production of pro-inflammatory cytokines and ultimately prolongs photoreceptor cell survival (Yang *et al.*, 2007; Zhang *et al.*, 2012; Martínez-Fernández de la Cámara *et al.*, 2014; Peng *et al.*, 2014). TA has also been found to reduce the number of microglial cells within the retina in response to a variety of different injurious stimuli (Uckermann *et al.*, 2005; Singhal *et al.*, 2010; Shen *et al.*, 2014). Taken together, these results suggest that the glucocorticoids are highly successful mediators of damaging microglial cell activation in the retina.

Microglia are not the only glial cell type in the retina. The retina also has glial cells known as 'Müller glial cells' – which again, as with all glial cells, serve as support cells for the neurons of the eye (Roche *et al.*, 2016). Spanning the width of the retina, these cells respond in a corticosteroid-specific manner. For example, TA has no influence on Müller cell gliosis

(Uckermann *et al.*, 2005) – a process occurring after neuronal damage. Reactive gliosis is characterised through Müller glial cell proliferation and changes in specific gene expression. This consequently may lead to glial scarring and inhibition of axonal regeneration (Dyer & Cepko, 2000). Alternately, DEX has been shown not only to supress reactive gliosis by Müller glial cells (Gu *et al.*, 2009), but through the GR, may also alter their mitochondrial metabolism (Psarra *et al.*, 2003). Two studies have also demonstrated that DEX and TA can modify the number of ion and water channels expressed by Müller glial cells, changing their osmotic swelling capacity, thereby aiding the resolution of edema (Smit-McBride *et al.*, 2011; Zhao *et al.*, 2011). The effects of glucocorticoids on retinal cell types other than photoreceptors reflects their use in clinical trials. Through modification of glial cell signalling, the glucocorticoids contribute to an anti-inflammatory-response in the retina, a process which has been proven conclusively to delay photoreceptor cell death (Yang *et al.*, 2007; Zhang *et al.*, 2012; Martínez-Fernández de la Cámara *et al.*, 2014; Peng *et al.*, 2014).

In addition to retinal cells, intravitreally administered steroids may impact other ocular cell types. In a comprehensive microarray study of the human trabecular meshwork, Fan *et al.* found that 1mg/mL of TA up-regulated 36 genes and down-regulated 21 others (Fan *et al.*, 2008). This was dose dependent, as 0.1mg/mL resulted in fewer gene alterations. Whilst gene alterations within trabecular cells may not impact on photoreceptors directly, they contribute to the overall health of the eye, as indicated by the association between their malfunction and glaucoma (Tektas & Lütjen-Drecoll, 2009). This paper by Fan *et al.* is also important for another reason. It directly compared genes altered by TA and dexamethasone in the same cell line. Even though the structural differences between these two glucocorticoid molecules are relatively small (Figure 1.2.1), they differentially regulate

several genes. TA changed the expression of 57 genes in total, 7 of which it shared with DEX; whilst DEX affected a separate 19 genes (Fan *et al.*, 2008).

All taken, the evidence presented in the preceding paragraphs serves to illustrate what little is known about the pro-survival signalling mechanisms employed by glucocorticoids within the retina. Nevertheless, glucocorticoid-induced increases in visual acuity is evident in near on all of the discussed studies and thus highlights the potential of these compounds to provide a successful therapy for certain inflammatory retinal diseases.

1.4. Sex steroids as retinal neuroprotectants

1.4.1. Sex steroids: Evidence for protection

Steroid hormones and their metabolites in the central nervous system have been shown to have neuroprotective properties (Compagnone, 2008; Borowicz *et al.*, 2011). Commonly referred to as 'neurosteroids', the sex steroids oestrogen and progesterone are now well documented to provide protection in several different systems. Progesterone, for instance, has been trialled for a treatment of spinal cord injury, brain ischemia, stroke, Alzheimer's disease and perhaps most prominently, traumatic brain injury (Gonzalez Deniselle *et al.* 2002a, 2002b; Gonzalez *et al.* 2005; Compagnone 2008; Espinosa-García *et al.* 2014; Yousuf *et al.* 2014; Qin *et al.* 2015). Indeed studies highlighting its viability in a number of different models of TBI have ensured progesterone's entry into human clinical trials for TBI (Stein 2011; Stein 2013; Stein *et al.* 2015). Alternately, oestrogen has also been trialled for protection against spinal cord injuries, cognitive decline following cerebral ischemia and

several neurodegenerative disorders (Chakrabarti *et al.*, 2014; Liu, Mu, *et al.*, 2015; Stanojlović *et al.*, 2015). These studies seem promising, making it likely that oestrogen will soon be assessed clinically.

Clearly, both progesterone and oestrogen are emerging as potential therapeutics in the treatment of neurodegenerative disorders. As such, research in to the protective properties of these compounds has moved to the retina. Oestrogen and its derivatives in particular have shown promising results in a number of systems and models of retinal disease. These include the s334ter-3 transgenic model of retinitis pigmentosa (Dykens *et al.*, 2004), light damaged rats (Yu *et al.*, 2004), 661W cone photoreceptor cells (Nixon & Simpkins, 2012), human Müller glial cells (Li *et al.*, 2006) and rat retinal neuronal primary cultures (Yu *et al.*, 2004; Li *et al.*, 2013) (Table 1.3.1). Interestingly, despite the success of progesterone treatment in disorders of the CNS, conflicting data exists as to the protective potential of progesterone in the eye. As such retinal studies have been slower to start. 'Norgestrel', a synthetic progesterone analogue has been shown to exert neuroprotective effects both *in vitro* and *ex vivo* (Byrne *et al.*, 2016); and in two distinct models of retinitis pigmentosa: the rd10 mouse model, in a preventative situation; and the light damage model, in a rescue situation (Doonan *et al.*, 2011; Doonan & Cotter, 2012). Progesterone was also shown to prevent against photoreceptor cell death in the rd1 mouse (Sánchez-Vallejo *et al.*, 2015) and to inhibit osmotic swelling following ocular ischemia (Neumann *et al.*, 2010; Allen *et al.*, 2015).

Whilst there have been no human clinical trials wherein oestrogen or progesterone has been specifically administered to preserve vision, some trials have been designed to take

into consideration hormone replacement therapy. This is where women are supplemented with exogenous hormones. In 2008 for example, a large scale study was carried out in 74,996 postmenopausal women. This study looked at the incidence of age-related macular degeneration (AMD) in those taking postmenopausal hormone (PMH) consisting of a combined treatment of progesterone and oestrogen; and those who had previously taken oral contraceptives. Most interestingly, PMH users had a notable 48% lower risk of neovascular AMD compared with those were not using PMH. Moreover, the risk of developing neovascular AMD was lowest amongst PMH users who had used oral contraceptives in the past (Feskanich *et al.*, 2008).

In 2001, a study of 58 women looked at the effect of the PMH 'Tibolone', a steroid drug with progesterone, oestrogen and weak androgenic actions. This research showed that the functional status of neurovisual transmissions was significantly improved after a three-month daily dose of 2.5mg Tibolone (Laçin *et al.*, 2001). Interestingly however, no difference in effects on visual acuity, IOP, tear functions and visual evoked potential were seen in a more prolonged experiment totalling six months, just a few years on (Verit *et al.*, 2006). Most recently, the effects of PHM were investigated with respect to blood flow and protection of the optic nerve. Researchers found that PMH-users (of both oestrogen-only and oestrogen plus progesterone PMH users) had a significantly increased retinal perfusion and greater preservation of the optic nerve fibre layer compared to control (Deschênes *et al.*, 2010). These studies suggest a neuroprotective role of PMH in the retina. Taken together with a number of neuronal based studies showing that PMH users have a decreased risk of developing neurodegenerative disease, e.g. Alzheimer's (Pintzka & Håberg, 2015), Parkinson's (Bourque *et al.*, 2009) and stroke (Hale & Shufelt, 2015), these

data lead us to conclude that the area of PMH supplementation for retinal protection post-menopause, deserves further attention.

1.4.1.1. Oestrogen specific neuroprotection

With regard to oestrogen-specific retinal neuroprotection, one of the earliest studies was carried out using the phytoestrogen 'Genistein'. This naturally occurring, oestrogen-like compound, was found to prevent neuronal degeneration in the inner retina of rats; as a result of ischemic injury (Hayashi *et al.*, 1997). The most potent endogenous oestrogen, 17β-estradiol, was soon after found to prevent excitotoxin, pregnenolone sulphate (PS)-induced retinal cell death in male Wistar rats, through prevention of a PS-induced reduction in pro-forms of caspase-3 and caspase-2 (Cascio *et al.*, 2002). 17β-estradiol was subsequently and sequentially found to protect against hydrogen peroxide-induced cell death in primary cell cultured retinal neurons (Yu *et al.*, 2004) and in ARPE-19 human retinal pigment epithelial cells (Yu *et al.*, 2005; Giddabasappa *et al.*, 2010); against retinal ganglion cell loss in the DBA/2J mouse model of inherited glaucoma (Zhou *et al.*, 2007); and against glutamate-induced neurotoxicity (5mM) in 661W cells, a mouse cone photoreceptor-like cell line.

In 2004, Yu *et al.* tested both 17α-estradiol and 17β-estradiol *in vivo*, using light-damaged, ovariectomized (an animal model for menopause), female Sprague-Dawley albino rats. Systemic administration of 17β-estradiol was found to significantly protect the structure and function of light-damaged rat retinas, inhibiting photoreceptor apoptosis. The positive results obtained both *in vitro* and *in vivo* for cellular protection following oestrogen administration, paved the way for human clinical studies of postmenopausal women taking

oestrogen-only PMH. Fraser-Bell *et al.* found that exogenous oestrogen was protective against soft drusen formation, associated with dry AMD (2006). This finding was similar to a study carried out around the same time by Haan *et al.*, whereby oestrogen was administered concomitantly with progestin to postmenopausal women. Combined, these steroids effectively conferred against soft drusen formation and neovascular AMD (2006). In 2012, clinical trials using oestrogen derivative 'phytoestrogen' for the treatment of post-menopausal dry eye verified the safety and efficacy of direct oestrogen-to-eye administration in humans (Scuderi *et al.*, 2012). All taken, these studies suggest that exogenous oestrogen should be further explored as a potential treatment for retinal cell survival.

1.4.1.2. Progesterone specific neuroprotection

Despite clear protective effects in the brain, conflicting studies exist as to the efficacy of progesterone in retinal cell neuroprotection. Early research into the actions of progesterone in the eye began in the 1970's (Chader & Reif-Lehrer, 1972; Macaione *et al.*, 1973). In 1977, one of the first major experiments using the light-damage model of retinal degeneration was carried out in ovariectomized albino rats. Rats were treated with 2.5mg progesterone before exposure to damaging light; however, no difference was seen in photoreceptor cell death in progesterone-treated rats compared to control (O'Steen, 1977). The negative results of this study ensured that interest in the neuroprotective efficacy of progesterone in the retina waned in following years, until its sudden reappearance in retinal literature in the mid 2000's (Káldi & Berta, 2004; Yu *et al.*, 2004; Swiatek-De Lange *et al.*, 2007). Much of this research demonstrated that progesterone could not protect against cell death in photoreceptors (Káldi & Berta, 2004; Yu *et al.*, 2004) and indeed may actually promote vascular endothelial growth factor (VEGF) induced

neovascularization within retina (Swiatek-De Lange *et al.*, 2007). And yet, despite these results, promising studies have recently been released detailing the potential of progesterone to suppress retinal cell death.

Research from our group has extrapolated the capabilities of the oral-contraceptive 'levonorgestrel' (commonly referred to as 'Norgestrel') to protect damaged photoreceptors (Doonan *et al.*, 2011; Doonan & Cotter, 2012; Byrne *et al.*, 2016). These studies verify previous literature looking at progesterone-induced protection in models of diabetic and ischemic retinopathies. In 2008, two 4mg/kg intraperitoneal injections of progesterone were shown to preserve the inner nuclear and nerve fibre layers (Table 1.3.1) in a rat model of retinal ischemia-reperfusion (Lu *et al.*, 2008). Following on from this, progesterone was shown to reduce swelling of retinal glial cells in models of diabetic and ischemic retinopathies – implying a consequential overall reduction of cytotoxic retinal edema (Neumann *et al.*, 2010) and most recently, proved to be protective to damaged photoreceptors in the rd1 mouse model (Sánchez-Vallejo *et al.*, 2015).

These retinal studies, along with research undertaken by the broader neuroscience research field (Gonzalez Deniselle *et al.*, 2002a, 2002b; Gonzalez *et al.*, 2005; Compagnone, 2008; Stein, 2011, 2013; Espinosa-García *et al.*, 2014; Yousuf *et al.*, 2014; Qin *et al.*, 2015; Stein *et al.*, 2015), provide ample evidence for progesterone's protective role in neurodegeneration. Indeed, in a recent paper by Allen *et al.*, authors detail how progesterone is protective following middle cerebral artery occlusion (MCAO) - causing transient ischemia in the retina and brain. Allen *et al.* also showed however, that progesterone cannot protect against rodent anterior ischemic optic neuropathy (rAION),

which induces permanent monocular optic nerve stroke (2015). Simply, they postulate that the disparity in these data may arise from mechanistic differences with injury type and the therapeutic action of progesterone. A hypothesis that may fit the conflicting results seen in other studies.

1.4.2. Sex steroids: Mechanism of action

Knowledge of the mechanisms underlying the protection provided by oestrogen, progesterone and their derivatives are arguably more advanced than that of glucocorticoids. However, the temporal sequencing of the precise intracellular signalling events remain to be resolved (Lebesgue *et al.*, 2009). In the following sections, we will attempt to divulge the potential mechanisms through which these steroids exert their neuroprotective effects.

1.4.2.1. Oestrogen specific mechanism of action

In brief, there are four possible mechanisms through which oestrogen may have its effects, though they are not mutually exclusive:

a. Genomic signalling via its cognate nuclear receptors, oestrogen receptors α and β (ERα/ERβ)
b. Non-genomic signalling through membrane receptors, i.e. its cognate receptor, GPR30, and atypical oestrogen receptors
c. Through its free radical scavenging properties
d. Where the molecule itself integrates into and stabilizes membranes (Figure 1.4.1)

The evidence for each of these four mechanisms, highlighting the studies completed in the retina, will be given in the following paragraphs. For additional information about the protective effects of oestrogen in brain diseases, the reader is referred to recent and comprehensive reviews (Brann *et al.*, 2007; Raz *et al.*, 2008; Lebesgue *et al.*, 2009).

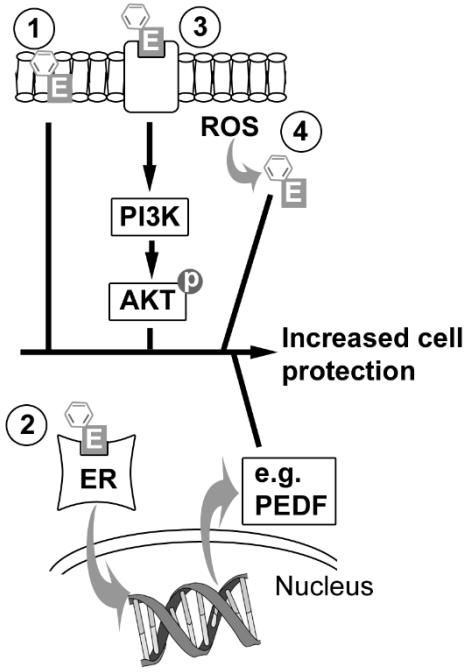


Figure 1.4.1. Recognised neuroprotective signaling pathways of oestrogen (E). (1) *Intercalation of oestrogen into cellular membranes, via its phenolic ring, stabilizes these membranes (Dicko* et al.*, 1999; Liang* et al.*, 2001; Dykens* et al.*, 2004).* ***(2)*** *Interaction with its cognate cytoplasmic receptors (oestrogen receptors: ER), which act as ligand dependent transcription factors, upregulating pro-survival factors, e.g. pigment epithelium derived factor (PEDF), and down regulating pro-death factors (Li* et al.*, 2006, 2013).* ***(3)*** *Interaction with membrane receptors resulting in changes in phosphorylation (p) of pro-survival signalling pathways, such as PI3K/AKT (Yu* et al.*, 2004; Lebesgue* et al.*, 2009; Nixon & Simpkins, 2012).* ***(4)*** *Scavenging of reactive oxygen species (ROS), thereby limiting oxidative stress (Moosmann & Behl, 1999; Brann* et al.*, 2007). (**1-3** have been confirmed in the retina and **4** is highly probable to occur there also.)*

Generally, it is accepted that oestrogen acts through its two nuclear receptors ERα/ERβ to up-regulate growth factors (Brann *et al.*, 2007). In three studies involving retinal pigment epithelial cells, ranging from the human ARPE-19 cell line to animal knock out mouse models; investigators found that ERβ was essential to conferring maximal protection from oestrogen (Elliot *et al.*, 2008, 2010; Giddabasappa *et al.*, 2010). However, although in these three cases ERβ was the critical receptor, the ultimate response to oestrogen varied from changing matrix metalloproeinase-2 activity (Elliot *et al.*, 2008, 2010), to the upregulation of antioxidant genes (Giddabasappa *et al.*, 2010).

In retinal ganglion cells, the situation is less clear. One study showed that 17β-estradiol (βE2) ligand binding to the ER was critical preventing glaucoma development in the DBA/2J mouse, a mouse model of an inherited (pigmentary) glaucoma. In this research, mice pre-treated with tamoxifen, an oestrogen receptor (ERα/β) antagonist, did not see any improvement in ganglion cell survival following βE2-administration (Zhou *et al.*, 2007). Similarly, two separate studies showed the capability of oestrogen receptor (ERα/β) inhibitor ICI 182-780, to reduce the protection conferred by oestrogen in two rat models of glaucoma (Hayashi *et al.*, 2007; Russo *et al.*, 2008).

Unfortunately, Kumar *et al.* found that the same inhibitor had no effect on the protection elicited by oestrogen in a retinal ganglion cell line (2005) - though the results of this study and all other studies employing the RGC-5 retinal ganglion cell, must now be regarded carefully. The RGC-5 cell line has since been found to be nearly identical to the 661W cone photoreceptor cell line and thus is likely not to be a representative of retinal ganglion cells *in vivo* (Krishnamoorthy *et al.*, 2013; Molecular Vision, 2013; Al-Ubaidi, 2014). On a similar note however, a large scale human clinical study (3842 participants) showed that in men, polymorphisms in ERβ were associated with a higher risk of open angle glaucoma (de Voogd *et al.*, 2008). All taken, whilst these studies implicate the oestrogen family of receptors in ganglion cell survival, they do not differentiate between ERα versus ERβ mediated signalling. Nor do they conclusively show the direct role that these receptors play in anti-apoptotic signalling. Indeed, in the following paragraphs, we will dissect the possibility that the actions of oestrogen in pro-survival signalling may instead be primarily independent of ERα/β.

Oestrogen is a female hormone. Therefore, to improve the suitability of using a feminizing[1] hormone as a therapy in neuronal disease for both sexes, efforts have ensued to generate non-feminizing derivative. These derivatives have been structurally altered to prevent their binding to ERα/β, for it is this interaction that will propagate the "feminizing" effects. Consequently, any such altered oestrogen derivative must now confer its protection in an ER-independent manner.

There is evidence for membrane oestrogen receptors performing a neuroprotective role within photoreceptors. Nixon and Simpkins used the 661W photoreceptor cell line to show that an agonist of GPR30 protected against a glutamate insult (2012). However, they also demonstrated that oestrogen and two of its analogues did not activate GPR30 or ERα/β. Besides GPR30, oestrogen is known to bind to other types of membrane receptors (Lebesgue *et al.*, 2009). Yu *et al.* demonstrated that the insulin receptor β (IRβ) was partly responsible for the pro-survival effects of oestrogen in primary cultures of retinal cells and in the rat light damage model (2004). Whilst they did not elucidate specifically how oestrogen activates IRβ, they hypothesised that Src may play a role. Downstream from this receptor, they demonstrated the phosphoinositide 3-kinase–AKT signalling pathway is important in protecting photoreceptors.

The studies discussed above all stand to highlight that the receptor with which oestrogen interacts is important in determining how protection is achieved. Nonetheless, this

[1] *Feminising effects of oestrogen include breast growth, decreases sexual libido and sperm formation, redistribution of body fat, skin softening and decreased hair growth ("Oestrogen Hormone Information (2) | The Gender Centre Inc.," 2016).*

interaction does not form the focus of every study in this area. There are two studies for example, which did not determine the receptor used by oestrogen in the retina, but still demonstrated a positive, neuroprotective effect. In the first, it was found that exogenous oestrogen resulted in the differential regulation of 69 genes in human Müller cell cultures (Li *et al.*, 2006). These included some genes with known links to survival signalling, such as mitogen-activated protein 3 and pigment epithelium derived factor. A separate study demonstrated different downstream effectors of this sex steroid (Li *et al.*, 2013). Pre-treatment of primary retinal cultures with oestrogen resulted in a downregulation of apoptotic BAX and caspase 3/9, which ultimately resulted in a reduction of mitochondrial-regulated cell death. Certainly, Zhou *et al.* found the signalling following 17β-estradiol (βE2) administration to the DBA/2J mouse model of glaucoma, to be diverse and far reaching. They showed that βE2 treatment reduced retinal ganglion cell apoptosis, preventing the subsequent loss of retinal neurofibers. They demonstrated the ability of βE2 to activate pro-survival AKT and cAMP-responsive-element-binding-protein, increase the expression of anti-oxidant molecule thioredoxin-1 and also to decrease the activation of mitogen-activated protein kinases and NF-κB. Finally, they found that βE2 administration successfully inhibited pro-inflammatory interleukin-18 release (Zhou *et al.*, 2007). All taken, these data suggest the critical role that oestrogen may play in the regulation of multiple biochemical events.

The final ways in which oestrogen can confer protection to retinal neurons is interestingly not through interacting with any specific receptor, but simply due to its molecular structure. This occurs via two distinct mechanisms. Oestrogen can act as a free radical scavenger (Moosmann & Behl, 1999; Brann *et al.*, 2007), thereby protecting against oxidative stress. Whilst this has yet to be proven in retinal tissues, the effect should not be

tissue specific. Alongside this, oestrogen is also known to protect cells through integrating into cellular membranes (Dicko *et al.*, 1999; Liang *et al.*, 2001). This mechanism was investigated in retinal cells by Dykens *et al.*, who found that MITO-4565, an oestrogen derivative, has protective properties in a model of retinitis pigmentosa (2004). It accomplishes this through integrating into cellular membranes and preventing the spread of lipid peroxidation, thus preserving membrane integrity. Key to the protection of cells was the ability of MITO-4565 to stabilize mitochondrial membranes, thereby reducing the likelihood of cells undergoing mitochondrial collapse and associated apoptosis. In order for an oestrogen derivative to possess this membrane intercalation capability, it must possess a steroid A-ring phenol (Dicko *et al.*, 1999; Liang *et al.*, 2001) (Figure 1.2.1). This was also found to hold true in the retina, as when Dykens *et al.* substituted the phenol ring of MITO-4565 with a methoxy group, its neuroprotective properties were abrogated (2004).

1.4.2.2. Progesterone specific mechanism of action

In amongst the multifaceted capabilities of progesterone, is its capacity to inhibit apoptosis and inflammation (Roglio *et al.* 2008; Choksuchat *et al.* 2009; Yu *et al.* 2010) – critical in the prevention of most retinal diseases. As with most neurosteroids, progesterone acts through specific binding to cellular receptors (Friberg *et al.*, 2009). Classical progesterone signalling occurs throughout the central nervous system through the classic progesterone receptors A and B (PR A/B). These receptors are widely expressed and can modulate a variety of different physiological functions (Wen *et al.*, 1994; Lange, 2008). Regulating gene transcription (Li *et al.*, 1997; Hanna *et al.*, 2010), PRs A/B are found at the plasma membrane but will localise to the nucleus upon ligand-binding (Lange, 2008). Just like oestrogen however, progesterone is a complex hormone and can also regulate its pro-survival activities through a variety of non-classical receptors (Pang and Thomas 2011;

Moussatche and Lyons 2012; Petersen *et al.* 2013; Qin *et al.* 2015). The progesterone receptor membrane component (PGRMC) family, featuring PGRMC1 and PGRMC2 (Cahill, 2007; Lösel *et al.*, 2008) and the Progestin and AdipoQ (PAQR) family of receptors, featuring membrane progesterone receptors α, β and γ (mPRα, mPRβ and mPRγ) (Zhu, Bond, *et al.*, 2003); have both been shown to facilitate survival signalling in neuronal cells (reviewed in Cahill, 2007; Brinton *et al.*, 2008; Pang & Thomas, 2011).

In addition to these 'typical' progesterone receptors, Neumann *et al.* demonstrated that in the retina, progesterone may act via atypical membrane receptors also. They identified these to be metabotropic glutamate receptors: the purinergic (P2Y1) receptor and the adenosine A1 receptor. They postulated that this is because exogenous progesterone results in the release of glutamate and ATP thereby activating these receptors and facilitating the reduction in glial cell swelling (2010). Therefore, these receptors are unlikely to form the apex of signalling in this model. All taken, it is probable that one receptor type does not act in isolation in response to progestins. Instead, it is likely that both the nuclear and membrane receptors are responsible for triggering different cell-survival signalling cascades, over different temporal scales. This would stand to reason, for this has already been shown to be the case with oestrogen.

Progesterone lacks oestrogen's phenol ring (Figure 1.2.1). Since it is this phenol ring that enables oestrogen to assimilate into cellular membranes (Dicko *et al.*, 1999; Liang *et al.*, 2001), progesterone has a limited capacity to integrate (Liang *et al.*, 2001). Progesterone, unlike oestrogen (Moosmann & Behl, 1999; Brann *et al.*, 2007), also has no proven antioxidant capabilities. Progesterone and its derivatives have however, been show to

induce an upregulation in pro-survival growth factors such as basic fibroblast growth factor (bFGF) (Guthridge *et al.*, 1992; Doonan *et al.*, 2011) and leukaemia inhibitory factor (Byrne *et al.*, 2016). In fact it is the relationship between progesterone and bFGF which plays a central role in the regulation of granulosa cell survival (Peluso & Pappalardo, 1999). We suggest therefore that it is progesterone's capability to promote this pro-survival signalling that will maintain cellular viability (Peluso & Pappalardo, 1999) and prevent the cleavage of pro-apoptotic PARP and Caspase 3 (Gómez-Vicente *et al.*, 2005; Miller *et al.*, 2006). The direct link between these pro-survival signalling events and their associated progesterone receptor interaction however, has not yet been elucidated.

1.5. Discussion

Retinal eye diseases are common and far reaching. Prevalent worldwide, the number of genetic mutations associated with retinal degeneration make the development of individual therapeutic gene-therapies a daunting and likely impossible task. Promising studies have emphasised the potential that both glucocorticoids and sex steroids have in providing retinal neuroprotection (Table 1.3.1). Thus we have endeavoured here to review the literature relating to these two distinct steroidal groups. Glucocorticoids have been trialled clinically for human retinal diseases. Arguably less is known of their signalling mechanisms than of the sex steroids. However, we hope that through their study, we can learn lessons to take through with us to develop the sex steroids as a treatment for retinal degenerations.

Outside of their structural differences, the main distinctions between these two steroidal groups lie in their clinical use and in their known signalling mechanisms (Figures 1.3.1, 1.4.1). For example, from examining glucocorticoid clinically, it is obvious that steroids provide enhanced therapeutic potential when administered via a slow release mechanism. Both DEX and FA benefited from incorporation into polymers, enabling enhanced retention within the vitreous (Haller *et al.*, 2010, 2011; Schwartz & Flynn, 2011). Through the use of slow release systems, fewer ophthalmological interventions are required by the patient, thereby reducing the probabilities of complications such as cataract development or increased intraocular pressure. If the non-feminising derivatives of oestrogen or the synthetic progestins are to be developed into clinical therapies for retinal degenerations, their potential would be improved through utilizing an equivalent delivery mechanism.

Despite the evidence that DEX can protect photoreceptors in the light damage model of retinal degeneration (Fu *et al.*, 1992; Wenzel *et al.*, 2001, 2003; Hao *et al.*, 2002; She *et al.*, 2008), these results have been disputed (Gu *et al.*, 2009) and as such, this protection is likely dependent on the process of AP-1-induced cell death (Figure 1.3.1). So, whilst glucocorticoids undoubtedly benefit the retina in inflammatory diseases, this may not translate into protection for retinal degenerations. On the other hand, estradiols protect photoreceptors in various retinal degeneration models (Dykens *et al.*, 2004; Yu *et al.*, 2004; Li *et al.*, 2006, 2013; Nixon & Simpkins, 2012). They possess four separate, well proven signalling mechanisms by which they protect neurons (Figure 1.4.1), three of which are proven to play a role in the retina (Yu *et al.*, 2004; Nixon & Simpkins, 2012; Li *et al.*, 2013). Given the amount of pre-clinical data on non-feminizing estradiols, it is probable that the sex-steroids could be of significant therapeutic value in some retinal diseases, but further research and subsequent clinical trials are needed.

Finally, the benefits of devoting some time to delineating the underlying signalling mechanisms must be stressed. Although the previous paragraph emphasized that there already appears to be ample evidence to pursue a clinical trial of estradiols in retinal degenerations, there are still many grey areas in steroidal pro-survival signalling. As an example it is appropriate to return to the role of DEX and AP-1 in photoreceptor degeneration. Without the follow-up studies by Gu *et al.* (2009) and Hao *et al.* (2002), it appeared that DEX was an ideal candidate for a retinal degeneration therapy (Wenzel *et al.*, 2001). Through comparing the three studies (Figure 1.3.1), it became apparent that DEX would only be appropriate when photoreceptor death was reliant on the activation of AP-1. The studies into developing non-feminizing estradiols exhibit a similar theme. Through extensive and detailed research, it was evident that the phenolic ring of oestrogen provides neuroprotection (Dicko *et al.*, 1999; Liang *et al.*, 2001; Dykens *et al.*, 2004), which was subsequently incorporated into effective derivatives. Whilst it is important to move forward with clinical trials using proven neuroprotective steroids, useful information may also be gained from continuing basic research into their signalling mechanisms.

In summary, the potential of glucocorticoids, estradiols and progestins to be neuroprotectants in retinal degenerations is evident from the literature. There are however valuable lessons about drug delivery to be ascertained from the administration of glucocorticoids in the treatment of inflammatory ocular conditions. These should be studies carefully and applied to the sex steroids oestrogen and progesterone, before commencement of large-scale clinical trials. Obviously there is still work to be done in explicating the exact mechanism of cell survival behind each of these compounds. However, in general, there appears to be a great deal of evidence supporting their use in retinal disease prevention. The current investigations into these steroids highlight that the

health of the retina as a whole is essential to photoreceptor maintenance and each of the steroids accomplishes this through interacting with different retinal cell types. Therefore, we suggest that there is great potential in developing at least one of these steroids in the quest to find an effective, neuroprotective based therapy for degenerative retinal diseases.

Chapter 2. Materials and Methods

2.1. Antibodies

Gene	Supplier	Catalogue #	Host	Dilution WB	Dilution IF
PARP	CST	9542	Rabbit Polyclonal	1:1000	
Caspase-3	CST	9662	Rabbit Polyclonal	1:1000	
Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199)	CST	4228	Rabbit Polyclonal	1:1000	
Beclin-1	CST	3738	Rabbit Polyclonal	1:1000	
LC3B	CST	2775	Rabbit Polyclonal	1:1000	
Phospho-Akt (Ser473)	CST	4060	Rabbit Monoclonal	1:2000	
Akt	CST	9272	Rabbit Polyclonal	1:1000	
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	CST	9106	Mouse Monoclonal	1:2000	
p44/42 MAPK (ERK1/2)	CST	4696	Mouse Monoclonal	1:1000	
Phospho-GSK-3β (Ser9)	CST	9336	Rabbit Polyclonal	1:1000	
GSK-3β	CST	9315	Rabbit Monoclonal	1:1000	1:200
GAPDH	CST	5174	Rabbit Monoclonal	1:1000	
PI3-Kinase p110α	BD	BD611398	Mouse Monoclonal	1:500	
Basic FGF	BD	BD610073	Mouse Monoclonal	1:250	
FGF2/basic FGF, clone bFM-2	Millipore	05-118	Mouse Monoclonal		1:200
PR A/B	Abcam	ab2764	Mouse monoclonal	1:200	1:100
PGRMC1	Sigma	HPA002877	Rabbit polyclonal	1:500	1:100
PGRMC2	Santa Cruz	sc-104609	Goat polyclonal	1:200	1:200
mPRα	Santa Cruz	sc-50111	Goat polyclonal	1:200	1:100
mPRβ	Abcam	ab46534	Rabbit polyclonal	1:500	1:100

mPRγ	Abcam	ab79517	Rabbit polyclonal	1:500	1:100
Tubulin	Sigma	T5168	Mouse monoclonal	1:10,000	
Actin	Sigma	A5441	Mouse monoclonal	1:10,000	
Histone H3	Abcam	ab1220	Mouse monoclonal	1:5000	
Calreticulin	Abcam	ab2907	Rabbit polyclonal	1:1000	
Rhodopsin	Millipore	AB9279	Rabbit		1:200
FITC PNA	Vector Labs	FL-1071	-		1:500
Iba1	Wako	019-19741	Rabbit polyclonal		1:500
Iba1	Novus Bio	NB100-1028	Goat polyclonal		1:400
CD68	AbD Serotec	MCA1957GA	Rat monoclonal		1:500
iNos	CST	13120	Rabbit monoclonal		1:200
MRC1	Abcam	AB64693	Rabbit polyclonal		1:1,000
Cone Arrestin	Millipore	AB15282	Rabbit polyclonal		1:1,000
GFAP	Sigma	G3893	Mouse monoclonal		1:300
Glutamine Synthetase	Millipore	MAB302	Mouse monoclonal		1:100
NFM	Abcam	AB7794	Mouse monoclonal		1:500
Syntaxin 1	Millipore	AB5820	Rabbit polyclonal		1:500
Calbindin	Swant	CB-38a	Rabbit monoclonal		1:500
CHX10	Santa Cruz	sc-21690	Goat polyclonal		1:100
Fractalkine	Abcam	AB25088	Rabbit polyclonal		1:1,000
CX3CR1	Abcam	AB8021	Rabbit polyclonal		1:100

Table 2.1.1. List of antibodies used throughout the course of this thesis. *WB: Western Blotting, IF: Immunofluorescence, CST: Cell Signalling Technology (Boston, MA, USA), BD: BD Biosciences Europe (Oxford, UK), Millipore: Millipore Ireland B.V. (Cork, Ireland), Abcam (Cambridge, UK), Sigma (Boston, MA, USA), Santa Cruz: Santa Cruz Biotechnology Inc. (Dublin, Ireland), Vector Labs: Vector Laboratories LTD (Peterborough, UK), Wako: Wako Chemicals USA, Inc. (Richmond, VA, USA), Novus Bio: Novus Biologicals (Abingdon, UK), AbD Serotec: Bio-Rad (Oxford, UK), Swant: Swiss Antibodies (Marly, Switzerland).*

2.2. Primers

Gene	Qiagen Primer	Product Size	RefSeq ID#
Actin	QT00095242	149	NM_007393
GAPDH	QT01658692	144	NM_008084
HPRT	QT00166768	168	NM_013556
bFGF	QT00128135	138	NM_008006
PR A/B	QT00114534	118	NM_008829
PGRMC1	QT00250887	131	NM_016783
PGRMC2	QT00258608	111	NM_027558
mPRα	QT00138145	78	NM_027995
mPRβ	QT00123886	67	NM_028829
mPRγ	QT01067612	67	NM_028748
iNOS	QT01547980	131	XM_001004823
Arginase	QT00134288	109	NM_007482
Mannose Receptor	QT00103012	132	NM_008625
HMGB1	QT00247786	78	NM_010439
IL-1α	QT00113505	110	NM_010439
MIP1Alpha	QT00248199	107	NM_011337
MIP1Beta	QT00154616	110	NM_013652
MCP1	QT00167832	118	NM_011333
MCP3	QT00171458	117	NM_013654
Fractalkine	QT00128345	98	NM_009142
CX3CR1	QT00259126	63	NM_009987
IL-1β	QT01048355	150	NM_008361
IL-6	QT00098875	128	NM_031168
TNF-α	QT00104006	112	NM_013693

Table 2.2.1. List of primers used throughout the course of this thesis. *All primers are from Qiagen (West Sussex, UK).*

2.3. Reagents, inhibitors and antagonists

Protein	Reagent/ Inhibitor/ Antagonist	Supplier	Catalogue #	Stock Concentration	Vehicle
GSK3β	SB216763	Tocris	1616	50mM	DMSO
PI3K/AKT	LY294002	CST	9001	50mM	DMSO
PKA	H-89	Sigma	B1427	50mM	DMSO
ERK1/2	UO126	Sigma	U120	50mM	DMSO
PGRMC1	AG205	Sigma	A1487	10mM	DMSO
Ryanodine Receptor	Dantrolene (Antagonist)	Sigma	D9175	10mM	DMSO
EGTA	Calcium Chelator	Sigma	E3889	0.5M	Water

Table 2.3.1. List of inhibitors used throughout the course of this thesis. *Tocris: Tocris Bioscience (Bristol, UK), CST: Cell Signalling Technology (Boston, MA, USA), Sigma (Boston, MA, USA).*

2.4. Mice

All animals were handled and maintained following the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were approved by University College Cork Animal Experimentation Ethics Committee and were supplied by the Biological Services Unit, University College Cork. All animals were humanely euthanized by cervical dislocation (Hwang & Iuvone, 2013). All experiments performed in the course of this study were carried out using both male and female C57BL/6 wild-type and homozygous rd10/rd10 (B6.CXBI-Pde6b^{rd10}/J) mice.

2.5. Retinal explant culture

C57BL/6 were sacrificed at postnatal day 30 (P30) by cervical dislocation and decapitated. The removed heads were wiped clean with tissue soaked in 70% ethanol and transported in to a laminar flow cabinet. From this point on, all handling was performed aseptically. The eyes were enucleated (Jin & Xiang, 2012) and placed in to R16 medium (recipe from P.A. Ekstrom, Wallenberg, Retina Centre, Lund University, Lund, Sweden). R16 media is made up by dissolving one vial of R16 Dry Powder Medium (Basal) *(*07490743A) (Invitrogen Life Technologies, Paisley, UK) in 800ml sterile dH_2O. This solution is made to a complete medium through hormones and vitamins supplementation (Table 2.5.1 – all from Sigma), 10% bovine serum albumin and 1% penicillin streptomycin (both Sigma) to a final volume of 1L and will last for up to three weeks at 4°C (Caffé *et al.*, 2002). The powder is composed of 41 ingredients that can be classified in salts, amino acids, sugars and vitamins (Table 2.5.2).

	Concentration		**Concentration**
$NaSeO_3$	30nM	**Thiocitic acid**	0.22µM
$MnCl_2$	5nM	**Retinol**	0.35µM
$CuSO_4$	10nM	**Retinyl acetate**	0.3µM
$NaHCO_3$	33.3µM	**Tocopherol**	2.32µM
Biotin	0.4µM	**Tocopherol acetate**	3.59µM
Ethanolamine	16.4µM	**Linoleic acid**	3.57µM
Transferrin	0.125µM	**Linolenic acid**	3.59µM
Progesterone	0.02µM	**L-Cysteine**	45µM
Insulin	3.33nM	**Glutathione**	3.25µM
T3	3.1nM	**Na Pyruvate**	454.4µM
Corticosterone	57.7nM	**Glutamine**	171µM
Thiamine HCL	8.2µM	**Vitamin C**	567.76µM
Vitamin B12	0.23µM		

Table 2.5.1. Supplements for R16 medium

	Concentration		Concentration
L-Alanine	23µM	**Glucose**	19.1mM
L-Arginine HCl	494µM	**D(+)-Galactose**	83µM
L-Asparagine H2O	23µM	**D(+)-Mannose**	56µM
L-Cystine Na_2	134µM	**Choline chloride**	43.5µM
L-Glycine	292µM	**Pyridoxal HCl**	13.4µM
L-Histidine HCl.H2O	158µM	**CaCl2.2H2O**	1.28mM
L-Isoleucine	546µM	**Fe(NO3)3.9H2O**	0.17µM
L-Leucine	562µM	**FeSO4.7H2O**	0.68µM
L-Lysine HCl	585µM	**KCl**	4.29mM
L-Methionine	142µM	**MgSO4.7H2O**	680µM
L-Phenylalanine	276µM	**NaH2PO4.2H2O**	610µM
L-Proline	68µM	**Na2HPO4**	230µM
L-Serine	292µM	**ZnSO4.7H2O**	0.70µM
L-Threonine	562µM	**Folic acid**	6.79µM
L-Tryptophan	55µM	**i-Inositol**	48.7µM
L-Tyrosine	275µM	**Nicotinamide**	22.2µM
L-Valine	562µM	**Hypoxanthine**	6.75µM
Putrescine	180µM	**Riboflavine**	0.74µM
L-Carnitine	12.4µM	**Thymidine**	0.67µM
NaCl	103mM	**D-calcium pantothenate**	5.77µM
Cytidine 5'-diphospho ethanolamine	1.28mg/ml	**Cytidine 5'-disphospho choline**	2.56mg/ml
Sodium phenol red	5.0mg/ml		

Table 2.5.2. Composition of R16 dry powder medium (basal)

The lens, anterior segment, vitreous body, retinal pigment epithelium and sclera were removed and the retina was flat mounted, photoreceptor side down on top of a nitrocellulose insert (Millipore, Billerica, MA) in six well culture dishes (Starstedt AG & Co., Wexford, Ireland). Explants were cultured in 1.2mL of complete R16 media. Explants were

pre-treated with 20µM Norgestrel (N2260) (Sigma), 0.5µM PKA specific inhibitor H89 (B1427) (Sigma), 10µM PGRMC1 specific inhibitor AG205 (A1487) (Sigma) or the equivalent dimethyl sulphoxide (DMSO) (D2650) (Sigma) control for 1 hour before treatment with 300µM of the nitric oxide donor sodium nitroprusside (SNP) (228710) (Sigma**)** over 24 hours.

2.6. Terminal dUTP nick-end Labelling (TUNEL) of fragmented DNA

DNA strand breaks in retinal cell nuclei were detected by terminal dUTP nick end-labelling (TUNEL) (Portera-Cailliau *et al.*, 1994). Retinal explants were fixed in 4% PFA for 10 minutes followed by cryoprotection in 30% sucrose overnight at 4°C. In all cases, four sections of 7µm were cut using a cryostat (Leica CM1950; Leica Co., Meath, Ireland) and placed on to one slide. 30µm sections were then sliced between these and methodically taken, ensuring sections were obtained from the entire retina. Frozen sections were incubated with terminal deoxynucleotidyl transferase (M1875) (Promega, Kilkenny, Ireland) and fluorescein-12-dUTP (#11373242910) (Roche, Lewes, UK) according to manufacturer's instructions and nuclei were counterstained with Hoechst 33342 (1µg/mL) (Sigma). Sections were incubated at 37°C for 1 hour in a humidified chamber and following several washes in phosphate-buffered saline (PBS; pH 7.4), were mounted in mowiol (Calbiochem, Nottingham, UK). Sections were viewed under a fluorescence microscope (Leica DM LB2). For each treatment, at least three animals were used and three fields per section (central, central-peripheral and peripheral retina) (x40 magnification) of at least three different

sections were evaluated. Incubated sections were taken from both the central and peripheral retina.

2.7. Cell culture and treatments

Experiments were carried out using the mouse photoreceptor-derived 661W cell line. This cell line was generously provided by Dr Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma, Health Sciences Centre, Oklahoma City, OK, USA) (Al-Ubaidi *et al.*, 2008). Validation of this cell line was carried out through RT-PCR analysis for cone specific opsin blue cone opsin (Opn1sw) and rod specific rhodopsin (Ait-Hmyed *et al.*, 2013). Opn1sw yielded a positive result confirming a cone cell phenotype (Figure 2.7.1).

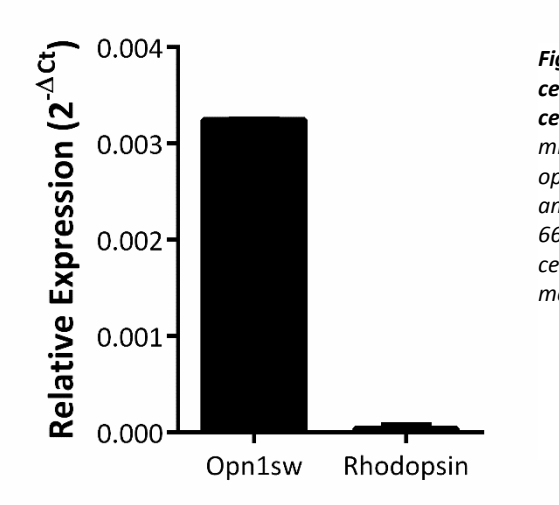


Figure 2.7.1. Validation of 661W cells as a cone photoreceptor-like cell line. *RT-qPCR analysis of mRNA encoding cone specific opsin blue cone opsin (Opn1sw) and rod specific Rhodopsin from 661W cone-photoreceptor like cells. Results are presented as mean ± SEM. N=3, n=4.*

2.7.1. 661W cell culture and treatment

Cells were cultured in Dulbecco's Modified Eagle's medium (D6429) (Sigma) supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin streptomycin (to make complete dulbecco's modified eagle's medium) and maintained at 37°C in a humidified 5% CO_2

atmosphere. To analyse the effects of Norgestrel (N2260) (Sigma) and/or a treatment on 661W cells by western blotting, one million cells were seeded in a T75 flask and allowed to attach overnight. To analyse the effects by RT-PCR, 7 x 10^5 cells were seeded in a 6 well plate and allowed to attach overnight. All cells were then washed three times with PBS and complete or serum-free medium supplemented with 20µM Norgestrel, treatment compound or the equivalent dimethyl sulfoxide (DMSO) (D2650) (Sigma) control was added. After incubation for the indicated times, cells for were detached using Accutase solution (A6964) (Sigma) and together with their supernatant, centrifuged to leave a whole cell pellet. Plates seeded with cells for RT-qPCR were washed and immediately placed at -80° until needed.

2.7.2. Culture of primary microglial cells

A protocol for isolating and culturing retinal microglia was adapted from a previously published protocol (Weigelt *et al.*, 2007). Briefly, retinas were dissected from the eyes of P16 rd10 mice, ensuring minimum contamination with vitreous body and retinal pigment epithelium. Four retinas were pooled, cut into small pieces and incubated for 40 min at 37°C in 1ml PBS with 1mg/ml collagenase type I (Sigma), 0.3mg/ml DNase I (Roche), and 0.2mg/ml hyaluronidase (Sigma). The cell suspension was filtered through a 70-µm cell strainer (Becton Dickinson). Cells were washed twice with 10ml DMEM/10% FBS/1% PS and resuspended in 15ml DMEM/10% (v/v) FBS/1% (v/v) PS. To isolate the mononuclear cells, the suspension was gently added to 15ml Ficoll paque premium reagent (GE Life Sciences) and centrifuged for 20 minutes at 2,000 rpm without the brake in a Beckmann GS-6R centrifuge. The interphase was removed carefully and washed twice with 10ml DMEM/10%

(v/v) FBS/1% (v/v) PS. The cells were cultured for 3 days, on either polylysine coated coverslips in 24 well plates or T25cm^2 flasks containing DMEM/10% (v/v) FBS/1% (v/v) PS.

2.7.3. Culture of 661W cells in microglial-conditioned media

A total of six retinas from three P16 rd10 mice were used to make the primary culture for the conditioned media experiment. Primary microglia were cultured in T25 flasks for 2 days before treatment with 20µM Norgestrel or vehicle (DMSO) for 24 hours. Cells were washed with PBS and cultured in fresh DMEM/10% (v/v) FBS/1% (v/v) PS for a further 24 hours. This conditioned media was collected and added to 661W cells (Roque *et al.*, 1999). 661W cells were seeded overnight in 96 well plates (Sarstedt) to allow for cell adherence. All cells were then washed three times in PBS and media replaced with either basal microglial media, or conditioned media from treated primary microglial cells (100µl/well). Cells were incubated for 20 hours before addition of MTS for a further 4 hours.

2.7.4. Co-culture of rd10 microglia with 661W cells

A total of sixteen retinas from eight P16 rd10 mice were used to make the primary culture for the co-culture experiment. Primary microglia were cultured on polylysine-coated coverslips in 24 well plates for 1 day before treating with Norgestrel or vehicle (DMSO). Primary cells were exposed to 20µM Norgestrel or vehicle control and left for 24 hours. Cells were washed with PBS prior to the addition of 661W cells that had been treated with Norgestrel or vehicle in the absence or presence of serum in the preceding 3 hours. 661W cells suspended in fresh DMEM/10% (v/v) FBS/1% PS (v/v) were added to the microglia at a density of 25 x 10^3 per well. Untreated microglia were also co-cultured with untreated 661W cells. Co-cultures of microglia and 661W cells were left for a further 21 hours before fixation in 4% (v/v) PFA.

2.7.5. Quantification from immunohistochemical sections and cells

Quantification of outer nuclear layer (ONL) thickness and outer segment length in retinal sections was carried out using ImageJ software. Average ONL thickness was measured by taking measurements from at least 20 sections per mouse. Per section, three distinct measurements were taken and averaged. Average outer segment length was measured by taking measurements from at least four sections per mouse. Per section, three distinct measurements of rod or cone outer segment length were taken and averaged. Three mice were analysed per group. Fluorescence intensity measurements of CD68, iNOS and MRC1 in primary microglia were performed using ImageJ software, by measuring points of intensity within single cells.

2.8. Western blot analysis

2.8.1. Whole cell lysis

Whole cells were centrifuged down to form a pellet, and solubilised with RIPA buffer: 50mM Tris-HCL pH 7.4, 150mM NaCl, 1mM Na_3VO_4, 1mM NaF, 1mM EGTA, 1% Nonidet P-40 (NP40) (v/v), 0.25% sodium deoxycholate, cocktail protease inhibitors (Roche, Welwyn, Hertforshire, UK) and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (0.2mM). Lysates were incubated on ice for 1 hour with occasional vortexing and debris was removed by centrifugation (10,000 x *g*) at 4°C. In all cases equivalent amounts of protein (15-50µg), as determined by the Bio-Rad Protein Assay (Bio-Rad, Oxford, UK) were resuspended in loading dye with Dithiothreitol (DTT) (Sigma) and boiled at 95°C for 5 minutes. This solution

was then centrifuged at 10,000 g for 1 min. Total supernatant was resolved using denaturing 12% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Membranes were blocked with 5% (w/v) bovine serum albumin (Sigma) in Tris-buffered saline/0.1% (v/v) Tween-20 (Sigma) for 1 hour at 18-20°C and incubated at 4° overnight with the appropriate dilution of primary antibody. Blots were then incubated with the corresponding peroxidase-conjugated secondary antibody (dilution 1:10,000) (LI-COR Biosciences UK Ltd, Cambridge, UK) for 1 hour. Antibody reactive bands were detected using a LICOR Odyssey infrared imaging. All experiments were carried out in triplicate (n=3 biological samples per condition). Western blots shown in results are representative images of three independent experiments, using different samples each time.

2.8.2. Subcellular fractionation

Subcellular fractionation was performed to detect movement of progesterone receptors using the subcellular protein fractionation kit (Thermo Scientific Pierce). Manufacturer's instructions were modified to ensure optimal amounts of protein were obtained from each fractionation step. In brief, for 661W cells, we assumed that 1 x 10^6 cells were the equivalent to 1µL packed cell volume. For cytoplasmic extraction, one tenth the recommended quantity of buffer was added and an additional vortexing step of 15s was carried out before gentle mixing for 10 minutes. When adding the membrane extraction buffer, one fifth the recommended quantity of buffer was added. Finally, for nuclear fraction extraction, RIPA lysate buffer was used as this was found to obtain better protein concentrations than the nuclear extraction buffers provided. RIPA lysate was added at a volume of 10µL per initial 1µL packed cell volume.

2.9. Immunofluorescence and live cell imaging

2.9.1. Cell seeding for 661W immunofluorescence

Cells were seeded overnight (25 x 10^3 per well) in 24 well plates (Starstedt AG & Co.) with glass coverslips. Cells were then washed with PBS, pH 7.4 before treated over the specified times. After treatment, cells washed again and fixed with 4% (v/v) PFA for 10 minutes at room temperature. Cells were subsequently blocked and permeabilised in 0.1% (v/v) Triton X-100 and 5% (v/v) donkey serum in PBS for 10 minutes at room temperature (18-20°C).

2.9.2. Fixation and sectioning of retinal explants

Following treatments, retinal explants were fixed at room temperature in 4% (v/v) paraformaldehyde (PFA) for 10 minutes (Samardzija *et al.*, 2012). Following washes, eyes were cryoprotected in 30% (w/v) sucrose in PBS overnight, at 4°C. Explants were dabbed dry, submerged and frozen in cryochrome (Thermo Scientific) and sectioned on a cryostat (Leica). Sections (7µm) were collected on superfrost glass slides (Fisher Scientific) and stored at -80°C. Before use, sections were permeabilised and blocked for half an hour in 0.1% (v/v) Triton X-100 and 5% (v/v) donkey serum in PBS at room temperature (18-20°C).

2.9.3. Fixation and sectioning of whole retinas

Eyes were enucleated and fixed at room temperature in 4% (v/v) PFA for 1.5 hours (Samardzija *et al.*, 2012). Following washes, eyes were cryoprotected in 15% (w/v) sucrose in PBS for 1 hour, 20% (w/v) sucrose for 1.5 hours and 30% (w/v) sucrose overnight, all at

4°C. Eyes were submerged and frozen in cryochrome and sectioned on a cryostat. Sections (7µm) were collected on superfrost glass slides (Fisher Scientific) and stored at -80°C. Before use, sections were permeabilised and blocked for half an hour in 0.1% Triton X-100 (v/v) and 5% (v/v) donkey serum in PBS at room temperature (18-20°C).

2.9.4. Immunofluorescence

After blocking, all sections and cells were incubated with primary antibody overnight at 4°C. Table 2.1.1 lists the details of all primary antibodies used. Following washes, coverslips and sections were incubated with conjugated secondary antibody (1:500) antibody (Alexa Fluor donkey anti-mouse/rabbit/goat with either a 488 or 594 fluorescent probe; Molecular Probes &/or FITC –PNA (Vector Labs)) and Hoechst (0.1 µg/ml) in the dark for 1h at room temperature. Eliminating the primary antibody in solution served as a negative control. Sections were mounted, and cell-seeded coverslips were mounted on to glass slides, both using Mowiol.

2.9.5. Microscopy

Retinal sections and cell preparations were viewed using a Leica DM LB2 microscope with Nikon Digital Sight DS-U2 camera, using 40x and 100x objectives. Images were taken using the software NIS-Elements version 3.0, Nikon, Japan. Immunofluorescence on retinal sections was performed on at least three mice of each group, at each time-point. Immunofluorescence on cell preparations was also performed in triplicate. Confocal micrographs were taken using an Olympus Fluoview FV1000 laser scanning confocal microscope, using 20x and 60x objectives. Images were taken using the software Olympus

Fluoview Ver 4.1a and are represented as single slices in the XY plane or maximum intensity projections from acquisition of z-stacks. Identical microscope settings were used when visualizing specific markers across all time points and treatments.

2.9.6. Live cell imaging: Visualisation of intracellular Ca^{2+}

Cells required for visualisation of intracellular Ca^{2+} were seeded overnight (25 x 10^3 per plate) in 35mm with 2mm micro-well glass bottomed dishes (MatTek, MA, USA). Cells were then washed three times in PBS before treatment. Cytoplasmic Ca^{2+} levels were determined using the probe Fluo-4 AM (Healy *et al.*, 2013). 30 minutes prior to treatment conclusion, 1µM Fluo-4 AM was added directly to the cells and incubated at 37°C in the dark. Cells were washed three times in warmed PBS and Ca^{2+} Fluo-4 AM cell loading medium added (see Flow Cytometry: Measurement of intracellular Ca^{2+} section). Plates were taken immediately to the confocal microscope and confocal micrographs imaged using an Olympus Fluoview FV1000 laser scanning confocal microscope, 20x objectives (Plank & Sussman, 2003). Images were taken using the software Olympus Fluoview Ver 4.1a and are represented as single slices in the XY plane. Identical microscope settings were used when visualising specific markers across all time points and treatments.

2.10.Total RNA isolation and RT-qPCR

Total RNA was isolated from whole cells using RNeasy Mini Kit (Qiagen, West Sussex, UK) following manufacturer's protocol. All samples were DNase treated using RNase free DNase set (Qiagen) and 240ng-1µg cDNA was synthesised using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions. The resultant cDNA was then diluted

1:5-6 with RNase free water (Sigma) and RT-PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) in a 384 well plate (Starstedt AG & Co.). Plates were run using the Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies Ltd., Paisley, UK) and each set of reactions included both a non-reverse transcription control and a no template sample negative control (data not shown). The protocol consisted of a cycling profile of 30s at 95°C, 60s at 60°C, and 30s at 72°C for 40 cycles. Qiagen QuantiTect Primer assays were used for all genes (Table 2.2.1). Melt curve analysis confirmed that a single PCR product was present and subsequent gene sequencing of progesterone receptors was carried out by GATC Biotech, London, United Kingdom. Relative changes in gene expression were quantified using the comparative Ct (ΔΔCt) method as described by Livak & Schmittgen (2001; Schmittgen & Livak, 2008). The Ct value of the gene of interest was normalised to an average of the three endogenous housekeeping genes (Actb, Gapdh and Hprt). This was then compared to the normalised control sample – i.e. the equivalent DMSO timed control or control mouse. Alteration in mRNA expression of target genes was defined as fold difference in the expression level in cells after treatment, relative to that of the control.

2.11. Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded (30 x 10^3) in 24 well plates (Starstedt) overnight to allow for cell adherence. Cells were stressed and treated with 20µM Norgestrel/DMSO control (500µl media). The supernatant was collected at the indicated times and samples were analysed for secreted bFGF using Abcam ELISA kit ab100670, according to manufacturer's instructions.

2.12.siRNA transfection

Cells were seeded overnight prior to transfection in 10cm petri dishes (750 x 10^3 per dish) (Starstedt) or 96 well plates (4 x 10^3 per well) (Starstedt) to allow for complete cell adherence. Qiagen GeneSolution siRNA (Table 2.12.1) was then transfected in to the cells using HiPerFect Transfection Reagent (Qiagen) (Table 2.12.2) and left for 24 – 48 hours before treatment.

Gene	Catalogue Number	Concentration (nM)	Incubation Time
FGF2	#GS14173	25	48
PGRMC1	#2968441	25	24
Allstars Negative Control	#1027280	10	As required

Table 2.12.1. GeneSolution siRNA: *Concentration and times used in siRNA Transfections*

Transfection Vessel	Area (cm^2)	HiPerFect (µL)	Total volume (mL)
96 Well Plate	0.3	0.36	0.1
24 Well Plate	2	2.4	0.5
6 Well Plate	10	12	2.4
T75	75	96	10

Table 2.12.2. HiPerFect Transfection Reagent: *Quantities used in siRNA Transfections*

2.13.Assessment of cell viability

MTS assay was used to quantify cellular viability (Penha *et al.*, 2013; Sobolewska *et al.*, 2013). Cells (4 x 10^3 per well) were seeded 6 hours prior to treatment with siRNA in 96 well plates (Starstedt). Cells used to quantify cellular viability after serum starvation and treatment were seeded overnight (2 x 10^3 per well 96 well plate). This allowed for cell adherence. All cells were washed three times in PBS before treatment. After 20 hours of treatment incubation, 20µl of MTS solution (Promega, Madison, WI, USA) was added to each well and incubated for 4 hours at 37°C. Viable cells reduce the yellow MTS solution to form formazan. Detection and quantification of the formazan crystals is then carried out with a microplate reader (Molecular Device Corporation, Spectramax Plus 384, Sunnyvale, CA, USA) at 490nm. 490nm readings taken from non-template wells (media and MTS, without cells) were deducted from actual cellular readings. A further reading at 650nm was also taken from all wells and deducted from the 490nm readings to account for any cellular debris. The quantity of formazan product as measured by the amount of 490nm absorbance, is directly proportional to the number of living cells in culture. Therefore, the absorbance of the formazan formed in 'control cell wells', i.e. non-serum-starved, healthy 661W cells, was taken as 100% viability. In experiments where cell death was evaluated after serum starvation, cell death was calculated as a percentage of non-stressed cells (0% cell death).

2.13.1. Cell viability following dose response inhibitor treatments

In all experiments entailing inhibitors, a control was carried out for every individual concentration, i.e. in an experiment where all cells were serum starved for 24 hours, cells treated with 0.5µM inhibitor plus Norgestrel were compared to cells treated with 0.5µM

inhibitor plus the equivalent amount of DMSO. In Figure 3.3.9, results obtained with 20µM Norgestrel administration were then graphed as a percentage of the viability of the DMSO control, where control viability was taken to be 100%. For graphical clarity however, only one DMSO 100% viability control bar has been shown.

2.14.Flow cytometry

2.14.1. Specific cell treatment for analysis by flow cytometry

Cells undergoing specific inhibition treatments for serum starvation and subsequent analysis by flow cytometry were seeded (1 x 10^6) in a T75 flask overnight. Cells were then washed three times with warmed PBS and pre-treated for 10 minutes in serum-free media supplemented with AG205 (1 - 10µM) (Qin *et al.*, 2015), dantrolene sodium salt (1µM) or calcium chelator EGTA (0.5mM) (Healy *et al.*, 2013). 20µM Norgestrel or the equivalent DMSO control was then added directly to the flasks. After incubation for the indicated times, cells were washed with PBS and detached using accutase solution (A6964) (Sigma). Cells were then spun down to leave a whole cell pellet.

2.14.2. Flow cytometry: Measurement of intracellular Ca^{2+}

Cytoplasmic Ca^{2+} levels were determined using the probe Fluo-4 AM. 30 minutes prior to treatment conclusion, 1µM Fluo-4 AM was added directly to the cells and incubated at 37°C in the dark. After 30 minutes incubation, cells were washed with PBS, detached and spun down to form a whole cell pellet. Cells were resuspended in Ca^{2+} Fluo-4 AM cell loading

medium: 10% HBSS (#14180-046) (Gibco), 1mM $CaCl_2 2H_2O$ (#223506) (Sigma), 1mM $MgCl_2$ (M8266) (Sigma), 1% foetal bovine serum (F7524) (Sigma), before analysis on a FACScan flow cytometer (Becton Dickinson, Oxford, UK). For experiments whereby the source of calcium was sought, cells were resuspended in Ca^{2+} Fluo-4 AM cell loading medium made up without $CaCl_2 2H_2O$. Intracellular Ca^{2+} production was measured at FL-1 (530nm) with excitation at 488nm. CellQuest software (Becton Dickinson) was used for data analysis. Healthy populations of cells were gated and a total of 10,000 gated events per sample were acquired. All FACS analyses were carried out in technical triplicates and all graphs represent data obtained from three independent experiments.

2.15. Norgestrel-supplemented diet

Norgestrel-supplemented and control diets were manufactured by Testdiet (Middlesex, UK). Norgestrel was added to the chow at a concentration of 0.05% (500ppm). Dam's of rd10 pups were given a Norgestrel-supplemented diet (LabDiet 5053, custom diet containing D(-)-Norgestrel (N2260) (Sigma)) when the pups were P10 to allow pups to receive Norgestrel in the milk. This equates to a daily intake of approximately 80mg/kg, assuming a 30g mouse consumes around 5g of food/day. Specialised diet soaked in water was also added to the cage in order for pups to receive Norgestrel directly, as soon as they began to eat. These pups were kept on the Norgestrel-supplemented diet post-weaning. With control litters, diet changes were replicated with a control diet (LabDiet 5053 control diet). Dams were given regular chow once pups were weaned. Supplementation of a mouse maternal diet with neuroprotective agents has previously been shown to provide neuroprotection in pups (Loren *et al.*, 2005).

2.16.Statistical analysis

Image Studio Lite Version 4.0 (LI-COR Biosciences UK Ltd, Cambridge, UK) was used to analyse all Western blots. Values in all graphs represented the mean ± standard error of the mean (SEM) and are representative of at least three individual experiments repeated in triplicate. Data were statistically analysed using the appropriate test (see individual figure legends) using Graph Pad, Prism 6. Values of $p<0.05$ were considered statistically significant.

Chapter 3. The synthetic progesterone ‘Norgestrel’ mediates its effects via basic fibroblast growth factor, protein kinase A and glycogen synthase kinase 3β signalling

3.1. Abstract

The synthetic progesterone 'Norgestrel', has been shown to have proven neuroprotective efficacy in two distinct models of Retinitis Pigmentosa: the rd10/rd10 (B6.CXBI-Pde6b^{rd10}/J) mouse model and the Balb/c light damage model. However, the cellular mechanism underlying this neuroprotection is still largely unknown. Therefore, this study aimed to examine the downstream signalling pathways associated with Norgestrel both *in vitro* and *ex vivo*.

In this work, we identify the potential of Norgestrel to rescue stressed 661W photoreceptor-like cells and *ex vivo* retinal explants from cell death over 24 hours. Norgestrel is hypothesised to work through an upregulation of neuroprotective basic fibroblast growth factor (bFGF). Analysis of 661W cells *in vitro* by real time polymerase chain reaction (rt-PCR), enzyme-linked immunosorbent assay (ELISA) and western blotting revealed an upregulation of bFGF in response to Norgestrel over 6 hours. Specific siRNA knock down of bFGF abrogated the protective properties of Norgestrel on damaged photoreceptors, thus highlighting the crucial importance of bFGF in Norgestrel-mediated protection. Furthermore, Norgestrel initiated a bFGF-dependent inactivation of glycogen synthase kinase 3β (GSK3β) through phosphorylation at serine 9. The effects of Norgestrel on GSK3β were dependent on Protein Kinase A (PKA) pathway activation. Specific inhibition of both the PKA and GSK3β pathways prevented Norgestrel-mediated neuroprotection of stressed photoreceptor cells *in vitro*. Involvement of the PKA pathway following Norgestrel treatment was also confirmed *ex vivo*. Therefore, these results indicate that the protective

efficacy of Norgestrel is at least in part, due to the bFGF-mediated activation of the PKA pathway, with subsequent inactivation of GSK3β.

3.2. Introduction

In 2009, a study of the 661W cone photoreceptor-like cell line identified the synthetic progestin 'Norgestrel'; a compound commonly found in the female oral contraceptive pill, as a neuroprotective agent to stressed photoreceptor-like cells *in vitro* (Doonan *et al.*, 2009). Successful treatment of two distinct models of RP: the rd10/rd10 (B6.CXBI-Pde6b^{rd10}/J) mouse model and the Balb/c light damage model followed (Doonan *et al.*, 2011) and Norgestrel was thus identified as a potential drug candidate for the treatment of Retinitis Pigmentosa (Doonan & Cotter, 2012). This study therefore aimed to elucidate the intracellular signalling events associated with Norgestrel-induced protection in stressed photoreceptor cells.

Progesterone is well documented to be protective in many different experimental models of disease and neurodegeneration (Gonzalez Deniselle *et al.*, 2002a, 2002b; Gonzalez *et al.*, 2005; Compagnone, 2008; Espinosa-García *et al.*, 2014; Yousuf *et al.*, 2014; Qin *et al.*, 2015). Affecting several different cellular processes, it can effectively inhibit apoptosis and inflammation (Roglio *et al.*, 2008; Choksuchat *et al.*, 2009; Yu *et al.*, 2010) – fundamental in its potential as a treatment of RP. Despite this, the mechanism by which progesterone works in the CNS and specifically in the retina, remains unclear. Progesterone acts through specific binding to cellular receptors (Friberg *et al.*, 2009) and there is evidence mounting to suggest a subsequent progesterone-induced cascade of hormones and growth factors (Luciano *et al.*, 1994; Peluso & Pappalardo, 1999). Indeed, it is the sequential expression of these factors that is believed to be what ultimately maintains cellular viability (Peluso & Pappalardo, 1999).

The relationship between progesterone and basic fibroblast growth factor (bFGF) plays a central role in the regulation of granulosa cell survival (Peluso & Pappalardo, 1999). Progesterone production promotes an increase in bFGF expression (Guthridge *et al.*, 1992), which in turn maintains cellular viability (Peluso & Pappalardo, 1999). However, bFGF is also known to be neuroprotective in its own right: in the retina, bFGF has been shown to promote photoreceptor cell survival both *in vitro* and *in vivo* (O'Driscoll *et al.*, 2007, 2008; Farrell *et al.*, 2011); whilst in mice, photoreceptor targeted FGF-receptor inactivation leads to retinal degeneration (Campochiaro *et al.*, 1996). Early studies on Norgestrel-injected mice implicated bFGF as a potential regulator of Norgestrel's pro-survival activities (Doonan *et al.*, 2011; Doonan & Cotter, 2012). Consequently, we proposed that Norgestrel could protect stressed photoreceptor cells through an upregulation of bFGF.

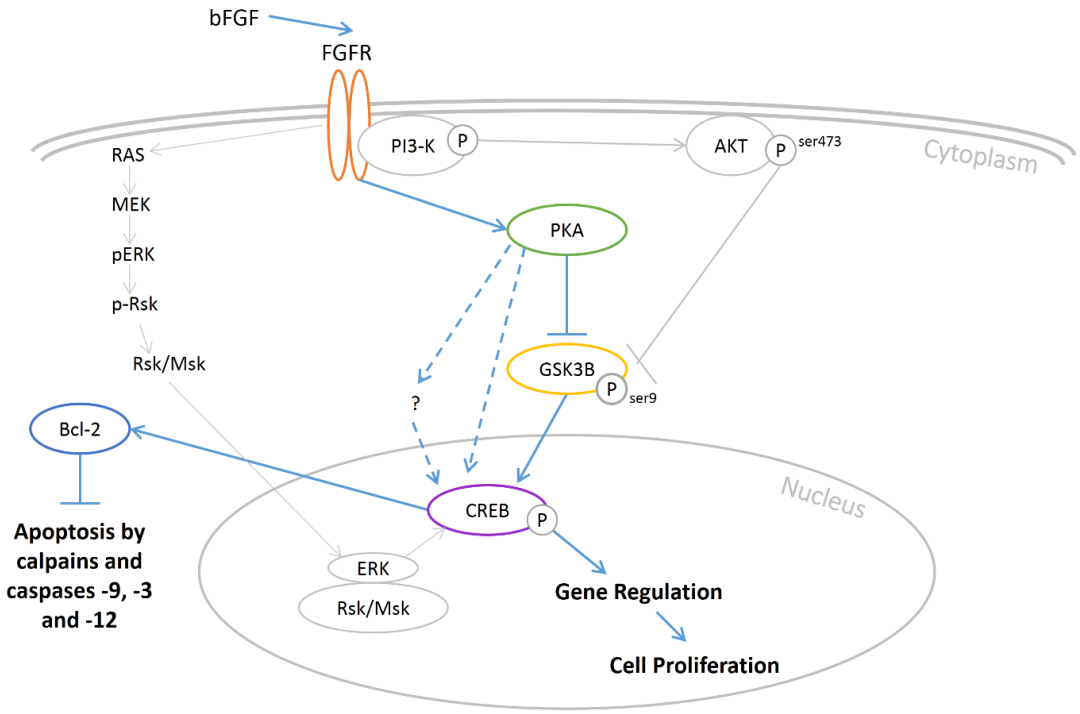


Figure 3.2.1. Pathways previously shown to be activated by basic fibroblast growth factor (bFGF). (Cao *et al.*, 1997; Cheng *et al.*, 1998; Désiré *et al.*, 2000; O'Driscoll *et al.*, 2007, 2008).

bFGF has been linked to several different pro-survival pathways (Figure 3.2.1). Shown in photoreceptor cells to stimulate the production of reactive oxygen species (ROS), it has been documented to activate the phosphatidylinositol 3-kinase-protein kinase B (PI3K-AKT) pathway, mediating neuroprotection (Farrell *et al.*, 2011). bFGF has also been linked to extracellular signal-regulated kinase (ERK) activation (Désiré *et al.*, 2000; O'Driscoll *et al.*, 2007, 2008) and to protein-kinase A (PKA) induced inactivation of glycogen synthase kinase 3β (GSK3β) (Cao *et al.*, 1997; Cheng *et al.*, 1998; O'Driscoll *et al.*, 2007). All taken, we hypothesised that Norgestrel may work to exert its neuroprotective effects through an upregulation of bFGF expression, with subsequent activation of pro-survival signalling pathways. This study aimed to examine this hypothesis in further detail.

3.3. Results

3.3.1. Norgestrel prevents cell death in stressed 661W photoreceptor-like cells *in vitro*

In Retinitis Pigmentosa, cones cells are known to die secondarily to rods (Samardzija *et al.*, 2012). One hypothesis as to why this occurs is that they lose their rod-derived trophic support (Léveillard *et al.*, 2004; Komeima *et al.*, 2006; Finnegan *et al.*, 2010). Though it is defined factors that are important to cone cell survival *in vivo*, rather than the mix of factors provided by serum; we have attempted to mimic this trophic loss *in vitro* by depriving cells of serum. Initiating cell death, serum starvation is an oft-used process by which to simulate photoreceptor activity and cell loss (Gómez-Vicente *et al.*, 2005; Mackey *et al.*, 2008; Guerin *et al.*, 2011). Treatment with Norgestrel can protect from photoreceptor cell death in two models of RP (Doonan *et al.*, 2011). However, in this study, we wished to determine if Norgestrel could also act to prevent from serum starved cell death *in vitro*. To this end, the immortalised 661W cone photoreceptor-like cell line was utilised.

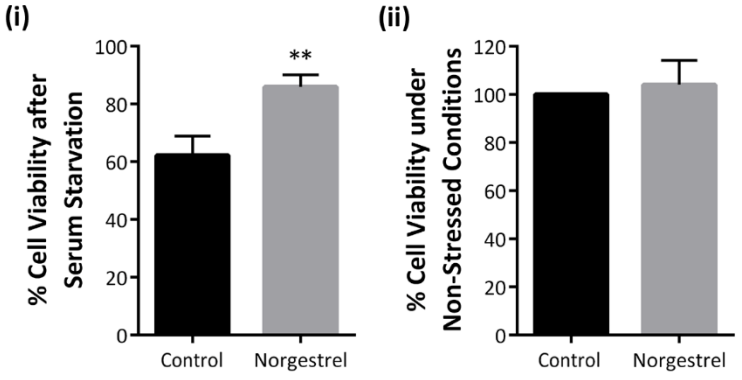


Figure 3.3.1. Treatment with 20μM Norgestrel significantly increased cellular viability of stressed 661W cone photoreceptor-like cells over 24 hours. (i) *Cells were treated with DMSO (Control) or 20μM Norgestrel (Norgestrel) in the absence* ***(i)*** *or presence* ***(ii)*** *of serum over 24 hours and percentage cell viability was assayed (n=4). Results are presented as mean ± SEM. Asterisks indicate significant difference (t-test, **p<0.01).*

661W cells were serum starved over 24 hours and treated with 20µM Norgestrel. Cell viability was then compared to non-serum starved control (100% viability) using the MTS assay; an assay frequently used in ocular studies as a measure of cellular proliferation and viability (Penha *et al.*, 2013; Sobolewska *et al.*, 2013). Norgestrel significantly increased cellular viability after serum deprivation (Figure 3.3.1i) and so it was demonstrated that Norgestrel is neuroprotective to stressed 661W cells.

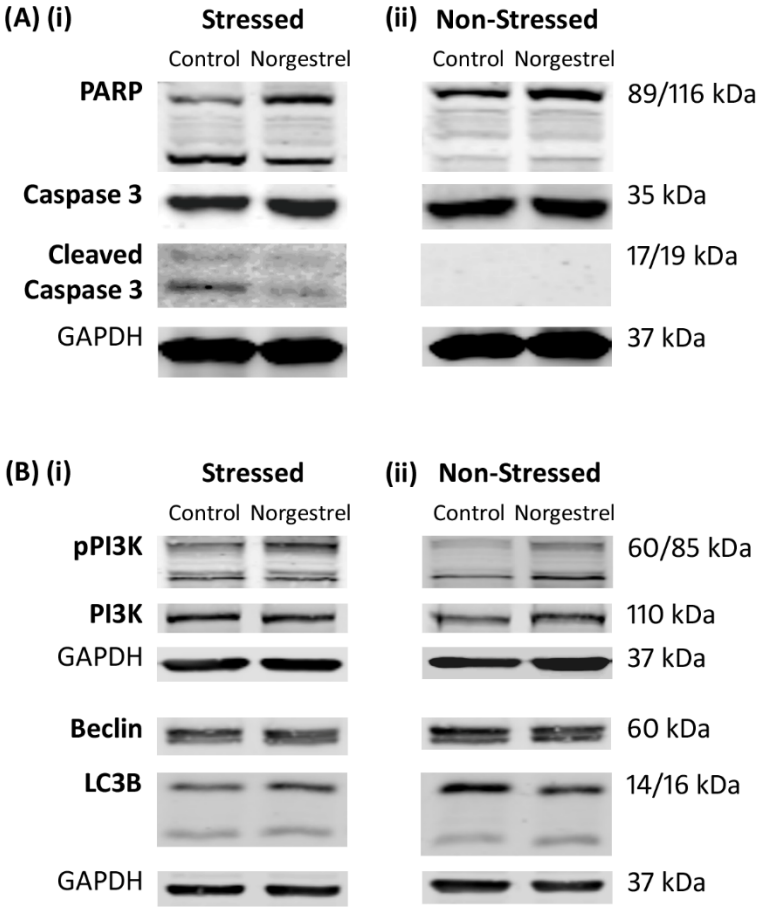


Figure 3.3.2. Norgestrel inactivates pro-apoptotic signalling pathways. (A) (i) *Western blotting demonstrates a decrease in PARP and Caspase-3 cleavage after 24 hours serum starvation (Stressed) in response to 20µM Norgestrel.* ***(B) (i)*** *No change in PI3K and Beclin/LC3B necrotic-associated pathways was determined in stressed cells treated with 20µM Norgestrel.* ***(A) (ii)*** *and* ***(B) (ii)*** *Western blotting revealed no changes in PARP, Caspase-3, PI3K, Beclin or LC3B in healthy cells (Non-Stressed). Equal loading of protein was demonstrated by probing for GAPDH. Blots are representative of three independent experiments.*

Analysis by western blotting confirmed this result. Treatment with Norgestrel decreased apoptotic induced cleavage of Poly (ADP-ribose) polymerase (PARP) and caspase-3 in

stressed cells comparison to DMSO-treated control (Figure 3.3.2Ai), though no changes were seen in the necrotic-associated Phosphoinositide 3-kinase (PI3K) and Beclin/Light Chain 3B (LC3B) pathways (Figure 3.3.2Bi). Norgestrel did not change cellular viability in non-stressed cells (Figure 3.3.1ii); nor could any changes be determined by western blotting (Figure 3.3.2A,Bii).

3.3.2. Norgestrel causes an increase in bFGF production over 6 hours in 661W cells

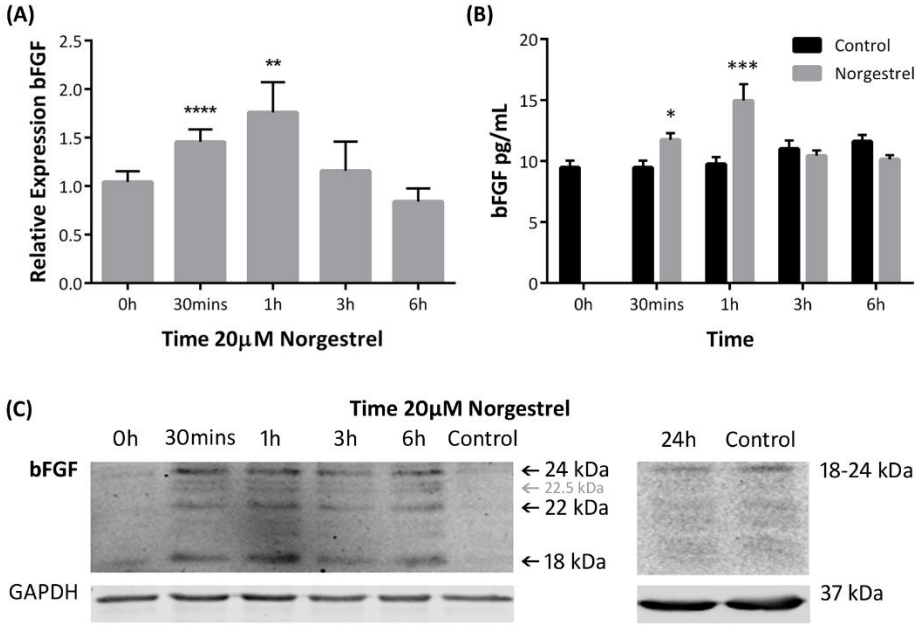


Figure 3.3.3. Treatment with 20µM Norgestrel significantly increased levels of bFGF over 6 hours. *Cells were serum starved and treated with 20µM Norgestrel or the equivalent DMSO control for the indicated time periods.* ***(A)*** *RT-qPCR analysis detected a significant increase in bFGF mRNA levels over 1 hour in response to Norgestrel. Values are relative to timed DMSO control.* ***(B)*** *Analysis by ELISA revealed treatment with Norgestrel triggers significant release of the secreted, 18kDa form of bFGF (pg/mL) over 1 hour.* ***(C)*** *Western blotting demonstrated an increase of three distinct isoforms of bFGF (18kDA, 22kDa, 24kDa) over 6 hours. There is also faint banding for the 22.5kDa form, which may be methylated. Graphical results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001). Equal loading of protein was demonstrated by probing for GAPDH. Blots are representative of three independent experiments.*

Evidence in the literature suggests a role of bFGF in photoreceptor cell survival (O'Driscoll *et al.*, 2007, 2008; Farrell *et al.*, 2011). Therefore, we sought to investigate if bFGF is up-

regulated in photoreceptor cells in response to Norgestrel. Cells were stressed by serum starvation and treated with 20µM Norgestrel or the equivalent DMSO control for the indicated time periods. Figure 3.3.3A demonstrates a significant upregulation of bFGF mRNA over 1 hour relative to timed DMSO control. A single copy gene, bFGF is capable of encoding a secreted 18 kDa low molecular weight (LMW) bFGF isoform (Yu *et al.*, 2007) and several non-secreted, high molecular weight (HMW) isoforms (Florkiewicz & Sommer, 1989; Florkiewicz, *et al.*, 1991a; 1991b; Arese *et al.*, 1999). Analysis by enzyme-linked immunosorbent assay (ELISA) (Figure 3.3.3B) confirmed that treatment with 20µM Norgestrel causes a significant increase in LMW bFGF in the supernatant of photoreceptor cells over 1 hour. Total intracellular levels of bFGF were evaluated by western blotting (Figure 3.3.3C), demonstrating an increase in three distinct isoforms of bFGF over 6 hours. Two faint bands were also observed in Norgestrel treated samples between 22 and 24kDa. Total protein levels of intracellular bFGF were back down by 24 hours (Figure 3.3.3C).

3.3.3. Transfection of 661W photoreceptor-like cells with small interfering ribonucleic acid targeted against bFGF abrogates Norgestrel-mediated protection of stressed cells

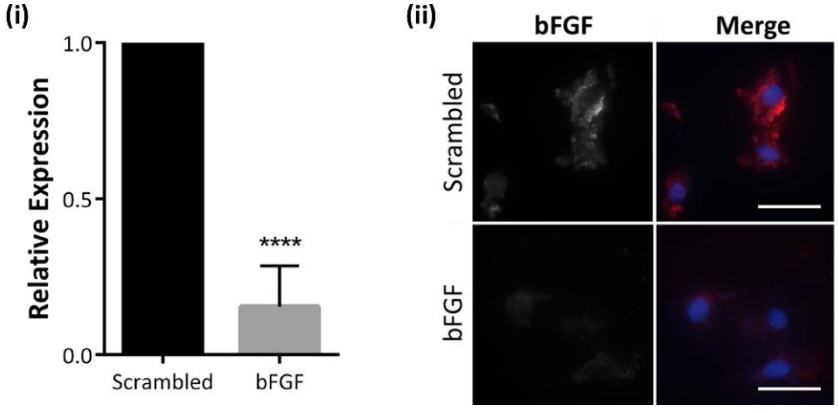


Figure 3.3.4. siRNA knock down of bFGF. *661W cells were transfected with siRNA targeted against bFGF (bFGF) or non-targeting scrambled control (Scrambled). Detection of bFGF mRNA levels by RT-qPCR **(i)** and immunofluorescence **(ii)**. Results are presented as mean ± SEM. Asterisks indicate significant difference (t-test, ****p<0.001). Scale bars 30µm.*

Norgestrel significantly inhibits 661W cells from serum starved cell death over 24 hours (Figure 3.3.1i, 3.3.5A). Having established that administration of Norgestrel causes an upregulation of bFGF over 6 hours (Figure 3.3.3), we then aimed to determine if bFGF is responsible for the neuroprotective effects afforded by Norgestrel. Photoreceptors were treated with small interfering ribonucleic acid (siRNA) against bFGF (Figure 3.3.4) and a significant decrease in bFGF expression (~80%) was verified through RT-qPCR (Figure 3.3.4i) and immunofluorescence (Figure 3.3.4ii).

Knock down of bFGF did not decrease cellular viability relative to non-targeting scrambled control (Scrambled) as measured by MTS assay (Figure 3.3.5B). siRNA treated, stressed cells were then treated with either DMSO or 20µM Norgestrel over 24 hours. No change in viability in these cells was determined compared to the relative DMSO control (Figure 3.3.5C).

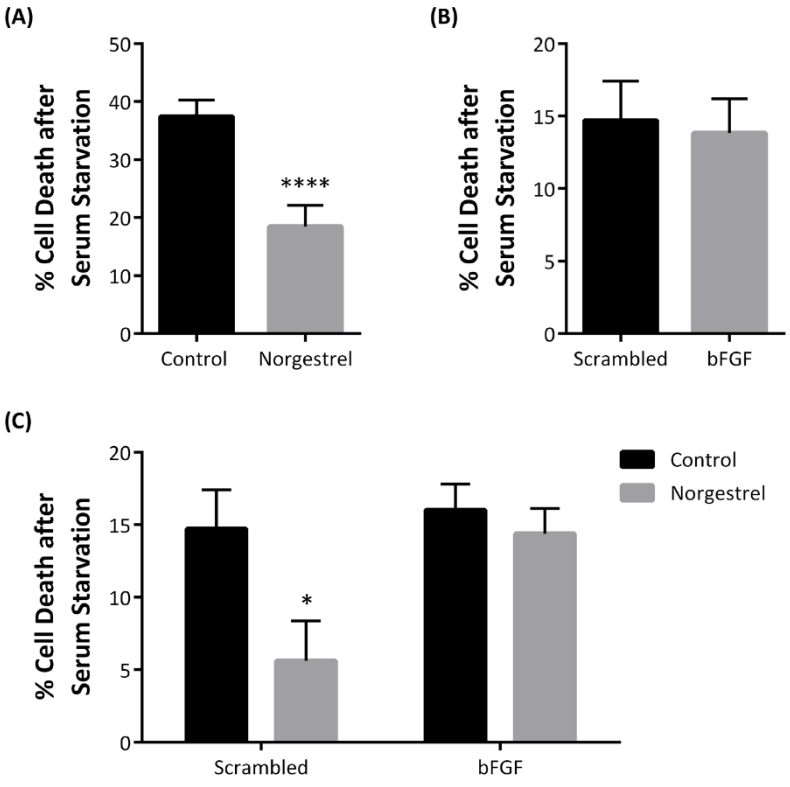


Figure 3.3.5. siRNA knock down of bFGF abrogates the neuroprotective effects of Norgestrel on stressed 661W cells. (A) *Administration of 20µM Norgestrel significantly decreases serum starved cell death over 24 hours.* ***(B, C)*** *661W cells were transfected with siRNA targeted against bFGF (bFGF) or non-targeting scrambled control (Scrambled).* ***(B)*** *Cells transfected with siRNA targeted against bFGF were assayed for cell death over 24 hours (n=4).* ***(C)*** *Transfected cells were serum starved over 24 hours in the presence of DMSO (Control) or 20µM Norgestrel (Norgestrel) and percentage cell death was assayed (n=4). Results are presented as mean ± SEM. Asterisks indicate significant difference (t-test, *p<0.05, ****p<0.001).*

3.3.4. Norgestrel mediates the inactivation of GSK3β in stressed photoreceptor cells through bFGF, but dos not activate AKT or ERK1/2

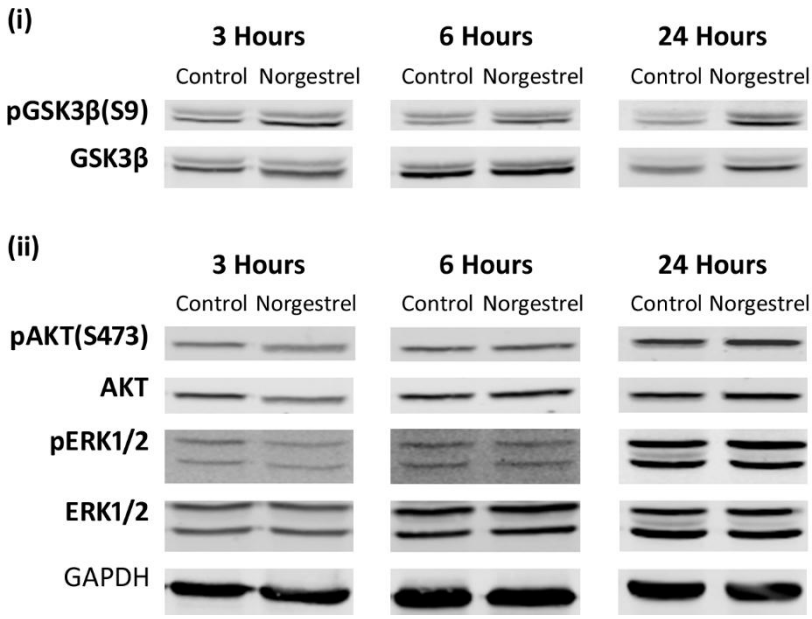


Figure 3.3.6. GSK3β is inactivated in stressed photoreceptor cells treated with 20μM Norgestrel. (i) *Cells were serum starved and treated with DMSO or 20μM Norgestrel for the indicated times. Western blotting revealed Norgestrel induced an inactivation of GSK3β through phosphorylation on serine 9 (GSK3β(S9)) over 24 hours.* ***(ii)*** *No changes in phosphorylation of AKT or ERK was seen in stressed cells. Equal loading of protein was demonstrated by probing for GAPDH. Blots are representative of three independent experiments.*

The PI3-kinase/AKT pathway, the MAP-kinase/ERK1/2 pathway and the PKA pathway are known to converge on GSK3β (Figure 3.2.1). Established as pro-survival pathways in photoreceptor cells, they are activated in response to bFGF (Hossain *et al.*, 2002; Mao & Lee, 2005; O'Driscoll *et al.*, 2007, 2008). Therefore, we hypothesised that Norgestrel was likely protecting cells from cell death through one or several of these systems. 661W cells were serum starved over 3, 6 and 24 hours in the presence of DMSO (control) or 20μM Norgestrel. The phosphorylation states of GSK3β, AKT and ERK1/2 were measured by western blotting (Figure 3.3.6). Treatment of stressed cells with 20μM Norgestrel induced a gradual increase in the phosphorylation of GSK3β on serine 9 (pGSK3β(S9)) over 24 hours (Figure 3.3.6i). No changes could be determined in stressed photoreceptor cells in the AKT or ERK1/2 pathways over 24 hours (Figure 3.3.6ii). No changes in any pathway was seen in non-stressed cells (Figure 3.3.7i, Figure 3.3.7ii).

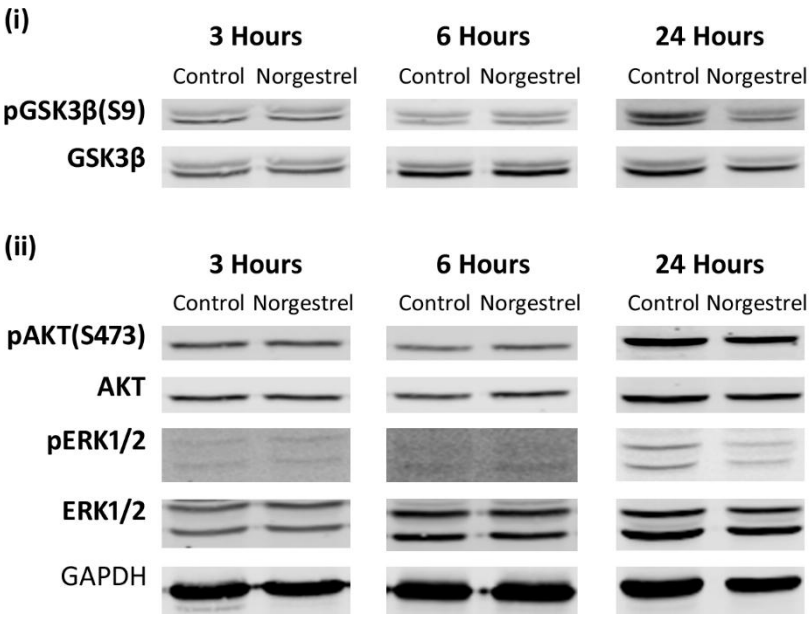


Figure 3.3.7. Norgestrel had no effect on phosphorylation of (i) GSK3β, (ii) AKT or ERK in non-stressed cells. *Equal loading of protein was demonstrated by probing for GAPDH. Blots are representative of three independent experiments.*

To determine if the changes seen in the phosphorylation state of GSK3β were bFGF-dependent, 661W photoreceptor-like cells were treated with siRNA against bFGF. siRNA-treated cells were then stressed by serum starvation and treated with either DMSO or 20μM Norgestrel over 24 hours. siRNA treated, stressed cells saw no change in levels of pGSK3β(S9) when treated with Norgestrel (Figure 3.3.8). Alternately, cells treated with a non-targeting scrambled siRNA retained the Norgestrel-mediated increase in both pGSK3β(S9) and total GSK3β seen in non-transfected, stressed, Norgestrel treated cells (Figure 3.3.8).

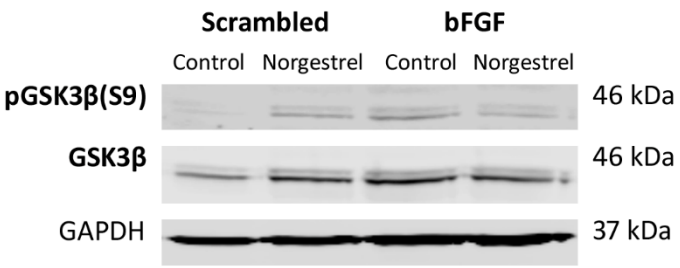


Figure 3.3.8. Norgestrel-induced phosphorylation of GSK3β is bFGF dependent. *661W cells were transfected with siRNA targeted against bFGF (bFGF) or non-targeting scrambled control (Scrambled) and serum starved over 24 hours in the presence of DMSO (Control) or 20μM Norgestrel (Norgestrel). Western blotting analysis showed no change in phosphorylation of GSK3β when treated with bFGF specific-siRNA (bFGF). Equal loading of protein was demonstrated by probing for GAPDH. Blots are representative of three independent experiments.*

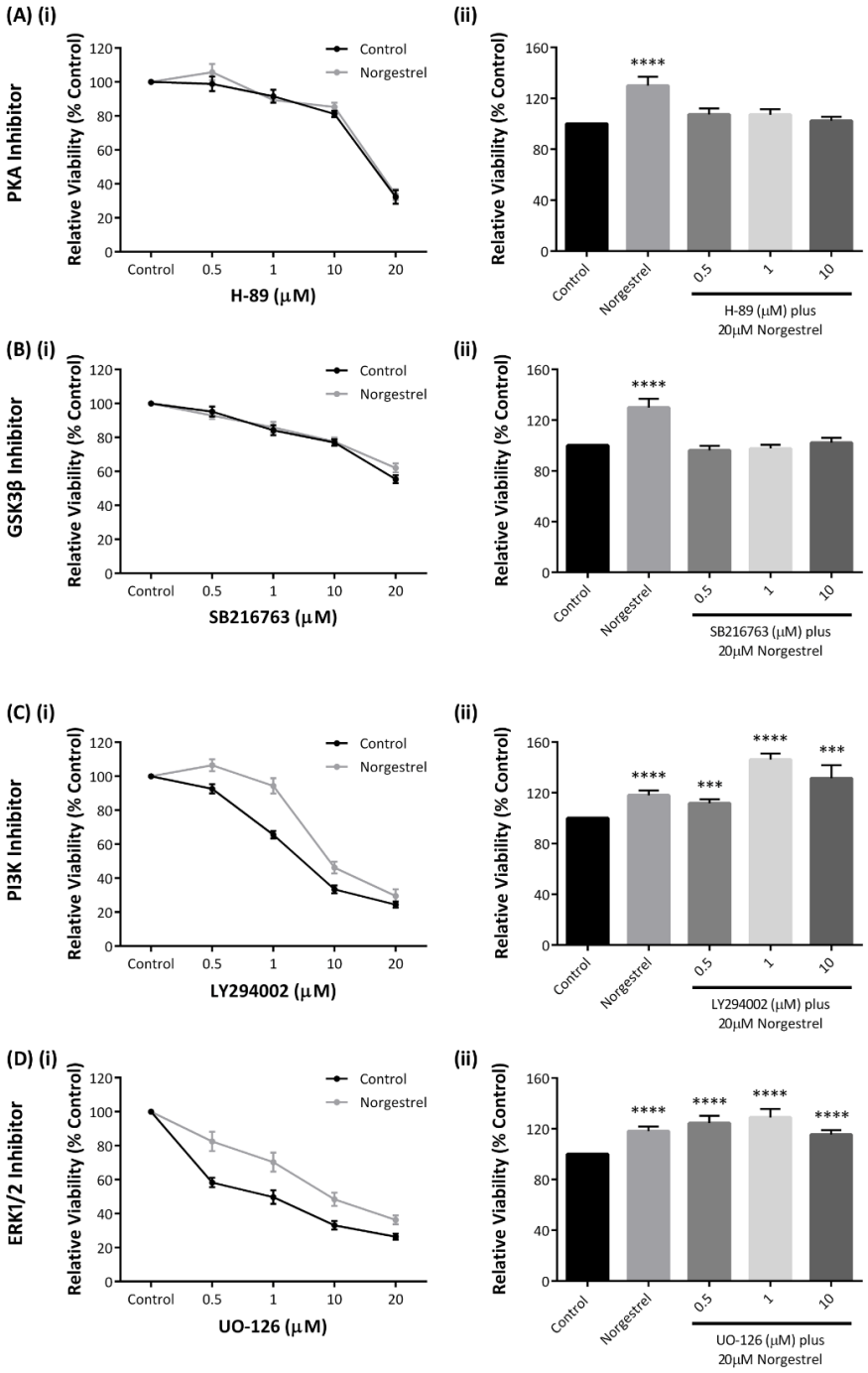


Figure 3.3.9. The neuroprotective effects of Norgestrel are mediated by protein kinase A. (A - D) (i) *Dose response curve measuring cell viability of 661W cells treated with increasing concentrations (μM) of inhibitors against Protein Kinase A (PKA) (H-89), Glycogen Synthase Kinase 3ϐ (GSK3ϐ) (SB216763), phosphatidylinositol 3-kinase (PI3K) (LY294002) and extracellular signal-related kinase 1/2 (ERK1/2) (UO-126), in the presence of Norgestrel (Norgestrel) or DMSO control (Control). Values are calculated as a percentage of viable cells, compared to the relevant 100% viable Norgestrel or DMSO control.* ***(A, B) (ii)*** *Abrogation of the Norgestrel-mediated rescue of photoreceptor cells was achieved through PKA* ***(A) (ii)*** *and GSK3ϐ* ***(B) (ii)*** *inhibition with H-89 and SB216763 respectively.* ***(C, D) (ii)*** *Norgestrel-induced neuroprotection was not blocked by PI3K inhibition with LY294002* ***(C) (ii)****, nor by UO-126 inhibition of ERK1/2 phosphorylation* ***(D) (ii)****. Values are expressed as a percentage of control viability. An individual control was carried out for each inhibitor concentration. This is represented by the single control bar shown. Results are presented as mean ± SEM, n=4. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001*

3.3.5. Inhibition of PKA but not AKT or ERK1/2 abolishes the neuroprotective effects of Norgestrel against serum starved cell death

In order to discount the actions of the AKT and ERK1/2 pathways in the protective actions of Norgestrel, 661W cells were stressed and treated with increasing concentrations of pathway-specific inhibitors over 24 hours (Figure 3.3.9). Cell viability was assessed by MTS assay and a dose response curve measuring cell viability with increasing concentrations of inhibitor (both in the absence and presence of Norgestrel) was constructed, using either Norgestrel or DMSO-treated cells as the relevant 100% control (Figure 3.3.9A-Di). Norgestrel did not protect photoreceptor cells from serum starved cell death when treated with either PKA-inhibitor H-89 or GSK3β-inhibitor SB216763 (Figure 3.3.9A,Bii), indicating that Norgestrel is working at least in part through PKA-mediated inhibition of GSK3β. Conversely, when stressed cells were treated with either AKT-inhibitor LY294002 or ERK1/2 inhibitor UO126, Norgestrel-mediated neuroprotection was conserved (Figure 3.3.9C,Dii).

3.3.6. The protection afforded to SNP-treated retinal explants by Norgestrel, is abrogated through specific inhibition of PKA by H-89

Postnatal day 30 (P30) explants from C57BL/6 mice, treated over 24 hours with 300µM nitric oxide donor Sodium Nitroprusside (SNP) (Doonan *et al.*, 2011) undergo cell death in all layers of the retina compared to untreated control (Figure 3.3.10i). SNP-induced cell death was successfully inhibited through 1 hour pre-treatment with 20µM Norgestrel (Figure 3.3.10ii). To determine if PKA was responsible for the protective properties of Norgestrel *ex vivo*, retinal explants were treated with PKA specific inhibitor H-89. Optimum inhibitor concentration was determined in 661W cells (Figure 3.3.9Ai), whereby 0.5µM H-

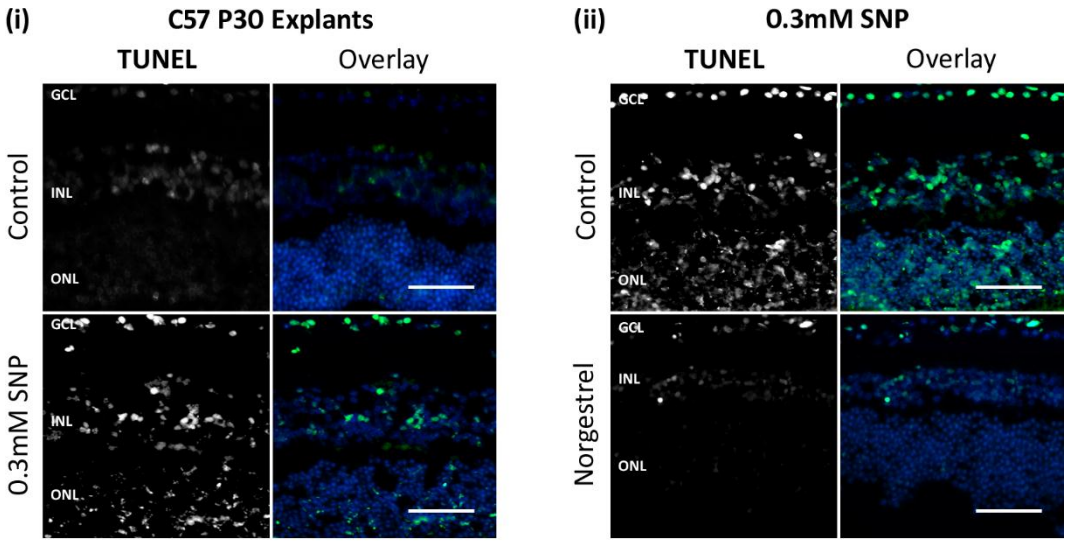


Figure 3.3.10. Norgestrel prevents SNP-induced cell death ex vivo. *Retinal explants from postnatal day 30 C57BL/6 mice were cultured over 24 hours. Apoptosis of retinal cells was detected by terminal dUTP nick end-labelling (TUNEL) of DNA strand breaks in cell nuclei.* ***(i)*** *Treatment with 0.3mM sodium nitroprusside (SNP) increases apoptotic cell death in all nuclear layers of the retina compared to untreated control.* ***(ii)*** *Treatment with 20µM Norgestrel inhibits SNP-induced apoptosis in all nuclear retinal layers. Results are representative of three independent experiments. GCL, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars 50µm.*

89 did not decrease cellular viability compared to control, yet still prevented Norgestrel-induced protection. Therefore, this concentration was carried forward for use in retinal explants. Explants incubated with 0.5µM H-89 alone over 24 hours did not show an increase in TUNEL-positive cells compared to control (Figure 3.3.11i). Explants were then pre-treated for 1 hour with 0.5µM H-89 and 20µM Norgestrel or equivalent DMSO control, before the addition of 300µM SNP for a further 24 hours. It was not possible to determine any major differences in TUNEL-positive staining across any of the PKA-inhibited explants, when treated with 20µM Norgestrel, compared to control (Figure 3.3.11ii).

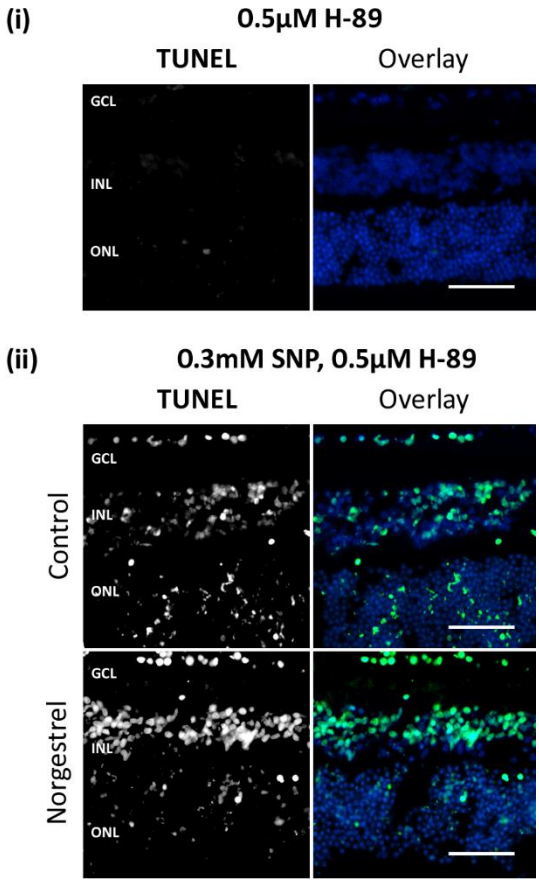


Figure 3.3.11. Specific inhibition of PKA by H-89 prevents Norgestrel-induced protection ex vivo. *Retinal explants from postnatal day 30 C57BL/6 mice were cultured over 24 hours. Apoptosis of retinal cells was detected by terminal dUTP nick end-labelling (TUNEL) of DNA strand breaks in cell nuclei.* ***(i)*** *Retinal explants were treated with 0.5µM PKA inhibitor H-89 over 24 hours.* ***(ii)*** *PKA inhibited, SNP-stressed explants treated with either 20µM Norgestrel or DMSO control demonstrated comparable TUNEL staining. Results are representative of three independent experiments. GCL, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars 50µm.*

3.4. Discussion

Studies from our laboratory identified Norgestrel, a synthetic progestin, as a potential therapeutic in the treatment of Retinitis Pigmentosa (RP) (Doonan *et al.*, 2011; Doonan & Cotter, 2012). However, to date, limited study has been carried out in to the potential downstream signalling targets of this compound. The present study provides the first evidence that Norgestrel initiates its pro-survival cellular activities both *in vitro* and *ex vivo* through bFGF-mediated activation of the protein kinase A (PKA) pathway.

Serum starvation of 661W cells induces cell death (Gómez-Vicente *et al.*, 2005). Poly (ADP-ribose) polymerase (PARP) and Caspase 3 cleavage are documented as key events in the initiation of apoptosis in photoreceptor cells (Gómez-Vicente *et al.*, 2005; Miller *et al.*, 2006; Sahaboglu *et al.*, 2013). Norgestrel was effectively able to prevent this cleavage in stressed cells (Figure 3.3.2Ai), though had no effect on non-apoptotic cell death pathways PI3K, Beclin and LC3B (Huang *et al.*, 2013; Wang *et al.*, 2013) (Figure 3.3.2Bi). Since Norgestrel significantly increased cellular viability in stressed cells by approximately 25% (Figure 3.3.1i) over 24 hours, we hypothesise that Norgestrel acts to protect 661W cells from cell death through prevention of apoptotic PARP and caspase-3 cleavage. However, due to emerging evidence suggesting that photoreceptor cell death in retinal degenerations may also involve non-apoptotic mechanisms (Sancho-Pelluz *et al.*, 2008; Arango-Gonzalez *et al.*, 2014), we cannot rule out the possibility that Norgestrel may protect against cell death through prevention of another non-apoptotic cell death pathway.

Intraperitoneal injection of Norgestrel to wild-type C57BL/6 mice and the rd10/rd10 (B6.CXBI-Pde6b^{rd10}/J) mouse model of RP causes an upregulation of basic fibroblast growth factor (bFGF) over just 3 hours (Doonan *et al.*, 2011). bFGF is a pleiotropic growth factor that regulates a variety of processes including cellular growth, angiogenesis, tissue injury healing and neuroprotection (Szabo *et al.* 1994; Ernst *et al.* 2001; Yoshida *et al.* 2004; O'Driscoll *et al.* 2007; O'Driscoll *et al.* 2008; Zhao *et al.* 2014; Wang *et al.* 2015). Most of these actions have been demonstrated with regard to the 18 kDa low molecular weight (LMW) bFGF isoform (Szabo *et al.*, 1994; Ernst *et al.*, 2001), which is secreted by cells via an unconventional ER/Golgi-independent pathway (Florkiewicz *et al.*, 1995) and activates cell membrane FGF receptors (Spivak-Kroizman *et al.*, 1994; Chlebova *et al.*, 2009). However, in many systems (e.g. cells exposed to stressors, or in some tissue such as the brain), the single copy bFGF gene additionally encodes non-secreted, high molecular weight (HMW) bFGF protein isoforms (Florkiewicz & Sommer, 1989; Florkiewicz *et al.*, 1991a, 1991b; Arese *et al.*, 1999). These are extended versions of 18 kDa LMW bFGF, containing two functional domains: a common 18 kDa amino acid sequence and an amino terminal extension (Florkiewicz *et al.*, 1991a; Vagner *et al.*, 1996; Arese *et al.*, 1999; Chlebova *et al.*, 2009). Each HMW isoform is a distinct gene product and is not a precursor of the 18 kDa isoform (Florkiewicz & Sommer, 1989; Florkiewicz *et al.*, 1991a; Arese *et al.*, 1999). Different cellular functions are attributable to differing bFGF isoforms (Pasumarthi *et al.* 1996; Davis *et al.* 1997; Joy *et al.* 1997; Grothe *et al.* 1998), suggesting that isoforms of bFGF may act through distinct pathways. Specifically, HMW isoforms appear to function intracellularly, independently of cell-surface FGF receptors (Arese *et al.*, 1999).

In this study, Norgestrel administration to stressed 661W cells caused a significant upregulation of bFGF mRNA expression (Figure 3.3.3A). Analysis by enzyme-linked

immunosorbent assay (ELISA) revealed a significant release of the LMW 18 kDa in to the cell culture supernatant over 1 hour (Figure 3.3.3B). This rapid, transient release is characteristic of LWM bFGF: LMW bFGF complexes with FGF receptors on the cell membrane, initiating internalisation of the ligand-receptor complex and subsequent trafficking back to the nucleus (Bikfalvi *et al.* 1995; Arese *et al.* 1999; Yu *et al.* 2007). Evaluation by western blotting revealed Norgestrel also caused an upregulation of intracellular HMW (22 and 24kDa) bFGF in stressed photoreceptor cells, in addition to LMW (18kDa) bFGF. This upregulation was maintained over 6 hours (Figure 3.3.3C). Two faint bands between 22 and 24kDa were also observed in Norgestrel treated samples. The HMW forms (22, 22.5, and 24 kDa) of bFGF may be post-translationally modified, resulting in a 1- to 2-kDa increase in apparent molecular mass (Pintucci *et al.*, 1996; Klein *et al.*, 2000). Consequently, we postulate that the extra banding may be accounted for by methylation of the HMW forms. The upregulation of HMW bFGF by Norgestrel is of interest, for it is HWM bFGF and not the secreted LMW bFGF, that have been shown to affect viability of cells under trophic factor deprivation (Quarto *et al.*, 1991; Bikfalvi *et al.*, 1995). Unsurprisingly, expression of bFGF was back down by 24 hours (Figure 3.3.3C). The half-life of bFGF is only 4.7 hours and all mitogenic activity will be lost after 24 hours (Shiba *et al.*, 2003). All taken, these results, along with previous knowledge from the literature, suggest that Norgestrel is initiating its pro-survival activity at least in part, through the upregulation of bFGF. The varying upregulation and release patterns of bFGF also imply that Norgestrel can initiate distinct cellular activities through the activation of different bFGF isoforms.

Administration of Norgestrel to stressed 661W cells causes an upregulation of bFGF (Figure 3.3.3) and significantly inhibits cell death (Figure 3.3.1). Consequently, we hypothesised that if Norgestrel was mediating its actions through bFGF, specific siRNA knock down of this

growth factor would abrogate the neuroprotection. Indeed, in the absence of bFGF (~80% siRNA knock down, Figure 3.3.4i), Norgestrel did not afford the same level of protection as the Norgestrel treated control (Figure 3.3.5C), demonstrating that Norgestrel-induced protection is bFGF-dependent.

Worthy of note, is that specific siRNA targeted to bFGF did not result in any decrease in cellular viability in comparison to control (Figure 3.3.5B). This is key to our understanding of Norgestrel's neuroprotective activities. Under conditions of trophic factor deprivation, photoreceptor cell death is initiated (Gómez-Vicente *et al.*, 2005; Mackey *et al.*, 2008) (Figure 3.3.1i). bFGF is known to be neuroprotective to many cells, including photoreceptors (O'Driscoll *et al.*, 2007, 2008; Del Río *et al.*, 2011; Doonan *et al.*, 2011; Florkiewicz *et al.*, 2011; Wang *et al.*, 2015). Stressed photoreceptors however, do not up-regulate bFGF transcription (Figure 3.3.3C, 0h control) without an external stimulus, e.g. Norgestrel. Protective bFGF is not produced under basal conditions in photoreceptor cells and so specific siRNA knock down of bFGF does not affect cellular viability (Figure 3.3.5B). This further substantiates the particular importance of Norgestrel-induced bFGF activation in stressed photoreceptor cells and its subsequent neuroprotective actions (Figure 3.3.5C).

It is well established in the literature that both Extracellular Signal-Regulated Kinase (ERK) and Protein Kinase B (AKT), can play a key role in multiple cellular processes in the retina. These include glucose metabolism, apoptosis, cell proliferation, transcription and cell migration (Yuan *et al.* 2012; Jin *et al.* 2013; Yang *et al.* 2013; Kucharska *et al.* 2014). Moreover, both AKT and ERK activation has been demonstrated to mediate the actions of bFGF (Eves *et al.*, 1998; Désiré *et al.*, 2000; Wahlin *et al.*, 2000; Kinkl *et al.*, 2001; Hossain *et*

al., 2002; Mao & Lee, 2005) and both pathways have been implicated in the neuroprotection of retinal neurons (Liu *et al.* 1998; Nakazawa *et al.* 2002; Soto *et al.* 2006). As a result, we decided to look at the activation of these pathways in response to Norgestrel. Norgestrel did not stimulate any change in the phosphorylation status of AKT or ERK1/2 in either stressed or non-stressed 661W cells over 24 hours (Figure 3.3.6ii, 3.3.7ii). This result was further validated through inhibitor studies with PI3K pathway inhibitor LY294002 (Figure 3.3.9C) and MAPK/ERK1/2 pathway inhibitor UO-126 (Figure 3.3.9D). Neither inhibitor had any effect on the capacity of Norgestrel to rescue photoreceptor cells from cell death. Therefore, we hypothesise that the actions of Norgestrel in the rescue of stressed 661W cells *in vitro* is independent of the PI3K and ERK1/2 pathways. This finding is consistent with several previous studies showing that neither pathway is critical in photoreceptor cell rescue following stress both *in vivo* and *in vitro* (O'Driscoll *et al.*, 2007, 2008; Bürgi *et al.*, 2009). The possibility of the involvement of PI3K and ERK1/2 in photoreceptor cell rescue following other cell stressors however, cannot be ruled out (Forkwa *et al.*, 2014; Makarev *et al.*, 2014).

Norgestrel increased levels of pGSK3β(S9) in stressed 661W cells over 24 hours (Figure 3.3.6i), a process known to prevent apoptosis (Li *et al.*, 2000; O'Driscoll *et al.*, 2007). Selective inhibition of the GSK3β pathway by SB216763 abolished the protective effects of Norgestrel on stressed 661W cells (Figure 3.3.9B). GSK3β is reported to be an essential downstream regulator of PI3K/AKT (Crowder & Freeman, 2000), ERK-associated pathway RSK (Torres *et al.*, 1999) and PKA (Li *et al.*, 2000) (Figure 3.2.1). However, given that the protective capabilities of Norgestrel appear to be independent of the AKT and ERK pathways (Figure 3.3.9C,D), we looked to see if Norgestrel was working through PKA-dependent phosphorylation of GSK3β. Treatment with PKA-selective inhibitor H-89 over 24

hours successfully prevented the protective capabilities of Norgestrel on stressed 661W cells (Figure 3.3.9A), implicating its role in Norgestrel-mediated neuroprotection. Interestingly, recently it has also been shown that increased total levels of GSK3β will also contribute to overall cell survival (Rådinger *et al.*, 2011). Norgestrel appears to elevate levels of total GSK3β in stressed 661W cells (Figure 3.3.6i). Therefore, we theorise that it is not only Norgestrel's ability to increase pGSK3β(S9) that contributes to its pro-survival capacity; but also its potential to maintain total levels of GSK3β.

bFGF can stimulate PKA activation in several retinal systems (Cheng *et al.*, 1998; O'Driscoll *et al.*, 2008; Farrell *et al.*, 2011). Therefore, in order to determine if the Norgestrel-induced phosphorylation of GSK3β is bFGF-dependent, cells were treated with specific siRNA targeted against bFGF. These cells were subsequently stressed and treated with Norgestrel and no ensuing change in pGSK3β could be determined. Alternately, cells treated with a non-targeting scrambled control retained the Norgestrel-mediated increase in both pGSK3β(S9) and total GSK3β (Figure 3.3.8). Thus, it is likely that Norgestrel is protecting through the bFGF-mediated, PKA-dependent inactivation of GSK3β.

Thus far, all results indicated that Norgestrel acts to protect 661W cone photoreceptor-like cells from apoptotic cell death through the PKA pathway. Therefore, in order to determine if Norgestrel also acts through the PKA pathway in the retina *ex vivo*, the nitric oxide donor sodium nitroprusside (SNP) was used as previously described to induce retinal cell death (Mastrodimou *et al.*, 2008; Doonan *et al.*, 2011). C57BL/6 retinal explants, cultured with 300µM of SNP over 24 hours (Doonan *et al.*, 2011), stained for large amounts of DNA strand breaks (TUNEL-positive staining) in all nuclear layers of the retina (Figure 3.3.10i).

Pre-treatment with Norgestrel successfully reduced TUNEL-positive staining in all retinal layers (Figure 3.3.10ii), suggesting that Norgestrel is protective against SNP-induced cell death in retinal explants. Stressed retinal explants treated with PKA inhibitor H-89 alone or in combination with Norgestrel showed similar levels of cell death (Figure 3.3.11ii). Therefore, we can confirm that PKA is involved in Norgestrel-mediated neuroprotection not only in stressed 661W cells, but also in SNP-stressed retinal cells, *ex vivo*.

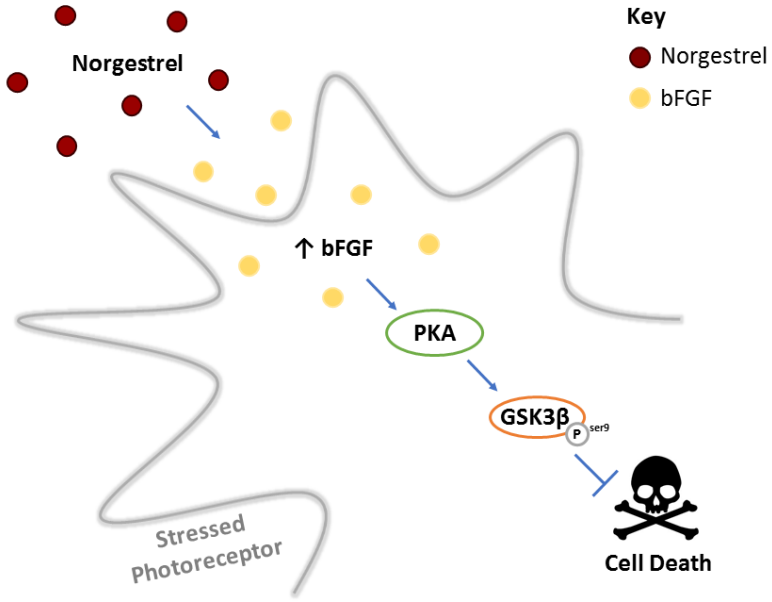


Figure 3.4.1. Schematic to show the hypothesised pro-survival mechanism of action of Norgestrel in stressed photoreceptor cells.

To conclude, we have shown that Norgestrel is capable of rescuing 661W cells from apoptotic cell death through prevention of PARP and Caspase-3 cleavage (Figure 3.3.2Ai). Norgestrel significantly up-regulates the expression of both secreted low molecular weight (LMW) bFGF and non-secreted high molecular weight (HMW) bFGF over 6 hours (Figure 3.3.3C). This upregulation is crucial in potentiating Norgestrel's pro-survival capabilities, for specific siRNA knock down of this growth factor abrogates Norgestrel's protective effects on stressed 661W cone photoreceptor-like cells *in vitro* (Figure 3.3.5C). Finally, we have

demonstrated that Norgestrel-induced upregulation of bFGF in stressed cells leads to subsequent inactivation of GSK3β through phosphorylation on serine 9 (Figure 3.3.8). This inactivation is hypothesised to be through PKA, for selective inhibition of the PKA pathway by H-89 prevented Norgestrel from protecting 661W cells against serum starved cell death (Figure 3.3.9Aii) and SNP-induced cell death in stressed retinal cells, *ex vivo* (Figure 3.3.11ii). A schematic has been provided in Figure 3.4.1. showing this hypothesised mechanism of action.

Chapter 4. Progesterone receptor signalling in retinal cell neuroprotection: Norgestrel mediates its protective effects through progesterone receptor membrane complex one

4.1. Abstract

'Norgestrel', identified as potential drug candidate for the treatment of the degenerative eye disease Retinitis Pigmentosa (RP), is a synthetic form of the female hormone progesterone. Consequently, it must signal through progesterone receptors. Unfortunately, no work has yet given a conclusive overview of the progesterone receptors present in the eye and so thus far, no work has looked at the Norgestrel's specific cellular target. Therefore, this study aimed to identify the receptor target of Norgestrel and begin to examine its potential mechanism of action in the retina.

In this work, we identify and characterise the expression of progesterone receptors present in the C57 wild type and rd10 mouse model of RP, and in the 661W cone photoreceptor cell line. Classical progesterone receptors A and B (PR A/B), progesterone receptor membrane components 1 and 2 (PGRMC1, PGRMC2) and membrane progesterone receptors (mPR) α, β and γ were found to be expressed. All receptors excluding PR A/B were also found in the 661W photoreceptor cell line. PGRMC1 is a key regulator of apoptosis and its expression is up-regulated in the degenerating rd10 mouse retina. Activated by Norgestrel through nuclear trafficking, siRNA knock down of PGRMC1 abrogated the protective properties of Norgestrel on damaged photoreceptors. Furthermore, specific inhibition of PGRMC1 by AG205 blocked Norgestrel-induced protection in stressed retinal explants. Therefore, we conclude that PGRMC1 is crucial to the neuroprotective effects of Norgestrel on stressed photoreceptors.

4.2. Introduction

In the previous chapter, we begin to elucidate the downstream signalling pathways associated with the neuroprotective properties of 'Norgestrel' in stressed photoreceptor cells. However, currently, very little is known as to the specific receptor associated with progesterone-induced retinal neuroprotection. This study therefore aimed to identify the receptor target of Norgestrel with the aim of further elucidating its specific mechanism of action.

Steroid hormones and their metabolites in the central nervous system (CNS) are commonly thought of as neurosteroids (Baulieu, 1991). Their neuroprotective properties have been defined in several different systems (Compagnone, 2008; Borowicz *et al.*, 2011). Progesterone is one of these neurosteroids, the effects of which are well documented in many different experimental models of disease and neurodegeneration: spinal cord injury, brain ischemia, stroke, Alzheimer's disease and perhaps most prominently, traumatic brain injury (TBI) (Gonzalez Deniselle *et al.* 2002a, 2002b; Gonzalez *et al.* 2005; Compagnone 2008; Espinosa-García *et al.* 2014; Yousuf *et al.* 2014; Qin *et al.* 2015). Furthermore, studies highlighting its safety and viability in a number of different models of TBI have ensured progesterone's entry into human clinical trials (Stein 2011; Stein 2013; Stein *et al.* 2015). Progesterone is believed to exert its neuroprotective effects through several different pathways. Perhaps most relevant to our studies is its ability to inhibit apoptosis and inflammation (Roglio *et al.* 2008; Choksuchat *et al.* 2009; Yu *et al.* 2010) – crucial in the protection of our degenerative rd10 mouse model.

The exact mechanism by which progesterone works in the CNS remains unclear. Similar to most neurosteroids, progesterone acts through specific binding to cellular receptors (Friberg *et al.*, 2009). The classic progesterone receptors A and B (PR A/B) are widely expressed in the central nervous system and have a variety of physiological functions (Wen *et al.*, 1994; Lange, 2008). Known as nuclear-localised receptors that regulate gene transcription (Li *et al.*, 1997), PR A/B has also been found localised to, or near the plasma membrane (Lange, 2008). Here they can interact with signal transduction kinases such as proto-oncogene tyrosine-protein kinase Src and can activate the mitogen activated protein kinase (MAPK) pathway (Faivre & Lange, 2007). Progesterone is a complex hormone however and there is growing evidence that progestins achieve their neuroprotective effects through non-classical progesterone receptors (Pang and Thomas 2011; Moussatche and Lyons 2012; Petersen *et al.* 2013; Qin *et al.* 2015). The progesterone receptor membrane component (PGRMC) family, featuring PGRMC1 and PGRMC2 (Cahill, 2007; Lösel *et al.*, 2008), is a prominent candidate for mediating the anti-apoptotic actions of progesterone, as is the Progestin and AdipoQ (PAQR) family of receptors (Zhu, Bond, *et al.*, 2003).

PGRMC1 is a small, complex molecule with multiple signalling domains (Petersen *et al.*, 2013). It is known to bind several steroid hormones, though progesterone is bound with the highest affinity (Meyer *et al.*, 1996) initiating a number of different responses. The signalling mechanisms through which PGRMC1 may act are incredibly diverse, for PGRMC1 has multiple partner proteins. These enable the regulation of functions such as damage repair, drug and hormone metabolism, apoptosis suppression, membrane trafficking and repression of gene transcription (Peluso *et al.* 2006; Cahill 2007; Peluso *et al.* 2008a, 2008b; Rohe *et al.* 2009; Peluso *et al.* 2012). One such binding partner is PGRMC2 (Intlekofer &

Petersen, 2011; Petersen *et al.*, 2013). Structurally similar to PGRMC1 (Cahill 2007; Wendler and Wehling 2013), there is little information available on PGRMC2 and its role in progesterone signalling is not yet clear. The key structural differences are in the N-terminus and transmembrane domain - where PGRMC1 possesses a progesterone binding site (Ashley *et al.*, 2006). PGRMC2 does not have this site and so cannot directly bind progesterone. Instead, it acts as a companion protein to PGRMC1 (Petersen *et al.*, 2013).

The Progestin and AdipoQ (PAQR) family of receptors, consisting of membrane progesterone receptors (mPR) α, β and γ (mPRα – PAQR7, mPRβ – PAQR8, mPRγ – PAQR5); were first cloned, identified and characterised in fish ovaries (Zhu, Rice, *et al.*, 2003). mPR mRNA has also since been isolated from several vertebrate species, including human and mouse (Zhu, Bond, *et al.*, 2003). Highly conserved in sequence and structural similarity, the mPRs are expressed on the plasma membrane of cells and bind progestins in a specific, displaceable, high-affinity, limited capacity manner – all characteristics of steroid membrane receptors (Zhu *et al.* 2003b; Thomas *et al.* 2007; Dressing *et al.* 2012). mPRs are expressed in both PR A/B positive and negative cells (Dressing and Thomas 2007; Pang and Thomas 2011). Thus it is now commonly accepted that mPRs can mediate progestin's anti-apoptotic effects in both a PR A/B dependent and independent manner (Dressing *et al.* 2012).

Unfortunately, studies looking specifically at PRs present in ocular tissue are limited (Wickham *et al.* 2000; Swiatek-De Lange *et al.* 2007). The classical PRs A and B (PR A/B), the membrane components PGRMC1 and PGRMC2 and the membrane PRs (mPRα, β, γ) are all well documented to be widely expressed in the CNS and exhibit anti-apoptotic effects both

in a variety of different cell lines and across several different diseases. For this reason, the major aim of this study was to determine which of these receptors are present in the retinal cells and specifically in the photoreceptors of the C57 wild type and rd10 mouse. From here, we hoped to gain insight in to which PRs are responsible for the neuroprotective effects of Norgestrel.

4.3. Results

4.3.1. Identification of progesterone receptors in the adult wild type C57 and degenerating rd10 mouse retina

The neuroprotective compound Norgestrel is a synthetic progestin. Hypothesised to work through progesterone receptors present in the eye, we sought to determine the occurrence of the various progesterone receptors in the retina of both the wild type (WT) C57BL/6 mice and the homozygous rd10/rd10 mice (B6.CXBI-Pde6b^{rd10}/J).

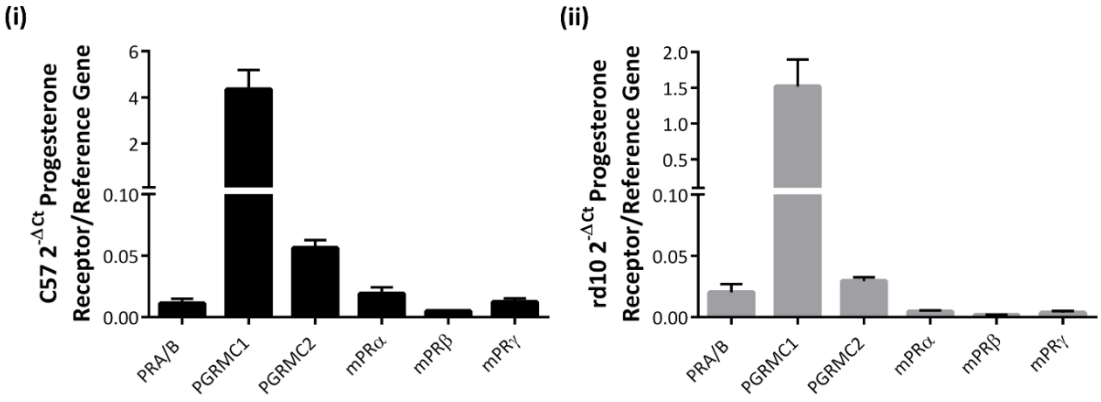


Figure 4.3.1. Progesterone receptor expression in the adult (i) C57 and (ii) rd10 retina. *RT-qPCR analysis detected similar levels of mRNAs encoding the classical progesterone receptor (PR A/B), progesterone receptor membrane complexes 1 and 2 (PGRMC1, PGRMC2) and all three membrane progesterone receptor isoforms, α, β and γ (mPRα, mPRβ, mPRγ) in the both the adult C57 wild type **(i)** and rd10 **(ii)** mouse retina. PGRMC1 was the most abundant PR in both retinas. Results are presented as mean ± SEM from three independent experiments.*

Quantitative RT-PCR detected the presence of all mRNAs encoding the classical progesterone receptor (PR A/B), progesterone receptor membrane complexes 1 and 2 (PGRMC1, PGRMC2) and all three membrane progesterone receptor isoforms, α, β and γ (mPRα, mPRβ, mPRγ) in both the C57 and rd10 adult mouse retina (Figure 4.3.1). RT-qPCR gene product sequencing confirmed the expression of specific progesterone receptors in the retinal tissue. PGRMC1 was the most highly expressed PR at the RNA level, with $2^{-\Delta ct}$

analysis yielding an RNA abundance roughly 80 (C57, Figure 4.3.1i) and 30 (rd10, Figure 4.3.1ii) times that of the next most abundant PR, PGRMC2. Western blotting for the progesterone receptors (PRs) confirmed the presence of all proteins in the retinas (Figure 4.3.2), although the relative abundance of each mPR isoform cannot be directly inferred from these blots. Western blotting also showed a significant increase in the presence of PGRMC1 in the degenerated rd10 retina in comparison to its aged matched WT control.

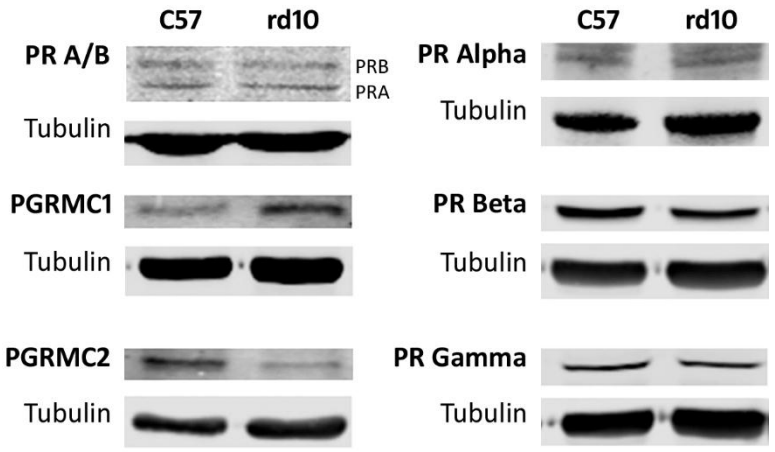


Figure 4.3.2. Protein expression of the progesterone receptors expression in the adult C57 and rd10 retinas. *Western blotting confirmed the presence of all progesterone receptors at the protein level. Detection of tubulin in western blots demonstrates equal loading of protein. Blots are representative of three independent experiments.*

4.3.2. PGRMC1 is localised predominantly in the photoreceptor layer

Immunohistochemical analysis showed differential expression of all progesterone receptors throughout the retina of both the C57 wild type and adult mouse retinas (Figure 4.3.3). PRA/B, mPRα and mPRβ were found predominately in both the inner and outer plexiform layers and photoreceptor outer segments. mPRα and mPRβ were also found in the ganglion cell layer (GCL). PGRMC2 co-localised with the nuclear stain of the ganglion cells and inner nuclear layer (INL), suggesting nuclear localisation in these cells. PGRMC1 and mPRγ were most abundantly expressed on the cell membranes in the photoreceptor cell layer (ONL). No co-localisation with the nuclear stain was apparent. PGRMC1 and mPRγ were also found in the photoreceptor outer segments and in the ganglion cell layer (GCL).

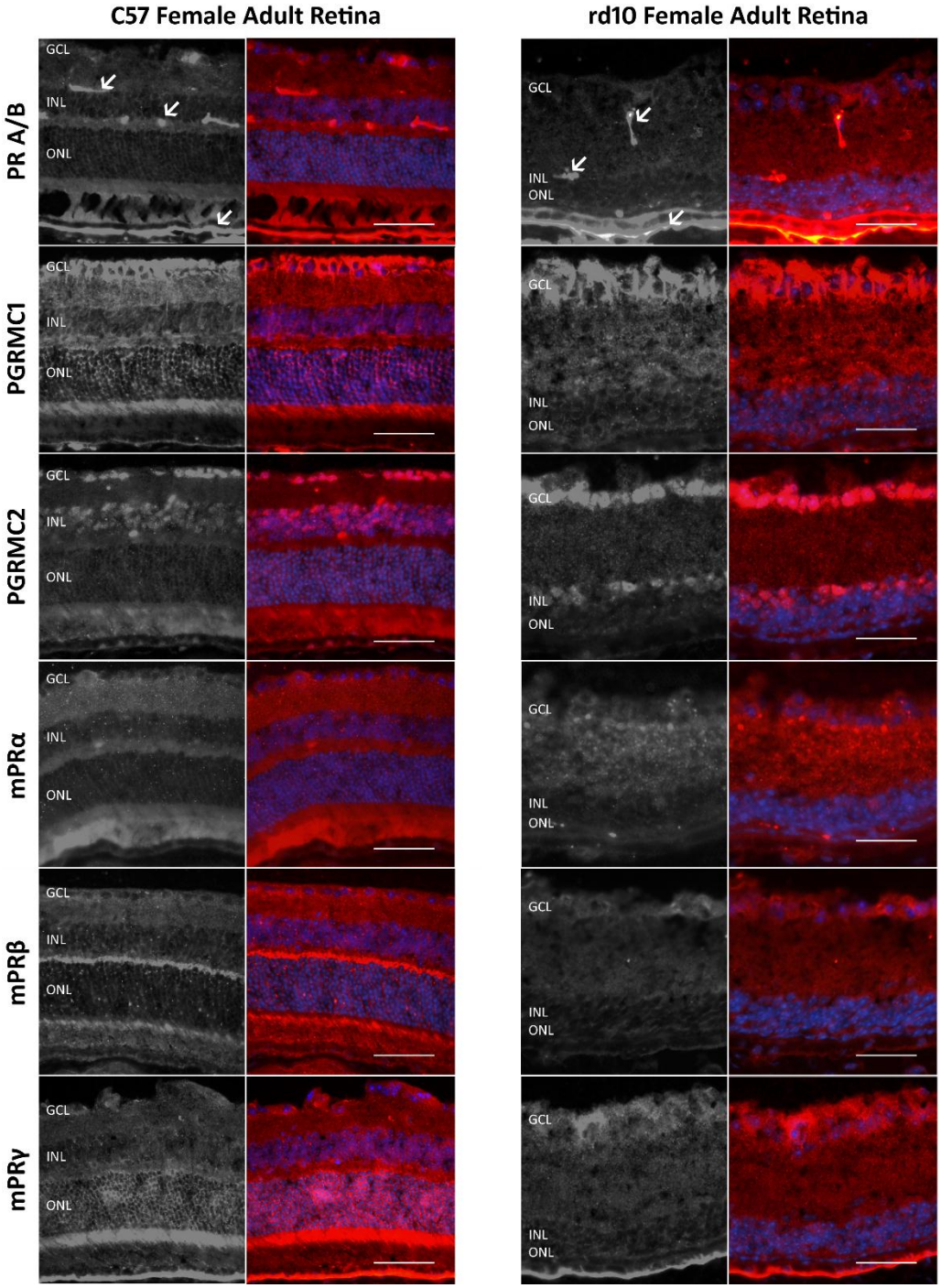


Figure 4.3.3. Immunohistochemical analysis revealed expression of all progesterone receptors throughout the C57 and rd10 mouse retina. *Specifically, PGRMC1 and mPRγ are the most prevalent progesterone receptors found in the photoreceptor cell layer (ONL). Arrows indicate non-specific binding of mouse secondary antibody to blood vessels. GCL, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars 50μm.*

Progesterone is considered to be a 'female hormone'. Therefore, to rule out any differences of expression between male and female progesterone receptor expression,

male C57 aged matched controls were also analysed (Figure 4.3.4). No differences in localisation or expression were determined. No staining of the cells was observed when incubated with secondary antibody alone (data not shown).

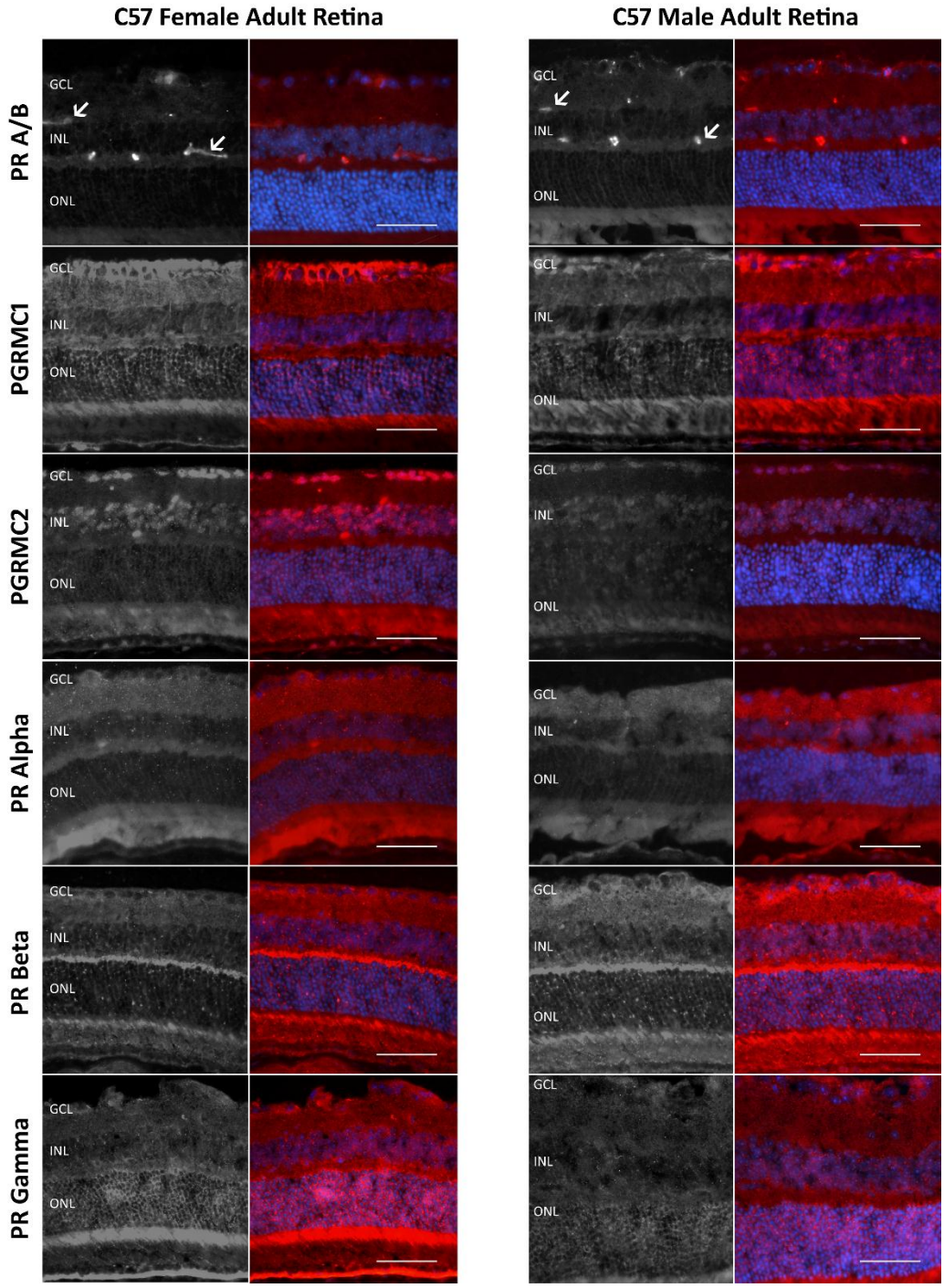


Figure 4.3.4. There is a similar expression profile of progesterone receptors in the male and female adult C57 retina. *Immunohistochemical analysis of receptors in the male and female adult C57 retina revealed expression of all progesterone receptors throughout the retina. Arrows indicate non-specific binding of mouse secondary antibody to blood vessels. GCL, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars 50µm.*

4.3.3. PGRMC1 is highly expressed in the rd10 mouse retina in development

Previous studies suggest that the mouse retina develops postnatally from P0, through eye opening at P12-P15 and up to full development at P18 (Young, 1984). The rd10 mouse then undergoes a large peak of photoreceptor cell loss from P18 (Samardzija *et al.*, 2012; Barone *et al.*, 2014; Roche *et al.*, 2016). Therefore, to look at the importance of the various PRs throughout this development, rd10 and WT retinas were analysed at P10, P15 and P22 for their relative quantity of PR expression. Quantitative RT-PCR detected the presence of all PR mRNA at all stages of development and degeneration (in the case of the rd10 mice) (Figure 4.3.5).

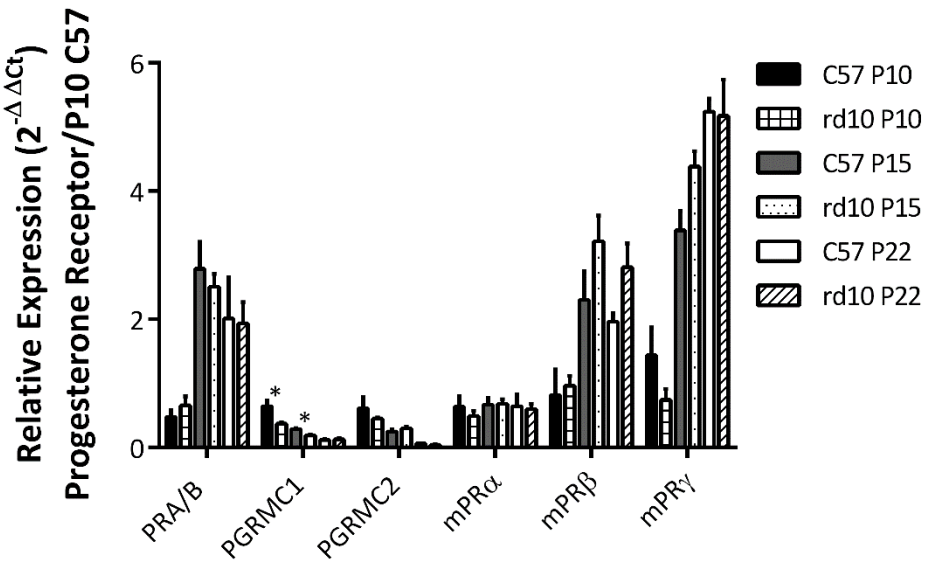


Figure 4.3.5. RT-qPCR analysis detected the presence of mRNAs encoding all progesterone receptors in the C57 and rd10 retina at P10, P15 and P22. *Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (t-test, * p<0.05).*

Western blotting for the PRs confirmed the presence of all PR protein in the developing retinas (Figure 4.3.6i). Densitometric analysis was performed (Figure 4.3.6ii) on all PRs excluding PR A/B, where blotting densities were deemed too little for accurate determination. Western blots revealed a decrease in protein expression of all PRs in the

degenerated rd10 (P22) in comparison to WT, except for PGRMC1 where a significant increase at both P15 and P22 was observed.

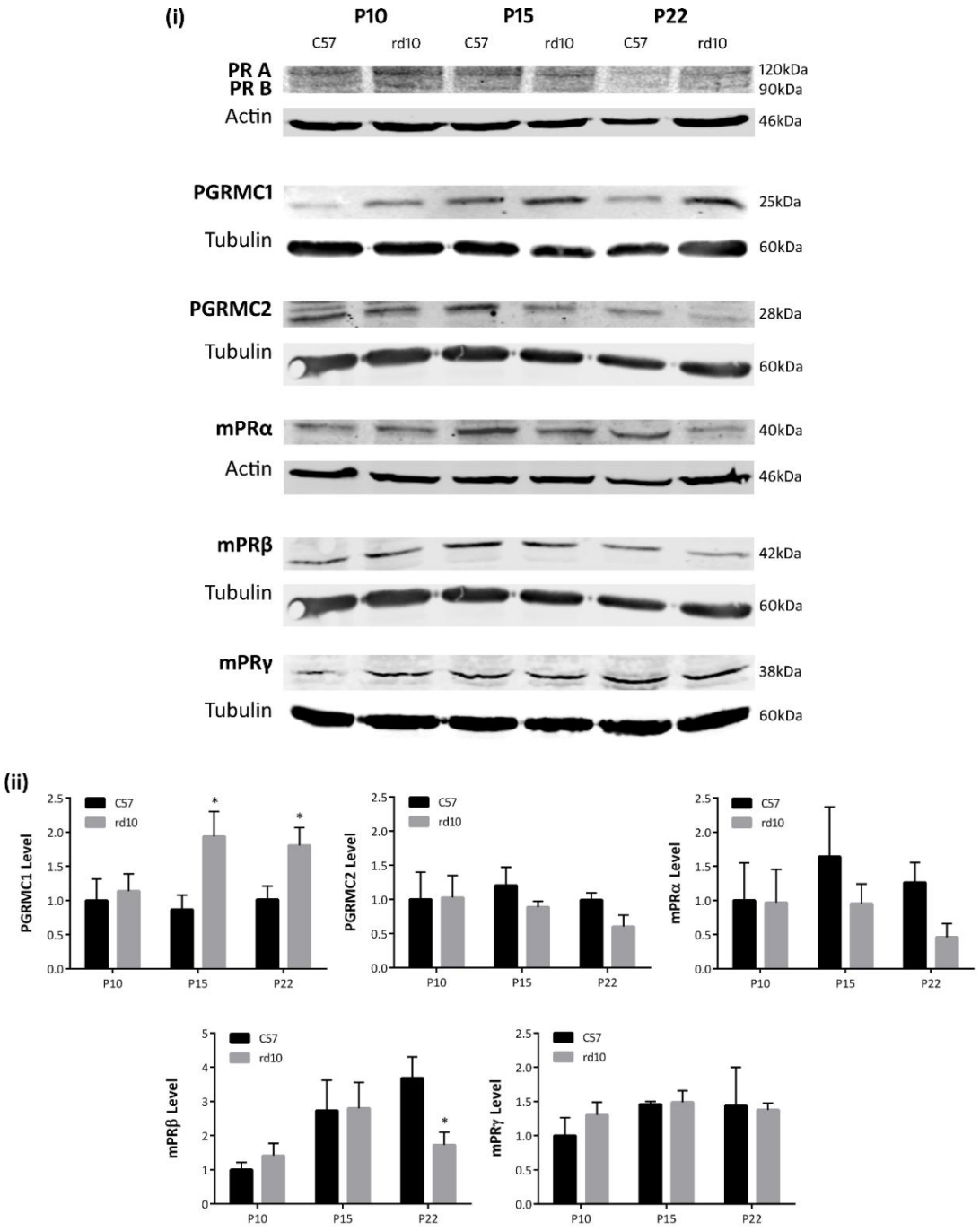


Figure 4.3.6. PGRMC1 expression is up-regulated in the rd10 mouse retina in comparison to its aged matched C57 wild type control. (i) *Western blotting confirmed the presence of all progesterone receptors in the retinas. Protein expression of PGRMC1 was increased in P15 and P22 rd10 retinas.* ***(ii)*** *Graphical representation of changes in progesterone receptor protein expression. PGRMC1 expression was significantly increased in the developing (P15) and degenerating (P22) rd10 retina. Values are relative to C57 P10 control and are expressed as mean ± SEM. Detection of actin and tubulin in western blots demonstrates equal loading of protein. Blots are representative of three independent experiments. Asterisks indicate significant difference (t-test, * p<0.05).*

4.3.4. Identification of progesterone receptors in 661W photoreceptor cell line

661W cells are used throughout this thesis as an *in vitro* model of photoreceptor-based retinal degeneration. Therefore, it was necessary to identify which PRs they express. RT-qPCR detected the presence of all PR mRNAs except for classical progesterone receptor (PR A/B) (Figure 4.3.7A). This protein was thus excluded from further analysis. PGRMC1 mRNA was most abundantly expressed receptor in this cell line. Expression of these proteins was confirmed by western blotting (Figure 4.3.7B) and immunohistochemical staining revealed

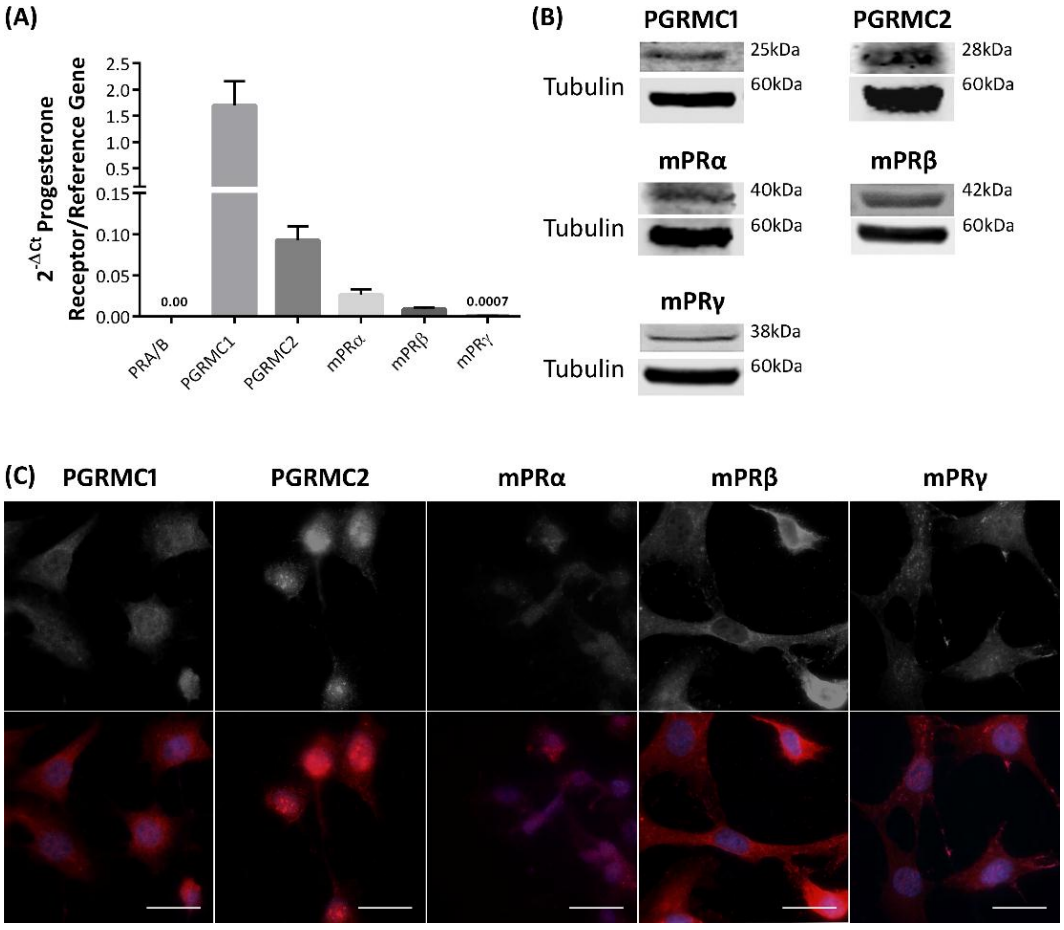


Figure 4.3.7. All progesterone receptors except classical progesterone receptor A/B are present in the 661W cone photoreceptor cell line. (A) *RT-qPCR analysis detected the presence of mRNAs encoding progesterone receptors PGRMC1, PGRMC2 and mPRs α, β and γ. PGRMC1 is the progesterone receptor most abundantly expressed at the mRNA level. Results are presented as mean ± SEM from three independent experiments.* ***(B)*** *Western blotting confirmed the presence of these progesterone receptors in photoreceptor cells. Detection of tubulin demonstrates equal loading of protein. Blots are representative of three independent experiments.* ***(C)*** *Immunohistochemical staining of progesterone receptors present in the 661W photoreceptor cells. PGRMC1 and mPRα, β and γ predominantly localise to the cell membrane whilst PGRMC2 is largely nuclear. Scale bars 50µm.*

that expression of PGRMC1 and mPRα, β and γ are predominantly localised on the cell membrane. PGRMC2 appears to be mostly nuclear (Figure 4.3.7C).

4.3.5. Treatment with Norgestrel over 6 days causes upregulation of all progesterone receptors

In order to determine if Norgestrel treatment induces an upregulation of progesterone receptor expression over time, 661W cone photoreceptor cells were treated with 20µM Norgestrel or equivalent DMSO control over 6 days. Quantitative RT-PCR analyses did not detect any significant changes in mRNA expression of any receptor over time (Figure 4.3.8A). Western blotting revealed an increase in protein expression of all progesterone receptors (Figure 4.3.8B), most evident in receptors PGRMC1 and PGRMC2.

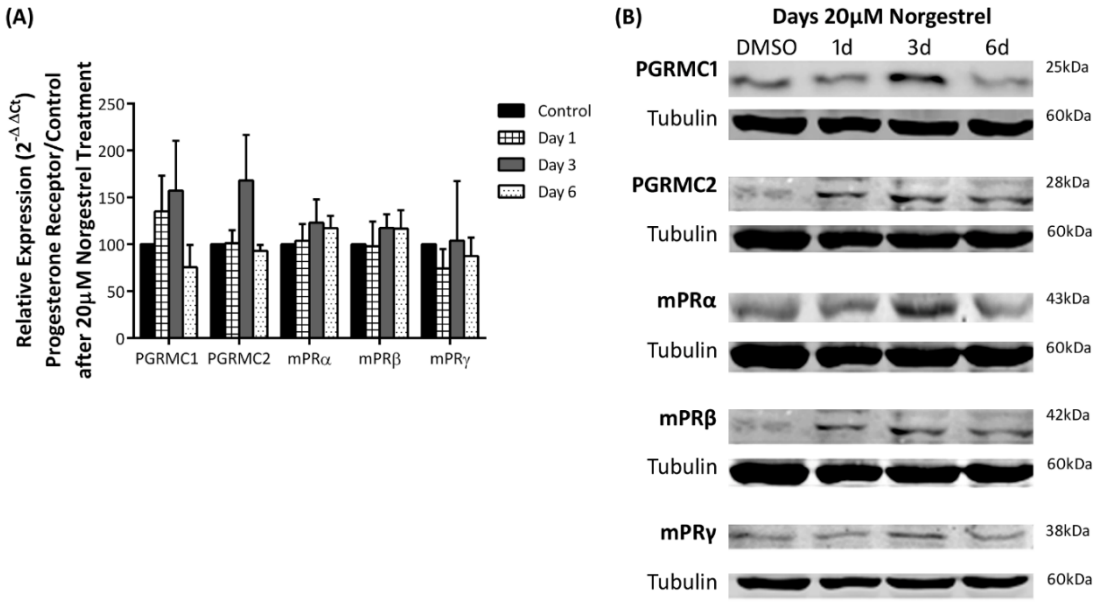


Figure 4.3.8. Norgestrel treatment induces an increase in progesterone receptor protein expression. *661W cells were treated over six days with 20µM Norgestrel and analysed for increases in progesterone receptor expression.* ***(A)*** *RT-qPCR analysis detected no significant increase in mRNA encoding progesterone receptors PGRMC1, PGRMC2 or mPRs α, β or γ. Results are presented as mean ± SEM from two independent experiments.* ***(B)*** *Western blotting revealed an increase in protein expression of all progesterone receptors. Detection of tubulin demonstrates equal loading of protein. Blots are representative of three independent experiments.*

4.3.6. Treatment with Norgestrel causes activation of PGRMC1, trafficking it in to the nucleus

Previous data consistently identified PGRMC1 as the most abundantly expressed PR in the retina of the degenerating rd10 mouse. Therefore, to investigate the potential role of PGRMC1 in the protection afforded by Norgestrel, we decided to look at activation of this receptor and its companion protein, PGRMC2, upon administration of the compound.

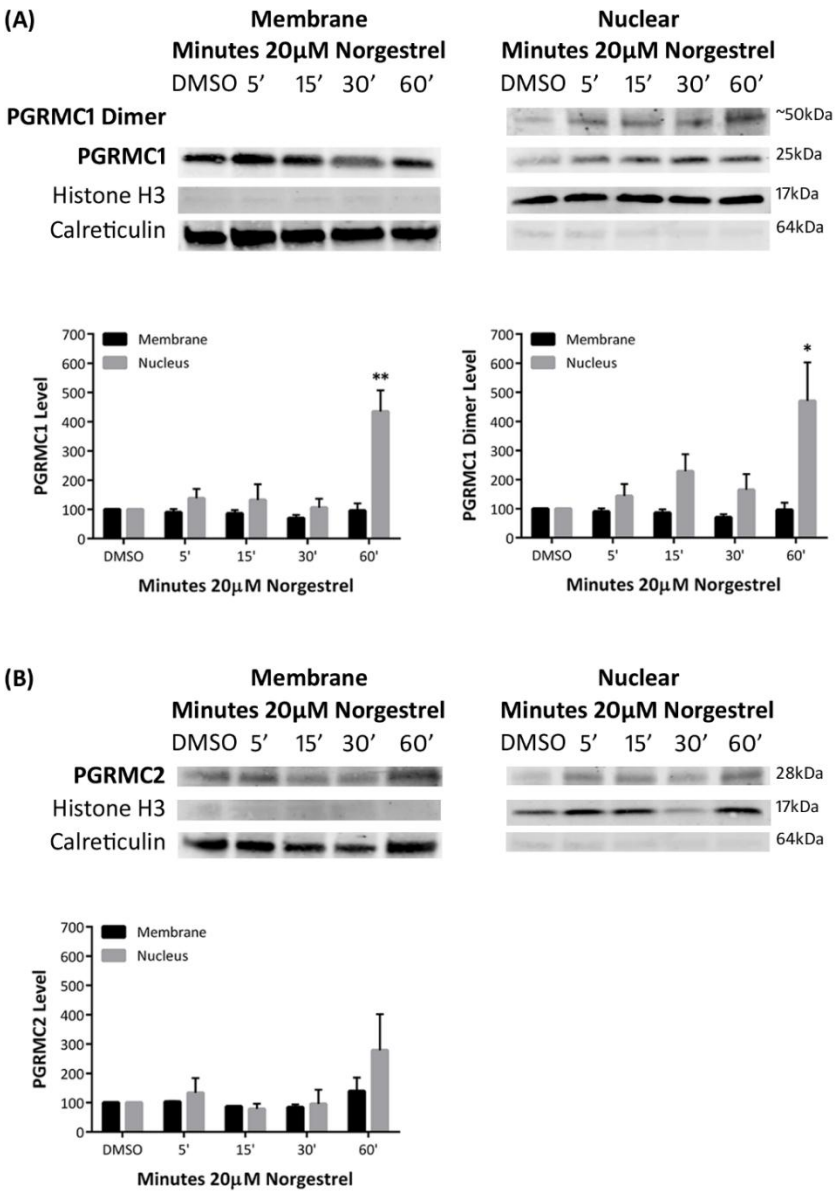


Figure 4.3.9. Treatment of 661W photoreceptor cells with 20µM Norgestrel causes activation of PGRMC1 with subsequent trafficking to the nucleus. (A) *Photoreceptor cells were treated with 20µM Norgestrel over 60 minutes and harvested for subcellular fractionation. Movement of PGRMC1 was detected by western blotting. Graphical analysis of western blots revealed both monomeric and dimerised nuclear PGRMC1 was significantly up-regulated over 60 minutes in comparison to membrane PGRMC1.* ***(B)*** *Movement of PGRMC1-companion protein PGRMC2 was also analysed by western blotting. Equal loading of membrane and nuclear fractions was demonstrated by probing for membrane localised calreticulin and nuclear localised histone H3 protein. Blots are representative of four independent experiments. Graphical analyses are in relative values and are expressed as mean ± SEM. Asterisks indicate significant difference (t-test, * p<0.05, ** p<0.01).*

Activation of PGRMC1 is initiated through ligand binding, causing a conformational change that allows the membrane complex to translocate to the nucleus (Peluso *et al.*, 2012). This is demonstrated clearly by western blotting (Figure 4.3.9A), showing that Norgestrel administration causes a significant increase in the quantity of both monomeric and dimerised PGRMC1 present in the nuclei of the photoreceptor cells. PGRMC2 is not significantly increased in the nucleus after 60 minutes of Norgestrel treatment (Figure 4.3.9B), leading us to conclude that Norgestrel may be driving its neuroprotective effects primarily through activation of PGRMC1. No changes in subcellular movement of the mPRs was determined by either western blotting (Figure 4.3.10A) or subsequent densitometric analysis (Figure 4.3.10B).

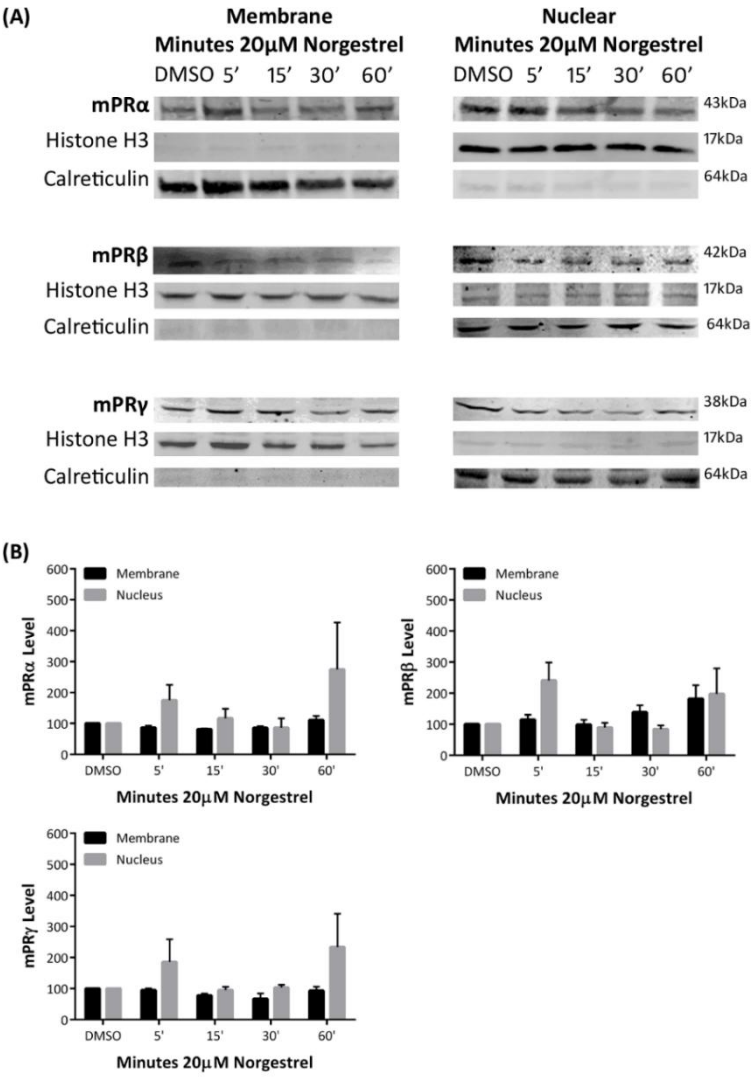


Figure 4.3.10. Treatment of 661W photoreceptor cells with 20μM Norgestrel does not cause any significant trafficking to the nucleus of mPRs α, β or γ. *Photoreceptor cells were treated with 20μM Norgestrel over 60 minutes and harvested for subcellular fractionation.* ***(A)*** *Movement of mPRs α, β and γ was detected by western blotting.* ***(B)*** *Graphical analysis of western blots revealed no significant movement of mPRs from the membrane in to the nucleus compared to DMSO control. Equal loading of membrane and nuclear fractions was demonstrated by probing for membrane localised calreticulin and nuclear localised histone H3 protein. Blots are representative of four independent experiments. Graphical analyses are in relative values and are expressed as mean ± SEM. Asterisks indicate significant difference (t-test, * p<0.05, ** p<0.01).*

4.3.7. PGRMC1 is responsible for the neuroprotective actions of Norgestrel in photoreceptor cells

In the previous chapter, we have shown that administration of 20µM Norgestrel can successfully prevent 661W cells from serum starved cell death over 24 hours (also shown in Figure 4.3.11A). Having established that PGRMC1 is activated by Norgestrel after just 60 minutes of exposure, we then wanted to determine if this membrane complex was responsible for the neuroprotective effects afforded by Norgestrel. Photoreceptors were treated with PGRMC1 siRNA and a significant decrease in PGRMC1 expression (~50%) was verified through RT-qPCR and western blotting (Figure 4.3.11B). Knock down of PGRMC1 also caused a significant decrease in cell viability as measured by MTS assay (Figure 4.3.11C). siRNA treated, stressed cells were then administered with 20µM Norgestrel over 24 hours. There was no change in viability in Norgestrel, PGRMC1 siRNA treated cells (Figure 4.3.11D).

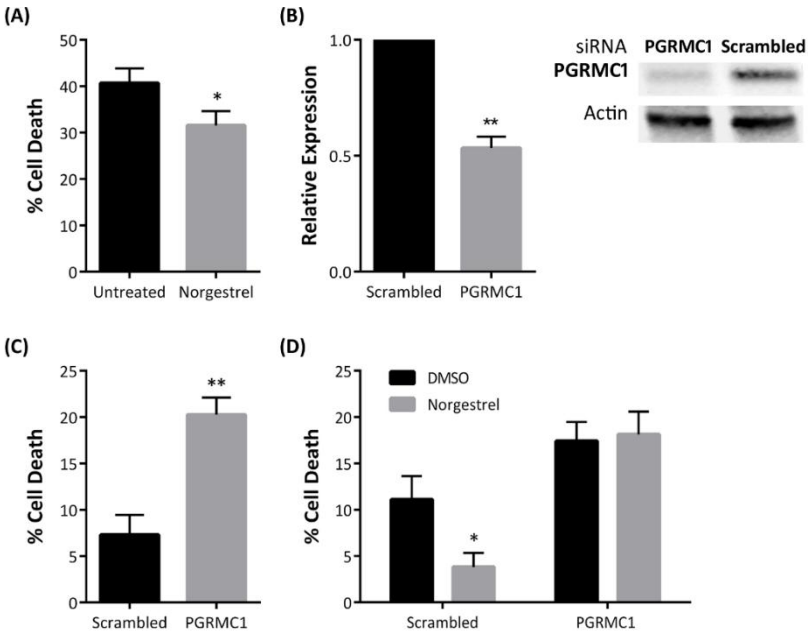


Figure 4.3.11. siRNA knock down of PGRMC1 abrogates the neuroprotective effects of Norgestrel on stressed photoreceptor cells. (A) *Treatment with 20µM Norgestrel significantly decreases cell death in stressed 661W photoreceptor cells over 24 hours.* ***(B-D)*** *661W cells were transfected with siRNA targeted against PGRMC1 (PGRMC1) or non-targeting scrambled control (Scrambled).* ***(B)*** *Detection of PGRMC1 mRNA and protein levels by RT-qPCR and western blot. Transfected cells were serum starved over 24 hours in the absence* ***(C)*** *or presence of* ***(D)*** *DMSO or 20µM Norgestrel and percentage cell death was assayed (n=4). Results are presented as mean ± SEM. Asterisks indicate significant difference (t-test, * p<0.05, **p<0.01).*

4.3.8. The protection afforded to SNP-treated retinal explants by Norgestrel, is abrogated through specific inhibition of PGRMC1 by AG205

In the previous chapter, we have shown that postnatal day 30 (P30) explants from C57BL/6 mice, treated over 24 hours with 300µM nitric oxide donor Sodium Nitroprusside (SNP) undergo apoptosis in all layers of the retina compared to untreated control (Figure 3.3.10i). SNP-induced apoptosis was successfully inhibited through 1-hour pre-treatment with 20µM Norgestrel (Figure 3.3.10ii). Consequently, to determine if PGRMC1 was responsible for the protective properties of Norgestrel *ex vivo*, retinal explants were treated with PGRMC1 specific inhibitor AG205. Preliminary experiments to determine optimum inhibitor concentration were undertaken in 661Ws (Figure 4.3.12). Stressed cells were treated with Norgestrel and AG205 and cellular viability was subsequently assessed by MTS assay. Norgestrel-induced protection was not afforded to AG205 treated cells (Figure 4.3.12ii). Treatment with up to 10µM AG205 did not decrease the viability of 661Ws (Figure 4.3.12i) and so this concentration was carried forward for use in retinal explants.

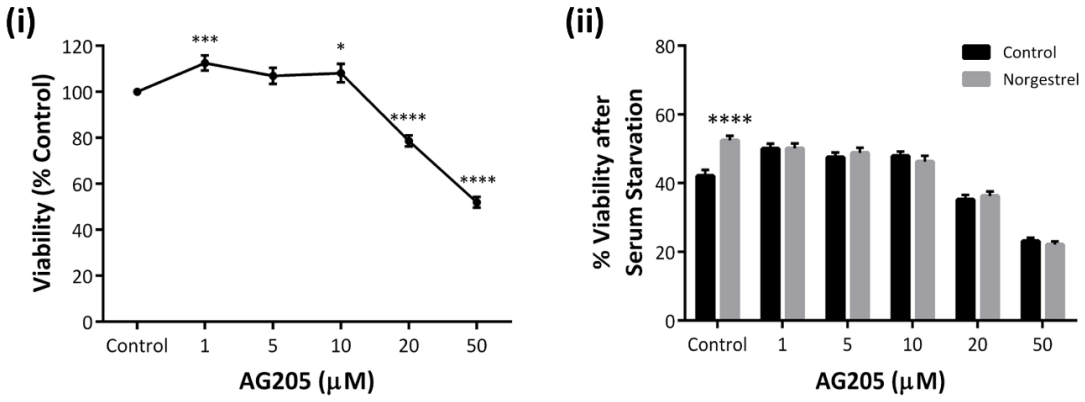


Figure 4.3.12. Specific inhibition of PGRMC1 by AG205 prevents Norgestrel-induced protection in stressed 661W photoreceptor cells. *661W cells were serum starved over 24 hours and treated with increasing concentrations of PGRMC1 specific inhibitor AG205.* ***(i)*** *Percentage cell death was assayed (n=4). AG205 decreases viability of stressed 661W cells in a dose dependent manner.* ***(ii)*** *AG205 treated cells were serum starved over 24 hours in the presence of DMSO (control) or 20µM Norgestrel. AG205 attenuates Norgestrel-induced cell survival. Results are presented as mean ± SEM. Asterisks indicate significant difference (t-test, * p<0.05, ***p<0.005, ****p<0.001).*

Explants incubated with 10µM AG205 alone over 24 hours did not show an increase in TUNEL-positive cells compared to control (Figure 4.3.13i). Explants were then pre-treated for 1 hour with 10µM AG205 and 20µM Norgestrel or equivalent DMSO control, before the addition of 300µM SNP for a further 24 hours. No difference in TUNEL staining could be determined in these explants when treated with 20µM Norgestrel, compared to control (Figure 4.3.13ii).

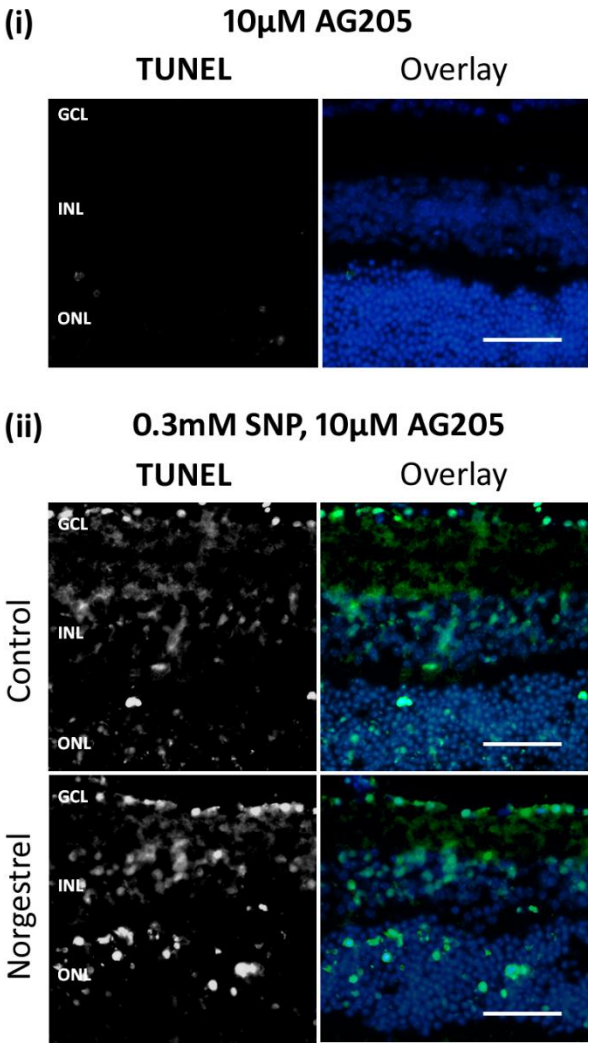


Figure 4.3.13. Specific inhibition of PGRMC1 by AG205 prevents Norgestrel-induced protection in an ex vivo model of retinal degeneration. *Retinal explants from postnatal day 30 C57BL/6 mice were cultured over 24 hours and treated with 10µM AG205 or equivalent control over 24 hours. Apoptosis of retinal cells was detected by terminal dUTP nick end-labelling (TUNEL) of DNA strand breaks in cell nuclei.* ***(i)*** *Treatment with 10µM AG-205 alone does not increase apoptotic cell death in any nuclear layer of the retina.* ***(ii)*** *PGRMC1 inhibited, SNP-stressed explants treated with either 20µM Norgestrel or DMSO control demonstrated comparable TUNEL staining. Results are representative of three independent experiments. GCL, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars 50µm.*

4.4. Discussion

The present study provides the first evidence that Norgestrel not only initiates its cellular activity through activation of PGRMC1, but it can also inhibit apoptotic cell death through this receptor. Both C57 wild type and rd10 mice retinas predominantly express PGRMC1 over any other PR (Figure 4.3.1). PGRMC1 is also significantly up-regulated at the protein level in both the degenerating (P15 – P22) (Figure 4.3.6) and fully degenerated (adult) mouse retina in comparison to wild type control (Figure 4.3.2). High expression of PGRMC1 is mimicked in the 661W photoreceptor cell line (Figure 4.3.7). All taken, this may suggest a role for PGRMC1 in retinal cell viability in the rd10 retina. Norgestrel causes a significant increase in nuclear PGRMC1 after 60 minutes of treatment (Figure 4.3.9A), which has implications for an increased amount of PGRMC1-dependent transcriptional activity. Crucially, through siRNA studies on 661W cells (Figure 4.3.11) and through specific PGRMC1 inhibitor work on retinal explants (Figure 4.3.13), we have demonstrated the critical role that PGRMC1 plays in Norgestrel-induced cell survival. Taken together, these results suggest a mechanism by which Norgestrel acts to exert its neuroprotective effects in stressed photoreceptor cells.

Progestins can elicit several different mechanisms of action. Many of these effects are attributed to binding to classical nuclear progesterone receptors A and B, regulating gene expression (Lange, 2008; Hanna *et al.*, 2010). Studies have shown that progesterone does indeed protect against apoptosis by binding to PR A/B (Friberg *et al.*, 2009). In support of this, mRNA for progesterone receptors is present in the human and rodent retina (Wickham *et al.*, 2000) and progestins are also metabolised in various retinal tissues (Lu *et al.*, 2008; Doonan *et al.*, 2011). However, we found low basal mRNA expression of classical

PR A/B in the retinas (Figure 4.3.1) with little detectable protein (Figure 4.3.2). The PR A/B transcript was also completely absent from the 661W cells (Figure 4.3.7) and thus it was concluded that the protective mechanisms of Norgestrel in the 661W photoreceptor cells could not be ascribed to this receptor.

The membrane progesterone receptors (α, β, γ) are non-classical membrane receptors that make up part of the Class II progestin and AdiopoQ receptor (PAQR) family of proteins (α – PAQR7, β – PAQR8, γ – PAQR5) (Kupchak *et al.*, 2009; Thomas & Pang, 2012; Petersen *et al.*, 2013). These mPRs are not structurally nor functionally related to either PGRMC1 or PGRMC2. Instead, studies have predicted them to be classical 7-transmembrane spanning G-protein coupled receptors (GPCR) (Zhu *et al.* 2003, Moussatche and Lyons 2012), linking them with the alkaline ceramidase protein family (Kupchak *et al.*, 2009). In effect, evidence that the mPRs may mediate signalling through a combination of both pathways is mounting (Moussatche & Lyons, 2012; Petersen *et al.*, 2013). The mPRs have specific and saturable high-affinity binding sites for progesterone (Petersen *et al.*, 2013) and are often associated with rapid signalling events leading to the progesterone dependent inhibition of cAMP (Zhu, Bond, *et al.*, 2003). In this study, we found no distinguishable change in expression of these receptors between the adult WT and rd10 retinitis pigmentosa mouse model (Figure 4.3.2). In fact, protein levels of mPRα and mPRβ were reduced in the degenerating rd10 retina (P22) (Figure 4.3.6), despite the fact that these receptors are not highly expressed in the photoreceptor layer, i.e. the layer suffering substantial cell loss in the rd10 animal (Figure 4.3.3). Therefore, we deemed it unlikely that these receptors could function as the major progesterone receptors responsible for Norgestrel's mechanism of action within the retina.

Both C57 and rd10 adult mice primarily express PGRMC1, a finding consistent across development (P10 – P22) of the adult eye (Figure 4.3.6) and in the 661W cone photoreceptor cell line (Figure 4.3.7). Expression of this receptor appears to be highly concentrated in the retinal outer nuclear layer (ONL), where the photoreceptors are found (Figure 4.3.3). Interestingly, this receptor appears to be up-regulated in rd10 mice in comparison to their aged matched control (Figure 4.3.2), despite the loss of photoreceptor cells. This could implicate compensation from other retinal cell types during retinal degeneration, in an attempt to promote retina-wide cell survival. This would also support the vast body of research that links PGRMC1 to cell survival in a wide variety of cell types (Crudden *et al.* 2006; Peluso *et al.* 2008a, 2008b; Thomas and Pang 2012; Qin *et al.* 2015), including retinal glial cells (Swiatek-De Lange *et al.*, 2007). Given that PGRMC1 showed the most profound change in expression in the degenerating rd10 retina (Figure 4.3.6) and is also the most abundantly expressed in the 661W photoreceptor cell line (Figure 4.3.7) and in the retina of the WT and rd10 (Figure 4.3.2) mice; we focussed our attention on PGRMC1 as the major progesterone receptor responsible for the actions of Norgestrel in the eye.

PGRMC1 is a member of the 'membrane associated progesterone receptor family' (MAPR), which is also a subclass of cytochrome b5 family of proteins (Cahill, 2007). In contrast to the mPRs, it comprises of a single transmembrane domain corresponding to amino acid residues 24-42 (Swiatek-De Lange *et al.*, 2007). In the literature, PGRMC1 is predominantly, and incorrectly, thought of as a membrane protein; as it is also detectable in the nucleus, generally in a dimerised form (Lösel *et al.* 2008; Peluso *et al.* 2008a, 2008b; Peluso *et al.* 2012). This PGRMC1 (often referred to as nuclear PGRMC1) is believed to be cell-cycle dependent (Lösel *et al.*, 2008) and progesterone-dependent transport from the membrane into the nuclear compartment is an essential part of the receptor's mechanism of action

(Peluso *et al.*, 2012). For this reason, we decided to look at transport of this receptor into the nucleus upon treatment of 20μM Norgestrel. We found that levels of both monomeric and dimerised nuclear PGRMC1 increased fivefold over 60 minutes compared to that of control (Figure 4.3.9A) (** $p<0.01$ monomeric, * $p<0.05$ dimerised). From this we can deduce that Norgestrel is binding to PGRMC1 at the plasma membrane: the receptor-progestin complex is then internalised and brought to the nucleus to initiate its mechanism of action. Although this trend was replicated with PGRMC1's binding partner PGRMC2 (Peluso *et al.*, 2014) (Figure 4.3.9B), the translocation was not significant. Therefore, we cannot decisively state whether the action of PGRMC1 in the nucleus is PGRMC2-dependent.

Worthy of note, is that the dimerised form of PGRMC1 was also found in the nucleus of Norgestrel-treated 661W cells (Figure 4.3.9A). This is interesting, given that this form of PGRMC1 is believed to be sumoylated (Peluso *et al.*, 2012) which is associated with the regulation of transcriptional activity (Geiss-Friedlander and Melchior 2007; Petersen *et al.* 2013). This hypothesis is in support of our idea that Norgestrel may function through PGRMC1 to affect transcriptional activity and thus cellular viability of stressed photoreceptor cells.

Norgestrel can rescue 661W cells from serum starved cell death (Figure 3.3.1). Thus, we theorised that if Norgestrel was mediating its neuroprotective effects through activation of PGRMC1, siRNA knock down of this receptor would abrogate the progesterone-induced protection. Indeed, in the absence of PGRMC1 (~50% siRNA knock down, Figure 4.3.11B) Norgestrel did not afford the same level of protection as the Norgestrel treated control.

This strongly suggests that Norgestrel acts to protect 661W cone photoreceptor cells from apoptotic cell death through activation of PGRMC1.

Of particular relevance was the finding that siRNA targeted to PGRMC1 results in a significant (** $p<0.01$) decrease in cellular viability (Figure 4.3.11C). This indicates that the membrane complex plays a key role in inhibition of cell death under conditions of stress. This is consistent with several recent studies showing decreased viability in cells treated with siRNA to PGRMC1 (Peluso *et al.*, 2014) and cells treated with high concentrations of PGRMC1-specific inhibitor AG-205 (Qin *et al.*, 2015). Interestingly, studies have shown that siRNA knock down of mPRα has no effect on cellular viability (Dressing *et al.*, 2012). This further corroborates the particular importance of PGRMC1 in Norgestrel-dependent regulation of cellular apoptosis and proliferation, over the mPRs.

Serum starvation of 661W cone photoreceptors induces cell death, in part, through the caspase pathway (Gómez-Vicente *et al.*, 2005). Depletion of PGRMC1 increases expression of caspases 3, 4 and 8 (Peluso *et al.*, 2012). Therefore, the ability of nuclear PGRMC1 protein to repress caspase expression could account, to some degree, for its ability to mediate the apoptotic pathway. In addition to this, there is a second set of PGRMC1-dependent genes, regulated by progesterone, that are involved in the suppression of apoptosis and mitosis. This finding was verified through bioinformatic analysis which revealed that genes involved in apoptosis are highly affected when PGRMC1 levels are depleted by targeted siRNA (Peluso *et al.*, 2012). This would thus be consistent with PGRMC1's role in viable cell maintenance (Crudden *et al.*, 2006; Peluso, Liu, *et al.*, 2008) and is in agreement with PGRMC1 being central to the anti-apoptotic role of Norgestrel in

661W photoreceptor cells. Future work will be aimed at providing further mechanistic insight in to the downstream signalling pathways of PGRMC1 following Norgestrel administration.

Lastly, with the idea that Norgestrel acts to protect 661W cone photoreceptor cells from apoptotic cell death through activation of PGRMC1, we looked to see if this was replicated in the retina *ex vivo*. Administration of 20µM Norgestrel to SNP-stressed retinal explants has previously been shown in this work to prevent against SNP-induced apoptosis (Figure 3.3.10B). Therefore, we hypothesised that if Norgestrel was working through PGRMC1 to rescue stressed retinal cells *ex vivo*, then treatment with the PGRMC1 specific inhibitor AG205 (Qin *et al.*, 2015) should abrogate this protection. Preliminary experiments with AG205 were carried out in stressed 661W cone photoreceptor cells to determine the optimum concentration to be used in explanting, i.e. the highest possible dose that did not decrease photoreceptor cell viability (Figure 4.3.12A). Moreover, Norgestrel could not act to protect cells from serum starved cell death when treated with AG205 (Figure 4.3.12B). This further validated our siRNA findings that specific knock down of PGRMC1 prevented the protective effects of Norgestrel in stressed photoreceptor cells (Figure 4.3.11D). Stressed retinal explants treated with PGRMC1 inhibitor AG205 alone or in combination with 20µM Norgestrel showed similar levels of apoptotic cell death (Figure 4.3.13ii). The results of this experiment therefore confirm that PGRMC1 is involved in Norgestrel-mediated neuroprotection in an *ex vivo* model of retinal degeneration.

To conclude, we have shown that although there are several progesterone receptors expressed in retinal cells, PGRMC1 is likely to be the critical progesterone receptor involved

in the neuroprotective effects of Norgestrel in the eye. Not only is PGRMC1 is expressed most abundantly in the photoreceptor cells (Figure 4.3.3), the cell type which ultimately undergo apoptosis in retinitis pigmentosa; its expression at the protein level is also up-regulated in the degenerated rd10 mouse retina (Figure 4.3.2). This suggests a role in cellular protection in the rd10 animal. We have clearly shown that PGRMC1 responds to Norgestrel treatment through subcellular fractionation studies (Figure 4.3.9A). The receptor is shuttled to the nucleus, where it has the ability to modulate cellular transcriptional activities. Finally, we have demonstrated that the loss of PGRMC1 abolishes the protective effects of Norgestrel to stressed photoreceptor cells (Figure 4.3.11D), whilst specific inhibition of PGRMC1 in an *ex vivo* model of retinal degeneration prevents Norgestrel-mediated neuroprotection (Figure 4.3.13ii). A summary of the results presented in this chapter is presented in Figure 4.4.1. Taken together, these data validate the significance of this receptor in the anti-apoptotic properties of this progestin.

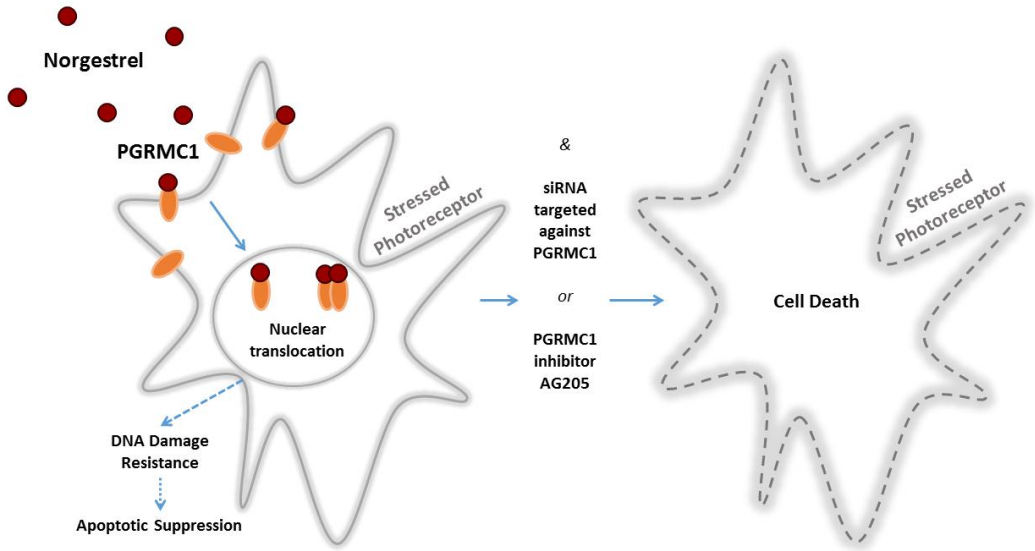


Figure 4.4.1. Schematic showing the protective effects of Norgestrel-PGRMC1 binding in photoreceptor cells. *This schematic details a summary of the results presented in this chapter.*

Chapter 5. Norgestrel induces a protective calcium influx in stressed photoreceptor cells through PGRMC1-induced activation of basic fibroblast growth factor

5.1. Abstract

So far in this work, we have identified the potential of Norgestrel to work through the activation of progesterone receptor membrane component 1 (PGRMC1) activation. In addition, we have shown that Norgestrel induces a neuroprotective upregulation of basic fibroblast growth factor (bFGF) in stressed photoreceptor cells. Until now however, the link between these two signalling events has not been determined. Using trophic factor deprivation of 661W photoreceptor-like cells, this work aimed to further elucidate the mechanism leading to Norgestrel-induced neuroprotection.

In the present study, we show by flow cytometry and live-cell immunofluorescence that Norgestrel induces an increase in cytosolic calcium in both healthy and stressed 661Ws over 24h. Specific PGRMC1 inhibition by AG205 showed this rise to be PGRMC1-dependent, primarily utilising calcium from extracellular stores. Calcium influx was also shown to be bFGF-dependent, for siRNA knock down of bFGF prevented Norgestrel-PGRMC1 induced changes in cytosolic calcium. Notably, we demonstrate PGRMC1-activation is necessary for Norgestrel-induced bFGF upregulation. We propose that Norgestrel protects through the following pathway: binding to and activating PGRMC1 expressed on the surface of photoreceptor cells, PGRMC1 activation drives bFGF upregulation and subsequent calcium influx. Importantly, raised intracellular calcium is critical to Norgestrel's protective efficacy, for calcium chelation by EGTA abrogates the protective effects of Norgestrel on stressed 661W cells *in vitro*.

5.2. Introduction

Progesterone signalling improves neuronal survival, reduces swelling, inhibits apoptosis and reduces damaging inflammatory processes (Gonzalez Deniselle *et al.* 2002a; Lu *et al.* 2008; Garay *et al.* 2011; Allen *et al.* 2015). Some of this signalling has been linked to changes in intracellular retinal cytosolic calcium concentrations ($[Ca^{2+}]_i$), both increased (Samadi *et al.* 2002; Koulen *et al.* 2008) and decreased (Luoma *et al.*, 2012). These fluctuations have important functional implications on a number of different pro-survival signalling events, for calcium is a multifaceted second messenger. Regulating a number of different cellular functions, calcium signalling is required to be highly versatile. In terms of timing, calcium can trigger exocytosis of vesicles from synaptic terminals within a number of microseconds. However, in order to drive gene transcription and proliferative events, calcium requires a longer signalling period: minutes to hours (Berridge *et al.*, 2003). Alternately to timing, differing cytosolic calcium concentrations will also differentially affect signalling. For example, a paper released in 2004 found that intermediate levels of $[Ca^{2+}]_i$ (50-200nM in rat hippocampal neurons) induced a long-term tolerance of ischemia, promoting calmodulin activation and increasing levels of pro-survival AKT and mitogen-activated protein kinase (MAPK). Neurons exposed to higher or lower levels of calcium died upon glucose and oxygen deprivation (Bickler & Fahlman, 2004). Interestingly, this paper also found that the protective, moderate increases in $[Ca^{2+}]_i$ increased the tolerance of subsequent larger increases in $[Ca^{2+}]_i$, which have previously been shown to induce cell death (Sharma and Rohrer 2004; Guerin *et al.* 2011).

One of the major challenges in the field at the moment is understanding how calcium signalling mechanisms control so many divergent processes. Dynamic changes in calcium concentrations and timing effects play a huge role. Still, these processes require tight regulation. In order to do this, cells adjust their $[Ca^{2+}]_i$ through a balancing act of 'on' and 'off' reactions. These reactions occur through a combination of buffers, pumps and exchangers: 'on' reactions bring calcium in to the cell whilst 'off' reactions remove calcium signals (Berridge *et al.*, 2003; Déliot & Constantin, 2015). Accordingly, the activity of calcium refers to signalling in a form of peaks and waves, from microseconds to hours and even days (Pinto *et al.*, 2016). Steroid binding to cellular receptors causes calcium ion influx during the 'on' period. These ions bind to various effectors in the cell, stimulating a variety of calcium-dependent processes. These events include steroid mediated activation of calcium-dependent PKC isoforms (Quest, 1996), modulation of the MAPK ERK-1/2 (Lee *et al.*, 2010) and activation of PKA (Sancho-Pelluz *et al.*, 2008), phosphoinositide 3-kinase (Revankar *et al.*, 2005) and protein kinase B/AKT (Yu *et al.*, 2004) pathways.

Time/concentration dynamics of calcium allow for heterogeneity in calcium-dependent pro-survival systems. However, upstream of these pathways is also an extensive machinery driving cytosolic influx. This machinery, comprised of neurotransmitters and their receptors, intracellular signalling factors and transcription factors to name a few (Pinto *et al.*, 2016), forms a diverse network of interactions. These interactions can then associate and drive a variety of different processes. In this study, we look at the potential of neuroprotective basic fibroblast growth factor (bFGF) (Désiré *et al.* 2000; O'Driscoll *et al.* 2007; O'Driscoll *et al.* 2008; Yang *et al.* 2014) to drive cytosolic calcium influx. bFGF has previously been shown to affect $[Ca^{2+}]_i$ through calcium channel modulation (Puro and Mano 1991; Rosenthal *et al.* 2005; Zamburlin *et al.* 2013). For example, bFGF-dependent

calcium influx through L- and N-type voltage-dependent calcium channels stimulates neurite outgrowth *ex vivo* (Zamburlin *et al.*, 2013), driving retinal axon extension through Ca^{2+}/calmodulin-dependent kinase II (Lom *et al.*, 1998). bFGF induced proliferation is also postulated to be calcium dependent (Puro & Mano, 1991). Consequently we hypothesised a link between bFGF-dependent neuroprotection and raised cytosolic calcium in stressed photoreceptors.

Unfortunately, the mechanisms which link moderate increases in calcium to cell survival are incompletely defined. Indeed, of even more uncertainty is the capacity for progesterone-induced calcium-dependent cell survival. Therefore, we tested a number of hypotheses: firstly, Norgestrel administration affects $[Ca^{2+}]_i$ in stressed photoreceptor cells. Secondly, this effect is PGRMC1-Norgestrel binding-dependent. Thirdly, Norgestrel acts through bFGF to affect $[Ca^{2+}]_i$. And fourthly, an altered cytosolic calcium concentration is necessary for Norgestrel-induced cell survival. Through this work, we hope to further elucidate the pro-survival pathway involved in Norgestrel's neuroprotective capabilities.

5.3. Results

5.3.1. Norgestrel induces an increase in intracellular Ca^{2+} levels in healthy 661W photoreceptor-like cells over 3 hours

Changes in $[Ca^{2+}]_i$ mediate many critical signalling processes in neurons (Berridge 1997; Koulen and Thrower 2001). Whilst some signalling is regulated by rapid and transient calcium increases, others are designed to produce longer-lasting global elevations of calcium (Berridge and Dupont 1994; Lopez *et al.* 2012). Knowing this, we wished to determine if changes in $[Ca^{2+}]_i$ occurred in photoreceptors in response to Norgestrel. Healthy 661W photoreceptor-like cells were treated with 20µM Norgestrel or equivalent DMSO control (control) over 3 and 24 hours. A healthy cell population was gated (Figure 5.3.1i) and an increase in intracellular calcium was detected using the specific intracellular calcium probe Fluo-4 AM. Norgestrel significantly increased $[Ca^{2+}]_i$ levels in healthy 661W cells over 3 hours but these changes were back down to control levels after 24 hours (Figure 5.3.1iii). All treatments are graphed compared to untreated control (Figure 5.3.1iii; unpaired t-test comparing Norgestrel treatments to timed DMSO control).

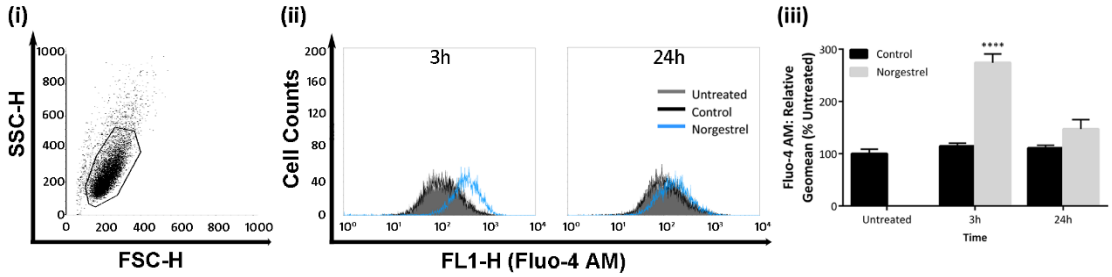


Figure 5.3.1. Norgestrel induces changes in intracellular calcium levels in healthy cells over 3 hours. *Calcium specific indicator, Fluo-4 AM was used to detect changes in intracellular calcium.* ***(i)*** *Healthy 661W cells were gated and treated with 20µM Norgestrel or equivalent DMSO control over 3 and 24 h.* ***(ii)*** *Fluo-4 AM fluorescence was measured using flow cytometry and plotted on a histogram. Y-axis represents cell counts, x-axis measures the level of fluorescence. A shift to the right along the x-axis represents an increase in Fluo-4 AM fluorescence and hence intracellular calcium level. Histograms show untreated cells (grey) compared to a timed DMSO control (black) or Norgestrel-treated cells (blue).* ***(iii)*** *Relative geomean of all treatments was determined and graphed compared to untreated cells (t-test comparing individual Norgestrel treatments to their timed DMSO control, ****p<0.001).*

5.3.2. Serum withdrawal induces an increase in intracellular Ca^{2+} levels in 661W photoreceptor-like cells

Previous studies detailed in this thesis have shown that the protective effects of Norgestrel on photoreceptor cells is dependent upon cellular stress. Deprivation of trophic factor support has been shown both by our group and throughout this thesis, to be a suitable *in vitro* model by which to study photoreceptor cells in the diseased state (Gómez-Vicente *et al.*, 2005). Interestingly, serum starvation induces a morphological change in 661W cells *in vitro* (Figure 5.3.2). After just 30 minutes the extended processes normally apparently in these cells withdraw. Processes in these cells stick to tissue culture plastic through a series of Ca^{2+} and Mg^{2+} bonds. Serum starvation induces a change in the ion composition of the media, thus this is likely what causes the cellular morphological change. This amoeboid morphology persists for approximately one hour before the cells appear to begin to adjust and settle again (Figure 5.3.2. 2h panel).

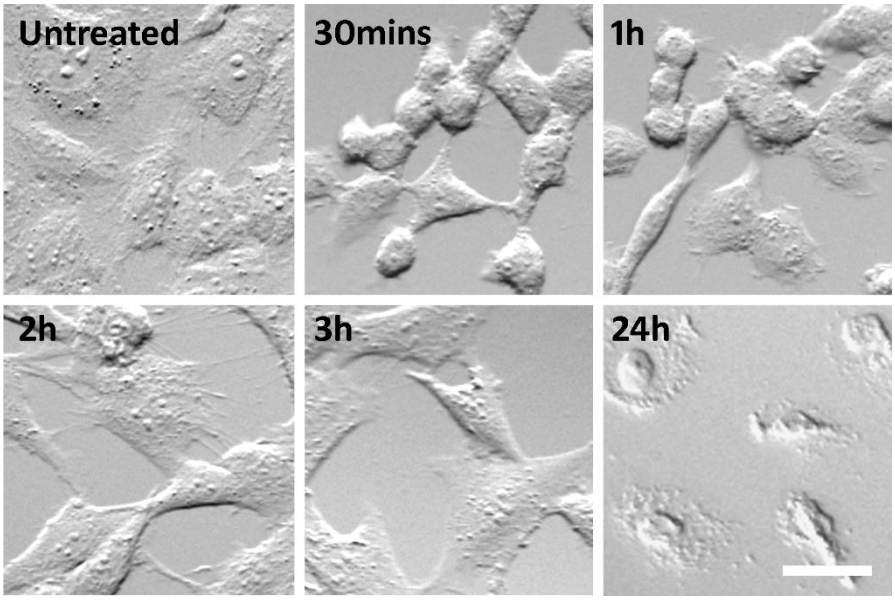


Figure 5.3.2. Live, representative confocal microscopic images of the changes in 661W cell morphology following serum starvation for the indicated times. *Serum starvation of causes an initial retraction of cellular processes. Cells begin to settle again, extending spindle like processes out after 2h serum starvation. Samples prepared by Alice Wyse Jackson. Confocal microscopic images generated by Dr Sarah Roche.*

661W cells were subsequently serum starved and the resulting changes in $[Ca^{2+}]_i$ measured using the specific intracellular calcium probe, Fluo-4 AM. Serum starvation alone significantly increased $[Ca^{2+}]_i$ in 661W cells three-fold over one hour compared to untreated control (Figure 5.3.3). This increase was maintained over three hours, but returned to normal, untreated levels after 24 hours of serum deprivation. All treatments are graphed compared to untreated control.

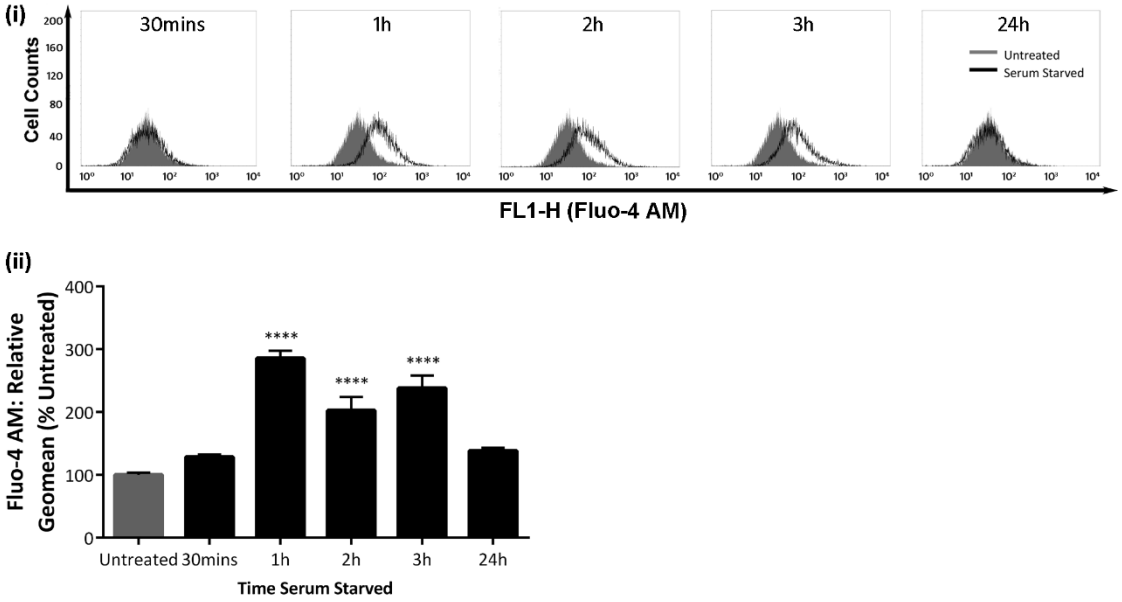


Figure 5.3.3. Serum starvation triggers cytosolic calcium influx in 661W cone photoreceptors cells over three hours. (i) *661W cells were serum starved over 24h and Fluo-4 AM fluorescence was measured using flow cytometry and plotted on a histogram. Y-axis represents cell counts, x-axis measures the level of fluorescence. A shift to the right along the x-axis represents an increase in Fluo-4 AM fluorescence and hence intracellular calcium level. All histograms show untreated control (grey line) compared to a timed serum starved sample (black line).* ***(ii)*** *Geometric mean of the histogram curve was quantified. Bar chart showing the effect of serum starvation on intracellular calcium (ANOVA followed by Dunnett's multiple comparisons test, ***p<0.005, ****p<0.001). Figures are representative of three independent experiments, repeated in triplicate. All results are presented as mean ± SEM. Asterisks indicate significant difference.*

Live imaging of intracellular calcium using the intracellular calcium probe, Fluo-4 AM in serum starved 661W cells corroborates this morphological change and supports the FACs quantification of intracellular calcium increases following serum starvation (Figure 5.3.4) – the changes in intracellular calcium are evident over 3 hours but are back down after 24 hours.

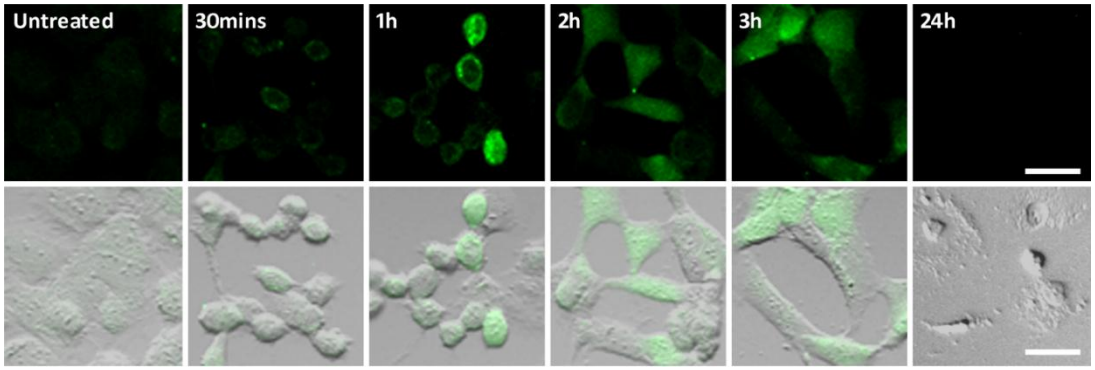


Figure 5.3.4. Changes in intracellular calcium induced by serum starvation are evident through live cell immunofluorescence by intracellular calcium probe, Fluo-4 AM. *Live imaging of intracellular calcium (Fluo-4 AM; green) in serum starved 661W cells compared to untreated control. Scale bars 30µm. Samples prepared by Alice Wyse Jackson. Confocal microscopic images generated by Dr Sarah Roche*

5.3.3. Norgestrel sustains an increase in intracellular Ca^{2+} levels over 24 hours

Progesterone signalling has been demonstrated to induce increases in cytosolic calcium (Samadi *et al.* 2002; Koulen *et al.* 2008). Therefore, further to the finding that serum

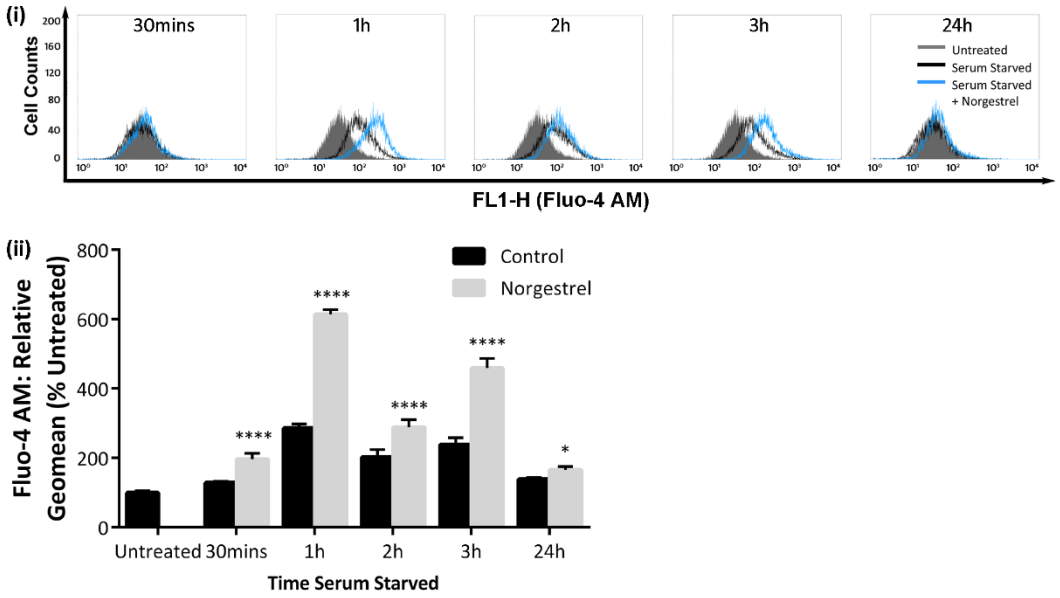


Figure 5.3.5. Norgestrel causes an increase in cytosolic calcium in stressed 661W cells over 24 hours. (i) *661W cells were serum starved over 24h and treated in the absence or presence of 20µM Norgestrel. An increase in intracellular calcium levels in response to Norgestrel was verified by flow cytometry using calcium specific indicator, Fluo-4 AM. Results were plotted on a histogram: Y-axis represents cell counts, x-axis measures the level of fluorescence. A shift to the right along the x-axis represents an increase in Fluo-4 AM fluorescence and hence intracellular calcium level.* ***(ii)*** *Geometric mean of the histogram curves was quantified and each Norgestrel-treated sample was normalised to the equivalent serum starved DMSO control (t-test, *p<0.05, ****p<0.001). Results are presented as mean ± SEM. Asterisks indicate significant difference. Figures are representative of three independent experiments, repeated in triplicate.*

deprivation drives an increase in $[Ca^{2+}]_i$; we sought to determine if administration of the neuroprotective synthetic progestin Norgestrel affected this signalling. 661W cells were stressed by serum starvation and treated with 20µM Norgestrel or the equivalent DMSO control for the indicated time periods. Norgestrel significantly induced a further increase in $[Ca^{2+}]_i$ over 24 hours compared to timed DMSO control (Figure 5.3.5). These changes were again evident through live cell immunofluorescence (Figure 5.3.6).

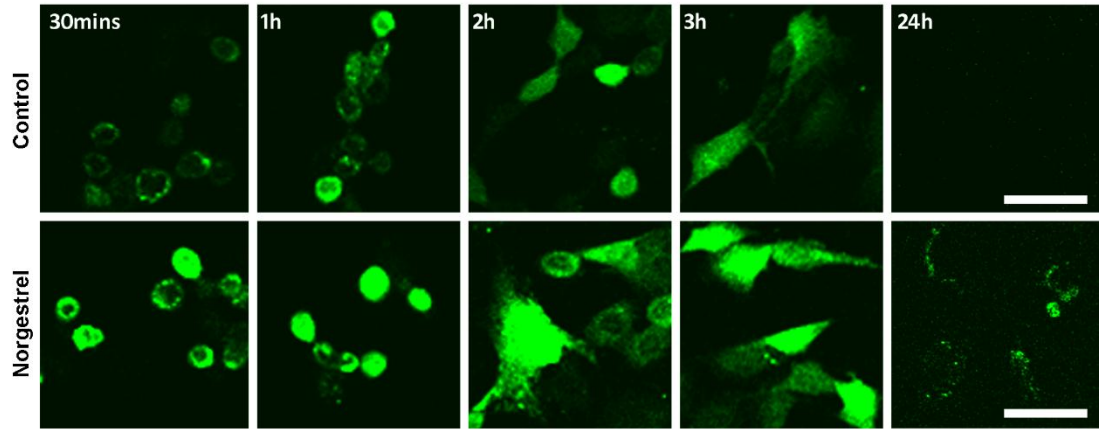


Figure 5.3.6. Changes in intracellular calcium following Norgestrel treatment are apparent in live imaging of cells. *Intracellular calcium was measured using Fluo-4 AM probe (green) in serum starved 661W cells treated with 20µM Norgestrel over the indicated time periods. Scale bars 30µm. Samples prepared by Alice Wyse Jackson. Confocal microscopic images generated by Dr Sarah Roche.*

5.3.4. Inhibition of progesterone receptor membrane complex 1 signalling abrogates Norgestrel-induced increases in intracellular Ca^{2+}

Whilst all known progesterone receptors are present in the retina, we have previously shown that Norgestrel signals predominantly through PGRMC1 (Chapter 4). Therefore, we hypothesised that the increase in $[Ca^{2+}]_i$ in Norgestrel-treated stressed 661W cells was due to signalling downstream of Norgestrel-PGRMC1. AG205 is a potent and specific inhibitor of PGRMC1 activity. Altering the spectroscopic properties of the PGRMC1–heme complex, it has also been shown to inhibit cell cycle progression (Ahmed *et al.*, 2010), likely affecting $[Ca^{2+}]_i$. Consequently, we trialled a number of different concentrations (1µM - 10µM) in

serum starved 661W cells to ensure $[Ca^{2+}]_i$ was not affected by the dose of AG205 used (Figure 5.3.7A). These concentrations have been previously shown in this thesis to have no effect on 661W cell viability (Figure 4.3.12). Analysis by flow cytometry showed that addition of 1µM AG205 to stressed 661W cells showed comparable levels of $[Ca^{2+}]_i$ compared to serum starved control (Figure 5.3.7A). This dose was also still able to effectively attenuate the protective effects of Norgestrel on stressed 661W cells, as measured by the MTS cell viability assay (Figure 5.3.7B). Thus the 1µM dose was taken forward to determine whether PGRMC1 was responsible for Norgestrel-induced increases in $[Ca^{2+}]_i$ in stressed 661W cells.

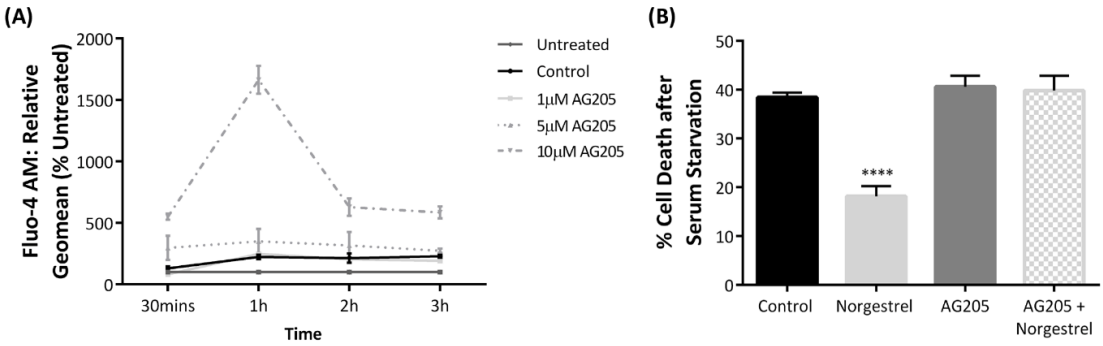


Figure 5.3.7. PGRMC1 inhibitor AG205 attenuates the protective effects of Norgestrel. (A) *PGRMC1 specific inhibitor AG205 causes a dose dependent increase in intracellular calcium in stressed 661W cells, as measured by flow cytometry using calcium specific indicator, Fluo-4 AM.* ***(B)*** *661W cells were serum starved over 24h and treated with 20µM Norgestrel or equivalent DMSO control in the absence or presence of 1µM AG205. Percentage cell death was assayed (n=4). AG205 attenuates Norgestrel-induced cell survival (ANOVA followed by Tukey's multiple comparisons test, ****p<0.001). Results are presented as mean ± SEM. Figures are representative of three independent experiments, repeated in triplicate.*

661W cells were serum starved and cultured in the absence and presence of 1µM AG205. After 10 minutes, 20µM Norgestrel or equivalent DMSO control was added directly to the flasks and incubated for the times indicated. Stressed cells pre-treated with 1µM AG205 do not exhibit any significant increase in $[Ca^{2+}]_i$ in response to 20µM Norgestrel over any time point up to three hours (Figure 5.3.8).

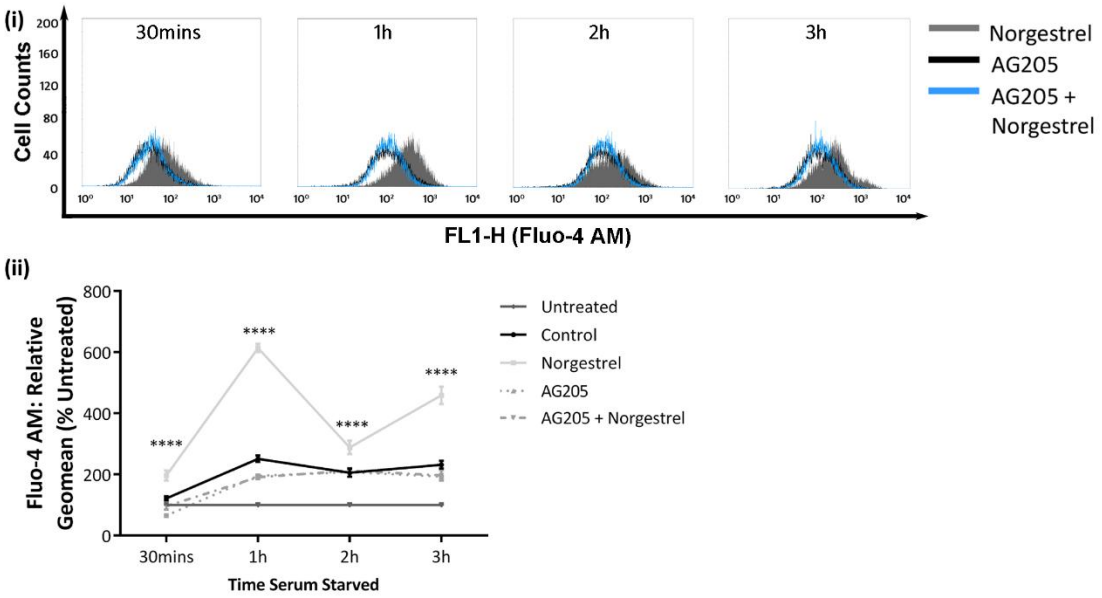


Figure 5.3.8. Norgestrel acts through PGRMC1 to induce an increase in cytosolic calcium. (i) *661W cells were serum starved over 3h and treated with 20µM Norgestrel and/or 1µM AG205. AG205 treatment prevented the Norgestrel-induced increase in intracellular calcium levels as determined by flow cytometry using calcium specific indicator, Fluo-4 AM. Results were plotted on a histogram: Y-axis represents cell counts, x-axis measures the level of fluorescence. A shift to the right along the x-axis represents an increase in Fluo-4 AM fluorescence and hence intracellular calcium level.* ***(ii)*** *Geometric mean of the histogram curves was quantified and each Norgestrel and/or AG205-treated sample was normalised to the equivalent serum starved DMSO control (t-test, ****p<0.001). Results are presented as mean ± SEM. Asterisks indicate significant difference. Figures are representative of three independent experiments, repeated in triplicate.*

5.3.5. Norgestrel signals through PGRMC1 to induce an upregulation of basic fibroblast growth factor

Previous studies identified bFGF signalling with sequential downstream targets of protein kinase A and glycogen synthase kinase 3β, as the major pro-survival pathway responsible for Norgestrel's neuroprotective efficacy in stressed photoreceptor cells. Since we know Norgestrel signals primarily through PGRMC1, we sought to determine if Norgestrel-PGRMC1 signalling was responsible for Norgestrel-induced increases in bFGF. 661W cells were serum starved and cultured in the absence and presence of 1µM PGRMC1 inhibitor AG205 for 10 minutes. After this pre-treatment, 20µM Norgestrel or equivalent DMSO control was added and cells were incubated for a further hour. Analysis by RT-qPCR showed that the Norgestrel-induced increase in bFGF mRNA in serum starved 661W cells, is

abrogated by pre-treatment with AG205 (Figure 5.3.9). From this, we suggest that Norgestrel-PGRMC1 signalling is responsible for the protective upregulation of bFGF.

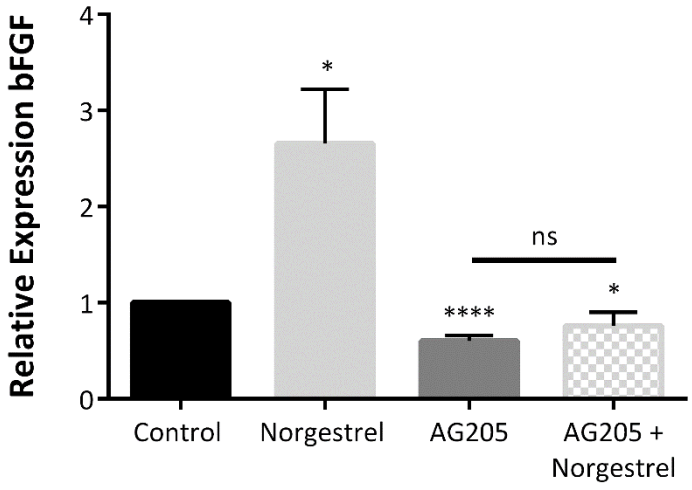


Figure 5.3.9. Norgestrel activates PGRMC1 to induce an upregulation of bFGF. *661W cells were serum starved for 1h and treated with 20µM Norgestrel and/or 1µM PGRMC1 specific inhibitor, AG205. RT-qPCR analysis detected a significant increase in bFGF mRNA levels over 1h in response to Norgestrel, relative to timed DMSO control. This increase was abrogated through treatment with 1µM AG205 (ANOVA followed by Tukey's multiple comparisons test, ns=no significance, *p<0.05, ****p<0.001). Results are presented as mean ± SEM. Asterisks indicate significant difference. Figure is representative of three independent experiments, repeated in triplicate.*

5.3.6. Norgestrel-induced increases in basic fibroblast growth factor drive subsequent increases in intracellular calcium

bFGF signalling has previously been shown to directly modulate calcium activity in the retina (Lom *et al.* 1998; Niu *et al.* 2004). Therefore, we sought to investigate the potential role that bFGF plays in Norgestrel-induced changes in $[Ca^{2+}]_i$. 661W cells were treated with siRNA against bFGF over 48 hours and a significant decrease in bFGF expression (~75%) was verified through RT-qPCR and immunofluorescence (Figure 5.3.10A). siRNA-treated stressed cells were then treated with 20µM Norgestrel or equivalent DMSO control for one hour and changes in $[Ca^{2+}]_i$ analysed using Fluo-4 AM. Stressed cells treated with siRNA against bFGF did not demonstrate the same Norgestrel-induced increase in $[Ca^{2+}]_i$ seen in the non-targeting scrambled control (Figure 5.3.10B).

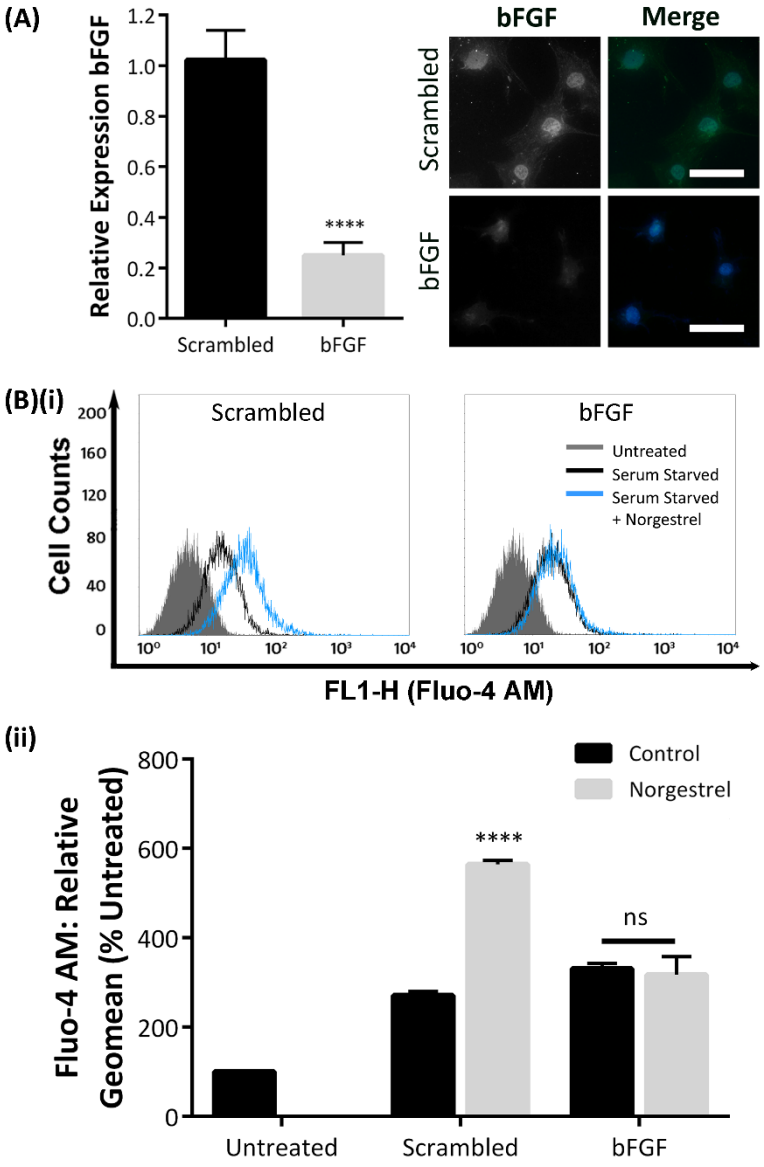


Figure 5.3.10. Norgestrel-induced upregulation of bFGF drives a subsequent calcium influx. (A, B) *661W cells were transfected with siRNA against bFGF or non-targeting scrambled control (Scrambled).* ***(A)*** *Detection of bFGF mRNA levels by RT-qPCR and immunofluorescence. Scale bars 30µm.* ***(B)*** *Transfected cells were serum starved for 1h in the presence of 20µM Norgestrel or equivalent DMSO control and intracellular calcium levels were measured as determined by flow cytometry using calcium specific indicator, Fluo-4 AM.* ***(B) (i)*** *Results were plotted on a histogram: Y-axis represents cell counts, x-axis measures the level of fluorescence. A shift to the right along the x-axis represents an increase in Fluo-4 AM fluorescence and hence intracellular calcium level.* ***(B) (ii)*** *Geometric mean of the histogram curves was quantified and each Norgestrel and/or AG205-treated sample was normalised to the equivalent serum starved DMSO control (t-test, ns=no significance, ****p<0.001). Results are presented as mean ± SEM. Asterisks indicate significant difference. Figures are representative of three independent experiments, repeated in triplicate*

5.3.7. Norgestrel induces an increase in intracellular calcium, predominantly from extracellular stores

Progesterone has previously been shown to evoke calcium signalling in retinal cells due to an activation of intracellular stores (Sharma & Rohrer, 2004) and also entry of extracellular calcium into the cell (Samadi *et al.*, 2002; Swiatek-De Lange *et al.*, 2007). However, the source of $[Ca^{2+}]_i$ in stressed 661W cells in response to Norgestrel is unknown. To evaluate this, the effect of both of IP3/ryanodine receptor antagonists (dantrolene) and an extracellular calcium chelator (EGTA) (Healy *et al.*, 2013) on Norgestrel-induced $[Ca^{2+}]_i$ elevations were examined. 661W cells were serum starved and pre-treated for ten minutes with 0.5mM EGTA or 1µM dantrolene. After this incubation period, cells were directly stimulated with 20µM Norgestrel or equivalent DMSO control for one hour before analysis by flow cytometry (Figure 5.3.11).

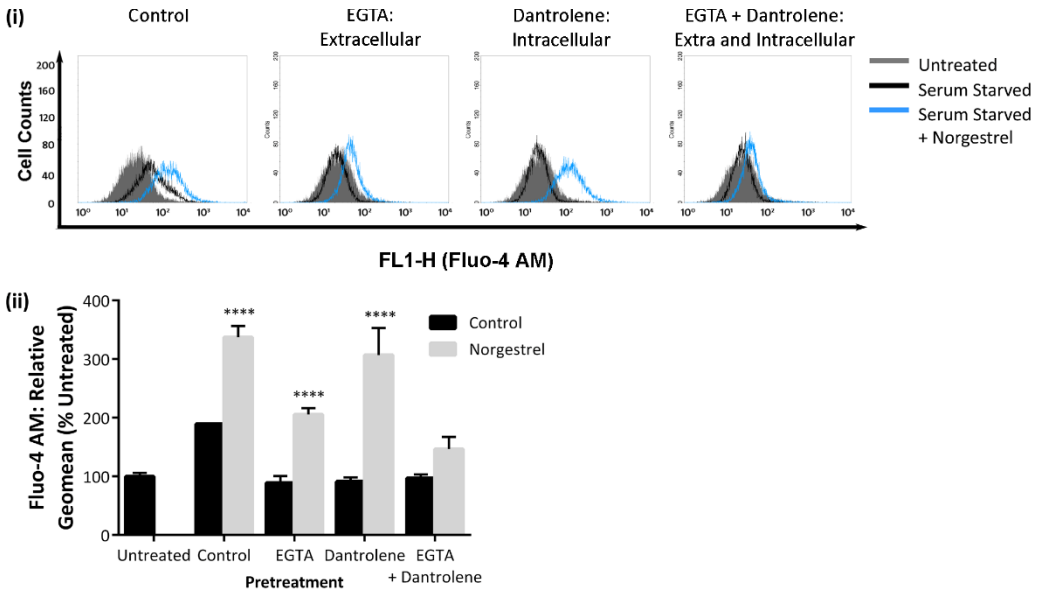


Figure 5.3.11. Norgestrel-induced increases in intracellular calcium come predominantly from extracellular stores. *661W cells were serum starved for 1h and treated with 0.5mM EGTA (extracellular calcium chelator) and/or 1µM dantrolene (antagonist of IP3/ryanodine receptors) in presence or absence of 20µM Norgestrel. Intracellular calcium levels were measured as determined by flow cytometry using calcium specific indicator, Fluo-4 AM.* ***(i)*** *Results were plotted on a histogram: Y-axis represents cell counts, x-axis measures the level of fluorescence. A shift to the right along the x-axis represents an increase in Fluo-4 AM fluorescence and hence intracellular calcium level.* ***(ii)*** *Geometric mean of the histogram curves was quantified and each sample was normalised to the 1h serum starved DMSO control (t-test, ***p<0.005, ****p<0.001). Results are presented as mean ± SEM. Asterisks indicate significant difference. All figures are representative of three independent experiments, repeated in triplicate.*

Cells pre-treated with 0.5mM EGTA did not show complete abrogation of Norgestrel-induced $[Ca^{2+}]_i$ increase. Despite this, there was still a significantly diminished response to Norgestrel and $[Ca^{2+}]_i$ increase was comparable to the levels of serum starved control (Figure 5.3.11). Alternately, cells pre-treated with 1µM dantrolene did not show any difference in $[Ca^{2+}]_i$ compared to non-pre-treated Norgestrel control. This intimates that the rise in $[Ca^{2+}]_i$ in stressed 661W cells in response to Norgestrel is not due to cytosolic influx from intracellular stores. A double control whereby both extracellular calcium was chelated and intracellular IP3/ryanodine receptors were antagonised was carried out to account for compensation between these stores. Stressed cells pre-treated with both 0.5mM EGTA and 1µM dantrolene showed only a slight increase in $[Ca^{2+}]_i$, compared to control. Taken together, these data suggest that Norgestrel-evoked calcium signalling utilises calcium predominantly from an extracellular source.

5.3.8. The protection afforded to Norgestrel-treated stressed 661W cells is abrogated through specific extracellular calcium chelation by EGTA

Norgestrel significantly inhibits 661W cells from serum starved cell death over 24 hours, as measured by the MTS cell viability assay (Figure 5.3.12A). Having established that administration of Norgestrel causes an upregulation of $[Ca^{2+}]_i$ over 24 hours (Figure 5.3.5) and that this increase is coming predominantly from extracellular stores (Figure 5.3.11), we then sought to determine whether $[Ca^{2+}]_i$ is required for the neuroprotective effects afforded by Norgestrel. Serum starved 661W cells were cultured with EGTA (0.5mM) and simultaneously treated with either DMSO or 20µM Norgestrel over 24 hours. No change in viability of these cells was determined compared to the relevant serum starved DMSO control (Figure 5.3.12A), thus EGTA abrogated Norgestrel's neuroprotective effects on

stressed 661W cells. Serum starved 661W cells cultured with EGTA and treated in the absence or presence of 20μM Norgestrel or equivalent DMSO control, were subsequently analysed by western blotting. Changes in levels of phosphorylated GSK3β on serine 9 (S9) and total GSK3β were measured. No discernible change in the phosphorylation state of GSK3β was evident in EGTA cultured cells following Norgestrel treatment, either by western blotting (Figure 5.3.12Bi) or by subsequent densiometric quantification (Figure 5.3.12Bii).

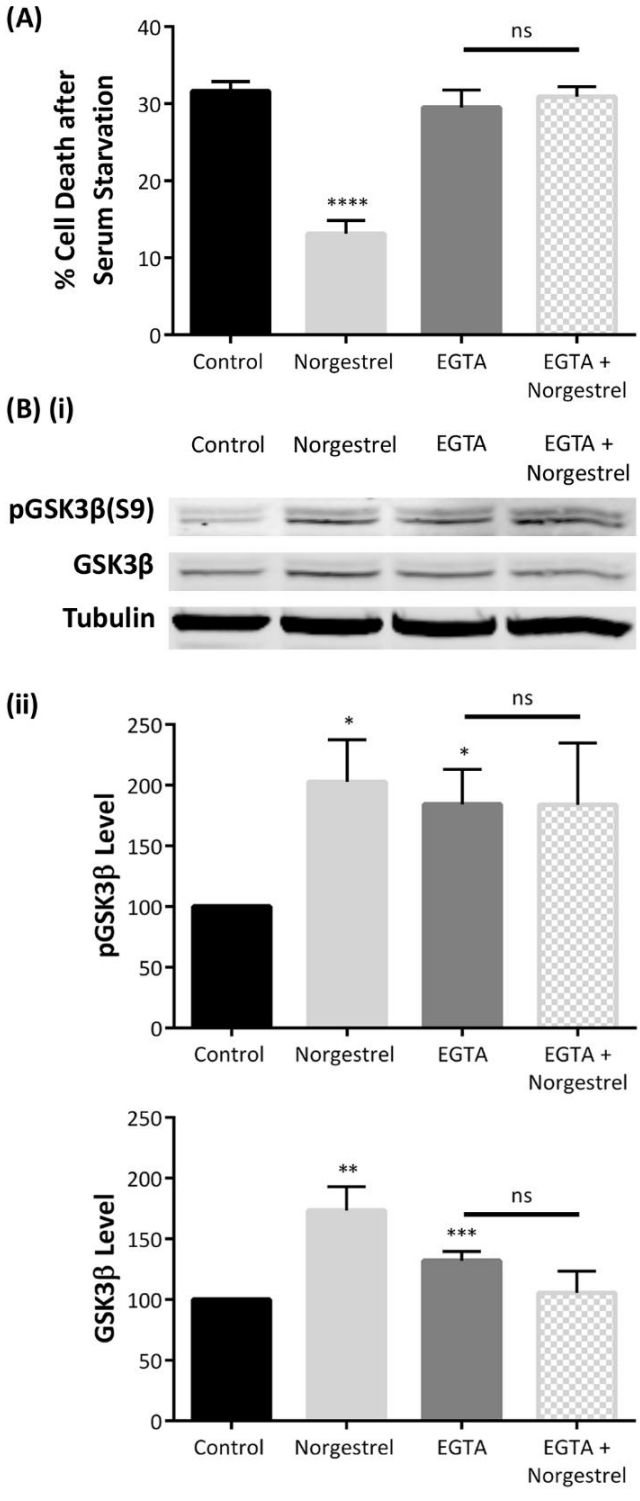


Figure 5.3.12. Extracellular calcium influx is necessary for Norgestrel-induced neuroprotection. *661W cells were serum starved for 24h and treated with 20μM Norgestrel or equivalent DMSO control in the absence or presence of 0.5mM EGTA.* ***(A)*** *Percentage cell death was assayed (n=4). EGTA attenuates Norgestrel-induced cell survival.* ***(B)*** *Western blotting revealed no significant difference in phosphorylation of GSK3β post EGTA treatment.* ***(ii)*** *Densiometric analysis of results presented in* ***(i)****. (ANOVA followed by Dunnett's multiple comparisons test, ns=no significance, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001). Results are presented as mean ± SEM. Asterisks indicate significant difference. All figures are representative of three independent experiments, repeated in triplicate.*

5.4. Discussion

In this study, we show that Norgestrel significantly increases intracellular calcium in photoreceptor-like cells following trophic factor deprivation (Figure 5.3.5, 5.3.6). It is often stated that sustained increases in intracellular calcium following neurological insult is responsible for neuronal cell death (Frasson *et al.* 1999; Sharma and Rohrer 2004; Rodríguez-Muela *et al.* 2014). However, this general assumption is hugely oversimplified. This is because the susceptibility of cells to calcium-induced neurotoxicity depends on a number of different factors (Friedman, 2006), for example the time span of calcium signalling vs. the concentration of intracellular cytosolic calcium (Berridge *et al.*, 2003). Indeed, evidence is now mounting to suggest a neuroprotective role of calcium in activating downstream pro-survival signalling (Bickler and Fahlman 2004; Swiatek-De Lange *et al.* 2007) and in regulation of cell cycle, proliferation, apoptosis, gene transcription, and cell migration (Berridge *et al.*, 2003; Déliot & Constantin, 2015).

Progesterone signalling has previously been shown to affect retinal cytosolic calcium concentrations. With such a variety of potential signalling capabilities however, the role that progesterone-induced calcium signalling plays in retinal neuroprotection is still unclear (Samadi *et al.* 2002; Luoma *et al.* 2012; Koulen *et al.* 2008). Despite this, progesterone, through modulation of calcium influx, has been shown to prevent osmotic swelling of retinal glial cells; a common feature of retinal oedema. Progesterone activates voltage gated calcium channels, causing $[Ca^{2+}]_i$ levels to rise. This initiates the glutamatergic-purinergic signalling cascade and consequently inhibits retinal glial cell swelling (Neumann *et al.*, 2010). Retinal progesterone-induced calcium signalling, is further corroborated in the

retina in a study showing that progesterone-PGRMC1 binding induces calcium influx in to Müller glial cells (Swiatek-De Lange *et al.*, 2007).

Since evidence presented in the literature showed that progesterone can drive a protective retinal calcium influx, we investigated if Norgestrel-PGRMC1 binding, already shown to be crucial in Norgestrel-induced neuroprotection; was responsible for the Norgestrel-induced increase in $[Ca^{2+}]_i$. siRNA abrogation of PGRMC1 has a significant detrimental effect on 661W cellular viability and thus PGRMC1 specific inhibitor AG205 was instead employed to block the effects of PGRMC1 in 661W cells. 1µM AG205 had no significant effect on cytosolic calcium levels (Figure 5.3.7A) but still prevented Norgestrel from protecting stressed cells *in vitro* (Figure 5.3.7B). Thus, using this AG205 pre-treatment, we found that Norgestrel-PGRMC1 binding was responsible for Norgestrel-induced $[Ca^{2+}]_i$ increases in stressed 661W cells (Figure 5.3.8).

In previous chapters, the actions of Norgestrel have been shown to act critically, through both PGRMC1 activation and bFGF upregulation, for knock down and inhibition of either PGRMC1 or bFGF, will prevent the protective actions of Norgestrel both *in vitro* and *ex vivo*. The link between these events however, has not yet been elucidated. In this work, we show in 661W cells that PGRMC1 activation by Norgestrel drives an influx and sequential rise in $[Ca^{2+}]_i$ (Figure 5.3.8). Previous research showed the potential of bFGF to modulate calcium channels, consequently affecting $[Ca^{2+}]_i$ (Puro and Mano 1991; Rosenthal *et al.* 2005; Zamburlin *et al.* 2013). Thus we proposed the following pathway of Norgestrel-induced neuroprotection: Norgestrel activates PGRMC1 expressed on the surface of photoreceptor cells. This activation drives the upregulation of bFGF which will subsequently affect $[Ca^{2+}]_i$.

In order to verify this hypothesis, stressed 661W cells treated with Norgestrel and/or PGRMC1 inhibitor AG205, were examined for bFGF upregulation. As in previous work, Norgestrel induced an increase in bFGF mRNA in stressed 661W cells over one hour (Figure 5.3.9). Cells pre-treated with AG205 however did not exhibit this same increase in bFGF mRNA (Figure 5.3.9). Therefore, we conclude that activation of PGRMC1 is necessary for the Norgestrel-induced upregulation of neuroprotective bFGF (Désiré *et al.* 2000; O'Driscoll *et al.* 2007; O'Driscoll *et al.* 2008; Yang *et al.* 2014). Moreover, we found that when the actions of bFGF are attenuated by siRNA (~75% siRNA knock down, Figure 5.3.10A), Norgestrel can no longer stimulate an increase in $[Ca^{2+}]_i$ (Figure 5.3.10B). These data suggest that Norgestrel-PGRMC1 binding leads to bFGF upregulation with a successive influx of cytosolic calcium.

Cytosolic calcium levels can be raised through calcium release from both intracellular and extracellular stores. These sources differ in their release mechanisms. Extracellular calcium influxes through activation of plasma membrane calcium channels. These include both voltage-gated and non-voltage gated channels, though calcium entry in excitable cells such as photoreceptors occurs mostly though voltage-gated channels (Berridge *et al.* 2003; Déliot and Constantin 2015). Alternately, intracellular calcium is released in to the cytosol through ligand gated receptor-operated calcium channels, the most recognised being the inositol triphosphate (IP_3) and ryanodine receptors found on the endoplasmic and sarcoplasmic reticulum (Bennett *et al.* 1996; Wójcik-piotrowicz *et al.* 2016). Release of calcium from either extra- or intracellular stores will lead to differential activation of cellular mechanisms, for many cellular activities are preferentially activated by different $[Ca^{2+}]_i$ (Berridge *et al.* 2003; Wójcik-piotrowicz *et al.* 2016). For example, internal stores hold a large but finite amount of calcium and thus will activate processes that do not

require sustained influxes of calcium from extracellular sources (Berridge and Dupont 1994; Bootman and Berridge 1995; Berridge 1997). For this reason, the source of calcium released in stressed 661W cells in response to Norgestrel administration was of interest.

Using the IP3/ryanodine receptor antagonist dantrolene and extracellular calcium chelators EGTA (Healy *et al.*, 2013) both individually and in combination, we found that the source of calcium released in stressed 661W cells in response to Norgestrel administration was primarily from extracellular sources (Figure 5.3.11). We conclude this for when calcium was chelated using EGTA, cells did not respond as readily to Norgestrel as in the stressed control and indeed $[Ca^{2+}]_i$ was only brought back to the levels of stressed 661W cells. This is interesting however, for when extracellular sources are blocked, there appears to be some compensation from intracellular stores. Using dantrolene and EGTA in combination, Norgestrel could not stimulate as large an increase in $[Ca^{2+}]_i$ as when EGTA was used alone (Figure 5.3.11B). Finally, having found that bFGF was responsible for the increase in $[Ca^{2+}]_i$ (Figure 5.3.10), we looked to see if this sequential calcium cascade was necessary for Norgestrel-induced 661W cell survival. When 661W cells are serum starved and treated with Norgestrel, cell viability is significantly rescued (Figure 5.3.12). Serum starved cells pre-treated with a concentration of EGTA that does not affect cell viability (0.5M) however, did not display an increase in cell viability when treated with Norgestrel. From this, we can conclude that a bFGF-mediated increase in $[Ca^{2+}]_i$ is necessary in Norgestrel's protective effects on stressed 661W cells.

Calcium is becoming more prevalent in the literature as a pro-survival signalling molecule, affecting both cellular proliferative and apoptotic processes (Berridge *et al.* 2003; Bickler

and Fahlman 2004; Swiatek-De Lange *et al.* 2007; Déliot and Constantin 2015). One potential mechanism by which calcium signalling may be neuroprotective in this system is by the calcium-dependent activation of adenylate cyclase (AC). AC, once activated, can

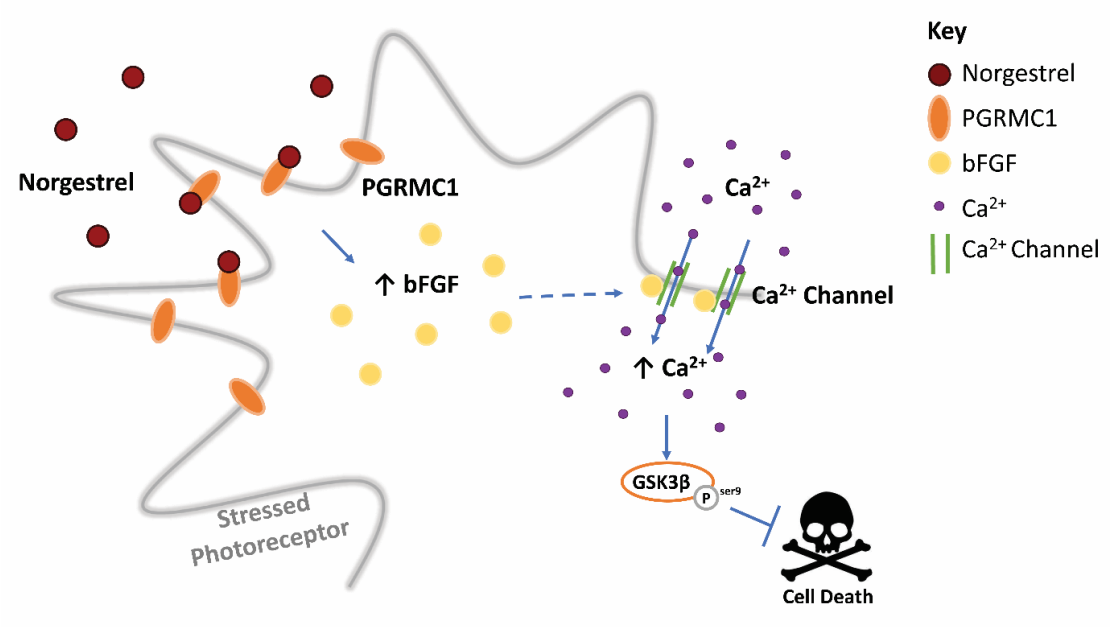


Figure 5.4.1. Schematic representation of the intracellular signalling events regulated by the synthetic progesterone Norgestrel. *The diagram illustrates only the pathways reported in this study to be affected by Norgestrel signalling. We propose that it is this pathway through which Norgestrel exerts its neuroprotective effects in photoreceptor cells in vitro.*

produce cAMP to mediate PKA activation (Sancho-Pelluz *et al.*, 2008), previously shown to be critical in the neuroprotective actions of Norgestrel on stressed photoreceptor cells (Chapter 3). Active PKA can then phosphorylate and activate cAMP response element binding protein (CREB), a transcription factor crucial for neuronal survival (Finkbeiner 2000; Mantamadiotis *et al.* 2002). Since bFGF, driven up by Norgestrel (Figure 5.3.9), can promote the phosphorylation of CREB via PKA (O'Driscoll *et al.*, 2007), we hypothesise that Norgestrel signals through the calcium dependent, cAMP-AC-PKA pathway to protect stressed photoreceptors. Further research will have to be carried out to substantiate this claim however.

Through this study, we have further elucidated the potential of Norgestrel to up-regulate pro-survival signalling activities in stressed photoreceptor cells through PGRMC1 receptor binding. A schematic overview of the results of this study is presented in Figure 5.4.1. We reveal a novel role for PGRMC1 activation in the upregulation of bFGF, a growth factor shown previously to be crucial to Norgestrel's neuroprotective capabilities. In addition to this, we show through specific siRNA knock down, that bFGF is responsible for the Norgestrel-induced increase in cytosolic calcium, in stressed 661W cells. Hypothesised to influx primarily from extracellular stores, we believe this increase in cytosolic calcium to be essential in regulating Norgestrel's pro-survival capacity, for calcium chelation by EGTA abrogates the protective properties of Norgestrel on stressed 661W cells *in vitro*.

Chapter 6. Norgestrel attenuates microglial-driven retinal degeneration and stimulates protective fractalkine-CX3CR1 signalling in the rd10 mouse model of retinitis pigmentosa

6.1. Abstract

Norgestrel provides significant protection to stressed photoreceptor cells. The retina however is a complex tissue with many different cell types. This study consequently aimed to see how Norgestrel's effects on photoreceptors affect their interactions with other retinal cells. Specifically, we look to Norgestrel's actions on microglia; for microglial-derived inflammation has been shown to potentiate retinal degeneration.

Nursing rd10 mothers were put on a Norgestrel supplemented diet (80mg/kg) when pups were aged P10 and upon weaning, pups remained on Norgestrel-supplemented diet. Retinal tissue was harvested from P15-P50. Norgestrel-treated rd10 mice showed a significant delay in disease progression up to P40. In this study, we delineate the potential of Norgestrel to act directly on microglia to reduce pro-inflammatory microglial activation. Observed at a time of significant photoreceptor protection, this implicates changes in microglial-response in Norgestrel-induced neuroprotection. Utilising primary cultures of P16 rd10 microglia and 661W photoreceptor-like cells, we show for the first time that Norgestrel directly down-regulates pro-inflammatory microglial activation, thereby reducing microglial-driven 661W cell death.

Photoreceptors communicate with microglia through a variety of cellular messengers. Expressed by damaged cells, chemokine and damage associated molecular pattern (DAMP) release stimulates pro-inflammatory and migratory microglial responses. Norgestrel effectively suppresses chemokine (MIP1α, MIP1β, MCP1, MCP3) and DAMP (HMGB1, IL-1α)

expression, repealing microglia and preventing pro-inflammatory IL-1β and TNFα release. Remarkably, Norgestrel up-regulates fractalkine-CX3CR1 signalling 1,000-fold at the RNA level, in the rd10 mouse. Fractalkine-CX3CR1 signalling has been shown to protect neurons by regulating retinal microglial activation and migration. Ultimately, these results present Norgestrel as a promising treatment for RP, with dual actions as a neuroprotective and anti-inflammatory agent in the retina.

6.2. Introduction

Throughout the course of this thesis, we have identified the capability of Norgestrel to directly work to protect photoreceptor cells through PGRMC1 activation (Chapter 4) with subsequent actions of bFGF (Chapter 3) and calcium signalling (Chapter 5). We are now beginning to understand more about the molecular mechanism driving this protection, however what has yet to be studied in detail is the effect that Norgestrel has on other cell types in the retina. Particularly, we still do not know if Norgestrel can work on the resident retinal macrophages, the microglia. Macrophages are essential in the clearance of cell debris, maintaining homestasis and facilitating tissue repair following injury in the CNS (Hanisch & Kettenman, 2007; Napoli & Neumann, 2009; Neumann, Kotter & Franklin 2009). However, macrophages have also been implicated in the pathology of many neurodegenerative diseases of the central nervous system, in which neuroinflammation is considered to be a hallmark (Amor et al., 2010). Previous studies have highlighted a detrimental role for microglia as drivers of retinal cell degeneration (Zhao et al., 2015, Peng et al., 2014, Yoshida 2013, Zeng et al., 2014). In mouse models of RP, studies have shown that when microglial cells are either genetically ablated (Zhao *et al.*, 2015), their phagocytic ability inhibited (Zhao *et al.*, 2015) or their pro-inflammatory activities dampened by anti-inflammatory drug administration (Peng *et al.*, 2014; Scholz *et al.*, 2015), RP disease progression is mitigated.

The aim of this study therefore, was to further our understanding of how Norgestrel works to provide neuroprotection in a mouse model of RP, the rd10 mouse, with particular interest being given to the response of retinal microglia to Norgestrel, if any. We knew that

the high IP dose of Norgestrel (100mg/kg) was protective to the diseased rd10 mouse (Doonan *et al.*, 2011). However in this study we wished to trial a less invasive technique. A Norgestrel-supplemented diet was developed by Testdiet (Middlesex, UK) and dams of rd10 pups were given the Norgestrel-supplemented diet *ad libitum* when the pups reached P10. This allowed the pups to receive Norgestrel in the milk. This technique has previously been proven effective in protection of the neonatal brain against a hypoxic-ischemic insult (Loren *et al.*, 2005). In this study, mouse dams were provided *ad libitum* access to drinking water with pomegranate juice during the last third of pregnancy and throughout the duration of litter suckling. At postnatal day 7, pups were exposed to a brain trauma and histological assessment was carried out after 1 week. Dietary supplementation with pomegranate juice resulted in markedly decreased brain tissue loss (>60%) and thus we concluded that a Norgestrel maternal dietary supplementation would likely be neuroprotective to the degenerating retina.

Using the Norgestrel-supplemented diet, we examined interactions between rd10 retinal microglia and photoreceptors *in vivo*. Photoreceptors communicate with microglia through a variety of cellular messages. Expressed by damaged cells, chemokine and damage associated molecular pattern (DAMP) release activates pro-inflammatory and migratory microglial responses. In this study, we therefore looked to see the potential of Norgestrel to suppress rd10 retinal chemokine (MIP1α, MIP1β, MCP1, MCP3) and DAMP (HMGB1, IL-1α) release, with consequential actions on pro-inflammatory release of IL-1β and TNFα, likely from microglia.

We also aimed to determine if Norgestrel had any effect on the release of neuroprotective fractalkine in the diseased rd10 retina. Fractalkine is a chemokine that signals specifically to the CX3CR1 receptor, known to be present on microglial cells. Unlike most other chemokines, fractalkine has two distinct roles: 1. As a transmembrane adhesion protein and 2. as a chemoattractant for CX3CR1-expressing target cells (Stievano *et al.*, 2004). Mouse fractalkine cDNA encodes a 395 amino acid chain that includes a signal sequence, a chemokine domain, a mucin stalk region, a transmembrane segment and a cytoplasmic tail (Harrison *et al.*, 2001; Mizoue *et al.*, 2001). Produced as a cell surface protein, in its full length form fractalkine protein has a molecular weight of approximately 95 - 100kDa, but cleavage can be induced by ADAM 10 and ADAM17 to generate the chemotactic 60 – 80kDa soluble form (Tsou *et al.*, 2001; Hundhausen *et al.*, 2007). Through regulation of this signalling, photoreceptors can dampen pro-inflammatory and migratory microglial responses which therefore makes the fractalkine-CX3CR1 pathway an ideal neuroprotective signalling pathway to target in preventing microglia-induced retinal degeneration.

6.3. Results

6.3.1. Norgestrel-supplemented diet from P10 results in significant protection of photoreceptors and outer segments in the rd10 mouse

Norgestrel has been shown to provide significant neuroprotection to the rd10 retina when administered by intra-peritoneal injection (Doonan *et al.*, 2011). In this study however, we sought to evaluate the protection offered when using the less invasive method of diet-supplementation.

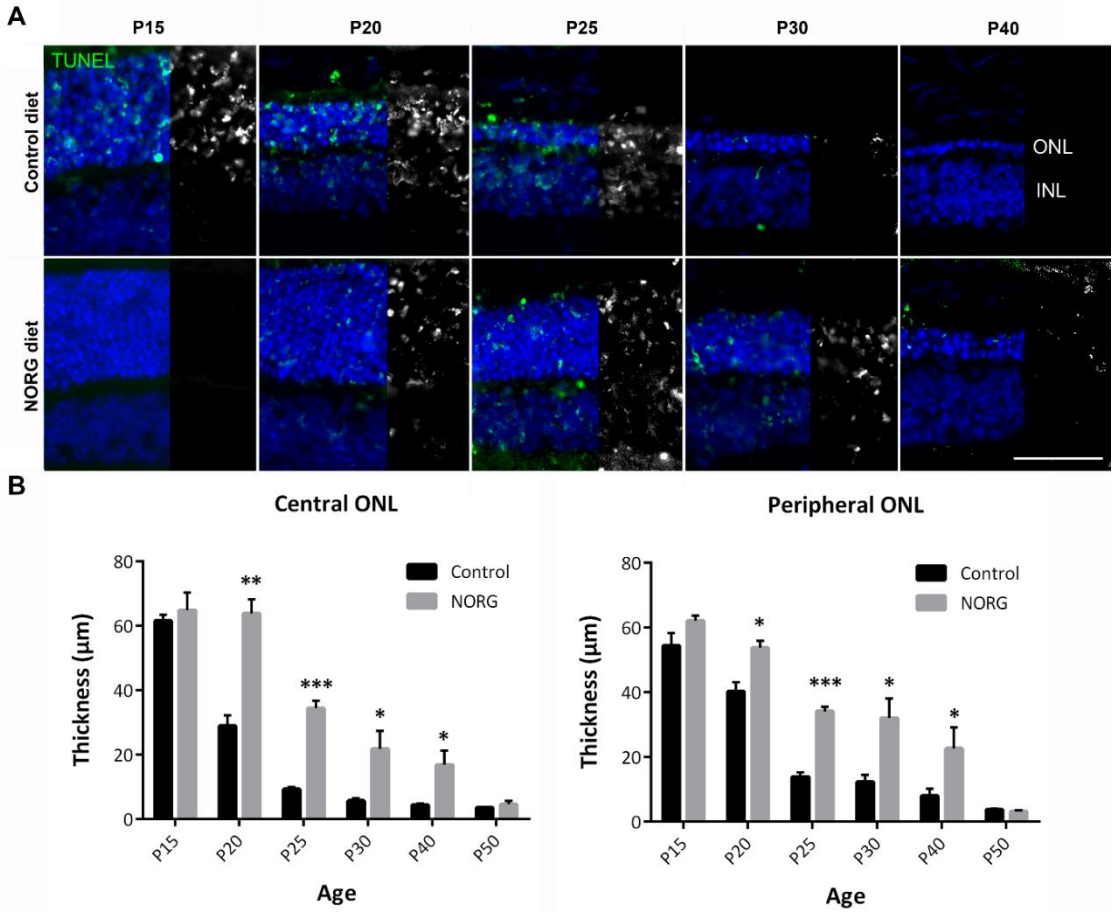


Figure 6.3.1. Norgestrel-supplemented diet significantly protects against photoreceptor cell death out to P40 in the rd10 retina. (A) *Apoptosis of retinal cells was detected by terminal dUTP nick end-labeling (TUNEL) of DNA strand breaks in cell nuclei. Norgestrel treatment (NORG diet) decreased TUNEL-positive staining in the central postnatal day (P) 15 and P20 retina compared to control mice (Control diet). TUNEL N=3, n=4. Hoechst (blue) staining reveals the cell layers in the retina. Scale bar 50µm.* ***(B)*** *Quantification of outer nuclear layer (ONL) thickness in the central and peripheral retina. Results are presented as mean ± SEM. Hoechst N=3, n=20. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ***p<0.005). INL, inner nuclear layer; ONL, outer nuclear layer.*

Dams of newborn rd10s were given a Norgestrel-supplemented diet when their pups reached post-natal day 10 (P10) and all rd10s continued to receive a Norgestrel-supplemented diet post-weaning. Control litters received an identical diet without Norgestrel. Norgestrel-supplemented rd10s demonstrated lower levels of TUNEL positivity in the outer nuclear layer (ONL) at P15 and P20 compared to control, coinciding with significant protection of photoreceptors (Figure 6.3.1A). As expected, control mice revealed a substantial loss of photoreceptors between P15 and P25 (Figure 6.3.1A) (Barhoum et al., 2008). Measurement of ONL thickness confirmed significant Norgestrel-induced ONL protection up to P40 in both the central and peripheral retina (Figure 6.3.1B).

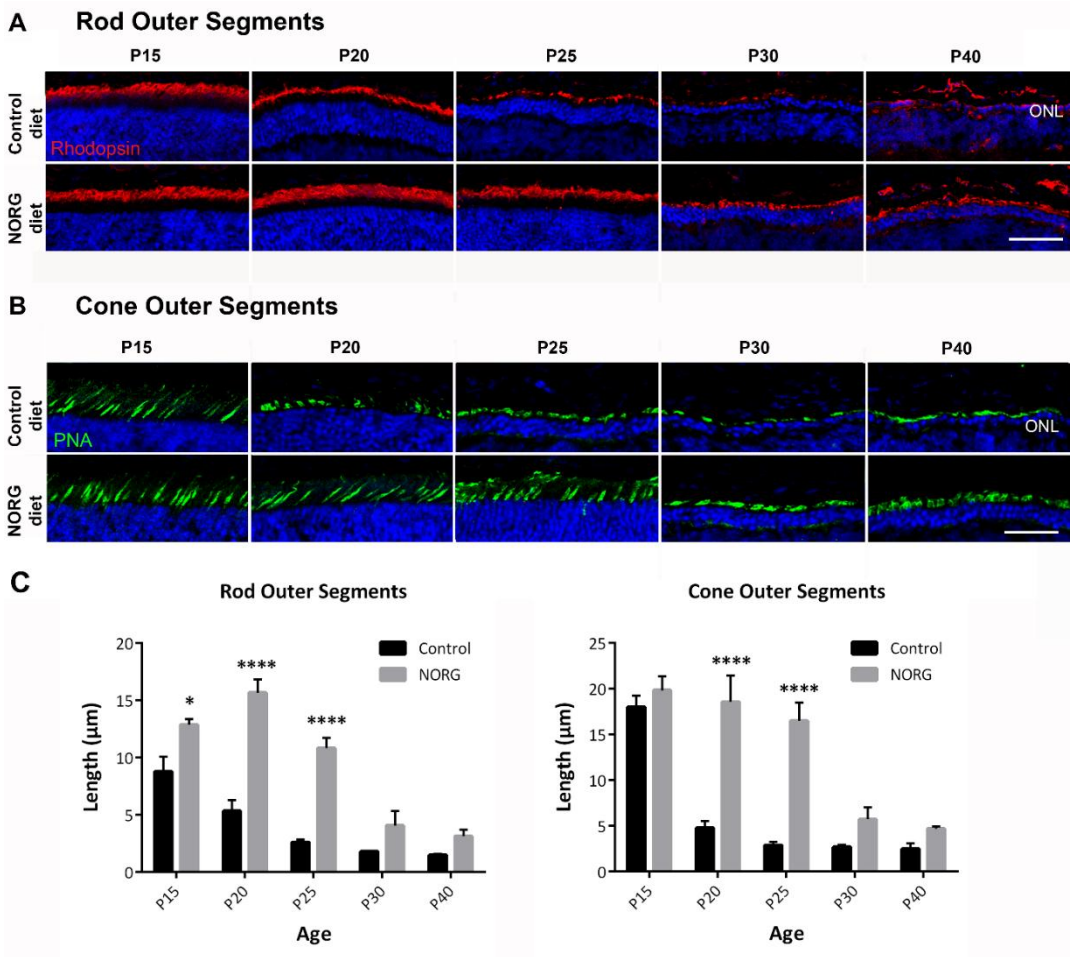


Figure 6.3.2. Norgestrel preserves rod and cone outer segment morphology in the central rd10 retina. *Confocal microscopic images of **(A)** rod (rhodopsin; red) and **(B)** cone outer segments (PNA; green) in the central retina from P15-P40 of control and NORG fed rd10 mice. Outer segments are shorter and sparser in the control from P15 for rods **(A)** and from P20 for cones **(B)**. Hoechst (blue) staining reveals the cell layers in the retina. Scale bar 50µm. **(C)** Quantification of rod and cone outer segment thickness in the central retina in control vs. NORG fed rd10 mice. Results are presented as mean ± SEM. N=3, n=4. Asterisks indicate significant difference (t-test, *p<0.05, ****p<0.001). Data generated by Dr Sarah Roche.*

In order to assess preservation of photoreceptor outer segments with Norgestrel diet, markers for rod (rhodopsin; red) and cone (PNA; green) outer segments were used on retinal sections. Outer segments were longer and denser in the central retina of Norgestrel treated mice compared to control (Figure 6.3.2A, B). This morphological change was observed from P15 for rods and from P20 for cones in the central retina (Figure 6.3.2A, B). Quantification of rod and cone outer segment length confirmed significant preservation up to P25 (Figure 6.3.2C).

6.3.2. Microglial response is significantly altered with Norgestrel treatment

Previous studies have highlighted a role for microglia in potentiating cell death in models of retinitis pigmentosa by driving inflammatory processes, resulting in photoreceptor loss (Peng *et al.*, 2014; Zhao *et al.*, 2015). We therefore sought to study microglial dynamics in rd10 mice following administration of a Norgestrel-supplemented diet. Activation states of macrophages (including resident retinal microglia) have been well characterized. Macrophages can both adopt and switch between pro-inflammatory (M1) and anti-inflammatory (M2) states (Mosser & Edwards, 2008) and thus specific state markers have been identified. Cluster of differentiation 68 (CD68) (Kobayashi *et al.*, 2013) and inducible nitric oxide synthase (iNOS) (Yang *et al.*, 2007) are markers for M1 macrophages. Markers of anti-inflammatory microglia (M2) have also been characterized, including mannose receptor (CD206/MRC1) (Karlstetter *et al.*, 2010) and arginase (Kobayashi *et al.*, 2013). CD68 is a lysosomal-associated membrane protein and scavenger receptor, expressed in phagocytic macrophages (Kobayashi *et al.*, 2013). iNOS is an enzyme responsible for the production of nitric oxide. Associated with neurotoxicity (Scholz *et al.*, 2015), it competes

with arginase - an enzyme that inhibits the production of nitric oxide to promote an M2 phenotype (Tenu *et al.*, 1999). MRC1 is involved in the recognition and endocytosis of glycoproteins on other cell types and the neutralization of pathogens (Gazi & Martinez-Pomares, 2009). Iba1 was used to identify microglia.

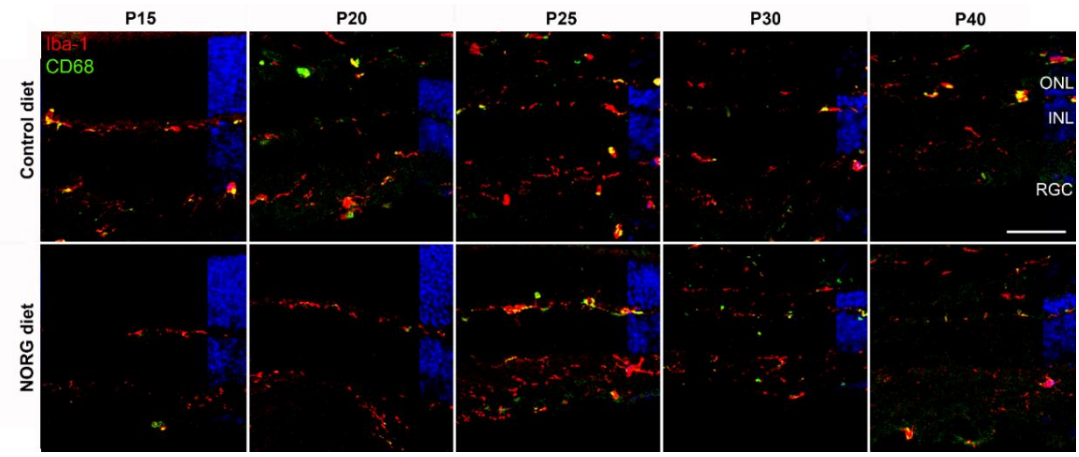


Figure 6.3.3. Norgestrel prevents microglial migration and dampens pro-inflammatory microglial activity in vivo. *Confocal microscopic images of microglia (Iba1; red) and pro-inflammatory (M1) microglia (CD68; green) in the central retina from P15-P40 control and NORG-fed rd10 mice. Hoechst (blue) staining reveals the cell layers in the retina. Scale bar 50µm. N=3, n=4. Data generated by Dr Sarah Roche.*

Amoeboid microglia could be observed surrounding the ONL at P15 and P20 in the control (Figure 6.3.3), during the peak of cell death (Figure 6.3.1A). Some of these microglia stained positively for CD68, indicative of an M1 phenotype. Interestingly, M1 microglia were observed in the inner plexiform layer (IPL) and retinal ganglion cell (RGC) layer, suggesting that microglia become activated even when not in close proximity to the ONL (Figure 6.3.3). In contrast, microglia appeared more ramified in morphology in Norgestrel-fed mice (P15, P20), showing less CD68 immunoreactivity than control: fewer M1 microglia were present in the Norgestrel-diet retinas (Figure 6.3.3). Correlating with significant photoreceptor protection (Figure 6.3.1A), this implicates microglial-driven inflammation in the cell death observed in rd10 mice.

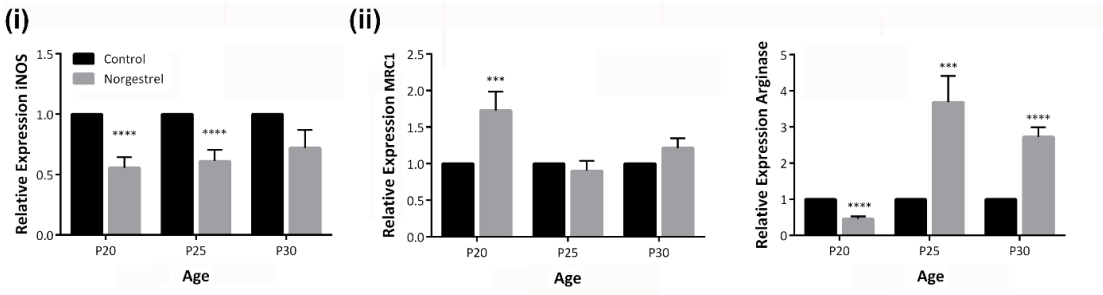


Figure 6.3.4. Norgestrel dampens pro-inflammatory microglial activity in vivo. *RT-qPCR analysis of mRNA encoding* ***(i)*** *pro-inflammatory (M1) and* ***(ii)*** *anti-inflammatory (M2) microglial markers in whole retina from P20-P30 control and NORG-fed rd10 mice. Results are presented as mean ± SEM. N=3, n=4. Asterisks indicate significant difference (t-test, ***p<0.005, ****p<0.001).*

This hypothesis is supported by the observation that phagocytic microglial activity (CD68) increased between P20 and P25 in mice on the Norgestrel diet, during a period of substantial photoreceptor loss (Figure 6.3.3). mRNA analysis was carried out on whole retina for M1 and M2 microglial state markers. This confirmed a protective, Norgestrel-driven, significant decrease in iNOS (M1) (P20, P25) (Figure 6.3.4i) and increase in M2 MRC1 (P20) and arginase (P25, P30) expression (Figure 6.3.4ii).

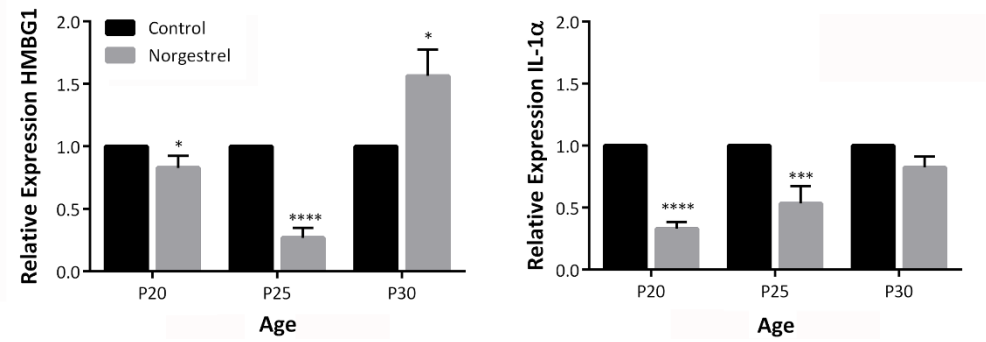


Figure 6.3.5. Norgestrel decreases DAMP release from photoreceptors. *RT-qPCR analysis of mRNA encoding danger associated molecular patterns (DAMP), high motility box group 1 (HMGB1) and interleukin-1α (IL-1α) in whole retina from P20-P30 control and Norgestrel-fed rd10 mice. Results are presented as mean ± SEM. N=3, n=4. Asterisks indicate significant difference (t-test, *p<0.05, ***p<0.005, ****p<0.001).*

High mobility group box 1 protein (HMGB1) and interleukin-1α (IL-1α) belong to the family of damage-associated molecular patterns (DAMPs), expressed and released by cells undergoing a loss of membrane integrity. These signals induce microglia to produce pro-

inflammatory cytokines and reactive oxygen species, resulting in worsened disease progression (Scaffidi *et al.*, 2002; Oppenheim & Yang, 2005; Chen *et al.*, 2007; Lugrin *et al.*, 2015). mRNA analysis of DAMP expression showed that Norgestrel induced a significant decrease in HMBG1 and IL-1α (Figure 6.3.5), coinciding with less M1 microglial activation at P20 (Figure 6.3.4i). Interestingly, at P25 when some microglia appear to have adopted an M1 phenotype with the Norgestrel diet (Figure 6.3.3), DAMP gene expression is still significantly decreased (Figure 6.3.5). This leads us to hypothesis the following scenario as likely: microglia migrate to the ONL to clear cellular debris, but respond instead to the cell death taking place. These microglia consequently take on an M1 phenotype, producing harmful inflammatory cytokines and reactive oxygen species, thus contributing to photoreceptor cell death.

6.3.3. Norgestrel significantly reduces rd10 microglial-driven, cell contact-dependent degeneration of 661W cells

In order to evaluate the contribution of microglia to the course of photoreceptor cell loss in the rd10 retina in more detail, we utilized primary cell cultures of rd10 retinal microglia. Primary rd10 microglial cultures were made using a previously established method (Weiglet 2007) and culture purity was confirmed. The absence of retinal astrocytes, Muller glia, rods, retinal ganglion, amacrine, horizontal and bipolar cells (Figure 6.3.6) in the cultures were confirmed by immunofluorescence. Although rare, some debris was present from these cell types was found in culture (Figure 6.3.6, inserts), however all isolated cells stained positively for Iba1 and so were confirmed to be pure microglia (Figure 6.3.6, 6.3.7).

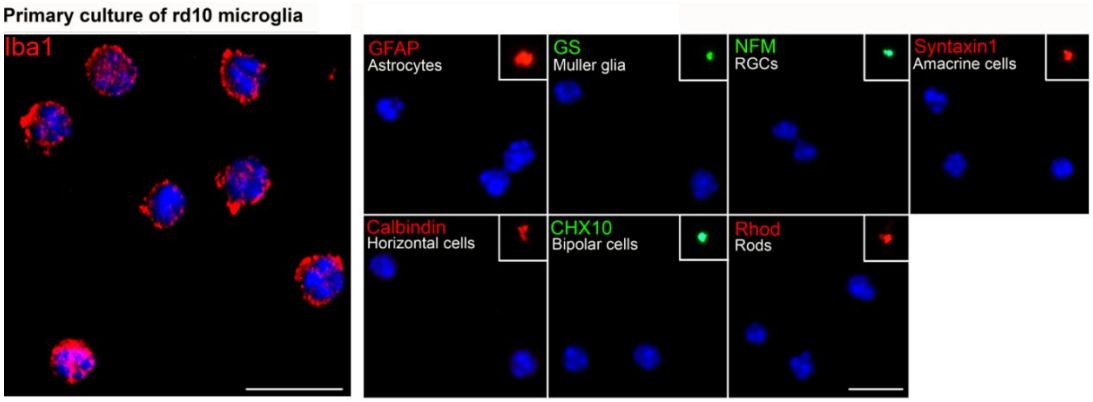


Figure 6.3.6. Characterisation of a pure microglial culture. *Primary microglial cells were cultured in vitro from post-natal day 16 rd10 retinas. Fluorescent microscopic images of primary rd10 microglial cells in vitro confirmed a pure microglial cell population (Iba1; Red). Hoechst (blue) staining reveals the cell nuclei. Antibodies targeted against astrocytes (GFAP; red), Müller glial cells (glutamate synthase (GS); green), retinal ganglion cells (RGCs) (neurofilament M (NFM); green), amacrine cells (syntaxin 1; red), horizontal cells (calbindin; red), bipolar cells (CEH10 homeodomain-containing homolog (CHX10); green) and rods (rhodopsin (Rhod); red) showed no positive staining within the isolated population of cells. Small quantities of non-nuclear (hoechst negative) debris from these cells was found in culture (see panel inserts). Scale bars 10µm. Figure generated by Dr Sarah Roche*

Due to the difficulty in isolating pure photoreceptor primary cultures (Roque *et al.*, 1999; Zhou *et al.*, 2012) and in order to assess the interaction between microglia and photoreceptors, we utilized the cone photoreceptor-like 661W cell line. Both throughout this thesis and in previous works published by our group, 661W cells have been used successfully as an *in vitro* model to study how photoreceptors respond to stressful stimuli and to understand how Norgestrel works in providing neuroprotection to these cells. Results from studies in 661W cells have consistently been validated *ex vivo* and so we believe it to be a suitable model for the study of photoreceptors *in vitro*. We first sought to determine the effect that released factors from rd10 microglia have on the viability of 661W cells, by culturing healthy 661W cells with conditioned media (CM) from rd10 microglia pre-treated with 20µM Norgestrel or equivalent DMSO control. 661W cells were cultured in microglial CM for 24 hours (h) and viability assessed using the MTS assay. Whilst CM from DMSO-treated microglia resulted in a ~14% increase in 661W cell death, CM from Norgestrel-treated microglia resulted in only a ~7% increase compared to untreated control

(Figure 6.3.7). This highlights a novel role for Norgestrel in modulating microglial immune responses in the rd10 retina.

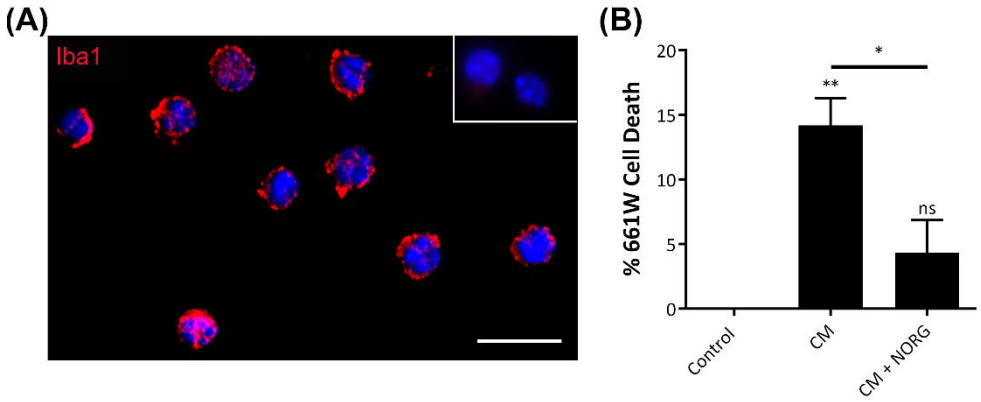


Figure 6.3.7. Norgestrel significantly abrogates rd10 microglial conditioned-media induced 661W cell death. (A) *Confocal microscopic XY image of primary rd10 microglial cells in vitro (Iba1; red). Secondary only control (panel insert) confirms specific staining of the antibody. Scale bar 10µm.* ***(B)*** *Conditioned media was collected from primary microglial cells cultured with (CM + NORG), or without (CM) 20µM Norgestrel over 24h. 661W cells were cultured over a further 24h in the absence (Control) or presence of conditioned media (CM, CM + NORG) and percentage cell death was assessed by the MTS assay. Results are presented as mean ± SEM. N=6, n=4. Asterisks indicate significant difference (One way ANOVA followed by Tukey's post-hoc test, *p<0.05, **p<0.01, ns=no significance).*

Direct cell-cell contact may regulate microglial responses (Duvall *et al.*, 1985; Lucas *et al.*, 2006). In particular, in the rd10 retina, microglia associate closely with cells in the ONL throughout the early stages of photoreceptor loss (Zhao *et al.*, 2015). Therefore, in order to assess the relationship between microglia and photoreceptors in a way that more closely resembles the *in vivo* state, we cultured microglia together with healthy 661W cells (example image of culture can be seen in Figure 6.3.8). To our knowledge, this has never before been carried out.

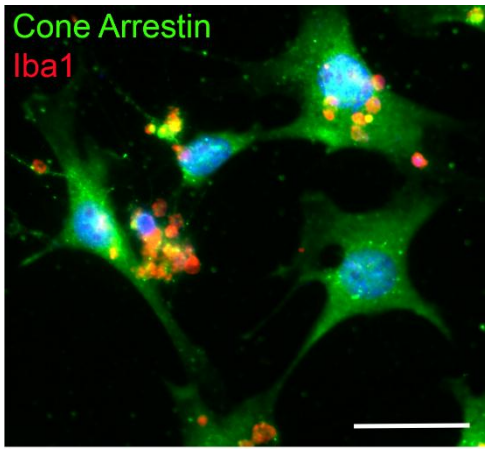


Figure 6.3.8. Co-culture of rd10 microglia and 661W cells. *Representative fluorescent microscopic image of primary rd10 microglial cells (Iba1; red) in culture with 661W cells (Cone arrestin; green). Hoechst (blue) staining reveals cell nuclei. Scale bar 30µm.*

Prior to co-culture, primary rd10 microglial and 661W cells were treated with 20µM Norgestrel or equivalent DMSO control (24h microglia, 3h for 661Ws). 661W cells were then lifted, added to the microglial cell culture and incubated for a further 24 hours. During this time, microglia appear to migrate and settle in clusters close to the 661W cells (Figure 6.3.8). Subsequent cell viability of co-cultured 661W cells was assessed using the TUNEL assay (Figure 6.3.9).

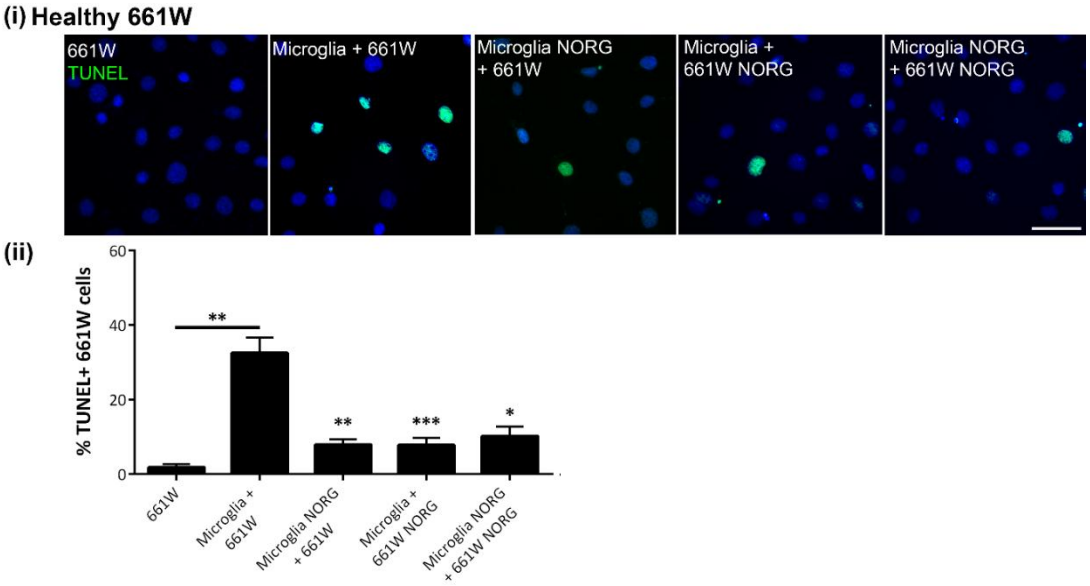


Figure 6.3.9. Norgestrel significantly abrogates rd10 microglial-induced death of healthy 661W cells. *661W cells were treated with either 20µM Norgestrel (661W NORG) or vehicle control (661W) over 3h. The treated 661W cells were then placed in culture with microglia that had been treated with (Microglia NORG) or without (Microglia) 20µM Norgestrel over 24h. Co-cultures were incubated for a further 24h. Apoptosis of 661W cells was detected by TUNEL of DNA strand breaks in cell nuclei. TUNEL N=6, n=3. Scale bars 50µm.* ***(i)*** *Representative images of TUNEL-positive (TUNEL+) staining in co-cultures.* ***(ii)*** *Quantification of TUNEL-positive 661W following Norgestrel or vehicle DMSO treatment and co-incubation with primary rd10 microglial cells pre-treated with Norgestrel or vehicle DMSO. All Norgestrel-treated co-cultures had significantly less TUNEL-positive 661W cells staining than equivalent vehicle controls. Results are presented as mean ± SEM. N=6, n=10. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ***p<0.005).*

After 24h of co-culture, ~32% of 661W cells were TUNEL-positive (TUNEL+) compared to ~1.8% of healthy 661W cells cultured in isolation (Figure 6.3.9). This strongly suggests that direct rd10 microglial-661W cell contact will result in higher levels of cell death, than when 661W cells are exposed to secreted microglial factors alone (conditioned media study, compare Figure 6.3.7B to 6.3.9). Treatment of microglia with 20µM Norgestrel prior to co-

culture significantly reduced their ability to damage the 661W cells (Figure 6.3.9), indicating that Norgestrel can work directly on microglia. Interestingly, pre-treatment of 661W cells with 20µM Norgestrel also resulted in increased protection of the photoreceptor-like cells Figure 6.3.9). Hence we demonstrate for the first time, Norgestrel's ability to prime undamaged photoreceptors against microglial-derived harmful stimuli.

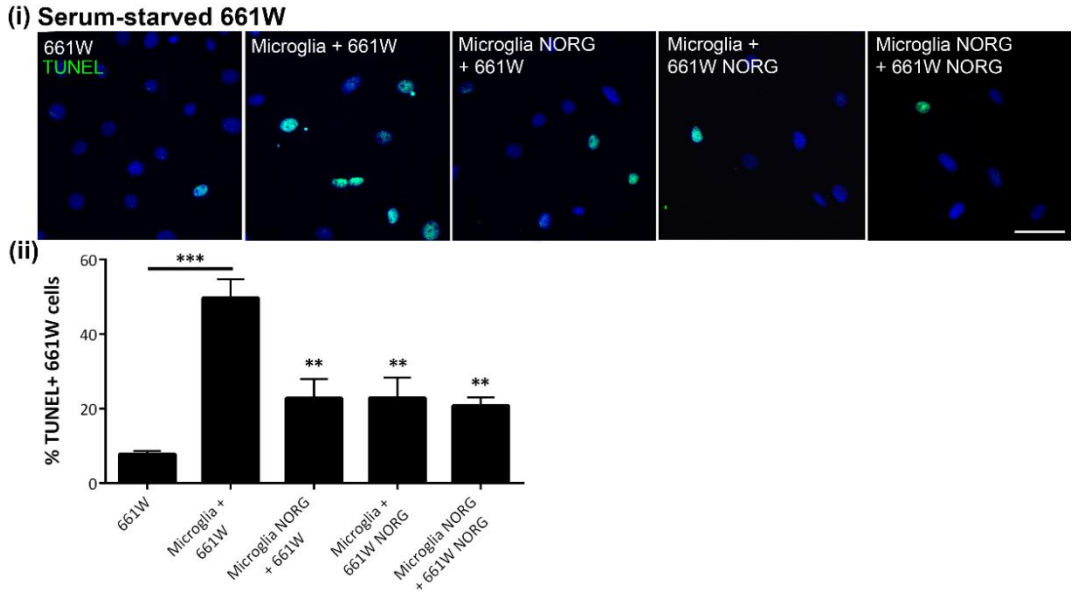


Figure 6.3.10. Norgestrel significantly abrogates rd10 microglial-induced death of stressed 661W cells. *661W cells were cultured in the absence of (serum starved 661W) serum over 3 h. These cells were simultaneously treated with either 20µM Norgestrel (661W NORG) or vehicle control (661W). The treated 661W cells were then placed in culture with microglia that had been treated with (Microglia NORG) or without (Microglia) 20µM Norgestrel over 24h. Co-cultures were incubated for a further 24h. Apoptosis of 661W cells was detected by TUNEL of DNA strand breaks in cell nuclei. TUNEL N=6, n=3. Scale bars 50µm.* ***(i)*** *Representative images of TUNEL-positive (TUNEL+) staining in co-cultures.* ***(ii)*** *Quantification of TUNEL-positive 661W cells cultured in the absence of (serum starved 661W) serum and following Norgestrel or vehicle DMSO treatment and co-incubation with primary rd10 microglial cells pre-treated with Norgestrel or vehicle DMSO. All Norgestrel-treated co-cultures had significantly less TUNEL-positive 661W cells staining than equivalent vehicle controls. Results are presented as mean ± SEM. N=6, n=10. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ***p<0.005).*

These data highlight a novel and important finding that Norgestrel can significantly reduce microglial-driven contact-dependent degeneration of healthy 661W cells. To further elucidate this relationship, we investigated the response of microglia to damaged photoreceptors, like those present in the rd10 mouse retina. The previous experiment was therefore replicated with 661W cells pre-exposed to a stressful stimulus, for it is possible that microglia would react differently when cultured with stressed 661W cells. Throughout

the course of this thesis, we have induced stress in 661W cells via serum-starvation over 24h. This results in an upregulation of pro-apoptotic pathways and ~40% cell death compared to control. Here, we serum starved 661W cells for 3h, resulting in ~8% cell death as assessed by the TUNEL assay (Figure 6.3.10ii). Addition of rd10 primary microglial cells to stressed 661Ws increased cell death to ~50% following 24h co-culture (as measured by TUNEL-positive (TUNEL+) staining). This was approximately a 42% increase in cell death compared to serum-starved 661W cells cultured in isolation (Figure 6.3.10).

When rd10 microglia are present in 661W cell cultures, cell death increased by ~30% and ~42% in healthy and serum starved 661W cells respectively. This implies that photoreceptors are more vulnerable to microglia-derived neurotoxic stimuli when they are already undergoing a stress response. Co-culture of microglia pre-treated with 20µM Norgestrel together with serum starved 661W cells significantly reduced the levels of 661W cell death (Figure 6.3.10ii). This suggests not only that Norgestrel can act directly on photoreceptors to protect against cell death, but also may work directly on microglia to both protect stressed photoreceptors from contact-dependent microglial-driven degeneration (Figure 6.3.10) and microglial-derived harmful stimuli (Figure 6.3.7B).

6.3.4. Norgestrel modulates pro- and anti-inflammatory states in microglia

Norgestrel can act directly on rd10 microglia and consequently prevent microglial-driven 661W cell death (Figures 6.3.7, 6.3.9, 6.3.10). Thus, we wished to elucidate on Norgestrel's mechanism of action on microglia. To do this, we first studied microglial progesterone

receptor expression, for we have previously shown that these receptors can mediate Norgestrel's neuroprotective effects. mRNA analysis of cultured rd10 microglia revealed similar expression patterns of progesterone membrane complexes 1 and 2 (PGRMC1, PGRMC2), and membrane progesterone receptors α, β and γ (mPRα, β, γ) (Figure 6.3.11A). Expression of the classical progesterone receptors A and B (PRA/B), was not detected in these cells (Figure 6.3.11A). Immunofluorescence confirmed expression of these receptors (Figure 6.3.11B).

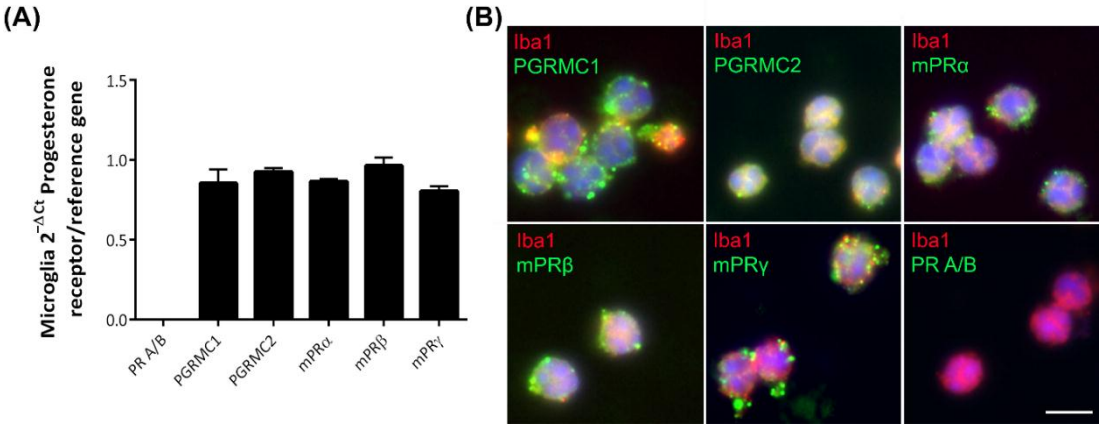


Figure 6.3.11. Classification of progesterone receptors present in rd10 microglia. (A) *RT-qPCR analysis detected similar levels of mRNAs encoding progesterone receptor membrane complexes 1 and 2 (PGRMC1, PGRMC2) and all three membrane progesterone receptor isoforms, α, β and γ (mPRα, mPRβ, mPRγ) in rd10 retinal microglial cells. Classical progesterone receptor (PR A/B) was not expressed. Results are presented as mean ± SEM.* ***(B)*** *Immunolabelling of protein confirmed the presence of these progesterone receptors in primary rd10 microglial cells. Scale bar 10µm.*

Since all progesterone receptors except classical progesterone receptors A/B (PR A/B) were present in rd10 microglia, it is likely that Norgestrel will act on these receptors to affect microglia directly. Primary rd10 microglia were subsequently treated with 20µM Norgestrel and analysed by immunofluorescence for M1 and M2 state markers. rd10 microglia treated with Norgestrel, revealed lower levels of CD68 and iNOS (M1) and higher levels of MRC1 (M2) compared to control (Figure 6.3.12A). Quantification of fluorescence intensity measurements confirmed a significant Norgestrel-induced decrease in M1 markers and

increase in M2 MRC1 (Figure 6.3.12B). Thus we hypothesise that Norgestrel dampens pro-inflammatory activity and stimulates anti-inflammatory states in rd10 microglia, resulting in increased protection of photoreceptors.

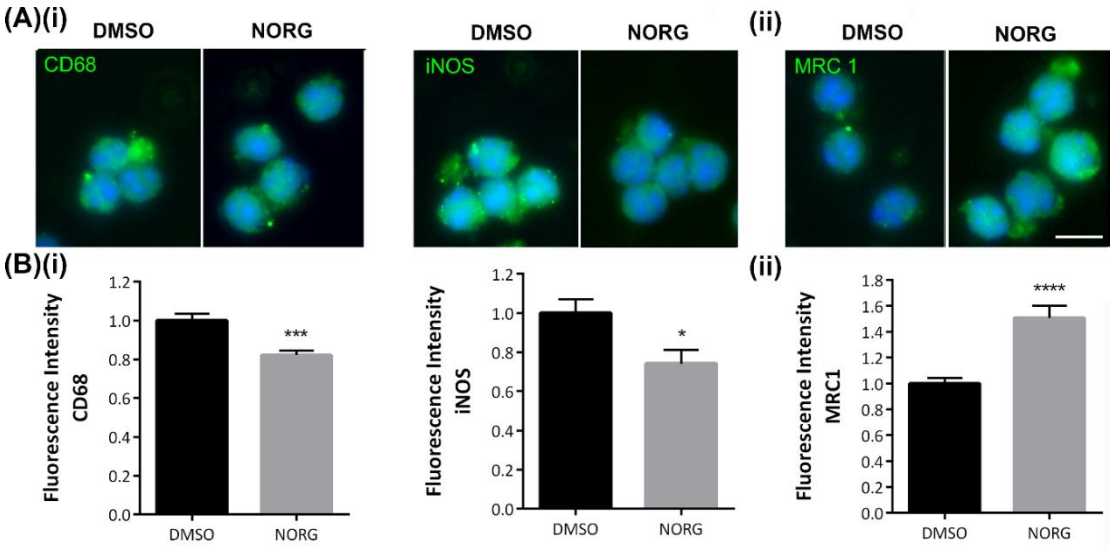


Figure 6.3.12. Norgestrel reduces pro-inflammatory and promotes anti-inflammatory phenotypes in rd10 microglia. (A) *Fluorescent microscopic images of primary rd10 microglia immunolabelled for* ***(A)(i)*** *pro-inflammatory (M1) markers CD68 and iNOS, and* ***(A)(ii)*** *anti-inflammatory (M2) activation state marker MRC1; after treatment with 20µM Norgestrel over 24h. Hoechst (blue) staining reveals the cell nuclei of the microglia. Scale bar 10µm.* ***(B)*** *Quantification of fluorescent intensity of* ***(B)(i)*** *CD68 and iNOS and* ***(B)(ii)*** *MRC1 in primary rd10 microglial cultures following Norgestrel treatment over 24h. Results are presented as mean ± SEM. N=4, n=4. (t-test, *p<0.05, ***p<0.005, ****p<0.001). Data generated by Dr Sarah Roche.*

6.3.5. Norgestrel acts as an anti-inflammatory in stressed 661W cells and in rd10 mice

Macrophage infiltration is extremely important in the progression of retinal disease (Zhao *et al.*, 2015). Chemokines, a family of structurally related cytokines are involved in the activation and directed migration of immune cells. Signalling to macrophages, these factors have been shown to drive microglial infiltration (Kunkel, 1999) and thus we looked at how Norgestrel treatment affected an array of CC chemokine gene expression: MIP-1α (CCL3), MIP-1β (CCL4), MCP1 (CCL2) and MCP3 (CCL7). These chemokines have all previously been

reported to be important in photoreceptor-microglial crosstalk (Rutar *et al.*, 2015; Syeda *et al.*, 2015) leading to retinal degeneration (Yang *et al.*, 2007; Scholz *et al.*, 2015).

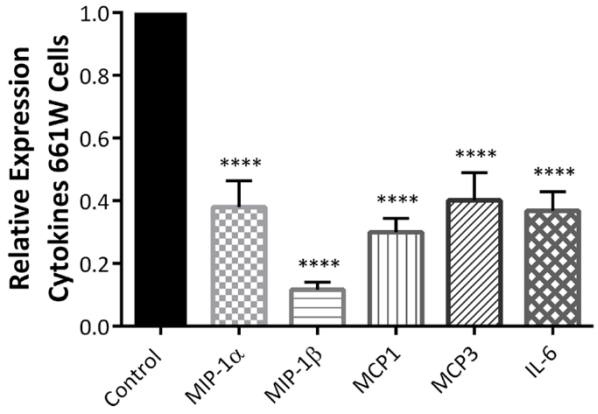


Figure 6.3.13. Norgestrel reduces inflammation in stressed 661W cells. *Retinal cytokine mRNA levels were measured by RT-qPCR analysis, in serum starved 661W cells treated with 20μM Norgestrel or DMSO control. Relative chemokine (macrophage inflammatory protein 1α and 1β (MIP-1α, MIP-1β), monocyte chemoattractant proteins 1 and 3 (MCP1, MCP3)) and pro-inflammatory cytokine interleukin 6 (IL-6)) mRNA expression levels in 661W cells. Only one control bar is shown for graphical clarity (Control). Results are presented as mean ± SEM. N=3, n=4. Asterisks indicate significant difference (t-test, ****p<0.001).*

Norgestrel successfully acts as an anti-inflammatory, downregulating chemokine gene expression both in stressed 661W cells (Figure 6.3.13) and during the early stages (P15 – P20) of rd10 disease progression (Figure 6.3.14i). Norgestrel-induced downregulation of CC chemokine gene expression concomitantly associated with reduced gene transcription for pro-inflammatory mediators TNFα, IL-1β and IL-6 (Figure 6.3.14ii). Likely secreted from retinal microglia, these cytokines are documented to act both directly (Scuderi *et al.*, 2015; Zhao *et al.*, 2015) and indirectly (Liu, Ye, *et al.*, 2015) on photoreceptors, potentiating cell death via pro-inflammatory mechanisms. A loss of photoreceptors at P25 correlates with an upregulation of chemokine gene expression (Figure 6.3.14ii) and a subsequent infiltration of microglia to the ONL (Figure 6.3.3). This is thought to be why there is an increase in pro-inflammatory gene transcription at P25 (Figure 6.3.14ii).

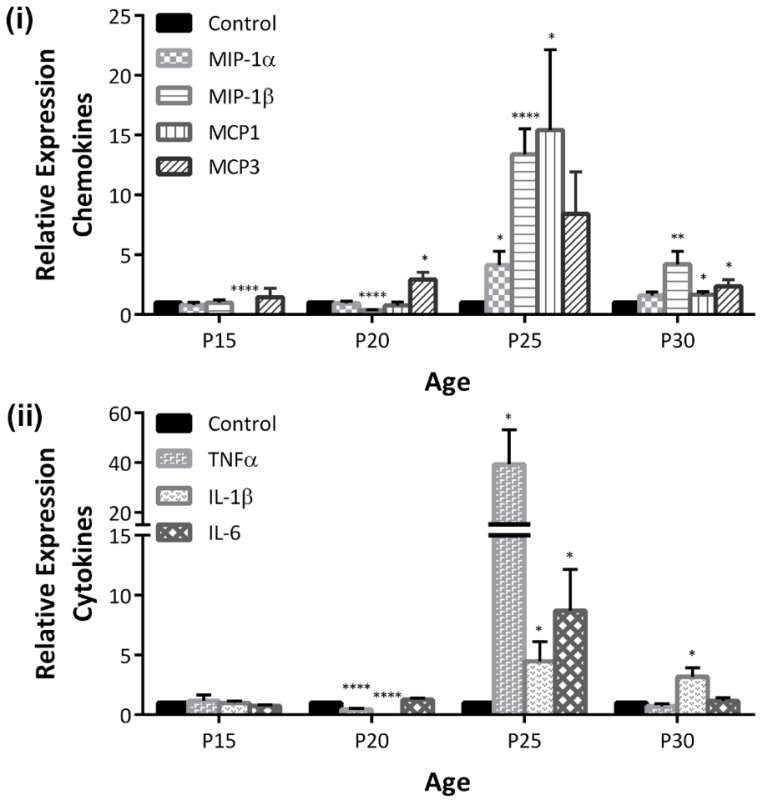


Figure 6.3.14. Norgestrel reduces inflammation in the rd10 retina. *Retinal cytokine mRNA levels were measured by RT-qPCR analysis, in post-natal (P) day 15-30 rd10 mice. Relative **(i)** chemokine (macrophage inflammatory protein 1α and 1β (MIP-1α, MIP-1β), monocyte chemoattractant proteins 1 and 3 (MCP1, MCP3)) and **(ii)** pro-inflammatory cytokine (tumour necrosis factor α (TNFα), interleukin 1β (IL-1β) and interleukin 6 (IL-6)) mRNA expression levels in retinas of rd10 mice supplemented with or without (Control) Norgestrel. Results are presented as mean ± SEM. N=3, n=4. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ****p<0.001).*

6.3.6. Norgestrel up-regulates CX3CR1-fractalkine signalling between microglia and photoreceptors

It is now recognised that it is not solely the actions of chemokines that contribute to the maintenance of microglia in a quiescent state; but also the interaction of microglia with other cell types (Lyons *et al.*, 2007, 2009; Costello *et al.*, 2011). Recent studies have shown that complementary distribution of the chemokine fractalkine (also known as CX3CL1) on neurons and its receptor CX3CR1 present on microglia, can modulate and attenuate pro-inflammatory microglial activation (Henkel *et al.*, 2009; Lyons *et al.*, 2009; Wang *et al.*,

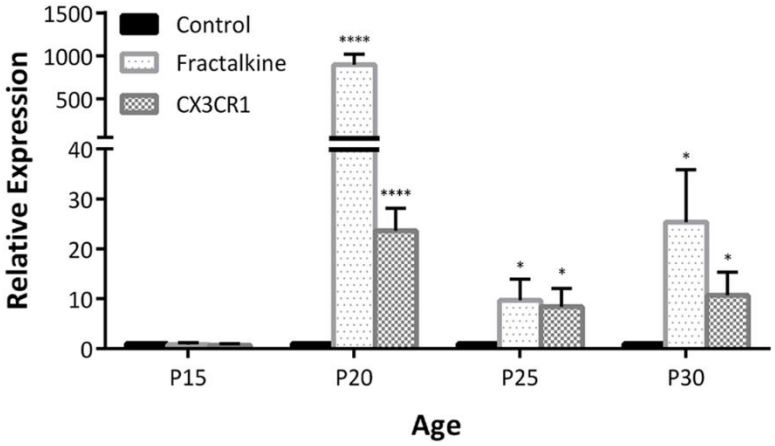


Figure 6.3.15. Fractalkine signalling is up 1000-fold in P20 Norgestrel-treated rd10s. *Retinal cytokine mRNA levels were measured by RT-qPCR analysis, in postnatal (P) day 15-30 rd10 mice. Relative mRNA expression levels in retinas of rd10 mice supplemented with or without (Control) Norgestrel. Results are presented as mean ± SEM. N=3, n=4. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ****p<0.001).*

2014). This pairing provides an ideal mechanism to mediate neural/microglial interactions (Harrison *et al.*, 1998). Therefore, we looked at the ability of Norgestrel to up-regulate the gene expression of fractalkine and its receptor CX3CR1, in the degenerating rd10 mouse retina. Norgestrel treatment significantly increased fractalkine levels in the P20 rd10 retina by over 1000-fold that of the control (Figure 6.3.15); coinciding with significant protection of the ONL (Figure 6.3.1). Interestingly, Norgestrel also up-regulated CX3CR1 gene expression at P20 (Figure 6.3.15), at a time when pro-inflammatory microglia are down in the retina (Figure 6.3.3, 6.3.4). Norgestrel-induced fractalkine signalling was dampened at later time points (P25, P30) compared to P20-Norgestrel treated mice, though gene expression of both fractalkine and receptor was still significantly over ten times that of the control (Figure 6.3.15). Dampened fractalkine signalling correlated with photoreceptor cell loss in the Norgestrel-treated mice (Figure 6.3.1).

6.3.7. Fractalkine is produced in the ONL of Norgestrel-treated mice

Fractalkine, a neuronal membrane-bound chemokine, can be proteolytically cleaved to shed a soluble chemoattractant domain (Bazan *et al.*, 1997; Hundhausen *et al.*, 2003). The two forms: soluble and full-length membrane-bound can therefore stimulate different

microglial activities. Membrane bound fractalkine maintains microglial in a quiescent state, preventing their infiltration towards neurons. Soluble fractalkine however, is more likely to activate microglial phagocytic activity and stimulate microglia migration (Zhang *et al.*, 2012). Since RT-qPCR studies showed a robust increase in fractalkine gene transcription in response to Norgestrel treatment from P20 onwards (Figure 6.3.15), we looked to see that this result was mimicked at the protein level. Fractalkine staining was increased in the ONL of all (P15 – P40) Norgestrel treated mice, compared to control (Figure 6.3.16). This staining was evident on the surface of the photoreceptors – indicative of an upregulation of full length membrane-bound forms.

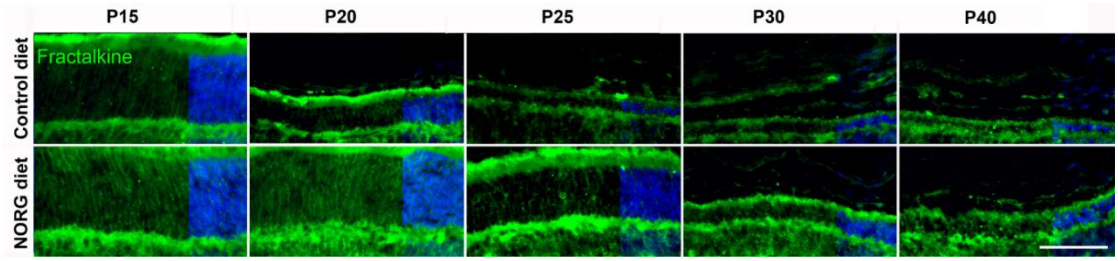


Figure 6.3.16. Fractalkine is evident in the ONL of Norgestrel-treated rd10 mice from P15. *Fluorescent microscopic images of fractalkine (green) in the central retina from post-natal day (P) 15-40 of control and Norgestrel (NORG) diet fed rd10 mice. Fractalkine staining is evident in the outer nuclear layer (ONL) of the retina in Norgestrel-supplemented mice. Hoechst (blue) staining reveals the cell layers in the retina. Scale bar 50µm. N=3, n=3.*

6.3.8. Fractalkine can signal directly to CX3CR1 receptors present on 661W cells

Norgestrel treatment of stressed 661W cells significantly up-regulated the gene expression of both fractalkine and, interestingly, CX3CR1 over 24 hours (Figure 6.3.17A). Recent literature suggests that fractalkine may also work on other cells apart from microglia, for the CX3CR1 receptor has been found upon a multitude of neuronal cells (London *et al.*, 2013; Dworzak *et al.*, 2015). Indeed, we identified expression of CX3CR1 at both the genomic (Figure 6.3.17Aii) and translational protein level (Figure 6.3.17B) in 661W cells.

Therefore, in order to identify if Norgestrel-induced fractalkine release could work directly on photoreceptors, we treated stressed 661W cells with increasing concentrations (0.01 – 100nM) of soluble recombinant fractalkine, over 24 hours. Fractalkine-treated stressed cells exhibited similar levels of neuroprotection to those treated with 20µM Norgestrel (Figure 6.3.17C), indicating that fractalkine's neuroprotective effects may be two tiered: both dampening microglial pro-inflammatory activation and working directly on stressed photoreceptors to induce cell survival.

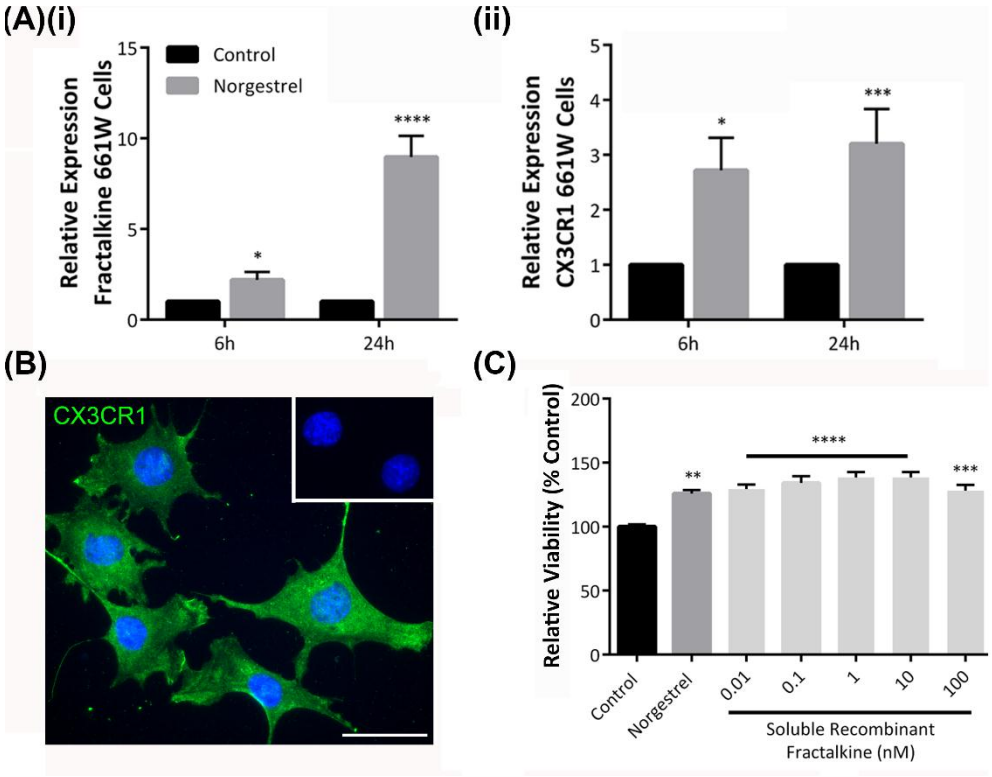


Figure 6.3.17. Fractalkine can act directly on 661W cells to prevent from serum starved cell death. (A) *661W cells were serum starved and treated with 20µM Norgestrel (Norgestrel) or the equivalent DMSO control (Control) over 6 and 24h. RT-qPCR analysis detected a significant increase in mRNA levels of both* ***(i)*** *fractalkine and* ***(ii)*** *its receptor CX3CR1 in starved cells treated with Norgestrel. N=6, n=4.* ***(B)*** *Representative fluorescent microscopic image of CX3CR1 (green) present in 661W cells. Secondary only control (panel insert) confirms specific staining of the receptor. Hoechst (blue) staining reveals the cell nuclei. Scale bar 30µm.* ***(C)*** *661W cells were serum starved over 24 hours and treated with 20µM Norgestrel (Norgestrel), increasing concentrations (nM) of soluble recombinant fractalkine, or the equivalent DMSO control (Control). Percentage cell viability was assayed and compared to 100% viable DMSO control. Results are presented as mean ± SEM. N=6, n=4. Asterisks indicate significant difference (t-test, *p<0.05, **p<0.01, ***p<0.005, ****p<0.001).*

6.4. Discussion

This research demonstrates the protective properties of the synthetic progestin 'Norgestrel', when administered via non-invasive diet supplementation. Previously shown to protect through an upregulation of basic fibroblast growth factor with subsequent actions on GSK3β (Chapter 3, 5), this study highlights a novel role for Norgestrel in acting directly upon microglial cells to dampen damaging inflammatory processes. This is important, as recent literature has shown that microglial cells may potentiate retinal disease (Arroba et al., 2011; Martínez-Fernández de la Cámara et al., 2015; Peng et al., 2014; Scholz et al., 2015; Zhao et al., 2015). We also show provide further mechanistic insight in to Norgestrel's neuroprotective effects, showing that Norgestrel drives up fractalkine expression in photoreceptors, dampening pro-inflammatory and migratory effects. In addition to this, we believe that fractalkine may also work to provide direct photoreceptor neuroprotection.

Reactive microglia are known to promote retinal cell death through inflammation (Yang *et al.*, 2007; Sivakumar *et al.*, 2011; Wang *et al.*, 2015). Initially involved in apoptotic cell clearance, microglia may accelerate photoreceptor cell loss through phagocytosis of stressed but viable neurons (Peng *et al.*, 2014; Zhao *et al.*, 2015). Certainly, in our study, we show microglial cells infiltrate the ONL during photoreceptor degeneration (Control, P20; Norgestrel-supplemented, P25) (Figure 6.3.3). Substantiating this, disease progression is significantly delayed when microglial cells are genetically ablated, or their phagocytic and pro-inflammatory activity dampened in the rd10 mouse (Peng *et al.*, 2014; Zhao *et al.*, 2015). Microglial cells exist in differing states of 'activation'. Known as the M1 'pro-

inflammatory' and M2 'anti-inflammatory' activation states, a greyscale exists – microglia may exist exhibiting properties of both (Mosser & Edwards, 2008; Cherry *et al.*, 2014). Therefore, to state conclusively that microglia are in the M1/M2 state is an over simplification and must be avoided. Our results however, suggest that Norgestrel can dampen M1 microglial activation and promote a protective M2 state (Figures 6.3.3, 6.3.4, 6.3.12). From the literature, this suggests that Norgestrel-induced M1 microglial inhibition is protective to rd10 retinas (Peng *et al.*, 2014).

As such, we designed this study with a number of questions in mind: Are rd10 retinal microglia predisposed to act as 'drivers' of degeneration, as the literature suggests; or, are they responding to signals released from mutated photoreceptor cells inducing a damaging M1 phenotype? Alternately, it may be that quiescent microglia migrate towards the ONL initially to clear debris (Pearson *et al.*, 1993). Once in contact with mutated photoreceptors however, they may then do they then adopt an M1 phenotype, further potentiating cell death through inflammation? In order to address the above questions, we utilised both *in vivo* models of retinal degeneration (rd10) and *in vitro* cultures of 661W cone photoreceptor-like cells and primary rd10 microglia both in isolation and in co-culture. These models enabled us to identify several novel mechanisms of action of Norgestrel.

Firstly, 661W cells, cultured with conditioned media from primary rd10 microglial cells, revealed Norgestrel's ability to act on microglial cells to attenuate damaging inflammatory factor secretion (Figure 6.3.7B), a process known to cause retinal cell death *in vivo* (Yang *et al.*, 2007; Sivakumar *et al.*, 2011; Zhou *et al.*, 2012; Dworzak *et al.*, 2015). Secondly, co-culture studies showed that microglia-photoreceptor contact induces photoreceptor cell

death significantly more than conditioned media alone (compare Figure 6.3.7B to 6.3.9ii). In addition to this, microglia induced cell death by ~30% and ~42% in healthy and serum starved 661W cells respectively, suggesting that photoreceptors are more vulnerable to microglia-derived neurotoxic stimuli when already stressed (compare Figure 6.3.9ii to 6.3.10ii). Thirdly, we provide mechanistic insight in to Norgestrel's effect on microglia both *in vivo* and *in vitro*, working directly to reduce pro-inflammatory M1 and stimulate anti-inflammatory M2 phenotypes (Figure 6.3.3, 6.3.4, 6.3.12), thus preserving photoreceptor cell viability. This finding was supported by RT-qPCR studies on whole retinas (Figure 6.3.14ii), showing Norgestrel lead to a significant decrease in pro-inflammatory TNFα and IL-1β gene expression, both expressed by retinal microglia (Wang et al., 2005).

Ultimately however, Norgestrel administration merely delays cell death in the rd10 mice (Figure 6.3.1). So what is driving this cell loss in the Norgestrel-treated mice? One potential theory is the loss of Norgestrel-induced fractalkine-CX3CR1 signalling at P25. Fractalkine can suppress TNFα release (Figure 6.3.14) from pro-inflammatory microglia (Figure 6.3.3, 6.3.4, P20) (Mizuno *et al.*, 2003) and fractalkine-CX3CR1 signalling has been shown to maintain quiescent microglia, preventing microglial migration and neurotoxicity (Mizuno *et al.*, 2003; Lyons *et al.*, 2009; Zhang *et al.*, 2012; Peng *et al.*, 2014). In the P20 rd10 retina, Norgestrel drives up fractalkine gene expression by a 1,000-fold change compared to control, however these levels are not maintained at P25 (Figure 6.3.15). Fractalkine appears to be mostly localised to the ONL and outer plexiform layer (OPL) (Figure 6.3.16). Since fractalkine signalling has been shown to prevent cell loss in a number of different disease models (Harrison *et al.*, 1998; Mattison *et al.*, 2013; Wang *et al.*, 2014; Cardona *et al.*, 2015; Dworzak *et al.*, 2015; Febinger *et al.*, 2015), we believe that there is a crucial importance of CX3CR1-fractalkine signalling in retinal neuroprotection. Thus when

Norgestrel-induced fractalkine signalling is lost at P25, microglial cells are able to infiltrate the ONL and drive disease progression (Figure 6.3.1, 6.3.3).

The fractalkine-CX3CR1 pathway is coming to the forefront of the retinal field due to a. its neuroprotective capacities and b. because of its differential signalling capabilities. To detail: fractalkine is unlike most other chemokines, in that it is a type I transmembrane adhesion protein. Therefore, it can act as a chemoattractant for target cells expressing its receptor CX3CR1 (Stievano *et al.*, 2004). Mouse fractalkine cDNA encodes a 395 amino acid chain that includes a signal sequence, a chemokine domain, a mucin stalk region, a transmembrane segment and a cytoplasmic tail. The chemokine domain contains binding and chemotactic regions whilst the mucin stalk region appears only to act as a spacer (Harrison *et al.*, 2001; Mizoue *et al.*, 2001). Produced as a cell surface protein, in its full length form fractalkine protein has a molecular weight of approximately 95 - 100kDa, but cleavage can be induced by ADAM 10 and ADAM17 to generate a 60 – 80kDa soluble form (Tsou *et al.*, 2001; Hundhausen *et al.*, 2007).

Fractalkine can be cleaved to form two distinct products and thus it can consequently act in two distinct manners. In a paper released in 2012, fractalkine was shown to have discrete roles dependent upon whether it was added in its soluble or full length form. Soluble fractalkine induced an increased amount of microglial migration to photoreceptors *in vitro*, causing an increased quantity of TUNEL-positive photoreceptor cells. Full length fractalkine however, was protective to stressed photoreceptors through a prevention of microglial migration (Zhang *et al.*, 2012).

This point may be significant in our capacity to understand what is happening at the P25 time point; when Norgestrel-induced neuroprotection is lost and pro-inflammatory microglia infiltrate the ONL. At P20, Norgestrel up-regulates fractalkine signalling over 1000-fold that of control. We postulate that it is this increase in fractalkine signalling that keeps the microglia in a quiescent state. At P20, fractalkine staining is predominantly localised to the cell membrane of photoreceptors; thus this is likely to be the full-length, membrane bound, protective fractalkine. Unfortunately, at P25 this staining is lost. There is still a significant increase of fractalkine at the mRNA level (approximately 10-15 fold), yet this is nowhere near the 1000-fold levels seen at the P20 time point. Corroborating this, at P25 membrane-bound fractalkine is no longer easily identifiable at the photoreceptors. Thus, we hypothesise the following: Norgestrel drives an upregulation of full-length fractalkine at P20, localised to the outer membranes of photoreceptors. At P25, the majority of Norgestrel-induced fractalkine transcription is lost and full length-fractalkine is cleaved to the soluble form. This will induce chemotaxis of CX3CR1-presenting microglia in to the ONL. Once here, these microglia will react to the damage of the diseased retina, engulfing both stressed and healthy photoreceptors alike. Work is continuing in our laboratory on fractalkine-CX3CR1 signalling following Norgestrel administration and the role of full length *vs.* soluble fractalkine in RP disease progression.

Interestingly, in our 661W cells, fractalkine also appears to have a direct role in cell viability: stressed cells treated with increasing concentrations of soluble recombinant fractalkine were afforded similar protection compared to stressed 661W cells treated with Norgestrel alone (Figure 6.3.17C). This is particularly interesting for it shows a mechanism by which fractalkine may work on photoreceptors directly through the CX3CR1 receptor (Figure 6.3.17Aii, B). This result may initially appear controversial, for CX3CR1 is often

stated to be present solely on microglia (Lyons *et al.*, 2009; Zhao *et al.*, 2015). However, evidence is mounting to show that the CX3CR1 receptor is also present on neurons and astrocytes (Meucci *et al.*, 2000; Gillard *et al.*, 2002; Hatori *et al.*, 2002; Hughes *et al.*, 2002; Mizuno *et al.*, 2003; Deiva *et al.*, 2004; Limatola *et al.*, 2005). Neuronal CX3CR1 appears to play a role in development and neuronal survival (Meucci *et al.*, 2000; Tong *et al.*, 2000; Gillard *et al.*, 2002; Mizuno *et al.*, 2003). Therefore, it would stand to reason that recombinant fractalkine is neuroprotective to stressed 661W cells *in vitro* (Figure 6.3.17C).

In conclusion, this study outlines for the first time, the neuroprotective properties of the synthetic progestin 'Norgestrel', in acting directly on retinal microglia to dampen damaging microglial activation *in vivo*. In addition to this, we reveal novel aspects to Norgestrel's protective effects within photoreceptors. The data presented suggest a critical role for Norgestrel-induced fractalkine signalling, not only in the dampening of microglial activation *in vivo* but also in the direct protection of damaged 661W photoreceptor-cells. Future studies will be aimed at further elucidating the role that fractalkine signalling plays in the neuroprotection associated with Norgestrel and considering therapeutic avenues that utilise a combination of drugs to include microglia-driven inflammation as a target. We believe direct photoreceptor neuroprotection should remain at the forefront of RP immunotherapy development, though a combined treatment with anti-inflammatory properties is still desirable. Targeting mutated photoreceptors directly would alleviate subsequent photoreceptor-microglial cell crosstalk, minimizing consequential microglial-driven photoreceptor cell loss. Additional anti-inflammatory properties may then further prevent microglial responses shown to exacerbate disease progression.



Chapter 7. Discussion



7. Discussion

Affecting 1 in 4000 individuals, retinitis pigmentosa (RP) is the most common genetically inherited ocular disease worldwide (Boughman *et al.*, 1980). Its genetic heterogeneity however makes it extremely complicated to treat at a genetic level. Consequently, a neuroprotective strategy remains at the forefront of retinal disease research. In 2009, our group followed along this line of research. A library of 1040 FDA approved drugs was screened for potential anti-apoptotic properties, using the retina-derived 661W photoreceptor cell line (Doonan *et al.*, 2009). The Alamar Blue assay, a fluorometric indicator of cellular metabolic activity; measured the cellular proliferation, cytotoxicity and viability of the cells after treatment with these compounds. This screen identified the synthetic progestin 'Norgestrel', a common component of the female oral contraceptive pill, for further analysis. Our group subsequently published the first study on the neuroprotective effects of Norgestrel in 2011. Here, they found Norgestrel to be neuroprotective to stressed or diseased retinas *in vivo* in both a preventative (through the Balb/c albino light damage model) and a rescue (using the rd10 mouse model of retinal degeneration) situation (Doonan *et al.*, 2011).

Progestogenic hormones are thought to modulate pro-survival pathways in the central nervous system (CNS) (Gonzalez Deniselle *et al.* 2002a, 2002b; Gonzalez *et al.* 2005; Compagnone 2008; Espinosa-García *et al.* 2014; Yousuf *et al.* 2014; Qin *et al.* 2015) and since these hormones are produced naturally in the body (Gans & De Jongh, 1961), their value as potential therapeutic agents is clear. However, despite promising studies in the CNS, research has presented conflicting results as to the capabilities of progesterone to act

as a neurotherapeutic in retinal eye disease. For example, progesterone administered by intraperitoneal injection (IP) (4mg/kg) protected the inner nuclear and nerve fibre layers in a model of retinal ischemia in rats (Lu *et al.*, 2008) but neither IP administration (60mg/kg for four days) nor daily dosage (2.5mg/day) with progesterone protected rat photoreceptors from cell death in models of light damage (O'Steen, 1977; Káldi & Berta, 2004). Conversely, Sánchez-Vallejo *et al.* (2015) recently showed that oral administration (100mg/kg administered *via* oral gavage) worked on several mechanistic levels to prevent photoreceptor cell death in the rd1 mouse model of RP. Specifically, in this thesis, we highlight the capability of Norgestrel to protect through non-invasive diet-supplementation (80mg/kg).

Obviously, there are inconsistencies in the literature regarding the protective capabilities of progesterone in the eye. This could be due to any number of different reasons. It may be simply that higher concentrations of the steroid are needed to induce retinal cell protection. For example, in attempting to protect from retinal degeneration in the light damage model, Káldi & Berta (2004) injected with progesterone (60mg/kg) for 4 days before light damage. This did not result in any significant preservation of the photoreceptor layer. Doonan *et al.* (2011) however achieved retinal neuroprotection through a 100mg/kg IP injection of Norgestrel just 1 hour prior to light damage. This dose was then repeated once every three days subsequently. In the rd genetic mouse models, successful progesterone/Norgestrel induced protection was achieved through administration of 100mg/kg on alternate days (IP injection, Doonan *et al.*, 2011; oral gavage, Sánchez-Vallejo *et al.*, 2015). Therefore, perhaps the conflicting results of these studies simply emphasise that consistently high concentrations of the drug are required to achieve retinal neuroprotection. Alternately, it may be that the protection ascribed to Norgestrel is

because Norgestrel is a synthetic progestin, not naturally produced in the body. Synthetic progestins are rapidly absorbed in the body in comparison to natural progesterone. With a longer half-life, they maintain a more stable level in the blood ("Progesterone - Pharmacy Reviewer," 2016) and thus it is probable that the higher concentrations of Norgestrel are available for longer.

Indeed, there may be other, mechanistic reasons behind the neuroprotective effects of Norgestrel. Basic fibroblast growth factor has previously been trialled as a potential therapeutic approach to retinal eye disease (O'Driscoll *et al.*, 2007, 2008). Shown to activate several pro-survival pathways, in this thesis we show for the first time the direct link between progesterone signalling and bFGF upregulation (Chapter 3, 4, 5). An early study carried out on the mouse mammary tumour line C4-HD suggested a crosstalk between steroid and growth factor signalling pathways (Lamb *et al.*, 1999). bFGF is also known to be produced in response to progesterone administration in non-neuronal tissues such as the ovaries and uterus (Rider *et al.*, 1997). However, to our knowledge, we are the only group to have demonstrated that that direct progesterone receptor stimulation will induce an upregulation in bFGF, crucial in Norgestrel-mediated neuroprotection (Chapter 3).

Norgestrel-mediated increases in bFGF drove the downstream pro-survival phosphorylation of GSK3β on serine 9 and this protein facilitated the actions of Norgestrel in the direct protection of stressed photoreceptors. This study, published in the European Journal of Neuroscience, Wyse-Jackson and Cotter (2016), was the first to establish a significant Norgestrel-mediated pro-survival signalling pathway. However, one of the advantages to

giving a blanket neuroprotective as opposed to simply administering, for example, bFGF as a therapeutic; is that steroid binding to specific receptors may activate a whole cascade of downstream intracellular signalling events. To explain: bFGF activation may be as a result of Norgestrel-binding to one specific receptor. However, Norgestrel may also have other beneficial effects through differential receptor activation.

Unfortunately, the retinal expression of progesterone receptors had not yet been fully studied. Before this work, all that was known was that both nuclear receptors A and B (PR A/B) were expressed in the retina (Wickham *et al.*, 2000) and that progesterone receptor membrane complex one (PGRMC1) was also expressed by retinal Müller cells, photoreceptors and the retinal pigment epithelium (Swiatek-De Lange *et al.*, 2007). Since androgen and oestrogen receptors had also been detected in the eye (Wickham *et al.*, 2000), it would appear that the retina is a bona fide target of progestogens and indeed steroids in general. Accordingly, we set out to characterise the progesterone receptors present in the retina, with the aim of exposing Norgestrel's specific cellular target.

In the following work (Chapter 4), we identified and characterized the complete expression of progesterone receptors present in the C57 wild type and rd10 mouse model of retinitis pigmentosa, and in 661W cone photoreceptor cells. In both mice retinas, regardless of genotype (C57 vs. rd10) or sex (male vs. female), classical progesterone receptors A and B (PR A/B), progesterone receptor membrane components 1 and 2 (PGRMC1, PGRMC2) and membrane progesterone receptors α, β and γ (mPR α, β and γ) were expressed. All receptors excluding PR A/B were also found in the 661W photoreceptor cell line. This work, published in the Journal of Neurochemistry (Wyse-Jackson *et al.*, 2016), showed that

Norgestrel appeared to work primarily through activation of PGRMC1, for specific siRNA transfection (*in vitro)* and receptor blockade (*ex vivo*) prevented its neuroprotective actions.

One thought as to the differing evidence behind progesterone in the CNS vs. the eye, is that that the protective effects of progesterone in the CNS are predominantly regulated by PR A/B (Schumacher *et al.*, 2014). However, since we show that PR A/B is only sparsely expressed in the retina - and is indeed the least prevalent progesterone receptor present (Chapter 4), perhaps progesterone signalling through PR A/B is only weakly neuroprotective to the diseased or damaged retina. This could conceivably explain why O'Steen (1977) and Káldi & Berta (2004) did not find any significant progesterone-induced retinal protection: it requires PR A/B activation, difficult when it is so modestly expressed. Alternately, Norgestrel, signalling through the abundantly expressed PGRMC1, can act in a significantly protective manner.

Interestingly, PGRMC1 in itself has been shown to be critical in cellular regulation (Lösel *et al.*, 2008; Peluso, Romak, *et al.*, 2008; Peluso *et al.*, 2010, 2014). In fact, in our own study, siRNA knock down significantly decreased cellular viability of the 661W photoreceptor cell line by 20% (Figure 4.3.11). Despite this, there is limited knowledge available on the downstream activities of PGRMC1 signalling. Research on the function of this receptor is further exacerbated in the case of ocular tissue, where only two such studies exist (Samadi *et al.*, 2002; Swiatek-De Lange *et al.*, 2007). PGRMC1 has been identified however, as a novel non-transcriptional mechanism of signal transduction in retinal Müller glial cells. In 2007, Swiatek-De Lange *et al.* found that activation of PGRMC1 caused a rapid elevation of

cytosolic [Ca^{2+}], which we show in this thesis to be protective to stressed 661W photoreceptor cells *in vitro* (Chapter 5). Furthermore, they showed that there is a PGRMC1-progesterone initiated, calcium-dependent induction of extracellular signal-regulated kinases (ERK); which has also been shown by our group to be up-regulated in the rd10 mouse model in response to IP administration of Norgestrel (Doonan *et al.*, 2011; Doonan & Cotter, 2012).

The first three results chapters of this thesis (Chapters 3, 4 and 5) nicely tie together one of the pro-survival signalling mechanisms of Norgestrel: from the activation and subsequent nuclear trafficking of cell membrane receptor PGRMC1, right through to the bFGF/calcium induced inactivation of pro-death protein GSK3β. The above work was crucial in identifying Norgestrel's protective mechanisms directly on photoreceptors. However, considering that the retina is a highly complex tissue composed of various cell types and inter-cellular interactions, we wished also to investigate the effects of Norgestrel on other retinal cell types. In particular, we were interested in exploring photoreceptor-microglia crosstalk in the context of Norgestrel-dependent neuroprotection.

Throughout the course of the three years that this thesis has spanned, more and more research has been published highlighting the critical role of microglia in the potentiation of retinal degeneration. In several ground-breaking studies by our own group (Roche *et al.*, 2016) and by others (Arroba *et al.*, 2011; Peng *et al.*, 2014; Zhao *et al.*, 2015), microglia have been shown to infiltrate in to the ONL in retinal degenerative models, ameliorating photoreceptor cell death. These studies critically show that when microglial activation is prevented, photoreceptor degeneration is slowed. However, no study has yet determined

whether microglia in a diseased retina, such as the rd10; become primed to target healthy photoreceptors during disease progression or if they are instead responding to a signal from dying photoreceptors. This being the case, they may then have subsequent unintentional but detrimental effects on nearby viable photoreceptors: we set out to dissect this conundrum.

Knowing that Norgestrel administration was protective against photoreceptor cell death, one of the major issues that we wished to overcome in this thesis was ease of administration. To date, all work on progesterone and Norgestrel-induced neuroprotection has used the invasive techniques of IP and oral gavage. These techniques have proven effective in preventing progressive retinal degenerations, however daily IP injection is time consuming, stressful for the animals and requires an administrative vehicle such as DMSO which can cause complications in itself. To emphasise this point, in the 2011 study, Norgestrel was made up in DMSO and injected intraperitoneally with peanut oil, a delivery vehicle used for lipophilic compounds. Whilst uncommon, about 1 in 20 mice suffered an adverse reaction to the mixture and had to be culled immediately as a result. With the complications of IP injection/oral gavage and also the time constraints of daily dosage in mind, we decided to trial a less invasive technique. The average adult mouse (18g – 30g, strain and sex dependent) eats 4-5g feed a day. A Norgestrel-supplemented diet was developed by Testdiet (Middlesex, UK) and dams of rd10 pups were given the Norgestrel-supplemented diet *ad libitum* when the pups reached P10. This allowed the pups to receive Norgestrel in the milk and, assuming that the average 30g mouse consumes around 5g of food/day, equates to an approximate daily dose of 80mg/kg of Norgestrel.

Norgestrel-diet supplementation (80mg/kg/day) (Chapter 6) worked beautifully to significantly protect the photoreceptor layer of rd10 mice out to P40. In the rd10 mouse, cell loss peaks between P18 and ~P21 (Gargini *et al.*, 2007; Barhoum *et al.*, 2008; Samardzija *et al.*, 2012; Roche *et al.*, 2016). However, we found that absolutely no cell loss took place between P15 and P20 in the Norgestrel-treated mice. Such a protection as this has not been previously seen in the rd10 mouse model. The fact that it was achieved through such an easy, non-invasive method makes it all the more astonishing.

Norgestrel-treated rd10 mice exhibited significantly dampened microglial reactivity and their migration in to the ONL was markedly reduced. We found that not only did Norgestrel work to protect damaged photoreceptors from cell death, but it could also work directly on rd10 microglia to prevent their entry in to the pro-inflammatory, damaging M1 state. Critically, through the use of novel primary rd10 microglia-photoreceptor co-cultures, we characterised for the first time the phagocytic activity of rd10 microglia. These cells, once exposed to an activating factor, will remain reactive and subsequently phagocytic. This is a vital point in the future of retinal degeneration treatment – for clearly more work is required in understanding what this initial signal is and how to prevent it. Most interestingly is that when this factor is removed (i.e. in the case of primary culture of rd10 microglia), they will still remain reactive and consequently engulf and damage healthy, viable cells. Evidently, Norgestrel acts as an anti-inflammatory in damaged photoreceptor cells. We therefore postulate that this sequentially reduces damaging inflammatory factor secretion from microglia and prevents their migration to the ONL.

In this thesis, several key molecular mechanisms regulating the neuroprotective properties of Norgestrel have been elucidated. Clearly, our understanding of how this synthetic progesterone analogue works directly on photoreceptor cells to delay and prevent cell death is expanding. However, an unexpected result to have come out of our research is that Norgestrel can also work on other cell types in the eye. In chapter 6, we show that rd10 microglia express progesterone receptors and through *ex vivo* primary cell culture, have demonstrated Norgestrel's ability to work directly on these cells to prevent pro-inflammatory M1 activation. Only now are we beginning to appreciate the fact that Norgestrel works in a highly protective, multi-faceted manner in the retina. As such, we hope to bring our working knowledge of how the compound works to a point that is ready for clinical trials. It is evident from our initial results with Norgestrel diet supplementation that Norgestrel will likely work optimally as a combination therapy, alongside a more potent anti-inflammatory. However, before this is started, we should discern what it is that Norgestrel cannot protect from; i.e. the signal or event causing the photoreceptor cell loss at P25. Once we know this, we can truly and honestly think of bringing this treatment to the clinic and closer to patients suffering from retinal degeneration.



Chapter 8. Bibliography



8. Bibliography

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