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Redox biology in retinal degeneration



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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March, 2019

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Abstract

Retinitis pigmentosa (RP) involves a group of hereditary diseases that cause progressive and severe visual impairment, with a prevalence of about 1 in 4,000 individuals worldwide. Unfortunately, there is currently no cure for RP and most patients become legally blind by age 40 due to the loss of retinal photoreceptors. The difficulty in finding a treatment relies on the complexity of its genetics. Although more than 3,000 mutations have been described to cause it, about 40-50% of RP cases still correspond to unknown patterns. In most cases, mutations affecting rods, which degenerate first, subsequently produce the death of the remaining photoreceptor cells, the cones.

Several therapeutic approaches have been studied during the last decades. In 2011, our group discovered the neuroprotective properties of 'Norgestrel', a synthetic progestin used in the female oral contraceptive pill, in the retina. Norgestrel was shown to protect against retinal cell death in three different models: *in vitro*, *in vivo* and *ex vivo*, using retinal explants. In fact, two mouse models were used in order to demonstrate such protection, the balb/c induced light damage model and the genetic rd10 (*Pde6b rd10*$^{-}$*/rd10*$^{-}$) model of RP. Since then, some components of its mechanism of action have been elucidated, as is the case of the receptor through which it works, the progesterone receptor membrane component 1 (PGRMC1); the neurotrophic factor basic fibroblast growth factor (bFGF) and its ability to reduce inflammation and gliosis in the diseased retina.

Reactive oxygen species (ROS), have been traditionally associated with cellular damage, and have been discovered to participate in signalling responses, including cellular responses that are protetective. The number of studies about their protective properties of ROS have increased in the last decades. Nevertheless, the accumulation of ROS and/or their persistance

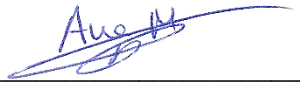
during time within cells have detrimental consequences. Antioxidant machinery is the defense mechanism that cells possess against harmful ROS. However, this system is not infallible. Worsening of several diseases including RP is known to be produced by the disregulation of intracellular ROS levels, which is known as 'oxidative stress'. In 2016, we demonstrated that Norgestrel effectively reduces the damaging ROS levels in the balb/c light damage mouse model.

In the current study, using the 661W cone photoreceptor-like cell line and retinal explants from rd10 mice, we demonstrated that ROS are used by Norgestrel to enhance cell survival. We found that such stimulation of pro-survival ROS levels occurs very rapidly, and is both PGRMC1 and bFGF-dependent (Chapter 3). Interestingly, we demonstrated that treatment with some antioxidants that prevent the up-regulation of ROS molecules, abrogates the Norgestrel-mediated neuroprotection and therefore, indicates that ROS are a crucial part of this survival response. However, little was known about the subsequent downstream mechanism in the Norgestrel-mediated signalling response that prevents cell death and thus, this study aimed to elucidate other processes that could be implicated in such a response (Chapter 4). Using the genetic rd10 mouse model of RP we additionally demonstrated that Norgestrel was able to reduce the levels of damaging ROS due to its antioxidant properties *via* stimulating the transcription factor nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2) and its effector protein, superoxide dismutase 2 (SOD2) (Chapter 5).

Taken together, this study highlights a dual nature of reactive oxygen species and we have demonstrated for the first time the implication of redox biology in the Norgestrel-mediated neuroprotection against retinal degeneration.

Declaration

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

Signed: 

Ana M. Ruiz López

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I have to confess that this has been the most challenging and difficult period of my life so far. Nevertheless, it has been great to put everything together and prove myself that I have been able to write a book in a language different to mine after all. However, without all the people I have had on my side during these four years, I wouldn't have got it. So thank you all.

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Abbreviations

661W:	Cone photoreceptor-like cell line
adRP:	Autosomal-dominant retinitis pigmentosa
AG-205:	Ethanone, 2-((1-(4-chlorophenyl)-1H-tetrazol-5-yl)thio)-1-(1,2,3,4,4a,9b-hexahydro-2,8-dimethyl-5H-pyrido(4,3-b)indol-5-yl)
Akt:	Protein kinase B
AMD:	Age-related macular degeneration
ANOVA:	Analysis of variance
arRP:	Autosomal-recessive retinitis pigmentosa
ATP:	Adenosine triphosphate
BDNF:	Brain-derived neurotrophic factor
bFGF:	Basic fibroblast growth factor (FGF2)
BSU:	Biological services unit
C57:	C57BL/6 Mice
Cas9:	CRISPR-associated protein 9
Ca^{2+}:	Calcium
cGMP:	Cyclic guanosine monophosphate
CNS:	Central nervous system
CNTF:	Ciliary neurotrophic factor
CoQ/Q:	Coenzime Q
COX:	Cyclooxygenase
CRISPR:	Clustered regularly interspaced short palindromic repeats
Cu^{2+}:	Cooper
DCFDA:	2′,7′-dichlorofluorescein diacetate
DHE:	Dihydroethidium
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DPI:	Diphenyleneiodonium
DUOX:	Dual oxidase
EDTA:	Ethylene diaminetetraacetic acid
EGTA:	Ethylene glycoltetraacetic acid
ER:	Endoplasmic reticulum
ETC:	Electron transport chain
FACS:	Fluorescence Activated Cell Sorting
FBS:	Foetal bovine serum

FDA: Food and Drug Administration (Federal Agency)

FGF2: Basic fibroblast growth factor (bFGF)

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GC: Guanylyl cyclase

GCL: Ganglion cell layer

GDP: Guanosine diphosphate

GFP: Green fluorescent protein

GFP+: Green fluorescent protein positive

gp91^{phox}: Glycoprotein 91 phagocyte oxidase

GPX: Glutathione peroxidase

gRNA: Guide RNA

GSH: Glutathione

GTP: Guanosine triphosphate

H_2O: Water

H_2O_2: Hydrogen peroxide

HPRT: Hypoxanthine-guanine phosphoribosyltransferase

h*ν*: Photon of light

INL: Inner nuclear layer

KO: Knockout

LIF: Leukemia inhibitory factor

MitoSOX: Mitogen activated protein kinase

MitoTEMPO: (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)2oxoethyl)triphenylphosphoniumchloride monohydrate

Mn^{2+}: Manganese

mPRα: Membrane progesterone receptor α (PAQR7)

mPRβ: Membrane progesterone receptor β (PAQR8)

mPRγ: Membrane progesterone receptor γ (PAQR5)

mRNA: Messenger ribonucleic acid

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

***n*=:** Number of biological replicates

Na^+: Sodium

NAC: N-acetyl-L-cysteine

NAD^+: Nicotinamide adenine dinucleotide

NADH: Reduced nicotinamide adenine dinucleotide

$NADP^+$: Nicotinamide adenine dinucleotide phosphate

NADPH: Reduced nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor-κB

NF-E2: Nuclear factor erythroid 2

NGF: Nerve growth factor

NORG: Norgestrel

NOX: NADPH oxidase

NOXA1: NOX activator 1

NOXO1: NOX organizer 1

Nrf2: NF-E2-related factor-2

NT-3: Neurotrophin-3

O_2: Molecular oxygen

$O_2^{\bullet-}$: Superoxide anion

OH•: Hydroxil radical

ONL: Outer nuclear layer

Opn1sw: Short-wave-sensitive opsin-1

p: Phosphorylation

P: Post-natal day

PPAR: Peroxisome proliferator-activated receptor

PAQR: Progestin and adipoQ receptor family

PBS: Phosphate-buffered saline

PDE: Phosphodiesterase

PDE6B: Phosphodiesterase-6B

PDGF: Platelet-derived growth factor

PEDF: Pigment epithelium-derived factor

PFA: Paraformaldehyde

PGRMC1: Progesterone receptor membrane component 1

PGRMC2: Progesterone receptor membrane component 2

phox: Phagocyte oxidase

PR A/B: Progesterone receptor A/B

ProTECT: Progesterone for Traumatic Brain Injury Experimental Clinical Treatment

PRX: Peroxiredoxin

R*: Metarhodopsin II

Rd1: rd1/rd1 Mice (*Pde6b rd1^{-}/rd1^{-}*)

Rd10: rd10/rd10 Mice (*Pde6b rd10^{-}/rd10^{-}*)

Redox: Reduction-oxidation

RGC: Retinal ganglion cell

RHO: Rhodopsin

RIPA: Radioimmunoprecipitation assay

ROS: Reactive oxygen species

RP:	Retinitis pigmentosa
RPE:	Retinal pigment epithelium
RPGR:	Retinitis pigmentosa GTPase regulator
RT:	Room temperature
RT-qPCR:	Real time quantitative polymerase chain reaction
SDS-PAGE:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM:	Mean ± standard error
siRNA:	Small interfering ribonucleic acid
SNP:	Sodium nitroprusside
SOD:	Superoxide dismutase
SOD1:	CuZn-SOD (cytoplasmic)
SOD2:	Mn-SOD (mitochondrial)
SS:	Serum starvation
TBI:	Traumatic brain injury
TBS-T:	Tris-buffered saline/0.1% Tween-20
TCA:	Tricarboxylic acid
Tiron:	4,5-dihydroxybenzene-1,3-disulfonate
TGF-β1:	Transforming growth factor β1
TRX:	Thioredoxin
TUNEL:	Terminal dUTP nick-end labellin
TUNEL+:	Terminal dUTP nick-end labelled positive
USH2A:	Usher's syndrome type IIA
UT:	Untreated
VAS2870:	3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-tria-zolo[4,5-d]pyrimidine
VEGF:	Vascular endothelial growth factor
Zn^{2+}:	Zinc

Publications and presentations

Publications

Ruiz-Lopez, A.M. (2017) The Young Researchers View (website) http://www.vision-research.eu/index.php?id=1132

Ruiz-Lopez, A.M., Roche, S.L., Wyse-Jackson, A.C., Moloney, J.N., Byrne, A.M., & Cotter, T.G. (2017) Pro-survival redox signalling in progesterone-mediated retinal neuroprotection. *Eur. J. Neurosci.*, **1**, 1–10.

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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., 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.

Presentations

Nov 2014: Retina Conference, Dublin, Ireland. *Poster Presentation.*

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

June 2016: International Cell Death Society Conference, Cork, Ireland. *Poster Presentation.*

July 2017: Young Researcher Vision Camp, Castle Wildenstein, Germany. *Poster Presentation.* ***1st place prize.***

Chapter 1: Introduction

1.1. The retina in health and disease

1.1.1. Visual processing

Vision is considered the most important function for quality of life in humans, and the eye is responsible for this function (Huberman & Niell, 2011; Hirneiss, 2014; Qiu *et al.*, 2014; Latham *et al.*, 2015). The human eye is a slightly asymmetrical sphere composed of various tissues. The retina, a complex and light-sensitive structure, is one of them and consists of multiple layers of interconnected neurons (Cuenca *et al.*, 2014; Ray *et al.*, 2016). The identification of different types of retinal neurons began with Santiago Ramón y Cajal in 1900, whose drawings are an incredible representation of such an organised structure (Masland, 2001; Llinás, 2003). This tissue contains both sensory neurons, the photoreceptors, that respond to light; and other neurons that form an intricate circuit for processing the signals that are sent to the brain through the optic nerve where the image is formed (Figure 1.1.1 and Figure 1.1.2) (Kolb, 2003; Roche *et al.*, 2016).

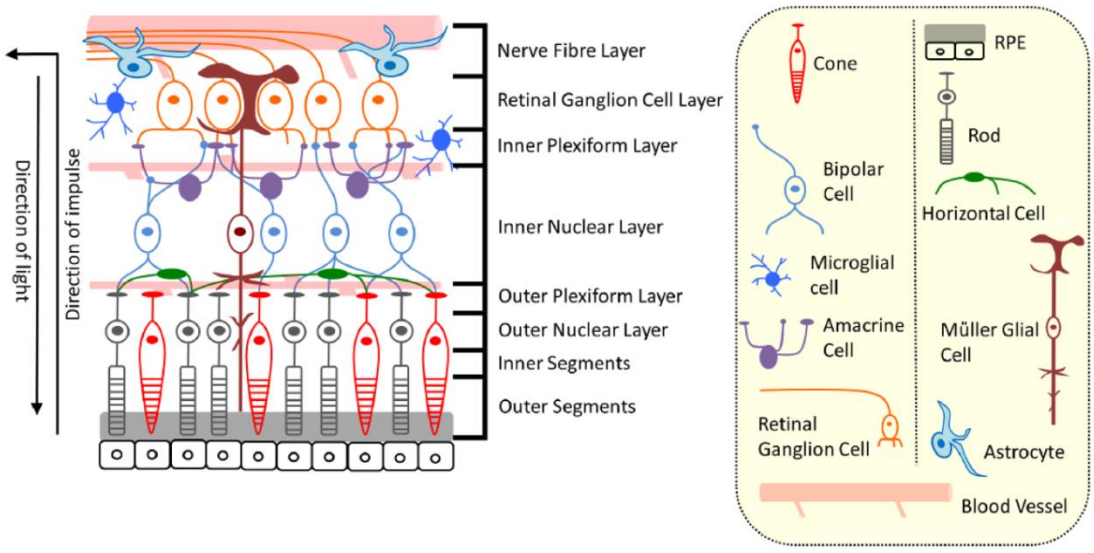


Figure 1.1.1. Schematic representation of the mouse retina. This schematic illustrates the variety of cell types and connections within the retina. Taken from Roche *et al.*, 2016.

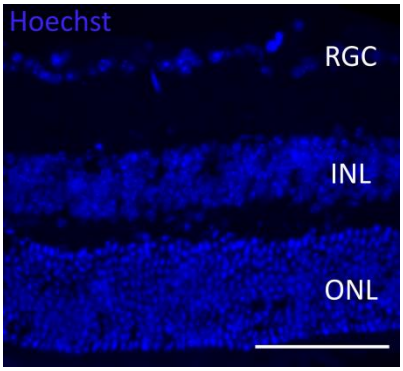


Figure 1.1.2. Nuclear layers within a wild-type C57 mouse retina. Fluorescent microscopic image of a C57BL/6 retinal section stained with Hoechst (blue) that reveals the cell layers present in the retina. ONL: outer nuclear layer, INL: inner nuclear layer, RGC: retinal ganglion cells. Scale bar 50 µm.

Visual processing takes place in a complex but organised system, starting with light that penetrates through several cell layers to reach the photoreceptors, where light and visual information is transformed into electrical signals (Marshel *et al.*, 2011; Ray *et al.*, 2016). Thus, neural signals are sent to the brain *via* different types of neurons (bipolar cells, retinal ganglion cells (RGC), horizontal cells and amacrine cells) through the optic nerve to neurons in the lateral geniculate nucleus, situated in the posterior thalamus (Marshel *et al.*, 2011). Subsequently, this output is transmitted predominately to the layer 4 of the primary visual cortex (Horton, 2006; Marshel *et al.*, 2011; Ray *et al.*, 2016). In addition, there are two types of photoreceptors in the retina: cones and rods, responsible for two different kinds of vision. Both types contain a photopigment in the outer segment which absorbs the light, leading to changes in the membrane potential that cause synaptic transmission within cells mentioned above (Curcio *et al.*, 1990; Lamb, 2013). Rod cells contain long cylindrical outer segments, have many disks and contain a high concentration of pigment (rhodopsin), responsible for dim-light vision. Such features enable rods to be extremely light sensitive, whilst cones require significantly brighter light in order to produce a signal and are primarily responsible for spatial acuity and colour vision (Warrant, 2009).

Interestingly, the human retina presents some differences when compared with the mouse retina as shown in figure 1.1.3. The most obvious difference is the size of the lens, which is substantially larger in the mouse retina. A key difference between the human and mouse retina is the presence of the macula in the human retina. The fovea is a cone-only region within the macula (Veleri *et al*., 2015) and the human retina contains about 120 million rod cells and 6 million cone cells concentrated mainly in the fovea. Thus this region contains a higher density of cones compared with the peripheral retina. In contrast, cones are equally distributed along the mouse retina. This difference between the human and mouse retina leads to peripheral to central degeneration in human retinitis pigmentosa (RP) and central to peripheral degeneration in the mouse RP, even though the disease in both species can be caused by identical genetic mutations.

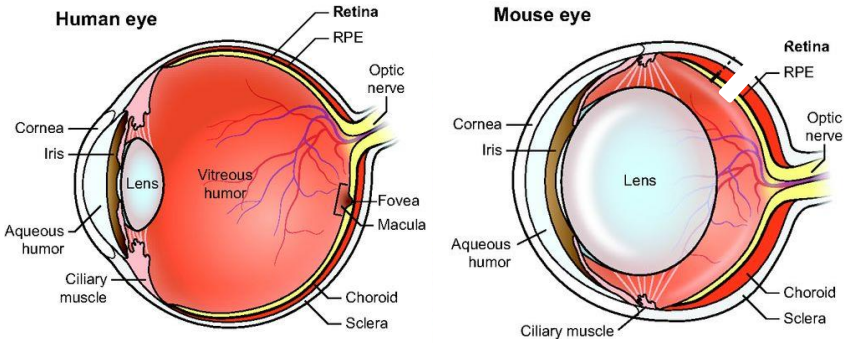


Figure 1.1.3. Difference between human and mouse retina. Modified from Veleri *et al.,* 2015.

The way in which visual inputs are converted in electrical signals is known as 'phototransduction' and consists of five steps represented in figure 1.1.4. The process takes place within the disk membrane of the photoreceptor outer segment and it starts when a light photon is absorbed by a rhodopsin molecule, transforming it into its enzymatically active conformation, metarhodopsin II (R*). Once activated, the signal is amplified through repeated contacts with the G-proteins, transducins, to further activate cyclic nucleotide phosphodiesterases (PDE), which results in the hydrolysis of multiple diffusible second messengers: the cyclic guanosine monophosphates (cGMP). Such hydrolysis closes cGMP-

gated channels due to the diminished cGMP concentration available for binding to the channels throughout the surface of the outer segment, decreasing the influx of Na^+ and Ca^{2+} ions and leading to hyperpolarisation of the cell and subsequent signalling (Pugh, 1999; Leskov *et al.*, 2000; Lamb & Pugh, 2006; Warrant, 2009).

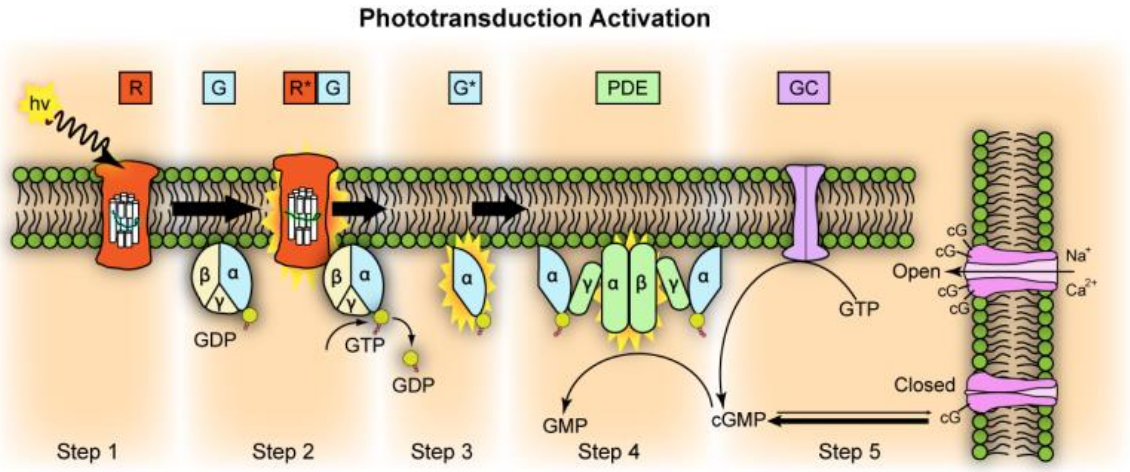


Figure 1.1.4. Representation of the phototransduction cascade. Shown is the outer membrane disk found in a rod cell. **Step 1, activation of rhodopsin:** Capture of a photon (hν) activates rhodopsin *via* a conformational change to R*. **Step 2, activation of the G-protein:** R* contacts with G-protein transducin (G) molecules that consist of three subunits: α, β and γ, with guanosine diphosphate (GDP) bound to the α subunit. This contact activates the molecule to G*, catalysing its activation by the release of bound GDP in exchange for cytoplasmic guanosine triphosphate (GTP). **Step 3, activation of the PDE:** This release expels the G-protein β and γ subunits leaving the α subunit. **Step 4, hydrolysis of cGMP:** G* α subunit binds inhibitory γ subunits of phosphodiesterase (PDE) activating its α and β subunits. Activated PDE hydrolyses cyclic guanosine monophosphates (cGMP). **Step 5, closure of ion channels:** Guanylyl cyclase (GC) synthesises cGMP. Reduced levels of cytosolic cGMP trigger cyclic nucleotide gated channels to close preventing further influx of Na^+ and Ca^{2+}. Modified from Pugh, 1999.

As a result of this mechanism, one molecule of R* induces hydrolysis of more than a few hundred thousand of cGMP molecules during the time period when it is active (Kawamura & Tachibanaki, 2008). At the cessation of light, R* is inactivated by phosphorylation of a rhodopsin kinase (Chen *et al.*, 1999; Kawamura & Tachibanaki, 2008). This process appears to be reasonably similar in mammalian cones and rods, only differing with respect to the photo-pigments used (Masland, 2001), which therefore, changes the rate of activation of both types of cells (Kawamura & Tachibanaki, 2008; Tachibanaki *et al.*, 2012; Lamb, 2013).

1.1.2. Retinitis pigmentosa

Due to its high structural and functional complexity, the retina is vulnerable to alterations from any sort of pathological injury. Inherited retinal diseases are a family of hereditary diseases where mutations affecting mainly photoreceptors lead to degeneration. Retinitis pigmentosa (RP) is included in these diseases (Fan *et al.*, 2006).

The term 'retinitis pigmentosa' was first used by the German physician Franciscus Donders in 1857 (Donders, 1857) and it includes a group of retinopathies characterised by an accumulation of retinal pigment deposits due to the loss of photoreceptors (rods and cones) and the adjacent retinal pigment epithelium (RPE) which leads to progressive blindness (Allard, 1983; Berson, 1993; Fan *et al.*, 2006; Hamel, 2006; Dias *et al.*, 2017). In most cases, the loss of rods causes the first symptom of RP: night blindness (nyctalopia). However, cones secondarily degenerate in the periphery of the retina causing a reduction of peripheral vision known as 'tunnel vision' (Hartong *et al.*, 2006; Cuenca *et al.*, 2014; Dias *et al.*, 2017; Athanasiou *et al.*, 2018), as shown in figure 1.1.5. By midlife, patients become legally blind when the defective regions gradually enlarge, affecting the central field as well (Allard, 1983; Pagon, 1988; Herse, 2005; Kennan *et al.*, 2005; Hamel, 2006). Furthermore, survival of cones appears to depend on rods and preservation of cones prevents patients becoming blind (Komeima *et al.*, 2006, 2007; Tao *et al.*, 2016). Nevertheless, there is currently no cure for RP. The prevalence of these disorders is 1:4000 individuals worldwide, being the most common cause of inherited blindness (Boughman *et al.*, 1980; Berson, 1993; Haim, 2002; Hartong *et al.*, 2006; Dias *et al.*, 2017).

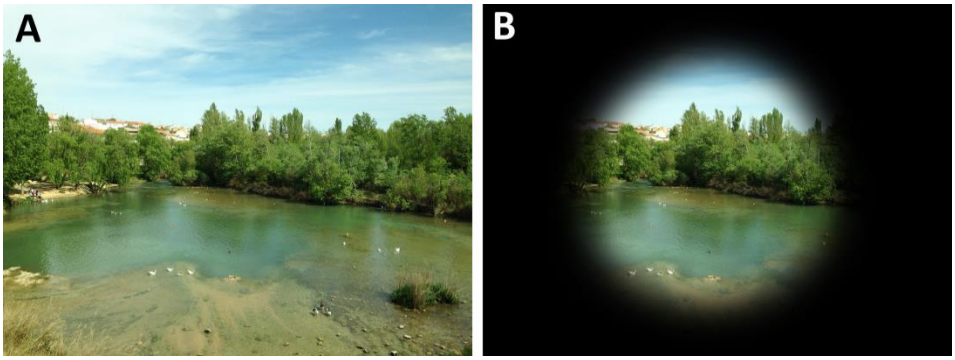


Figure 1.1.5. Progression of visual field loss in retinitis pigmentosa. (A) Normal vision. **(B)** Vision of a patient with RP: loss of peripheral vision (tunnel vision).

Age-related macular degeneration (AMD) disorders are another type of inherited retinal diseases which are characterized by progressive destruction of the macula, produced by abnormalities in the RPE (Fan *et al*., 2006; Winkler *et al*., 2007). Thus, AMD is related to loss of central vision rather than loss of peripheral which occurs in RP.

RP has been reported to be inherited in all the three Mendelian modes: autosomal-dominant (adRP, about 15-25% of the cases), autosomal-recessive (arRP, 5-20%) and X-linked (5-15%) (Bunker *et al.*, 1984; Wang *et al.*, 2005; Hartong *et al.*, 2006; Athanasiou *et al.*, 2018), although about 40-50% of cases correspond to unknown patterns as represented in figure 1.1.6 (Dias *et al*., 2017). The genetics of this disease is highly complex due to the fact that about 70 genes are involved and more than 3,000 mutations are known to cause it (Guadagni *et al.*, 2015; Cheng *et al.*, 2016; Dias *et al.*, 2017). Due to such broad genetic complexity of RP, gene-specific therapies are challenging (Cuenca *et al.*, 2014). The major causative genes (Figure 1.1.6) are the rhodopsin (*RHO*) gene that leads to about 25% of adRP; the Usher's syndrome type IIA (*USH2A*) gene, which might cover about 15% of recessive disease; and the retinitis pigmentosa GTPase regulator (*RPGR*) gene that causes about 70% of X-linked cases (Wang *et al.*, 2005; Hartong *et al.*, 2006; Daiger *et al.*, 2013). These three genes are found in about 30% of all cases of RP (Hartong *et al.*, 2006; Dias *et al.*, 2017).

Among the mutated genes that cause arRP, we find the gene that encodes the rod cGMP-phosphodiesterase-6B subunit β (*Pde6b*) which is an essential component in the phototransduction cascade (Cote, 2004; Kennan *et al.*, 2005). In fact, a correct function of this subunit is crucial for the whole protein function (Ullah *et al.*, 2016; Gopalakrishna *et al.*, 2017). The rod *Pde6b* gene is located in the chromosome 5 and it has 22 exons. *Pde6b* mutations represent about 5% of arRP cases (Figure 1.1.6).

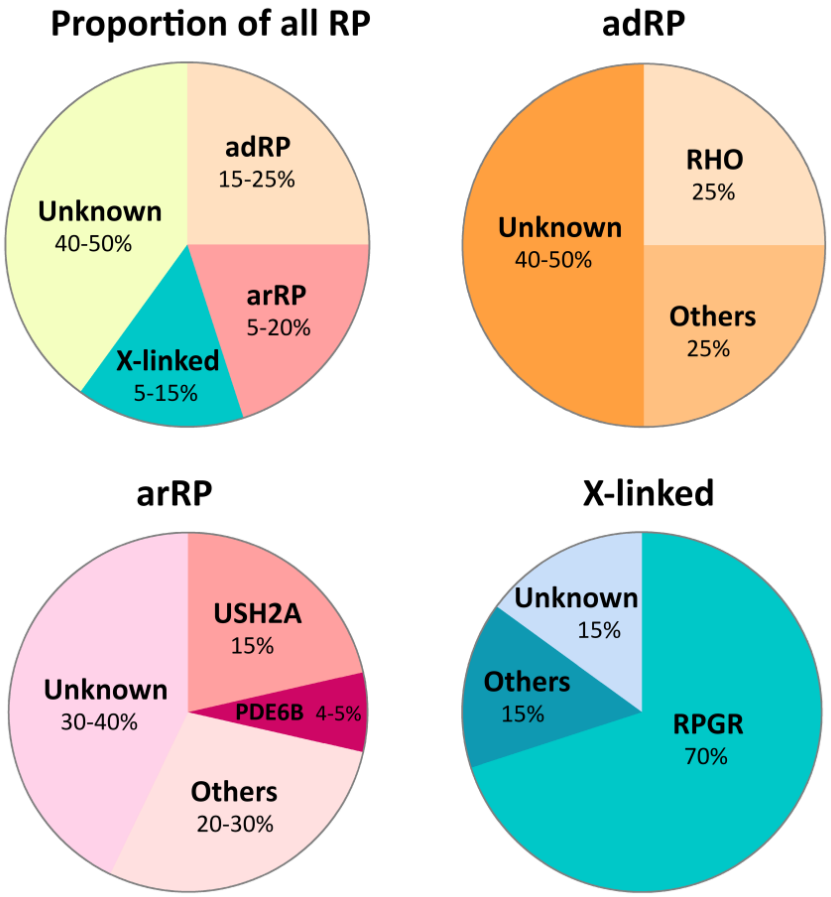


Figure 1.1.6. Genes and their relative contribution to retinitis pigmentosa. The inheritance of RP consists of autosomal-dominant (adRP), autosomal-recessive (arRP), X-linked and unknown patterns. The main causative genes for them are rhodoposin (*RHO*), Usher's syndrome type IIA (*USH2A*) and retinitis pigmentosa GTPase regulator (*RPGR*). Phosphodiesterase-6B (*Pde6b*) is also of interest throughout this thesis.

1.1.3. Genetic mouse models of retinitis pigmentosa

Despite our understanding of the genetic abnormalities leading to RP, the mechanism by which many mutations, including the ones previously described, lead to cell death has yet to be fully elucidated. Unfortunately, the direct study of human RP is limited given the difficulty in obtaining relevant human tissue samples. As a result, animal models that present the same mutations found in humans and therefore are a good representation of the human disease both genetically and phenotypically have become as an invaluable tool for studies of RP.

Perhaps the best characterised mouse model of retinal degeneration is the naturally occurring rd1 mouse (*Pde6b rd1*$^{-}$*/rd1*$^{-}$) since Keeler described it in 1924 (Keeler, 1924). However, in 1990 it was determined that degeneration in the rd1 mouse model is caused by a point mutation in exon 7 of the β subunit of the *Pde6b* gene (Bowes *et al.*, 1990; Pittler & Baehr, 1991). Such mutation leads to a rapid loss of rod photoreceptors starting at postnatal day (P)9 which ends at P18. A subsequent cone cell death is detected at P20 (Portera-cailliau *et al.*, 1994; Barhoum *et al.*, 2008; Greferath *et al.*, 2009). At P30, the majority of cones have also degenerated and thereafter, the degeneration becomes slower. Carter-Dawson *et al.* showed that as a consequence of a slower degeneration, 1.5% cones remain at P600 (18 months of age) (Carter-Dawson *et al.*, 1979). Although this model has been widely used (Doonan *et al.*, 2003; Hart *et al.*, 2005; Komeima *et al.*, 2006; Usui *et al.*, 2009), it presents some limitations in the study of RP due to the rapid loss of photoreceptors which coincides with retinal development (Sanyal & Bal, 1973; Carter-Dawson *et al.*, 1979).

Alternately, another mouse model, the rd10 strain (*Pde6b rd10*$^{-}$*/rd10*$^{-}$), with a missense mutation in exon 13 of the same gene (*Pde6b*) was described (Chang *et al.*, 2002, 2007; Han *et al.*, 2013). The mutation is naturally produced by the exchange of an arginine by a cysteine at position 560 (Arg560Cys) of the protein (Chang *et al.*, 2007). This mutation results in a slower retinal degeneration, starting with the death of rods around P18, peaking at P25 and finishing at P50 with the subsequent death of cones (Chang *et al.*, 2002; Gargini *et al.*, 2007; Barhoum *et al.*, 2008; Samardzija *et al.*, 2012; Roche *et al.*, 2016). This reason and the fact that this mutation also causes RP in humans (McLaughlin *et al.*, 1993), make the rd10 mouse a suitable model for studying retinal degeneration and developing therapeutic approaches for patients affected by hereditary retinal dystrophies. In this model, degeneration of photoreceptors follows a clear gradient from the central to the peripheral regions of the retina (Gargini *et al.*, 2007; Roche *et al.*, 2016) and it also occurs more rapidly in the central retina in comparison to the periphery (Barhoum *et al.*, 2008). As seen in human RP, rods are lost first with cones persisting into adulthood (Gargini *et al.*, 2007; Barhoum *et al.*, 2008; Roche *et al.*, 2016).

In addition, not only photoreceptors are affected in the rd10 model, but also other retinal cells such as amacrine cells and bipolar cells, as well as photoreceptor synapses (Barhoum *et al.*, 2008; Puthussery *et al.*, 2009; Roche *et al.*, 2016). According to this, glial cells, which can be divided into two types: microglia and macroglia (Müller glia and astrocytes), have been shown to respond dramatically during this period in the rd10 retina (Roche *et al.*, 2016, 2017a, 2017b; Roche & Wyse-Jackson *et al.*, 2016).

1.2. Progesterone-induced neuroprotection

Functions of female steroid hormones in the central nervous system (CNS) were classically associated with reproductive processes and sexual behaviour. In 1968 it was recognised that these molecules can reach the brain (McEwen *et al.*, 1968). However, it has become clear in the last decades that they play alternative roles that have nothing to do with endocrine functions. For example, they can affect electrical activity in the brain and contribute to neuroprotection in the CNS (De Nicola, 1993; Joëls, 1997; Azcoitia *et al.*, 1999; McEwen, 1999; Schumacher *et al.*, 2000). Studies *in vitro*, *in vivo* and in patients affected by diseases such as Alzheimer's disease, have demonstrated their important neuroprotective effects (Henderson, 1997; McEwen, 1999; Vongher & Frye, 1999; Brinton, 2001).

Among the hundred different types of steroids found in nature, the majority of them in the human body are derived from the membrane lipid cholesterol (Conn *et al.*, 1950; Sanderson, 2006; De Nicola *et al.*, 2017). Steroid hormones can be divided into three groups depending on the place where are synthesised: corticosteroids (made in the adrenal cortex), sex steroids (made in the gonads or placenta), and neurosteroids, when they are made by neurons and other cells pertaining to the CNS (Joëls, 1997; Sanderson, 2006; De Nicola *et al.*, 2017). These three groups have been related with neuroprotection during years (De Nicola, 1993; Cox, 1995; Joëls, 1997; Mannella *et al.*, 2009; Guennoun *et al.*, 2015). In this context, many of them have been proved to protect in some retinal diseases. Glucocorticoids, a group of corticosteroids, have been shown to induce photoreceptor survival (Fu *et al.*, 1992; Wenzel *et al.*, 2001, 2003; Bhisitkul *et al.*, 2008; Glybina *et al.*, 2009). Several human clinical trials of varying degrees of success have been also performed and all looked at the potential for glucocorticoids to act as anti-inflammatories in the eye as reviewed by Cebeci & Kir (Cebeci & Kir, 2015). In all of the aforementioned trials, visual acuity was improved through

corticosteroid administration. However, the mechanism of action by which this occurs, be it directly or indirectly on the photoreceptors, has not yet been elucidated.

Progesterone, the progestin on which we are going to focus in this study, is included in the second and third group, sex steroids and neurosteroids (Baulieu *et al.*, 2001; Guennoun *et al.*, 2015; Sánchez-Vallejo *et al.*, 2015). Progesterone is a gonadal hormone synthesised primarily by the ovary in females, and the testes and adrenal cortex in the male (Singh & Su, 2013; Sánchez-Vallejo *et al.*, 2015). Although progesterone levels are normally higher in females, males also present substantial levels of this hormone (Oettel & Mukhopadhyay, 2004; Singh & Su, 2013) and thus, may be equally important in both. This hormone has been found to have multiple functions in the CNS including the modulation of neuroprotection, neuroplasticity, neurogenesis and neuroinflammation (Petersen *et al.*, 2013). Treatment with progesterone produces regenerative effects in brain injury models (Schumacher *et al.*, 2000), such as the traumatic brain injury (TBI). In fact, studies with progesterone have achieved promising results for the treatment of TBI in phases II and III of clinical trials (Wright *et al.*, 2007; Xiao *et al.*, 2008).

Given the high complexity of RP genetics, a 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 diseases (Doonan & Cotter, 2012; Trifunovic *et al.*, 2012). Protective and regenerative effects of progesterone make it a promising solution to many of them.

1.2.1. Norgestrel

Preclinical studies have raised great hope for the use of progesterone as a potential therapeutic agent due to its effectiveness on different diseases including spinal cord injury, brain ischemia, stroke, Alzheimer's disease, multiple scleroris and TBI (Gonzalez Deniselle *et al.*, 2003; Gonzalez *et al.*, 2005; Compagnone, 2008; Espinosa-García *et al.*, 2014; Yousuf *et al.*, 2014; Qin *et al.*, 2015; De Nicola *et al.*, 2017). The Progesterone for Traumatic Brain Injury Experimental Clinical Treatment (ProTECT) study was carried out in order to establish drug safety (Wright *et al.*, 2007). Following that, more clinical trials have been performed to demonstrate progesterone treatment reduces mortality in patients with TBI (Stein, 2011, 2013; Schumacher *et al.*, 2016). As a result of such neuroprotection, progesterone effects were also studied in eye diseases, such as diabetic and ischemic retinopathies (Lu *et al.*, 2008; Neumann *et al.*, 2010), and specifically, retinal diseases (Feskanich *et al.*, 2008; Sánchez-Vallejo *et al.*, 2015). Sánchez-Vallejo *et al.* showed that oral administration of this hormone during several days, delays cell death happening in the rd1 mouse model (Sánchez-Vallejo *et al.*, 2015).

Furthermore, the role that progesterone plays in neuroprotection has become clear given that its synthesis is up-regulated in response to the injury produced by these diseases (Schumacher *et al.*, 2016). Taken together, experimental observations strongly suggest that an increase in progesterone production by gonads and by neural cells might be part of a physiological process of neuroprotective responses. For this reason, progesterone and its synthetic analogues offer considerable promises as neuroprotectant agents.

One of these synthetic analogues is levonorgestrel, commonly referred to as 'Norgestrel', which presents a similar structure as progesterone. Norgestrel, and the rest of steroids,

contains a four cycloalkane ring core (Figure 1.2.1). This compound is Food and Drug Administration (FDA) approved and is found at concentrations of 0.075-0.35 mg (daily dosage) in versions of the progesterone-only mini pill (Doonan & Cotter, 2012). Norgestrel, like other synthetic progestins, has been widely used in the reproductive medicine given that it is rapidly absorbed and has a longer half-life compared with progesterone, which allows the maintenance of stable levels in the blood.

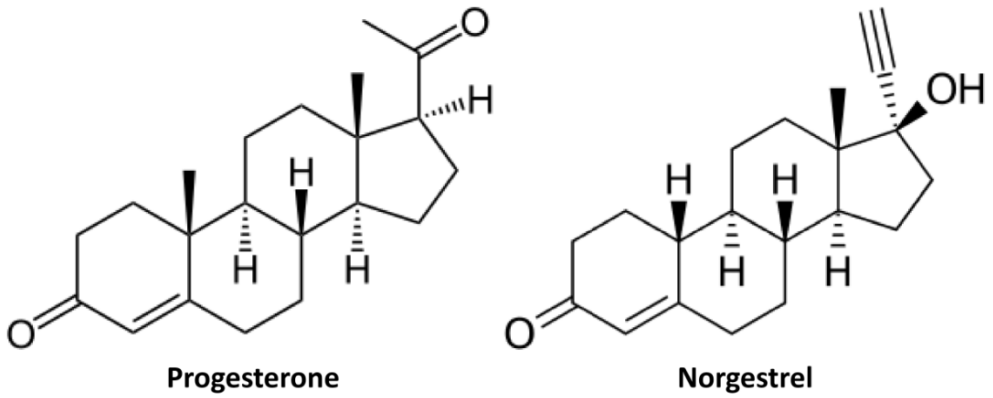


Figure 1.2.1. Chemical structures of progesterone and Norgestrel.

Our group has demonstrated Norgestrel's ability to protect damaged photoreceptors in three different models: *in vitro*, *in vivo* and *ex vivo*, using retinal explants (Doonan *et al.*, 2011; Doonan & Cotter, 2012; Byrne *et al.*, 2016a, 2016b; Roche & Wyse-Jackson *et al.*, 2016; Wyse Jackson *et al.*, 2016a, 2016b; Wyse Jackson & Cotter, 2016; Roche *et al.*, 2017a, 2017b; Ruiz-Lopez *et al.*, 2017). Doonan *et al.* firstly showed that intraperitoneal administration of Norgestrel preserved photoreceptor cells in both a light-induced damage model and rd10 mice (Doonan *et al.*, 2011). Since then, our knowledge about this compound has increased and our interest has foccused on elucidating its mechanism of action.

1.2.2. Progesterone and Norgestrel: mechanism of action

As with most neurosteroids, progesterone acts through specific binding to cellular receptors (Friberg *et al.*, 2009). Progesterone receptors A and B (PR A/B) have been classically thought to be responsible for progesterone signalling in CNS. These receptors are widely expressed and can modulate a variety of different physiological functions (Wen *et al.*, 1994; Lange, 2008). However, progesterone, as a complex hormone, can also act through a variety of non-classical receptors (Moussatche & Lyons, 2012; Petersen *et al.*, 2013; Qin *et al.*, 2015). These are: the progesterone receptor membrane component (PGRMC) family, featuring PGRMC1 and PGRMC2 (Cahill, 2007, 2017; Lösel *et al.*, 2007; Peluso *et al.*, 2014) and the progestin and adipoQ receptor (PAQR) family (Zhu *et al.*, 2003; Petersen *et al.*, 2013), featuring membrane progesterone receptors α, β and γ (mPRα, mPRβ and mPRγ).

Given both classical and non-classical receptors are important in the progesterone-mediated signalling, they are widely distributed in many tissues (Graham & Clarke, 2002; Han *et al.*, 2006), including the brain (Kato *et al.*, 1994; Petersen *et al.*, 2013; Singh & Su, 2013). This ubiquitous expression highlights their importance throughout the body. Progesterone binding to the receptor produces its dimerization and the subsequent interaction with several co-regulatory proteins (Singh & Su, 2013; Grimm *et al.*, 2016). This results in a response that can be generated by causing changes in gene transcription through transcription factors (Li *et al.*, 1997; Faivre *et al.*, 2008) or *via* non-genomic mechanisms by the interaction with signal transduction kinases and the use of second messengers (Boonyaratanakornkit *et al.*, 2001; Jacobsen & Horwitz, 2012; Singh & Su, 2013).

Studies about the different PRs present in the retina and their mechanism of action are very limited (Swiatek-De Lange *et al.*, 2007). Nevertheless, in 2016 our group discovered that the

receptor target by which Norgestrel exerts its action is the PGRMC1 receptor in the 661W cone photoreceptor-like cell line and in both C57 and rd10 mouse retina (Wyse Jackson *et al.*, 2016a). Interestingly, PGRMC1 expression was equal in male and female mice, demonstrating progesterone signalling plays and important role in both. PGRMC1 localises in the plasma membrane but following ligand binding, it is activated and translocated to the nucleus (Peluso *et al.*, 2012; Wyse Jackson *et al.*, 2016a). Nuclear PGRMC1 might be associated with regulation of transcriptional activity (Peluso *et al.*, 2012). However, its function inside the nucleus following Norgestrel treatment is still unknown.

One of the main effects discovered of Norgestrel-mediated response was the up-regulation of neurotrophic factors (Doonan *et al.*, 2011; Doonan & Cotter, 2012; Byrne *et al.*, 2016a; Wyse Jackson & Cotter, 2016; Ruiz-Lopez *et al.*, 2017). Cytokines and neurotrophic factors are regarded as a possible therapeutic modality due to their effects in increasing proliferation and survival in different cell types and tissues (Kolomeyer & Zarbin, 2014). This is of interest given that such effects are also important in the treatment of retinal diseases (Désiré *et al.*, 2000; Yip & So, 2000; Arroba *et al.*, 2009, 2011; Bürgi *et al.*, 2009; Zheng *et al.*, 2009; He *et al.*, 2014; Kolomeyer & Zarbin, 2014; Arroba & Valverde, 2015). There is much evidence in the literature about how progesterone-mediated stimulation of these trophic factors induces neuroprotection (Schumacher *et al.*, 2000; Gonzalez Deniselle *et al.*, 2003; Gonzalez *et al.*, 2005; Deutsch *et al.*, 2013; Kolomeyer & Zarbin, 2014; Melcangi *et al.*, 2014). Therefore, the fact that the basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) are part of the Norgestrel-mediated survival response is not surprising (Doonan *et al.*, 2011; Byrne *et al.*, 2016a; Wyse Jackson & Cotter, 2016; Ruiz-Lopez *et al.*, 2017).

1.3. Reactive oxygen species

The term 'reactive oxygen species' (ROS) describes a group of molecules and free radicals (chemical species with one unpaired electron) that are generally metabolic by-products constantly formed as a consequence of endogenous physiological processes that involve oxygen consumption (Gilbert, 1994; Andreyev *et al.*, 2005; Winterbourn, 2008). Since molecular oxygen (O_2) possesses two unpaired electrons with the same spin in the outer shell, known as triplet state, is a not reactive molecule. However, when one of the two unpaired electrons is excited, its spin changes and the resulting species becomes a powerful oxidant that reacts with other pairs of electrons (Turrens, 2003). These reactions in which an exchange of electrons is produced are commonly known as 'reduction-oxidation' (redox) reactions as represented in figure 1.3.1.

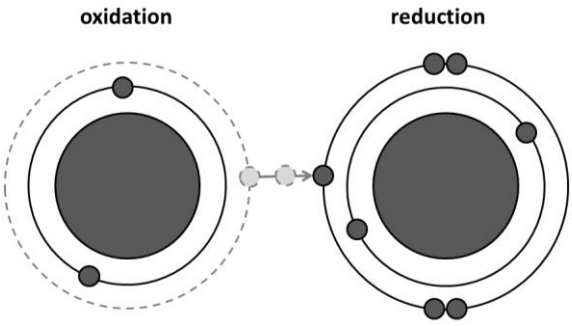


Figure 1.3.1. Schematic representation of reduction-oxidation (redox) reactions. Oxidation is the loss of electrons by a molecule, atom or ion with the subsequent reduction or gain of electrons by other.

ROS molecules are the product of the partial reduction of oxygen. Superoxide anion ($O_2^{\bullet -}$) results from the one-electron O_2 reduction and it is the precursor of most of the other ROS types (Knowles *et al.*, 1969; Turrens, 2003). Spontaneous or non-spontaneous superoxide dismutation produces uncharged hydrogen peroxide (H_2O_2) which can be partially reduced to hydroxyl radical (OH•) or fully reduced to form water (Davies, 1995; Keyer & Imlay, 1996; Turrens, 2003; Winterbourn, 2008).

ROS have long been associated with activation of death pathways and cellular damage (Chance, 1979; Gorman *et al.*, 1997; Curtin *et al.*, 2002; Rhee, 2006; Battistelli *et al.*, 2016). In fact, both $O_2^{\bullet-}$ and OH• are unstable radicals that are responsible of such detrimental effects, especially OH•, which is considered one of the strongest oxidants in nature. ROS implication has also been documented in neurodegenerative diseases leading to mitochondrial dysfunction (England *et al.*, 2006; Gandhi & Abramov, 2012; Kim *et al.*, 2015; Di Meo *et al.*, 2016), and more specifically, in the worsening of retinal diseases including RP (Carmody *et al.*, 1999; Cingolani *et al.*, 2005; Carmody & Cotter, 2007; Winkler *et al.*, 2007; Usui *et al.*, 2009; Lange & Bainbridge, 2012; Eshaq *et al.*, 2014; Campochiaro *et al.*, 2015; Tao *et al.*, 2016). The retina is a tissue highly affected by ROS given its elevated oxygen consumption rate (Eshaq *et al.*, 2014) in order to convert light into electrical inputs as described before. Furthermore, some studies have also linked the photoreceptor cell death produced by the exposure to bright light with an increase in ROS production as reviewed by Winkler *et al.* (Winkler *et al.*, 2007). It was then assumed that the faster the elimination of these toxic molecules, the better for the cell.

Over the last few years, however, our understanding about these molecules has dramatically changed. It is true that ROS are related with a great variety of diseases, but that is just an oversimplification of their importance within the cell (Figure 1.3.2). Contrary to what was thought, ROS were discovered to be a double-edged sword, playing a crucial role in cells by working as second messengers in signal transduction (Burdon, 1995; Rhee, 2006; D'Autréaux & Toledano, 2007; Groeger *et al.*, 2009b; Benitez-Alfonso *et al.*, 2011; Finkel, 2011; Gough & Cotter, 2011). This change in the perception of these molecules is the reason why our interest about such unexpected function has considerably increased in the last decades.

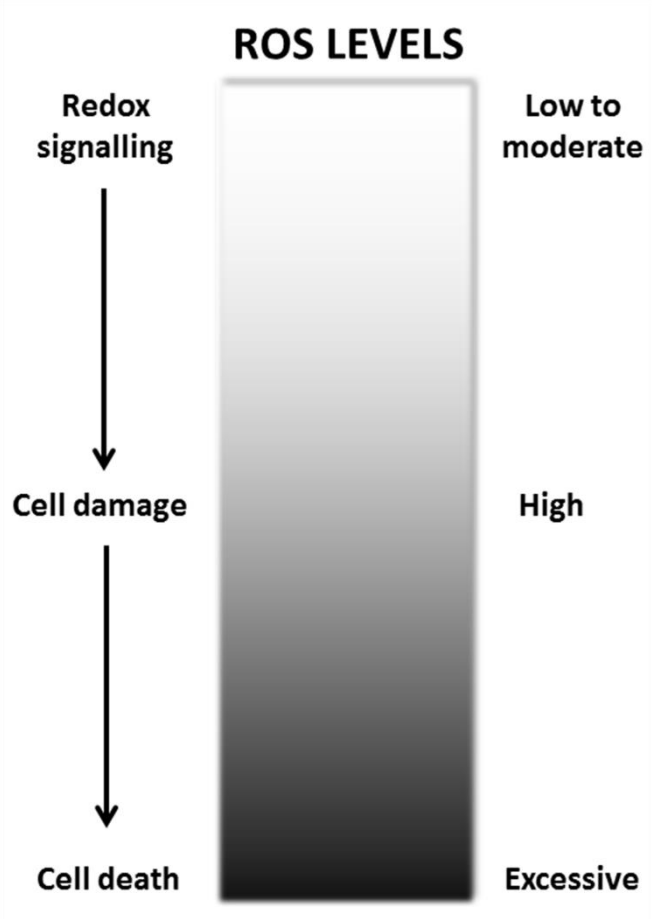


Figure 1.3.2. Dual effect produced by ROS in cells. Low or moderate ROS levels are able to act as second messenger in cellular signalling to mediate pro-survival response. However, they become toxic when their levels are excessive, leading to cell death.

Low or moderate levels of ROS can participate in pro-survival signalling pathways, but it is the increase in their production, their persistence during the time or their presence in a wrong time or place, which turns them into potent pro-death molecules (Trachootham *et al.*, 2008; Nathan & Cunningham-Bussel, 2013). Thus, cells use small amount of ROS to amplify the signal and make it more effective.

Nevertheless, only hydrogen peroxide is suggested to accomplish such function given its stable non-radical condition which means that it reacts poorly or not at all with most biological molecules (Winterbourn, 2013). H_2O_2 is a freely diffusible molecule which allows

it to modulate physiological responses such as cell proliferation, differentiation, and migration across membranes (Rhee, 2006; Finkel, 2011; Reczek & Chandel, 2015). It is important to understand H_2O_2-mediated signalling is produced by its interaction with downstream target molecules, especially by the oxidation of critical residues on proteins or by activating protein kinases (Rhee, 2006). For example, the tyrosine kinase Scr becomes active when H_2O_2 oxidises two of its cysteine residues (Giannoni *et al.*, 2005). Proteins, therefore, are believed to be the main target of ROS (Davies, 2005).

1.3.1. Intracellular sources of ROS: NOX proteins

Living organisms produce ROS from different systems localised in several parts of the cell: the cytosol, the plasma membrane, mitochondria, the endoplasmic reticulum (ER) and peroxisomes (Figure 1.3.3). Cytosolic ROS are mainly formed by enzymes such as xanthine oxidases or soluble components including catecholamines and flavins. Peroxisomes and ER are known to produce ROS as part of their metabolic functions (Finkel, 2011; Di Meo *et al.*, 2016). Peroxisomes are known to perform fatty acid α- and β-oxidation and the synthesis of lipid compounds. Such reactions induce the formation of ROS as by-products (Antonenkov *et al.*, 2010). In the same way, among the multiple functions of the ER, the introduction of double bonds in fatty acids or the formation of intramolecular disulfide bonds during the protein folding, are known to involve oxidation reactions and therefore, to produce ROS (Hwang *et al.*, 1992; Di Meo *et al.*, 2016). Free radicals can also be formed by membrane associated proteins such as lipoxygenases and cyclooxygenases (COX) enzymes during the arachidonic acid oxidation (Nogawa *et al.*, 1997; Farrell *et al.*, 2011; Holmström & Finkel, 2014).

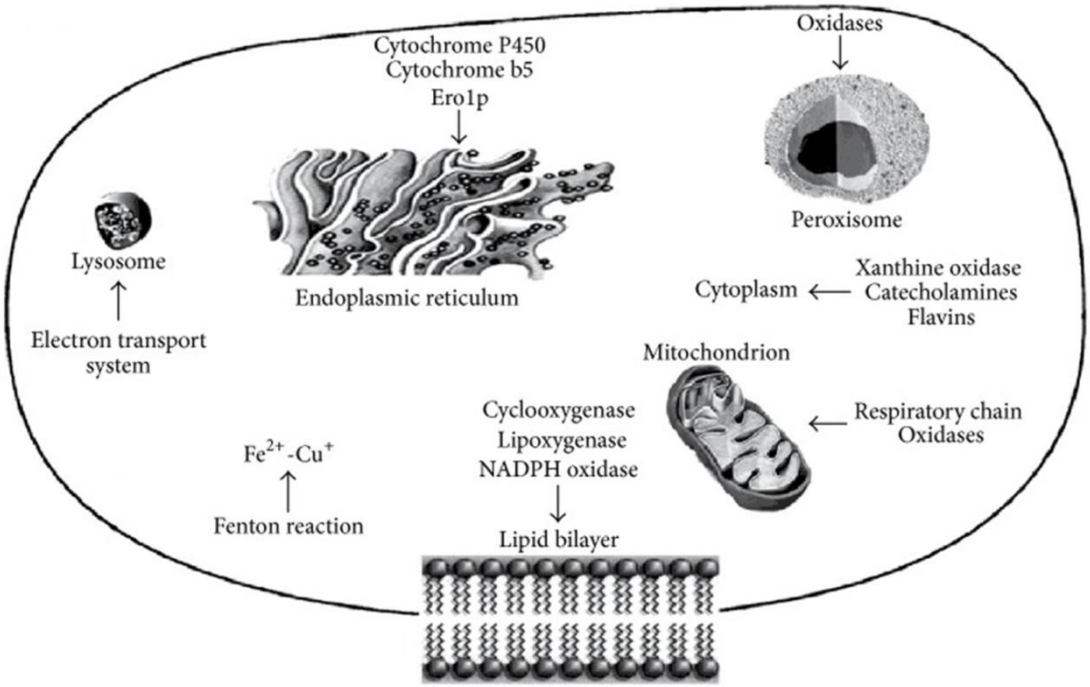


Figure 1.3.3. Intracellular sources of ROS. Representation of the different ROS sources and their localization within the cell. Taken from Di Meo *et al.*, 2016.

However, the main source of ROS located on the plasma membrane is the enzymatic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family of proteins. Differing from other sources, their only known function is the formation of ROS and hence, these molecules are not generated as by-products by NOX enzymes (Bedard & Krause, 2007; Takac *et al.*, 2012; Brandes *et al.*, 2014a). These proteins were first described in leukocytes where they produce ROS as part of their essential role in host defence. Once activated, neutrophils are able to internalise microorganisms by phagocytosis and inactivate them through the secretion of enzymes and the production of ROS (Holmström & Finkel, 2014). We now know that these proteins are also expressed in non-phagocytic cells.

The NOX family consists of seven transmembrane members (NOX1-5, DUOX1-2), differing mainly in their organ-specific expression, control of their activity and the type of ROS they

release (Katsuyama, 2010; Brandes *et al.*, 2014a; Moloney & Cotter, 2017) as represented in figure 1.3.4. NOX2, originally termed glycoprotein 91 phagocyte oxidase ($gp91^{phox}$), was the first NOX member to be identified in phagocytes. Catalytic subunit NOX2 does not generate superoxide on its own, but recruits different components to do so: the transmembrane subunit $p22^{phox}$, three cytosolic proteins, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ and a small GTP-binding protein (Rac) (Katsuyama, 2010; Holmström & Finkel, 2014).

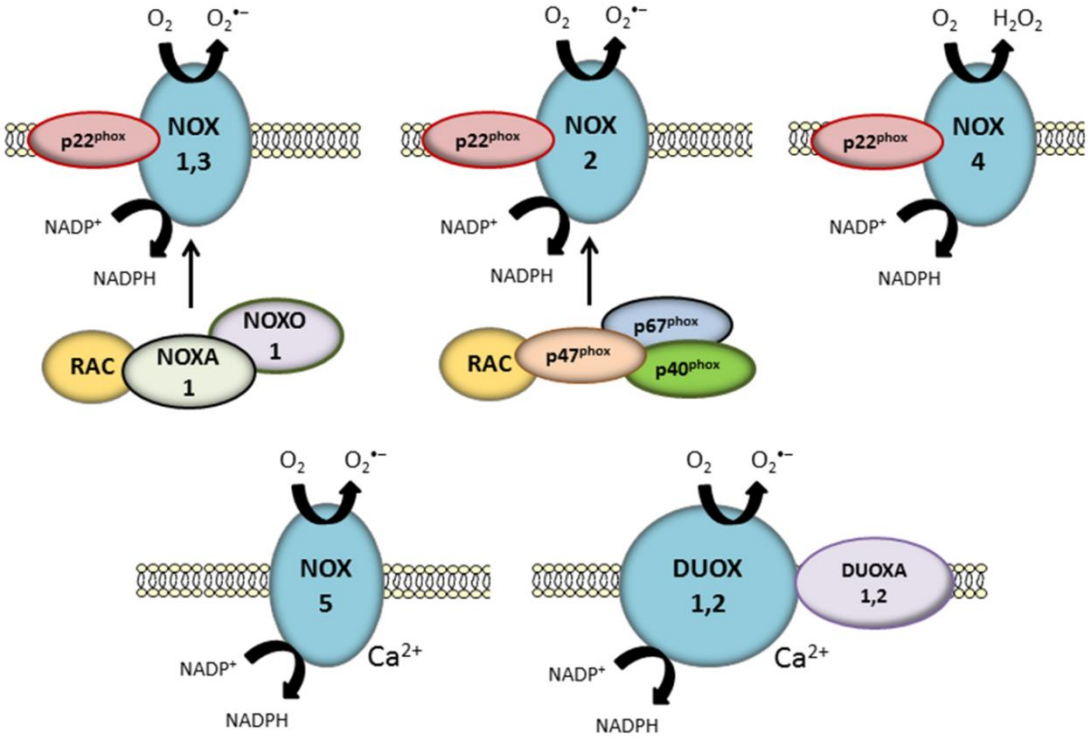


Figure 1.3.4. ROS production by NOX isoforms. NOX1-5 and DUOX1-2 are the seven members of the NOX protein family. Upon activation through different subunits, the catalytic subunit removes an electron from cytosolic NADPH and transfers it to O_2, to produce superoxide ($O_2^{\bullet-}$) or hydrogen peroxide (H_2O_2) as is the case of NOX4. The transmembrane subunit $p22^{phox}$ is required for activation of NOX1-4 whereas NOX1-3 activation also requires the recruitment of other cytosolic subunits. NOX5 appears to only require Ca^{2+} for activation and DUOX1- 2 also require another transmembrane protein known as DUOXA1-2.

Although all NOX isoforms are structurally similar, each is activated by specific mechanisms and regulatory subunits and has specific subcellular localization. Subunits $p47^{phox}$ and $p67^{phox}$ have their homologs NOX organizer 1 (NOXO1) and NOX activator 1 (NOXA1), respectively, when it comes to the regulation of both NOX1 and NOX3. Despite

the fact that NOX1-4 interact with $p22^{phox}$, NOX4 appears to be somewhat different to the other NOXs (Martyn *et al.*, 2006) and it seems to be constitutively active (von Löhneysen *et al.*, 2012). In contrast to other isoforms, NOX4 produces H_2O_2 rather than $O_2^{\bullet-}$ given a modification in its unique tertiary structure (Martyn *et al.*, 2006; Takac *et al.*, 2011; von Löhneysen *et al.*, 2012; Nisimoto *et al.*, 2014). NOX5 also differs to the others in its regulation, which appears to only require Ca^{2+} to produce $O_2^{\bullet-}$. In addition, DUOX1-2 maturation and function seem to depend on other transmembrane factors known as DOUXA1-2, whose function is not completely understood (Morand *et al.*, 2009).

Regarding their expression, NOX enzymes are also different. NOX1 is highly expressed in the colon epithelium and smooth muscle cells (Katsuyama, 2010; Takac *et al.*, 2012) whereas NOX2 is mainly found in endothelial and phagocytic cells (Di Meo *et al.*, 2016), and NOX3 is required for the formation of otoliths in the inner ear (Bánfi *et al.*, 2004). NOX4 expression was first observed in the kidney amongst other tissues (Shiose *et al.*, 2001). However, we now know that this NOX isoform is ubiquitously expressed in a large variety of cell types (Xu *et al.*, 2018), generally exceeding the expression of other NOX proteins (Ago *et al.*, 2004). Expression of NOX5 has been reported in several tissues including the spleen, stomach, testis, uterus and endothelial cells, while DUOX1-2 are expressed mainly in the thyroid and the respiratory epithelia (Takac *et al.*, 2012; Moloney & Cotter, 2017). Given that NOX5 is not expressed in rodents owing to a genetic deletion, few studies have been performed and its role and function are still not well understood (Takac *et al.*, 2012).

NOX enzymes activity is therefore exerted by protein-protein interactions or calcium, with the exception of NOX4, whose activity is controlled by its expression (von Löhneysen *et al.*, 2012; Brandes *et al.*, 2014a). Furthermore, their activity as ROS generators is tightly

regulated given that the amount of ROS produced can be both beneficial and detrimental as it has been discussed above. Growth factors and cytokines are worth being described as activators of NOX function, taking into account the large amount of studies reporting such effect (ten Freyhaus *et al.*, 2006; Lee *et al.*, 2007; Hecker *et al.*, 2009; Sancho *et al.*, 2009; Schröder *et al.*, 2011; Stanic *et al.*, 2012; Takac *et al.*, 2012; Brandes *et al.*, 2014a; Holmström & Finkel, 2014). These studies demonstrate that neurotrophic factors utilise ROS production as part of their signalling response. For instance, Hecker *et al.* showed that the transforming growth factor beta 1 (TGF-β1) induces NOX4-mediated hydrogen peroxide production to repair functions of myofibroblasts in response to lung injury (Hecker *et al.*, 2009) and ten Freyhaus *et al.* demonstrated that NOX inhbition abrogates platelet-derived growth factor (PDGF)-dependent smooth muscle cell chemotaxis (ten Freyhaus *et al.*, 2006). In fact, the absence of growth factors is also responsible for increased ROS levels (Lee *et al.*, 2010; Li *et al.*, 2013). In this context, our laboratory has well defined the lack of growth factors caused by serum withdrawal as a main cause to produce ROS from NOX proteins in a cone photoreceptor cell line (Mackey *et al.*, 2008; Groeger *et al.*, 2009a).

Such stimulation of NOX-dependent ROS formation induced by neurotrophic factors can induce either proliferation or cell death, depending on the amount of ROS formed. Diseases such as Alzheimer's and Parkinson's diseases or retinal degeneration, have been documented to be caused in part by an elevated ROS generation from NOX proteins (Usui *et al.*, 2009; Song *et al.*, 2016; Tao *et al.*, 2016; Ma *et al.*, 2017; Rastogi *et al.*, 2017). In fact, Usui *et al.* pointed NOX proteins as the responsible source of ROS production in the cone cell death occuring in retinitis pigmentosa. They demonstrated a longer preservation of cones in the rd1 mouse model when NOXs were inhibited (Usui *et al.*, 2009). Nevertheless, NOX-generated ROS have been more often associated with pro-survival signal transduction cascades (Brown & Griendling, 2009; Groeger *et al.*, 2009a; Takac *et al.*, 2012; Hakami *et al.*, 2015; Stanicka *et al.*, 2015; Accetta *et al.*, 2016; Moloney & Cotter, 2017; Moloney *et al.*, 2017a, 2017b).

Their role in pro-survival mechanisms is not surprising given that their only known function is the synthesis of ROS molecules. This intracellular source of ROS is, therefore, an ideal point of regulation when it comes to control of ROS generation.

1.3.2. Intracellular sources of ROS: Mitochondria

The mitochondrion is the other large contributor to ROS generation within most mammalian cells. Mitochondria use oxygen to generate energy, in the form of adenosine triphosphate (ATP), by using intermediate metabolites from a metabolic process known as 'tricarboxylic acid (TCA) cycle', which implies the removal of one electron of those metabolites in order to transfer it to the electron transport chain (ETC), resulting in the reduction of O_2 to form $O_2^{\bullet-}$ (Forman & Kennedy, 1974; Mclennan & Esposti, 2000; Nickel *et al.*, 2014; Sabharwal & Schumacker, 2014). Electrons are transferred from nicotinamide adenine dinucleotide (NADH) to O_2 through the four protein complexes which form the respiratory chain: NADH dehydrogenase, succinate dehydrogenase or succinate coenzyme Q (CoQ or Q) reductase, cytochrome *c* oxidoreductase, and cytochrome *c* oxidase (Figure 1.3.5). The reduction of O_2 to two molecules of H_2O takes place in the complex IV. This flow of electrons through all these complexes creates a concentration gradient of protons that is used to produce the energy carrier molecule ATP at the matrix side by the complex V or ATP synthase (Murphy, 2009).

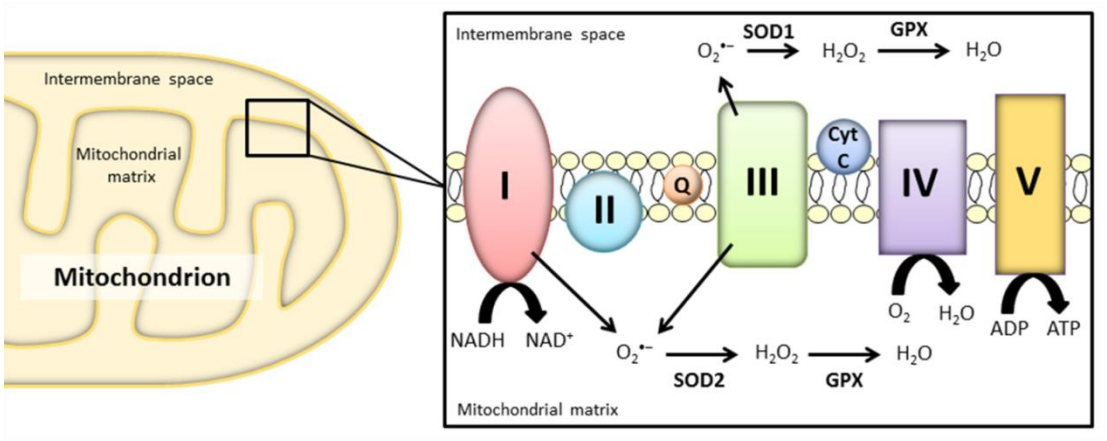


Figure 1.3.5. Mitochondrial ROS production. The inner mitochondrial membrane-bound respiratory chain consists of complex I-V. **Complex I** (NADH dehydrogenase), **II** (succinate dehydrogenase or succinate coenzyme Q reductase), **III** (cytochrome *c* oxidorectuctase), **IV** (cytochrome *c* oxidase) and **V** (ATP synthase). $O_2^{\bullet-}$ is produced at complex I and III during the transfer of electrons from NADH to O_2. This process ends with the generation of ATP at the matrix side. Mitochondrial antioxidants manganese SOD (SOD2) in the mitochondrial matrix and copper/zinc SOD (SOD1), localised in the intermembrane space or cytoplasm, converts $O_2^{\bullet-}$ into membrane permeable H_2O_2. H_2O_2 is reduced to water by glutathione peroxidases (GPX).

Superoxide anion can be produced by at least eleven different sites of mitochondria, mainly at the complexes I and III (Brand *et al.*, 2004, 2016) of the respiratory chain. Complex I is believed to produce most of the $O_2^{\bullet-}$ generated from mitochondria, which is mainly in the matrix side of the inner membrane (St-Pierre *et al.*, 2002). For this reason, several inhibitors to study mitochondrial involvement in ROS production have been designed to target specifically complex I, as is the case of Rotenone (Curtin *et al.*, 2002; Brand *et al.*, 2004), which will be discussed in the chapter 4 of this thesis.

Contrary to NOX enzymes, ROS are generated as by-products of mitochondrial function, which makes difficult their regulation to act in signalling responses. Thus, mitochondrial ROS have been generally associated with aging (Choksi *et al.*, 2008; Liochev, 2013) and other mechanisms related with cell damage (Sanz & Bernardi, 2016). Disease worsening happens due to the progressive accumulation of free radicals, which leads to decline cellular

function and subsequent cell death. For example, some compounds used to block the electron transport chain in mitochondria (e.g. Rotenone) result in increased ROS levels that cause the death of experimental animals in minutes or hours as reviewed by Sanz & Bernardi (Sanz & Bernardi, 2016). Other studies have also demonstrated that oxidative stress produces damage in mitochondrial DNA, which subsequently kills the cell (Yakes & Houten, 1997; Shokolenko *et al.*, 2009). Yakes & Houten hypothesised that ROS-damaged mitochondrial DNA, characterised in several degenerative diseases, leads to decreased mitochondrial function, increased mitochondrial-generated ROS and persistent mitochondrial DNA damage (Yakes & Houten, 1997). Low quantities of ROS, specifically superoxide and hydrogen peroxide, are reported to be unable to cause mutations in DNA by themselves (Gutteridge, 1982). However, they can both generate more ROS types, such as OH•, that eventually induce DNA damage. Unfortunately, what is not very clear yet is whether ROS are cause, consequence or both of mitochondrial dysfunction (Sanz & Bernardi, 2016).

On the other hand, little is known about mitochondrial-mediated redox signalling. This is in part due to the fact that mitochondrial ROS are less well regulated that the ones generated by NOX enzymes (Dröge, 2002), and therefore, they have not been considered for study over the years. The majority of the studies regarding mitochondrial ROS signalling have been performed in endothelial cells and they have been suggested as an amplification method to sustain ROS signalling outside mitochondria (Zhang & Gutterman, 2006; Harel *et al.*, 2017). Some studies suggest that mitochondrial ROS are important in several physiological processes, such as the control of insulin release in pancreatic β-cells (Sakai *et al.*, 2003) or the elimination of bacteria by macrophages (West *et al.*, 2011). Thus, these observations elucidate another area of investigation within mitochondria.

1.3.3. Regulation of ROS: antioxidants

Since different amounts of ROS levels have opposite functions as described above, tight regulation is critical to their role in cellular signalling. Among these regulatory mechanisms which involve repair mechanisms and physical defences, cells also possess an antioxidant defence (Valko *et al.*, 2007). Biological antioxidants are referred to any molecule that is able to delay or prevent the oxidation of a substrate (Pisoschi & Pop, 2015). This system includes enzymatic and non-enzymatic proteins. Superoxide dismutate (SOD) enzymes were the first enzymatic antioxidants to be discovered by McCord & Fridovich in 1969 (McCord & Fridovich, 1969). Since then, other components of this antioxidant machinery have been described. Glutathione (GSH), flavonoids and vitamins (A, C and E) constitute the non-enzymatic antioxidants (Poprac *et al.*, 2017) whilst enzymatic antioxidants are mainly SODs, glutathione peroxidases (GPX), superoxide reductases, glutathione reductases, peroxiredoxins (PRX), thioredoxins (TRX) and catalases (Usui, Oveson, *et al.*, 2009; Martínez-Fernández de la Cámara *et al.*, 2013; Moloney & Cotter, 2017). This defence system is primarily regulated at the transcriptional level. Initial effect of oxidative stress involves the activation of several antioxidant transcriptional factors such as the nuclear factor kappa B (NF-κB) or the nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2) that modulate the expression of antioxidant enzymes (Kaspar *et al.*, 2009; Ray *et al.*, 2012; Xiong *et al.*, 2015; Marengo *et al.*, 2016). In fact, SODs are well-known effector proteins of Nrf2 (Gwarzo, 2009; Kaspar *et al.*, 2009; Niture *et al.*, 2013).

Under normal physiological conditions, there is a balance between ROS generation and ROS scavenging as shown in figure 1.3.6. Unbalance between ROS production and antioxidants favouring the first one, implies the occurrence of several diseases as previously described.

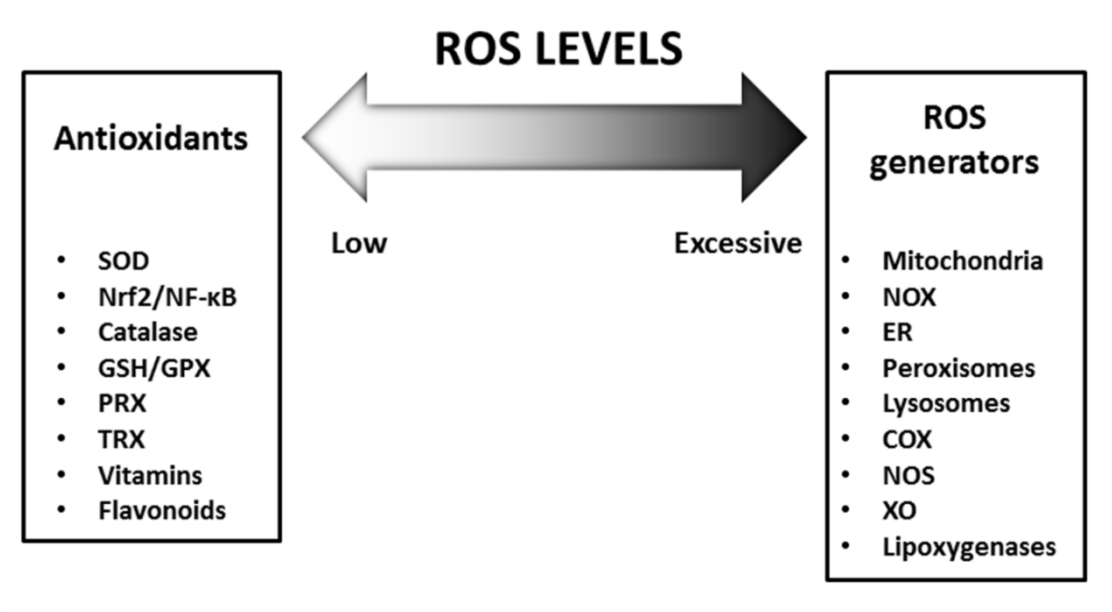


Figure 1.3.6. Schematic representation of redox homeostasis within cells. When ROS levels become excessive from different cellular sources, antioxidants are able to reduce them. SOD: superoxide dismutase; GSH: glutathione; GPX: glutathione peroxidase; PRX: peroxiredoxin; TRX: thioredoxin; NOX: NADPH oxidase; ER: endoplasmic reticulum; COX: cyclooxygenase; NOS: nitric oxide synthase; XO: xanthine oxidase.

Thus, the maintenance of redox homeostasis within cells is essential for their survival. A disruption in redox state can happen either by an increase in ROS production or due to a reduction in the antioxidant activity (Valko *et al.*, 2007). In the same way, a decrease in intracellular ROS levels when they become pathological, can be the consequence of reduced ROS generation or increased antioxidant machinery (Cadenas, 1997; Valko *et al.*, 2007; Moloney & Cotter, 2017).

Antioxidants are responsible for the degradation of ROS (mainly $O_2^{\bullet-}$ and H_2O_2) to prevent oxidative damage. SOD proteins are metalloenzymes that contain copper (Cu^{2+}), zinc (Zn^{2+}) or manganese (Mn^{2+}) in their catalytic centre and they are the most important antioxidants within this defence mechanism (Fukai & Ushio-Fukai, 2011). Intracellular members of this family of proteins are CuZn-SOD (SOD1) and Mn-SOD (SOD2), which are located in the

cytosol and mitochondrial matrix, respectively (Zelko *et al.*, 2002). The third member of SOD enzymes (SOD3) has not been discussed in this thesis given its extracellular location (Pisoschi & Pop, 2015; Moloney & Cotter, 2017). Both SOD1 and SOD2 are in charge of turning superoxide anion to H_2O_2. Given their importance within living organisms, it has been reported that knockout (KO) of SOD2 dramatically shortens lifespan of mice and the fruit fly, *Drosophila melanogaster* (Van Remmen *et al.*, 2003). Since OH• and other potent oxidants can be formed from H_2O_2 reduction, enzymes that effectively reduce this molecule into water (H_2O) are needed. Enzymes that accomplish such function are GPXs, PRX, TRX and catalases (Pisoschi & Pop, 2015; Reczek & Chandel, 2015).

In addition, some natural substances also act as antioxidants within the cell (López-Alarcón & Denicola, 2013). For example, ascorbic acid, also known as vitamin C, is able to scavenge hydroxyl radical and superoxide anion, preventing the oxidative decay of essential biomolecules (Du *et al.*, 2012). Similar properties have also been described in the case of vitamin E or α-tocopherol, although vitamin C efficiency has been reported as the greatest (Pisoschi & Pop, 2015). Nonetheless, these two vitamins can work together to reduce oxidative damage (Du *et al.*, 2012). These two antioxidants can be synthesised by most species of plants and animals, but not by humans, even though they have retained an absolute requirement for it, consequently, they must be taken in the diet (Benzie, 2000). Thus, our antioxidant defence system is widely influenced by nutrition owing to our lack of endogenous ascorbic acid and tocopherol production.

1.3.4. Antioxidants as therapeutic molecules

In this context, synthetic antioxidants have also been developed. Antioxidants Tiron, a vitamin E analog; N-acetyl-L-cysteine (NAC), a precursor of the reduced glutathione; or the mitochondria-targeted antioxidant MitoTEMPO; are some examples that will be used throughout this thesis. All three have been shown to effectively reduce intracellular ROS levels (Curtin *et al.*, 2002; Groeger, Mackey, *et al.*, 2009; Dikalova *et al.*, 2010; Woolley *et al.*, 2013; Bordt & Polster, 2014; Taherian *et al.*, 2014; McCarthy & Kenny, 2016; Zhang *et al.*, 2017). McCarthy & Kenny demonstrated that targeting mitochondrial ROS through the use of MitoTEMPO, reduced endothelial dysfunction and identified mitochondria-targeted antioxidants as potential therapeutic candidates (McCarthy & Kenny, 2016).

Antioxidant supplementation has been used therefore, as a therapeutic approach to combat ROS-related diseases. There are evidences in the literature about the success of exogenous antioxidant addition in the treatment of some diseases, including RP (Jarrett *et al.*, 2006; Komeima *et al.*, 2006, 2007; Valko *et al.*, 2007; Dong *et al.*, 2008; Trachootham *et al.*, 2008; Lu *et al.*, 2009; Usui *et al.*, 2009, 2011). Komeima *et al.* demonstrated that rd1 mice treated with a mixture of antioxidants, including vitamin C and E, showed significantly greater cone photoreceptor survival and antioxidant-treated mice preserved cone function compared with the vehicle-treated controls (Komeima *et al.*, 2006, 2007). In fact, studies from the same group show that increased levels of SOD2 and catalase are able to reduce cone cell death in rd1 and rd10 mice (Usui *et al.*, 2009, 2011). These results are very promising for the treatment of late stages of RP when oxidative stress plays an essential role for the cone degeneration (Shen *et al.*, 2005). However, the majority of these strategies have failed so far (Nickel *et al.*, 2014; Sanz & Bernardi, 2016). The idea that increased ROS levels require their total abolishment through the use of a high amount of antioxidants might be erroneous and can be harmful for cells (Sheu *et al.*, 2006). In biological systems, nutrients may become

toxins and *vice versa* in certain circumstances (Bickham & Smolen, 1994). We cannot forget low ROS levels play a fundamental role in cell signalling. For this reason, a compound that could up-regulate the intracellular antioxidant machinery would be desirable so that cells could autoregulate their ROS levels.

In this context, studies from our laboratory demonstrated that the FDA-approved Rosiglitazone, a member of the thiazolidinedione family of synthetic peroxisome proliferator-activated receptor (PPAR) agonists, was neuroprotective *in vitro* (661W cells) and *in vivo* (C57 and balb/c mice) against oxidative stress (Doonan *et al.*, 2009). PPARs are a family of nuclear hormone receptors that modulate a variety of functions, including anti-inflammatory activity and resistance to insulin (Shiojiri *et al.*, 2002). This compound was able to up-regulate intracellular SOD2 expression to reduce the quantity of ROS produced by stress and therefore, enhance photoreceptor survival.

Furthermore, numerous studies have shown that antioxidant activity can be also increased by progesterone and synthetic progestin treatment (Capel *et al.*, 1981; Moorthy *et al.*, 2005; Bednarek-Tupikowska *et al.*, 2006; Unfer *et al.*, 2015; Zhang *et al.*, 2017). SODs and Nrf2 protein levels were mainly up-regulated by such treatment. According to this, our laboratory demonstrated in 2016 that Norgestrel is able to modulate Nrf2 and SOD2 expression in order to reduce oxidative damage in an induced light damage mouse model (Byrne *et al.*, 2016b). However, its implication in redox responses in a mouse model of inherited retinitis pigmentosa has not yet been described.

1.4. Objectives

Retinal diseases have been difficult to treat so far given that little was known about the mechanisms that lead to blindness. During the last decades, mutations in a great number of genes have been discovered to be responsible for the majority of these diseases. Unfortunately, this is not enough. In some cases, such as retinitis pigmentosa, the fact that the genetic cause of about 40-50% of RP cases is still unknown (Figure 1.1.6), suggests there is still work to be done in the understanding of this disease. This, together with the complexity of its genetics, makes the development of individual therapeutic gene-therapies an impractical task.

Despite the fact that there is currently no cure for RP, promising approaches have been studied. Progesterone has gained attention given its successfully neuroprotective effects on the brain and the retina (Gonzalez *et al.*, 2005; Lu *et al.*, 2008; De Nicola *et al.*, 2013; Deutsch *et al.*, 2013; Singh & Su, 2013; Allen *et al.*, 2015; Sánchez-Vallejo *et al.*, 2015; Schumacher *et al.*, 2016). In this context, we have investigated the progesterone-analogue, Norgestrel, as a possible effective treatment for RP since it was discovered to rescue photoreceptors *in vitro* and *in vivo* (Doonan *et al.*, 2011; Doonan & Cotter, 2012; Byrne *et al.*, 2016a, 2016b; Roche & Wyse-Jackson *et al.*, 2016; Wyse Jackson & Cotter, 2016; Wyse Jackson *et al.*, 2016a, 2016b; Roche *et al.*, 2017a, 2017b). After all these studies, we have understood the great potential of Norgestrel as a therapeutic compound in retinal diseases. This is mainly due to its effects on a wide variety of mechanisms, which include anti-inflammatory properties, the up-regulation of some neurotrophic factors and the reduction of harmful reactive oxygen species in an induced light damage model.

Since ROS have been described as important signalling molecules as well as toxic (Figure 1.3.2), the number of studies on such area has increased. The presence of high levels of ROS in diseases like RP has been demonstrated to have detrimental consequences (Punzo *et al.*, 2012; Campochiaro *et al.*, 2015). However, the fact that ROS are able to protect photoreceptors against stress was a surprising finding as shown in previous studies from this laboratory (Groeger *et al.*, 2009a,2009b, 2012; Bhatt *et al.*, 2010). The aims of this project therefore, were firstly to investigate the role that ROS play at different time points in the Norgestrel-mediated neuroprotective response in a model of retinitis pigmentosa and their relationship with the known components of the Norgestrel-mediated response, such as the progesterone receptor PGRMC1 (Wyse Jackson *et al.*, 2016a) and the neurotrophic factor bFGF (Doonan *et al.*, 2011; Wyse Jackson & Cotter, 2016). We also wished to examine the possible cellular source or sources of such response, given that ROS molecules can be produced from different components within the cell (Figure 1.3.3-1.3.5). It has been previously shown that NOX enzymes produce pro-survival ROS following serum withdrawal in the 661W cone photoreceptor cell line (Groeger *et al.*, 2009a), whereas COX proteins are responsible of such increase upon the exogenous treatment of bFGF in the same cell line (Farrell *et al.*, 2011).

The final aim of this study was to elucidate whether these molecules are regulated by any component of the endogenous antioxidant machinery (Figure 1.3.6) in a model of retinitis pigmentosa. Several studies have linked progesterone treatment with an increase in antioxidants (Capel *et al.*, 1981; Moorthy *et al.*, 2005; Bednarek-Tupikowska *et al.*, 2006; Unfer *et al.*, 2015; Zhang *et al.*, 2017). Our laboratory has also demonstrated that Norgestrel is able to increase antioxidants Nrf2 and SOD2 in order to enhance photoreceptor survival in the balb/c induced light damage mouse model (Byrne *et al.*, 2016b). However, this has not been studied in a genetic model of RP yet.

Chapter 2: Material and Methods

2.1. Cell culture and treatments

The mouse photoreceptor-derived 661W cell line generously provided by Dr Muayyad Al-Ubaidi (Department of Biomedical Engineering, Cullen Eye Institute, University of Houston, Houston, TX USA) was used in the experiments throughout this thesis (Al-Ubaidi *et al.*, 2008). To ensure the identity of the cell line as a cone-like cell line, RT-qPCR analysis for cone specific blue opsin (Opn1sw) and rod specific rhodopsin was performed as previously described (Wyse Jackson *et al.*, 2016a). Expression of Opn1sw confirmed a cone cell phenotype (Figure 2.1.1).

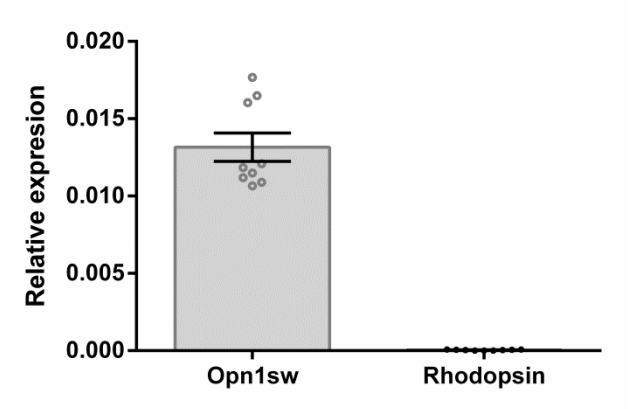


Figure 2.1.1. Validation of 661W cells as a cone photoreceptor-like cell line. Cone specific blue opsin (Opn1sw) and rod specific Rhodopsin mRNA levels were analysed by RT-qPCR in 661W cone-photoreceptor like cells. Results are presented as mean ± SEM.

Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, D6429, Sigma, Dublin, Ireland), supplemented with 10% foetal bovine serum (FBS, F7524, Sigma) and 1% penicillin/streptomycin (P0781, Sigma). Cultures were maintained in a sterile humidified atmosphere at 37°C and 5% CO_2. 500,000 cells were seeded in a T75 cm^2 flask (Starstedt AG & Co, Nümbrecht, Germany) and allowed to attach overnight. Cells were then washed three times with phosphate-buffered saline (PBS, pH 7.4) prior to addition of 20 µM Norgestrel (N2260, Sigma) or the equivalent dimethyl sulfoxide (DMSO, D2650, Sigma) in complete (for untreated cells) or serum-free medium. After incubation at indicated times, cells were washed in PBS and detached using Accutase solution (A6964, Sigma).

2.2. Reagents, inhibitors and chemicals

Table 2.2.1. List of inhibitors and modulators used throughout the course of this thesis. R&D: R&D Systems (Abingdon, UK).

Reagent	Function	Supplier	Cat #	Conc.	Vehicle	Duration
AG-205	PGRMC1	Sigma	A1487	1 µM	DMSO	10 min
NAC	Antioxidant	Sigma	A7250	100 µM	H_2O	1 h
Tiron	Antioxidant	Sigma	172553	1 mM	H_2O	1 h
DPI	NOX	Sigma	D2926	1 µM	DMSO	1 h
VAS2870	NOX	Sigma	SML0273	1 µM	DMSO	1 h
Rotenone	Mitochondria	R&D	3616	20 µM	DMSO	1 h
Diclofenac	COX	Sigma	D6899	1 µM	H_2O	1 h
MitoTEMPO	Antioxidant	Sigma	SML0737	5 µM	H_2O	1 h

2.3. Primers

Table 2.3.1. List of primers used throughout the course of this thesis. All primers are from Qiagen (West Sussex, UK).

Gene	Qiagen primer	Product size (bp)	Ref Seq ID #
β-Actin	QT00095242	149	NM_007393
GAPDH	QT00199388	144	NM_008084 XM_001003314 XM_990238 NM_001001303 NM_001289726
HPRT	QT00166768	168	NM_013556
OPN1SW	QT00257292	107	NM_007538
Rhodopsin	QT00099022	78	NM_145383 XM_006505860 XM_006505861

bFGF	QT00128135	138	NM_008006
NOX2	QT00139797	146	NM_007807 XM_006527565
NOX4	QT00126042	75	NM_015760 NM_001285833 NM_001285835 XM_006508010 XM_006508011 XM_006508012
SOD1	QT00165039	88	NM_011434 XM_128337
SOD2	QT00161707	159	NM_013671
NRF2	QT00095270	77	NM_010902

2.4. Antibodies

Table 2.4.1. List of antibodies used throughout the course of this thesis. WB: Western Blotting, IF: Immunofluorescence. BD: BD Biosciences Europe (Oxford, UK), Novus Bio: Novus Biologicals (Abingdon, UK), Santa Cruz: Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

Gene	Supplier	Cat #	Host	Dilution WB	Dilution IF
β-Actin	Sigma	A5441	Mouse monoclonal		1:500
bFGF	BD	610073	Mouse monoclonal		1:250
NOX2	BD	611414	Mouse monoclonal	1:500	1:200
NOX4	Novus	NB110-58851	Rabbit polyclonal	1:500	1:200
$p22^{phox}$	Santa Cruz	CS9	Mouse monoclonal		1:250
SOD1	Abcam	13498	Rabbit polyclonal	1:500	
SOD2	Abcam	13533	Rabbit polyclonal	1:500	1:200
pNrf2 (S40)	Abcam	76026	Rabbit monoclonal	1:500	1:100
Nrf2	Abcam	31163	Rabbit polyclonal	1:500	

2.5. Flow cytometry

Cells were incubated with the following probes for 30 min at 37°C to measure intracellular ROS production: 50 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA, #35845, Sigma) or 0.5 μM dihydroethidium conjugate with hexyl triphenylphosphonium (MitoSOX Red, M36008, Invitrogen, Eugene, OR, USA). The difference between these probes is that these compounds were detected at either FL-1 (530 nm) or FL-2 (585 nm), respectively, by a FACScan flow cytometer (Becton Dickinson, Oxford, UK) with ROS production indicated by an increase in fluorescence. All samples were gated using the healthy population of the untreated sample and 10,000 gated events per sample were collected in the experiments. Each experimental condition was performed in technical triplicates and all graphs represent data obtained from at least three independent experiments. CellQuest software (Becton Dickinson) was used for data analysis.

2.6. MTS assay for cell viability

A colorimetric tetrazolium (MTS) assay (G3580; Cell Titer 96 AQueous One Solution kit, Promega, Madison, WI, USA) was used to determine the number of viable cells (Penha *et al.*, 2013; Wyse Jackson *et al.*, 2016b). 4,000 cells per well were seeded in 96-well culture plates (Starstedt). At 24 h post seeding, cells were washed and treated for times indicated. 20 μl of MTS solution was added to each well and incubated at 37°C in dark for the same time as treatment, leaving it for a maximum of 4 h in the case of 6, 24 or 48 h treatment. The quantity of formazan product as measured by absorbance at 490 nm with a Spectramax Plus 384 microplate reader (Molecular Device Corporation, Sunnyvale, CA, USA) is directly proportional to the number of living cells in culture. A further reading at 650 nm was also taken from all wells and deducted from the 490 nm readings to account for any cellular

debris. Untreated healthy cells were used as the 100% viable control. In all experiments entailing inhibitors, a control was carried out for every individual concentration. All samples were carried out in 6 replicates in a total of at least three independent experiments.

2.7. Small interfering RNA (siRNA) silencing

661W cells (300,000) were seeded in a T75 flask (Starstedt) the day before transfection. Both 25 nM FGF2 siRNA (GS14173, Qiagen) and AllStars Negative Control siRNA (Qiagen) were transfected and incubated for 24-48 h before treatment using HiPerFect Transfection Reagent (Qiagen). Mock-transfected cells (treated with HiPerFect Transfection Reagent alone) were included as a control to determine effects of transfection process (Figure 2.7.1) and negative control siRNA (Scrambled) was used to detect non-specific changes in phenotype or gene expression. Cells were then washed three times in PBS followed by treatment with 20 µM Norgestrel or DMSO (vehicle) in serum-free medium. siRNA knockdown was validated by RT-qPCR and immunofluorescence.

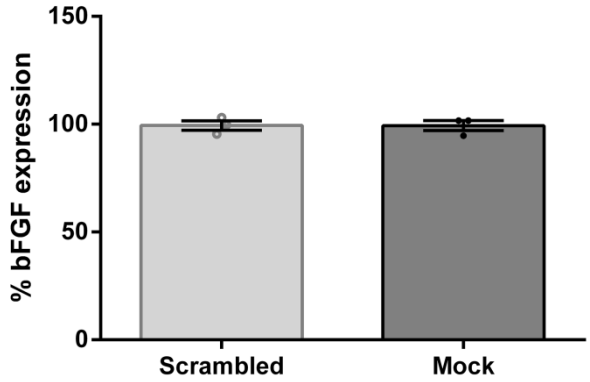


Figure 2.7.1. bFGF expression was not affected by transfection process itself. AllStar Negative Control siRNA (Scrambled) and cells treated with transfection reagent only (Mock) confirmed non-specific effects on bFGF expression produced by the transfection reagent or process, analysed by RT-qPCR.

2.8. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR- associated proteins (Cas) system

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) is a natural RNA-guided DNA nuclease system used by archaea and bacteria for the degradation of foreign genetic material (Mojica *et al.*, 2005). Cells were seeded (100,000) in antibiotic-free standard growth DMEM (Sigma) in a 6-well culture plate (Starstedt) and allowed to attach overnight. 5 µl of UltraCruz® Transfection Reagent (sc-395739, Santa Cruz) were added to 1 µg of NOX4 Double Nickase Plasmid (m) (sc-424443-NIC, Santa Cruz) (Figure 2.8.1) and incubate it for 48 h. The addition of transfection reagent alone was used as a negative control. After transfection, cells were treated with 1 µg/ml puromycin antibiotic for 6 days. Colonies were analysed to confirm complete allelic knockouts by immunofluorescence and western blotting.

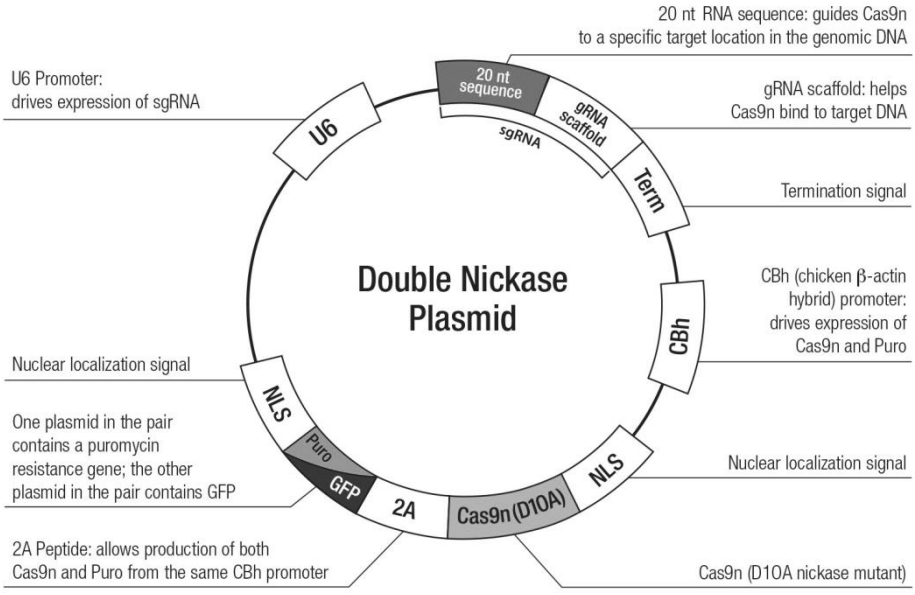


Figure 2.8.1. Double Nickase Plasmid from Santa Cruz. Double Nickase Plasmid consist of a pair of plasmids each encoding a D10A mutated Cas9 nuclease and a target-specific 20 nt guide RNA (gRNA or sgRNA) designed to knockout gene expression with greater specificity than its CRISPR/Cas9 KO counterpart. One plasmid in the pair contains a puromycin resistance gene for selection and the other, a green fluorescent protein (GFP) marker to visually confirm transfection. Modified from Santa Cruz Biotechnology Inc. website.

2.9. 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 (License Number AE19130/P009). Experiments were approved by University College Cork Animal Experimentation Ethics Committee. Both male and female homozygous rd10/rd10 mice (B6.CXBI-Pde6b$^{rd10/J}$) were used and C57BL/6 mice (C57) were used as controls. Mice were supplied by the Biological Services Unit (BSU), University College Cork and were humanely euthanised by cervical dislocation (Hwang & Iuvone 2013).

2.9.1. Retinal explant culture

C57BL/6 and rd10 mice were sacrificed at postnatal day 30 (P30) or P15, respectively, 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. 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 800 ml sterile dH_2O. This solution is made to a complete medium through hormones and vitamins supplementation (Table 2.9.1 – all from Sigma), 10% bovine serum albumin and 1% penicillin streptomycin (both Sigma) to a final volume of 1,000 ml and will last for up to three weeks at 4°C (Caffé *et al.*, 2001). The powder is composed of 41 ingredients that can be classified in salts, amino acids, sugars and vitamins (Table 2.9.2).

Table 2.9.1. Supplements for R16 medium. Conc. (final concentration).

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

Table 2.9.2. Composition of R16 dry powder medium (basal). Conc. (final concentration).

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

The retina was flat mounted, photoreceptor side down on top of a nitrocellulose insert (Millipore, Billerica, MA) in 6-well culture plates (Starstedt) as shown in figure 2.9.1. Explants were cultured in 1.2 ml of complete R16 media and treated with 20 µM Norgestrel or the equivalent DMSO for the times indicated, using a total number of 4 mice per treatment.

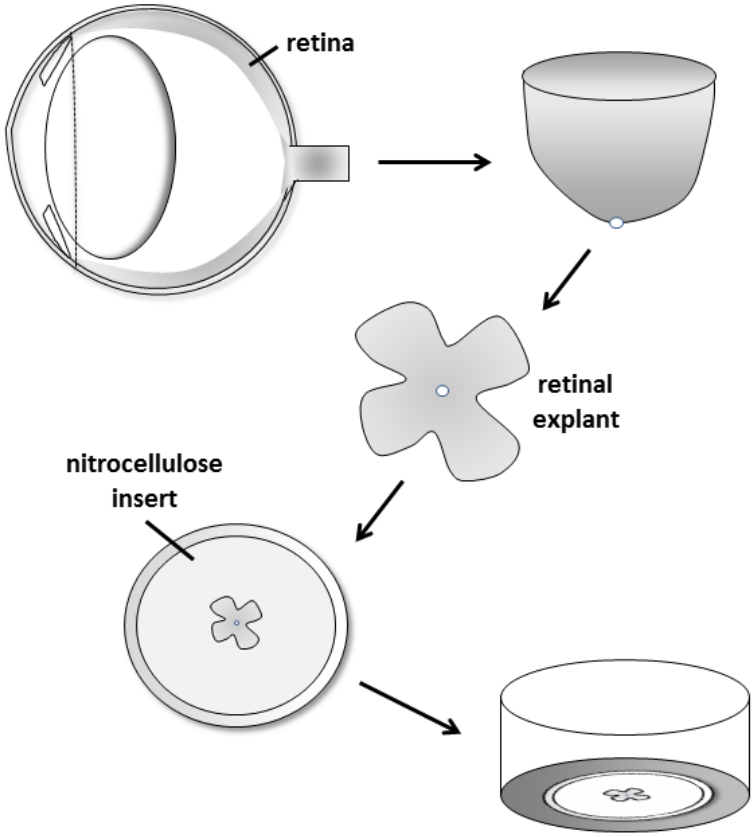


Figure 2.9.1. Schematic representation of retinal explant culture. The lens, anterior segment, vitreous body, retinal pigment epithelium and sclera were removed and the retina was flat mounted on a nitrocellulose insert and culture with media in 6-well culture dishes.

2.9.2. 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% (500 ppm). Dams of rd10

pups were given a Norgestrel-supplemented diet (LabDiet 5053, custom diet containing Norgestrel) when the pups were P10 to allow pups to receive Norgestrel in the milk. This equates to a daily intake of approximately 80 mg/kg, assuming a 30 g mouse consumes around 5 g 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 also given 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).

2.9.3. FACS analysis in retinal explants

After treatment, retinal explants were transferred to a trypsin-EDTA solution 0.25% (T4049, Sigma) containing 50 µL deoxyribonuclease II from bovine spleen (10,000 units/ml; D8764, Sigma) and maintained at 37°C for 15 min. Single-cell suspensions were made by pipetting up and down ten times using a P1000 pipette and large debris was allowed to settle. Then, 4 ml of single-cell suspension were collected and placed into a FACS tube (BD Biosciences) and incubated with 50 µM DCFDA for 15 min at 37°C. Fluorescence was measured using a Becton-Dickinson FACScan flow cytometer.

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

Localization of dying cells within retinal explants was assessed with the terminal dUTP nick end-labelling (TUNEL) assay (Portera-cailliau *et al.*, 1994). Retinal explants were fixed in 4% paraformaldehyde (PFA) for 30 min followed by cryoprotection in 15% sucrose in 1xPBS for 1 h, 20% sucrose for 1 h and 30% sucrose overnight, all at 4 °C. Eyes were

submerged and frozen in cryochrome (Thermo Scientific, Waltham, US). Sections of 7 μm were cut using a cryostat (Leica CM1950; Leica Co., Meath, Ireland) and collected on superfrost glass slides (Fisher Scientific, Waltham, US) and stored at −80 °C. Retinal sections were then permeabilised with 0.1% Triton X for 2 min followed by incubation with terminal deoxynucleotidyl transferase (M1875; Promega, Wisconsin, US) and fluorescein-12-dUTP (#11373242910; Roche, Lewes, UK) according to manufacturer's instructions. Nuclei were counterstained with Hoechst 33342 (1 μg/mL). Sections were incubated at 37°C for 1 h in a humidified chamber. After three washes with PBS, sections were mounted in Mowiol and viewed under a fluorescence microscope (LeicaDMLB2). For each treatment, at least three animals were used and two fields (x40 magnification) per section of at least three different sections were evaluated. Quantification was performed using ImageJ software. Per section, three distinct measurements were taken and averaged from at least four sections per mouse.

2.9.5. Quantification from immunohistochemical sections

Quantification of outer nuclear layer (ONL) thickness in retinal sections was carried out using ImageJ software. Average ONL thickness, where photoreceptor nuclei are located through the use of Hoechst 33342, 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. Three mice were analysed per group.

2.10. RNA isolation, cDNA synthesis and quantitative real time PCR (RT-qPCR)

Total RNA isolation from 661W cells and retinal explants was performed using an RNeasy Mini Kit (Qiagen) following manufacturer's protocol, including DNase treatment. 1 µg of cDNA was synthesised using QuantiTect Reverse Transcription Kit (Qiagen) to perform RT-qPCR with QuantiTect Primer Assays and QuantiTect SYBR Green PCR Kit (Qiagen) in 96 well-plates (Roche, West Sussex, UK). Samples were run in triplicates using LightCycler®96 Instrument (Roche) whose protocol include: denaturing at 95°C for 10 min, followed by 40 cycles of 10 s at 95°C, 10 s at 60°C for and 10 s at 72°C. mRNA values (Ct) were obtained using LightCycler®96 Application Software (Roche) and normalised to the average of three housekeeping genes (β-Actin, GAPDH and HPRT). Relative changes in gene expression were quantified using the comparative Ct (ΔΔCt) method (Livak & Schmittgen, 2001).

2.11. Western blot analysis

Whole cell pellets and whole retinas were lysed in RIPA buffer: Tris–HCl (50 mM, pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, NaCl (150 mM), EGTA (1 mM), sodium orthovanadate (1 mM), sodium fluoride (1 mM), cocktail protease inhibitors (#11836153001, Sigma) and phenylmethanesulfonyl fluoride (1 mM). Cell and tissue lysates were centrifuged at 20,800 g at 4°C for 15 min and 30 min, respectively, and protein concentration was measured using a Bradford assay (Bio-Rad, Hercules, CA, USA). 30-50 µg of protein per sample were resolved using denaturing 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose membrane (GE10600001, Sigma). Total protein levels were analysed using REVERT total protein stain (LI-COR Biosciences, Lincoln, US) as per manufacturer's instructions and imaged on a LI-

COR scanner in the 700 channel as previously shown (Roche & Wyse-Jackson et al., 2016, Roche et al., 2017a). Membranes were blocked with 5% milk in Tris-buffered saline/0.1% Tween-20 (TBS-T) and incubated overnight with primary antibodies in blocking buffer. Table 2.4.1 lists the details of all primary antibodies used. Membranes were then probed with IRDye® 800 goat anti-mouse (C60726-02, LI-COR) or IRDye® 800 goat anti-rabbit (C61103-06, LI-COR) secondary antibody (1:10,000). The signal was detected with an Odyssey infrared imaging system. Western blots shown in results are representative images of three independent experiments.

2.12. Immunofluorescence

25,000 cells were seeded on glass coverslips overnight in 24-well plates (Starstedt) prior treatment. Cell were then washed in PBS and fixed with 4% PFA for 20 min at room temperature (RT). Cells and retinal sections were blocked for 30 min at RT with 5% donkey serum (containing 0.1% Triton X-100 for permeabilization). Coverslips and sections were subsequently incubated with primary antibody in 5% donkey serum overnight at 4°C. Table 2.4.1 lists the details of all primary antibodies used. After washes, conjugated secondary antibody (1:500; donkey anti-mouse/goat/rabbit with either a 488 or 594 fluorescent probe, Life Technologies, Eugene, OR, USA) was used for 1.5 h in dark at RT. All coverslips were incubated with Hoechst 33342 (1 μg/ml) for 10 min at RT in dark to counterstain the nuclei. Coverslips were mounted on to glass slides using Mowiol and imaged using a fluorescent microscope Leica DM LB2 (Leica Co., Wetzlar, Germany).

2.13. Statistical analysis

Numerical data were compiled using Microsoft Excel (Microsoft, Dublin, Ireland) and expressed as mean ± standard error (SEM) from at least three separate experiments, with each being performed in triplicate. In the case of two sample comparisons (e.g. vehicle vs. Norgestrel), significant differences across groups were assessed with an unpaired Student's *t*-test using the GraphPad Prism 6 software. One-way ANOVA and post hoc Tukey's test were used for multiple comparisons and post hoc Dunnett's test was used when groups were only compared with one control group (e.g. untreated, scrambled). Differences were considered significant if $P < 0.05$ indicated on the graphs with asterisks (*).

Chapter 3: Pro-survival redox signalling in progesterone-mediated retinal neuroprotection

3.1. Abstract

Retinitis pigmentosa (RP) is characterised by photoreceptor cell loss. Despite a substantial understanding of the mechanisms leading to cell death, an effective therapeutic strategy is sought. Our laboratory has previously demonstrated the neuroprotective properties of Norgestrel, a progesterone analogue, in the degenerating retina, mediated in part by the neurotrophic factor basic fibroblast growth factor (bFGF). In other retinal studies, we have also presented a pro-survival role for reactive oxygen species (ROS), downstream of bFGF. Thus, we hypothesised that Norgestrel utilises bFGF-driven ROS production to promote photoreceptor survival. Using the 661W photoreceptor-like cell line, we now show that Norgestrel, working through progesterone receptor membrane complex 1 (PGRMC1); generates an early burst of pro-survival bFGF-induced ROS. Using the rd10 mouse model of RP, we confirm that Norgestrel induces a similar early pro-survival increase in retinal ROS. Norgestrel-driven protection in the rd10 retina was attenuated in the presence of antioxidants. This study, therefore, presents an essential role for ROS signalling in Norgestrel-mediated neuroprotection *in vitro* and demonstrates that Norgestrel employs a similar pro-survival mechanism in the degenerating retina.

3.2. Introduction

Reactive oxygen species (ROS) are constantly being generated under normal conditions in aerobic respiration and for many years, they were described simply as destructive by-products of the process (Chance, 1979; Rhee, 2006; Trachootham *et al.*, 2008; Ray *et al.*, 2012; Song *et al.*, 2017). ROS accumulation has been associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease through induction of cellular damage and mitochondrial dysfunction, worsening disease progression (Gandhi & Abramov, 2012; Kim *et al.*, 2015). In retinitis pigmentosa (RP), apoptosis has also been related with a high production of ROS (Carmody & Cotter, 2000; Tao, 2016; Usui *et al.*, 2009). Despite the fact mutations causing RP mainly affect rods leading to apoptosis, cones are subsequently affected by such loss. The most accepted causes of cones cell death include the lack of growth factors production by rods (Lavail *et al.*, 1992; Corrochano *et al.*, 2008; Doonan *et al.*, 2012) and the increase in the toxic ROS levels as a consequence of the loss of rods (Shen *et al.*, 2005; Komeima *et al.*, 2006; Usui *et al.*, 2009; Campochiaro & Mir, 2017).

In recent years, however, our understanding of these molecules has changed and they are no longer associated solely with damage. It has become increasingly clear that ROS signalling is also involved in pro-survival mechanisms. It is acknowledged that ROS function as intracellular signalling molecules, when present in moderate quantities (Remacle *et al.*, 1995; Rhee, 2006; Trachootham *et al.*, 2008; Ray *et al.*, 2012; Moloney & Cotter, 2017). Acting as second messengers, ROS often act in a pro-survival capacity (Groeger *et al.*, 2009b; Trachootham *et al.*, 2008), with previous studies from our group highlighting a protective role for ROS in the retina (Bhatt *et al.*, 2010; Farrell *et al.*, 2011; Groeger *et al.*, 2009a, 2012; Mackey *et al.*, 2008). This is of interest, for the retina has high oxygen consumption. In addition, given that the retina is constantly converting light into neural

signals (Eshaq *et al.*, 2014), ROS are consequently produced from endogenous sources (Bhatt *et al.*, 2010; Kim *et al.*, 2015; Nishimura & Hara, 2016).

Cytokines and neurotrophic factors have shown promise as therapeutics for neurodegenerative disorders, due to their neuroprotective effects and their ability to delay degeneration in RP and a variety of other diseases (Kolomeyer & Zarbin, 2014). The main neurotrophic factors that are produced by the RPE and therefore, are important in the treatment of RP are vascular endothelial growth factor (VEGF), pigment epithelium-derived factor (PEDF), platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and basic fibroblast growth factor (bFGF) (Kolomeyer & Zarbin, 2014). Several studies suggest that these molecules can modulate the production of ROS in different systems (Ilatovskaya *et al.*, 2013; Kirkland *et al.*, 2007; Lo & Cruz, 1995), including various cell types of the retina (He *et al.*, 2014; Zheng *et al.*, 2009). Indeed, we have shown that bFGF is linked to ROS synthesis in the retina (Groeger *et al.*, 2012; O'Driscoll *et al.*, 2008). More recent studies from our group have presented the synthetic progesterone, Norgestrel, as a potential therapeutic for retinal degeneration (Byrne *et al.*, 2016a, 2016b; Doonan *et al.*, 2011; Roche & Wyse-Jackson *et al.*, 2016, Wyse-Jackson & Cotter, 2016; Roche *et al.*, 2017a, 2017b). Working primarily through activation of progesterone receptor membrane complex 1 (PGRMC1) (Wyse Jackson *et al.*, 2016a, 2016b), we have shown Norgestrel's protective effects are dependent on bFGF production.

Therefore, in light of the above studies, we hypothesised that Norgestrel utilises bFGF-driven ROS production to promote photoreceptor survival. The current study was designed to examine the role of ROS in Norgestrel-mediated neuroprotection.

3.3. Results

3.3.1. Serum deprivation induces a pro-survival production of ROS in 661W cells

The cessation of trophic support has been related with the death of cones after rods die as a consequence of mutations in RP (Komeima *et al.*, 2006, 2008; Finnegan *et al.*, 2010; Athanasiou *et al.*, 2018). Thus, serum starvation (SS) has been used as a model of cellular stress. It has been reported that ROS are produced as a consequence of serum deprivation in the cone-photoreceptor 661W cell line (Mackey *et al.*, 2008) and other cell lines (Li *et al.*, 2013). In order to corroborate this, cells were incubated with 50 µM 2',7'-dichlorofluorescin diacetate (DCFDA, Figure 3.3.1), one of the most commonly used probes for detecting intracellular ROS levels (Eruslanov & Kusmartsev, 2010; Karlsson *et al.*, 2010). For the flow cytometry analyses, untreated 661W cells (UT) were used to gate the population used in all subsequent experiments (Figure 3.3.2).

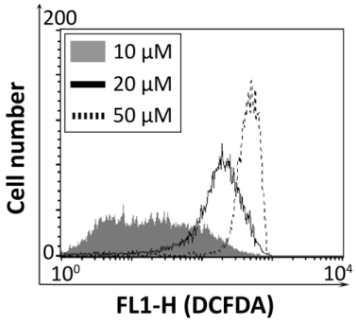


Figure 3.3.1. DCFDA was used to detect intracellular ROS levels. Different concentrations were analysed by flow cytometry in order to find an optimal peak of fluorescence. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. 50 µM was used in all subsequent experiments.

According with the results obtained by Mackey *et al.* and Li *et al.*, SS produces a rapid increase in the ROS production *in vitro* (Mackey *et al.*, 2008; Li *et al.*, 2013). A significant increase in ROS was observed in this study at 30 min and up to 1 h in serum-starved cells compared to vehicle control (one-way ANOVA, serum starvation effect, $F_{4,39} = 66.91$, $P < 0.0001$; Dunnett's *post hoc* test, 30 min: $P < 0.0001$; 1 h: $P = 0.003$; $n = 3$ per group; Figure 3.3.3).

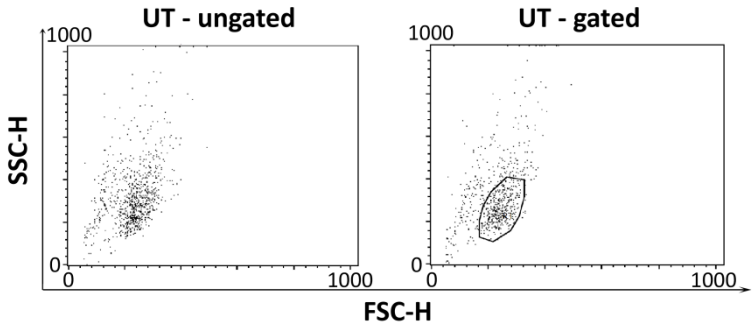


Figure 3.3.2. Untreated 661W cells (UT) were used to gate the population analysed in all subsequent experiments.

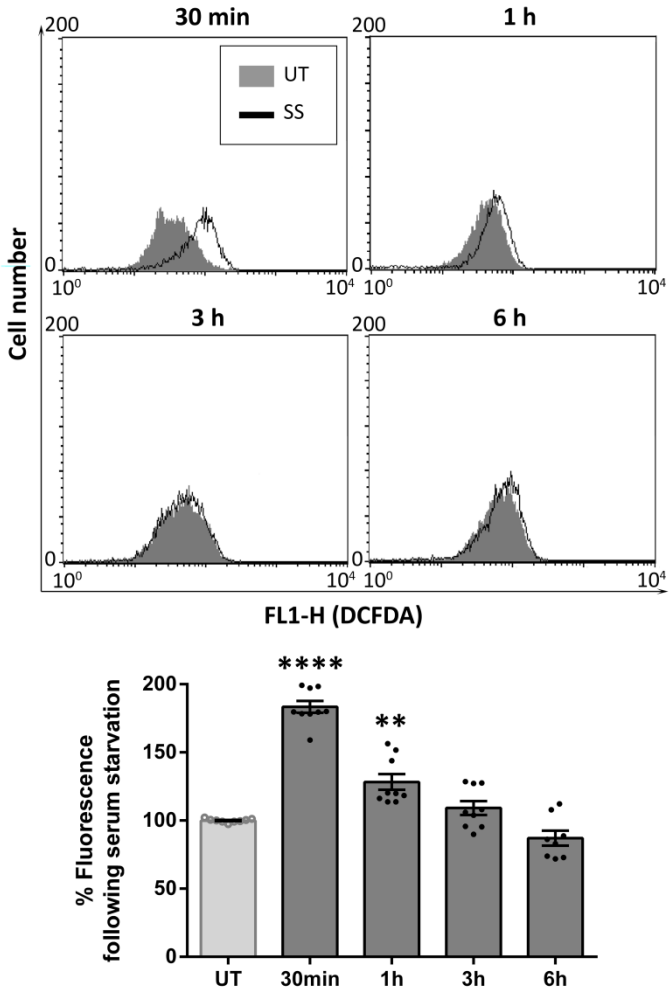


Figure 3.3.3. Serum starvation increases ROS levels in 661W cells. DCFDA probe was used to detect ROS levels by flow cytometry and plotted on a histogram. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (Dunnett's *post hoc* test, comparing all time points to the UT control). Cells following serum starvation (SS) increased ROS levels at 30 min and up to 1 h. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (** $P < 0.01$, **** $P < 0.0001$).

Increased ROS production has been described to be related to autophagy, a mechanism that can isolate damaged organelles and cellular biomolecules to subsequently increase in the survival of the remaining cells, in some cell types (Li *et al.*, 2013). Interestingly, Mackey *et al.* also demonstrated that the increase in ROS levels activates the protein kinase B (Akt) survival pathway (Mackey *et al.*, 2008). Therefore, in order to establish whether this burst of ROS is beneficial to 661W cells in this study, different concentrations of the antioxidants sodium 4,5-dihydroxybenzene-1,3-disulfonate (Tiron) or N-acetyl-L-cysteine (NAC) were used to inhibit the increase in ROS (Taherian *et al.*, 2014). 1mM and 100 µM of Tiron and NAC, respectively, showed no cell death compared with the vehicle (Figure 3.3.4(i)) and significantly decreased ROS levels (independent samples *t*-test, Tiron: $t_{16} = 7.313$, $P < 0.0001$; NAC: $t_{16} = 2.324$, $P = 0.0336$, $n = 3$ per group; Figure 3.3.4(ii) and Figure 3.3.5A).

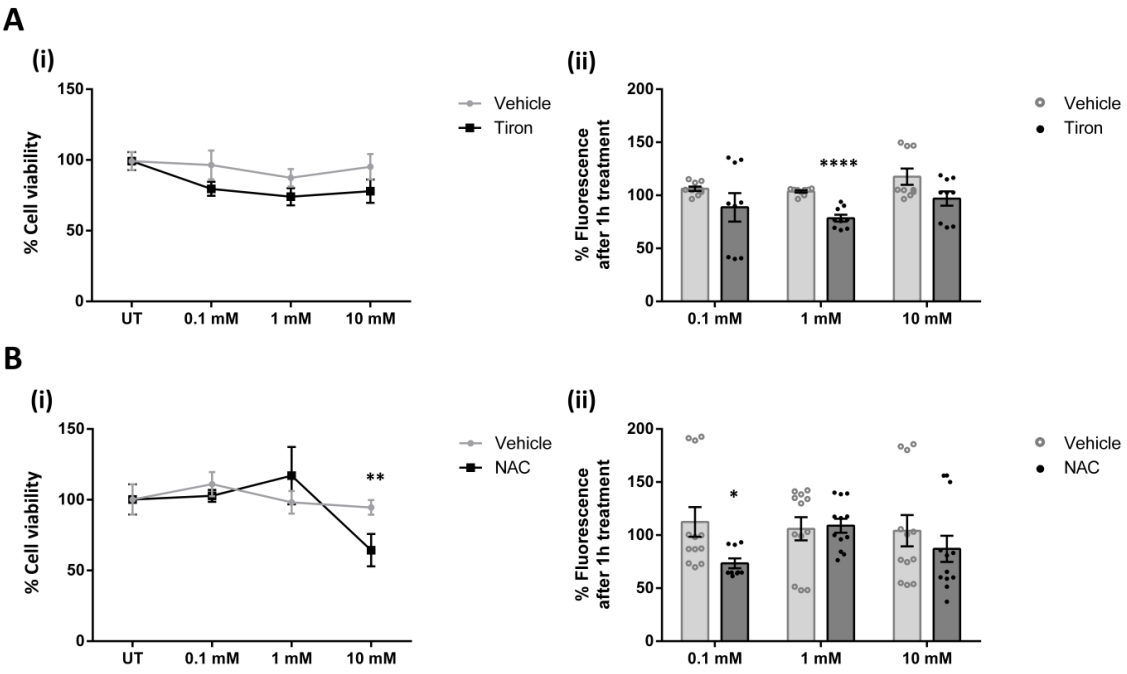


Figure 3.3.4. Tiron and NAC decrease ROS formation in 661W cells. Dose-response curve measuring cell viability **(i)** and DCFDA fluorescence **(ii)** of 661W cells treated with increasing concentrations of antioxidants, Tiron **(A)** and NAC **(B)** (*t*-test comparing individual treatments to their vehicle control) for 1 h in completed media. FACS analysis: *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the x-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed (*t*-test comparing individual treatments to their timed vehicle control). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$**, $P < 0.01$, **** $P < 0.0001$).

Cells pre-treated with vehicle and maintained in serum-free media for 30 min displayed a decrease in cellular viability compared to untreated cells (one-way ANOVA, serum starvation effect, $F_{3,88} = 30.63$, $P = 0.0008$; Tukey's HDS *post hoc* test, $P < 0.0001$; $n = 4$ per group; Figure 3.3.5B). Pre-treatment with Tiron or NAC resulted in a further decrease in cell viability (Tukey's HDS *post hoc* test, Tiron: $P < 0.0001$; NAC: $P < 0.0001$). These data suggest that the presence of ROS is essential for cells to survive normally, given that cells treated with Tiron or NAC showed even more cell death than cells treated with vehicle control only (Tukey's HDS *post hoc* test, Tiron: $P = 0.004$; NAC: $P = 0.0411$), corroborating previous studies (Mackey *et al.*, 2008; Groeger, *et al.*, 2009a, 2009b; Farrell *et al.*, 2011).

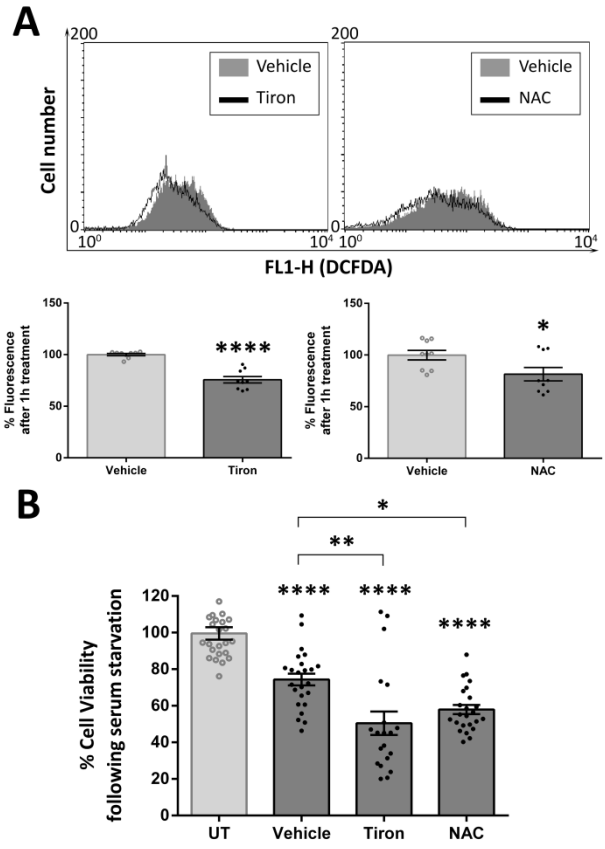


Figure 3.3.5. Serum deprivation induces a pro-survival production of ROS in 661W cells. (A) Antioxidants Tiron and NAC decreased the ROS production in 661W cells (*t*-test comparing individual treatments to their vehicle control). *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. **(B)** Changes in cell viability were measured by the MTS assay and graphed as a percentage of 100% viable UT control (Tukey's HDS *post hoc* test for multiple comparisons). SS (vehicle) decreased cell viability at 30 min. Stressed cells pre-treated with antioxidants showed a further decrease in survival compared with the vehicle. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

3.3.2. Norgestrel induces a pro-survival production of ROS in serum-deprived 661W cells

Previous studies have documented the protective effects of progesterone in the retina (Allen *et al.*, 2015; Sánchez-Vallejo *et al.*, 2015), including the progesterone analogue Norgestrel in the stressed 661W photoreceptor-like cell line (Doonan *et al.*, 2009, 2011; Wyse Jackson & Cotter, 2016). However, ROS implication in this response has not been studied yet. Here, we sought to understand the role that ROS play in the Norgestrel-mediated protection. By means of flow cytometry analyses, Norgestrel significantly increased ROS levels at 30 min following serum-starvation compared to vehicle control (independent samples *t*-test, $t_{18} = 3.277$, $P = 0.0042$; n = 3-4 per group; Figure 3.3.6A). The use of DMSO as vehicle also increased ROS levels at all time points compared with the UT cells (Figure 3.3.6A), possibly through its anti-inflammatory effects as previously described (Elisia *et al.*, 2016). For this reason, these serum starved samples are not comparable with samples in figure 3.3.3.

Norgestrel has been demonstrated to protect 661W cells against the cell death produced by 24 h following the stress caused by nitric oxide donor sodium nitroprusside (SNP) or SS (Doonan *et al.*, 2011; Wyse Jackson & Cotter, 2016). In this study, MTS assay was used to analyse cellular viability and proliferation with Norgestrel over time. Norgestrel enhanced cell survival at all time points studied (independent samples *t*-test, 30 min: $t_{34} = 3.503$, $P = 0.0015$; 1 h: $t_{34} = 3.805$, $P = 0.0006$; 3 h: $t_{34} = 4.182$, $P = 0.0002$; 6 h: $t_{34} = 2.644$, $P = 0.0105$; n = 3-4 per group; Figure 3.3.6B), suggesting that ROS production is linked to an increase in cell survival.

In order to test this hypothesis, the antioxidants Tiron and NAC were again used to block the increase in ROS 1 h prior to Norgestrel treatment. Cells pre-treated with vehicle (H_2O) were

protected by Norgestrel following 30 min of serum deprivation (independent samples *t*-test, $t_{33} = 3.002$, $P = 0.0051$; $n = 3$ per group; Figure 3.3.6C). However, Norgestrel was no longer protective in the presence of the antioxidants compared with the vehicle only (Tiron: $t_{40} = 3.916$, $P = 0.0003$; NAC: $t_{41} = 6.204$, $P < 0.0001$; $n = 4$ per group). Thus, the protective effects of Norgestrel in serum-deprived 661W cells are dependent on ROS production.

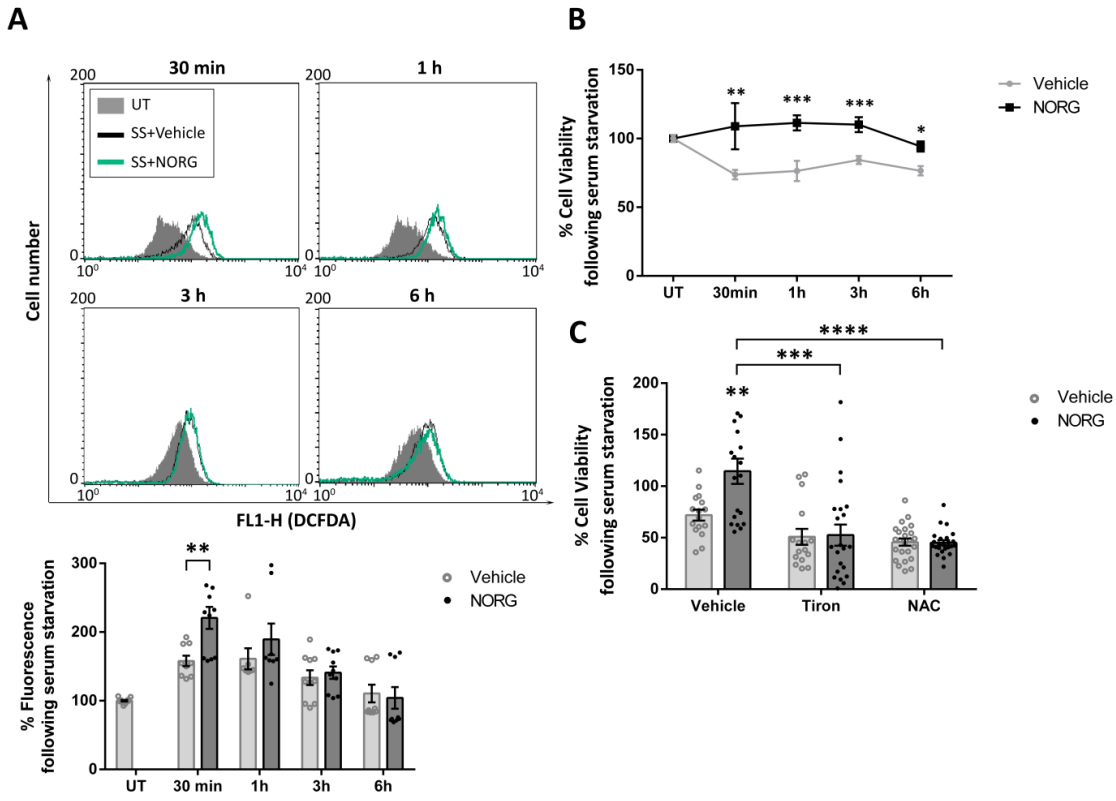


Figure 3.3.6. Norgestrel induces a pro-survival production of ROS in serum-deprived 661W cells. (A) FACS analysis of 661W cells treated with 20 µM Norgestrel or vehicle control (DMSO) following SS. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the x-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual Norgestrel treatments to their timed vehicle control). ROS production was increased by Norgestrel at 30 min. **(B)** MTS assay showed Norgestrel enhanced cell survival in all time points studied compared with the vehicle control (*t*-test comparing individual Norgestrel treatments to their timed vehicle control). **(C)** Antioxidants were used to reduce ROS production following serum starvation. Norgestrel was able to rescue cells pre-treated with the vehicle (H_2O), whereas Norgestrel was no longer protective in the presence of antioxidans (*t*-test comparing individual treatments). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

3.3.3. Norgestrel accelerates the early survival response in stressed 661W cells

The early ROS-mediated response which takes place in the first minutes after serum deprivation has been documented to induce cell survival (Groeger et al., 2009a; Mackey et al., 2008). Furthermore, according with some studies, the exogenous addition of hydrogen peroxide (H_2O_2) can activate the transcription factor nuclear factor kappa B (NF-κB) (Schreck *et al.*, 1991; Kaul & Forman, 1996; Forman, 2007) and prolongs the activation of the survival Akt pathway, delays the activation of the cell death cascade and therefore, enhances survival of 661W cells (Mackey *et al.*, 2008). In this context, Norgestrel-mediated neuroprotection in 661W cells also involves the increase in the levels of pro-survival ROS as seen in figure 3.3.6. Thus, to examine the effects of Norgestrel on this early pro-survival burst of ROS compared with the response produced by the serum deprivation itself, cells were serum-starved and treated with either Norgestrel or vehicle (DMSO) for shorter time points of 1-20 min. Norgestrel treatment increased ROS levels from 5 min (independent samples *t*-test, $t\ 16 = 7.748$, $P < 0.0001$; $n = 3$ per group; Figure 3.3.7A) compared to serum starved control (vehicle).

The study of the whole response over time showed that increased levels of ROS were maintained up to 30 min following Norgestrel treatment (10 min: $t\ 16 = 2.172$, $P = 0.0463$; 20 min: $t\ 16 = 3.665$, $P = 0.0021$; 30 min: $t\ 18 = 3.277$, $P = 0.0042$; $n = 3$ per group; Figure 3.3.7B) but no difference in response was detected beyond this point. These data show that Norgestrel enhances the early pro-survival response of increased ROS levels in serum-starved 661W cells in a quicker manner compared with the serum-starved control, accelerating the pro-survival signalling to enhance cell survival.

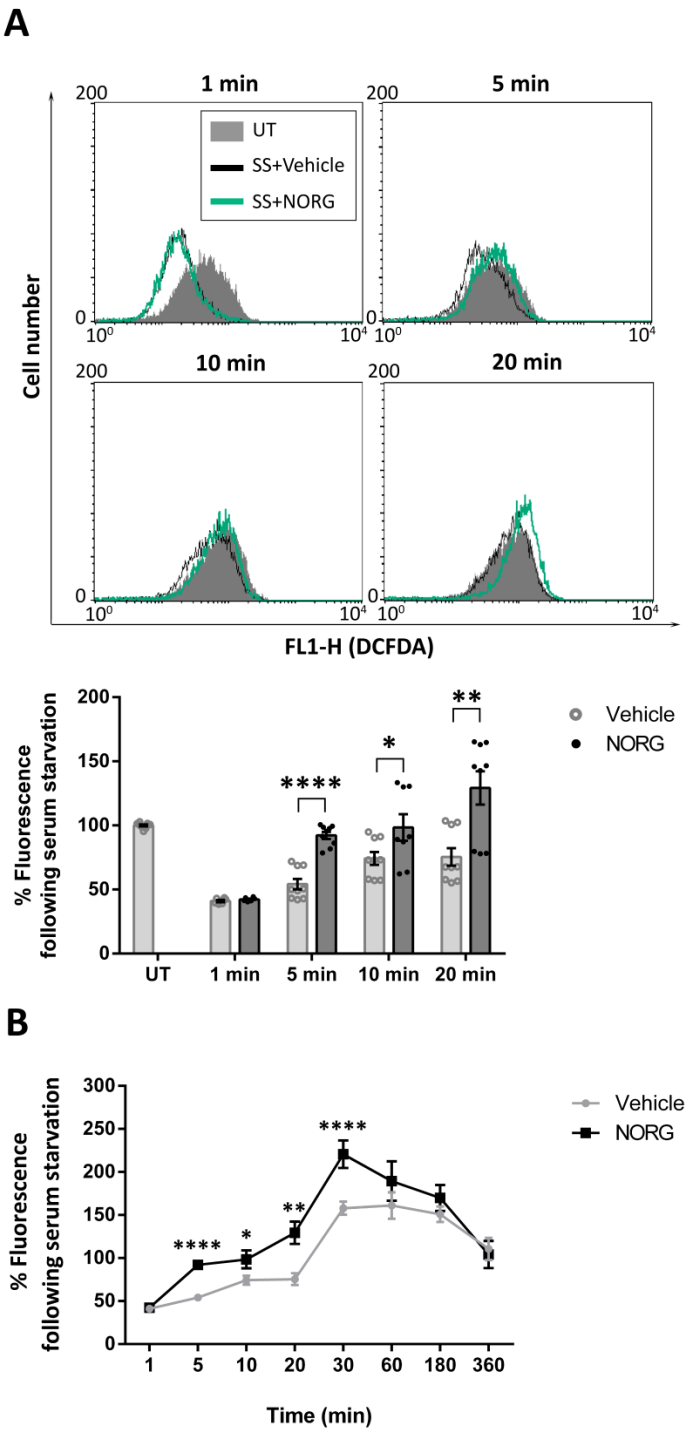


Figure 3.3.7. Norgestrel accelerates the early survival response in stressed 661W cells. (A) Various time points up to 30 min were analysed following Norgestrel treatment by flow cytometry and plotted on a histogram. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual Norgestrel treatments to their timed vehicle control). Norgestrel induced a significantly higher response compared with vehicle from 5 min. **(B)** Timeline of ROS levels in response to Norgestrel in stressed 661W cells. Norgestrel stimulated increased ROS production from 5 min and up to 30 min when levels reverted to normal. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

3.3.4. Norgestrel signals through PGRMC1 to mediate redox signalling

Previous studies have shown that PGRMC1, a membrane-localised progesterone receptor, is responsible for the neuroprotective actions of progesterone and Norgestrel (Cahill, 2007; Wyse Jackson *et al.*, 2016a). Therefore, FACS analysis was performed in order to investigate its role in ROS production. The specific inhibitor AG-205 was used to block PGRMC1 in stressed 661W cells in a concentration that does not affect cellular viability (Wyse Jackson *et al.*, 2016b). The burst of ROS produced by Norgestrel (independent samples *t*-test, $t_{16} = 4.096$, $P = 0.0008$; $n = 3$ per group; Fig. 3.3.8) was abrogated when PGRMC1 was inhibited as detected by decreased DCFDA fluorescence intensity ($t_{16} = 0.9794$, $P = 0.342$; $n = 3$ per group), suggesting the importance of the receptor in this response.

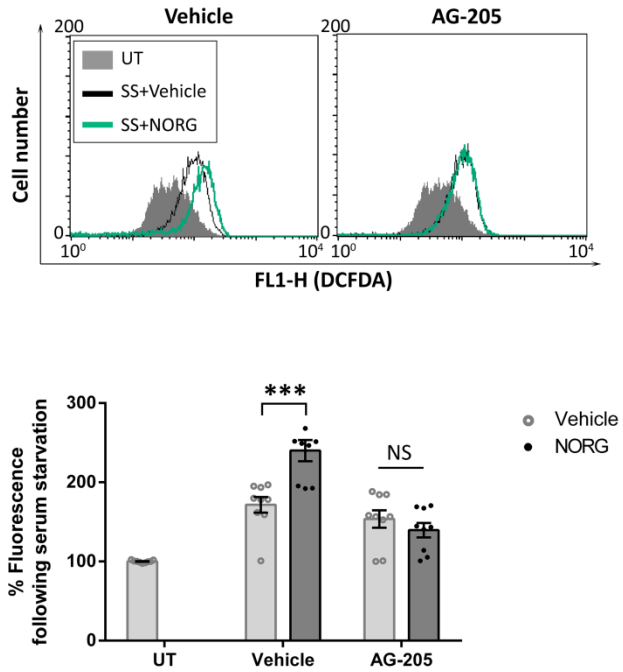


Figure 3.3.8. Norgestrel signals through progesterone receptor membrane component 1 (PGRMC1) to mediate redox signalling. 661W cells were treated with the PGRMC1 specific inhibitor AG-205 prior to serum starvation for 30 min and Norgestrel treatment. DMSO was used as vehicle control. DCFDA probe was used to detect ROS levels by flow cytometry and plotted on a histogram. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual treatments). PGRMC1 inhibition abrogated the burst of ROS produced by Norgestrel. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant, *** $P < 0.001$).

3.3.5. Inhibition of bFGF abrogates the Norgestrel-induced increase in ROS in serum-starved 661W cells

We have previously shown that Norgestrel elicits its neuroprotective effects in 661W cells at least in part by up-regulating the growth factor, basic fibroblast growth factor (bFGF) (Doonan *et al.*, 2011; Wyse Jackson & Cotter, 2016). In other studies, addition of bFGF led to increased ROS production in the photoreceptors of retinal explants (O'Driscoll *et al.*, 2007, 2008; Farrell *et al.*, 2011). We therefore sought to establish if Norgestrel-induced increase in ROS was dependent on bFGF activity. Firstly, we show that serum starvation alone in 661W cells does not up-regulate bFGF expression (one-way ANOVA, serum starvation effect, $F_{4,51} = 1.924$, $P = 0.1206$; $n = 3$-4 per group; Figure 3.3.9(i)). However, when treated with Norgestrel, 661W cells displayed increased expression of bFGF following 30 min of SS (independent samples *t*-test, $t_{21} = 2.844$, $P = 0.0097$; $n = 4$ per group; Figure 3.3.9(ii)).

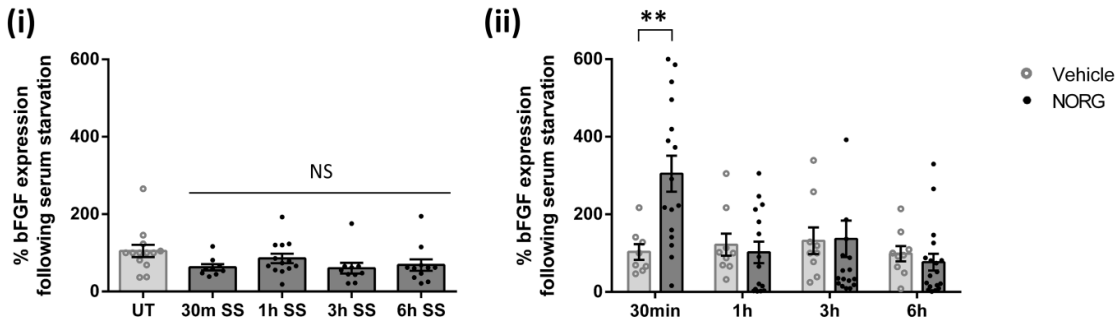


Figure 3.3.9. Norgestrel up-regulates bFGF expression in serum-starved 661W cells. bFGF mRNA levels were measured in 661W following SS (**(i)**, Dunnett's post hoc test, comparing all time points to the UT control) and treated with Norgestrel over time by RT-PCR (**(ii)**, *t*-test comparing individual Norgestrel treatments to their timed vehicle control). bFGF up-regulation only took place in the presence of Norgestrel. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant, ** $P < 0.01$).

To understand whether bFGF is involved in the redox response produced by Norgestrel at 30 min, siRNA was used to knockdown bFGF over 48 h. A significant reduction in bFGF expression was verified through RT-qPCR (one-way ANOVA, time of incubation siRNA, *F*

$_{2,15}$ = 65.95, $P < 0.0001$; Dunnett's *post hoc* test, 24 h: $P = 0.0002$; 48 h: $P < 0.0001$; $n = 2$ per group; Figure 3.3.10A) and by immunofluorescence (Figure 3.3.10B).

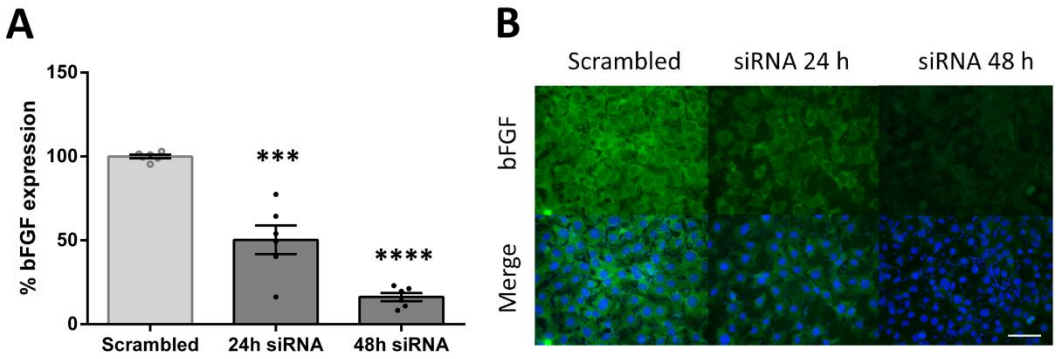


Figure 3.3.10. Successful siRNA knockdown of bFGF. bFGF levels detected by RT-PCR **(A)** and immunofluorescence **(B)** in 661W cells transfected with siRNA targeted against bFGF. Scale bar 30 µm. Hoechst staining reveals cell nuclei. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (*** $P < 0.001$, **** $P < 0.0001$).

siRNA-treated cells were then serum-starved and treated with either DMSO (vehicle) or 20 µM Norgestrel for 30 min before ROS were measured using DCFDA. Norgestrel was unable to increase ROS when bFGF was knocked down, suggesting that this response is bFGF-dependent (independent samples *t*-test, $t_{37} = 0.2197$, $P = 0.8277$; $n = 5$ per group; Figure 3.3.11).

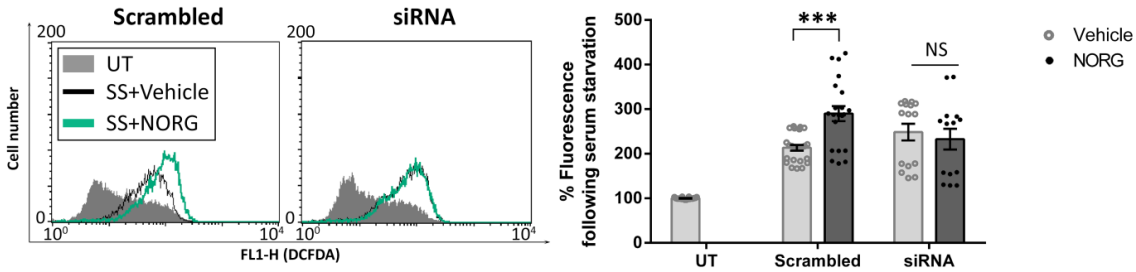


Figure 3.3.11. Inhibition of bFGF abrogates Norgestrel-induced increases in ROS in serum-starved 661W cells. FACS analysis of cells transfected with bFGF siRNA treated with DMSO or Norgestrel. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual treatments). bFGF knockdown prevented the increase of ROS produced by Norgestrel (scrambled vs siRNA). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant, *** $P < 0.001$).

3.3.6. Norgestrel induces an increase in ROS production in photoreceptors *ex vivo*

Our understanding of RP and the development of therapeutic strategies have improved due to the use of several animal models of this disease. One of such models is the rd10 mouse, which is a good approach for studying autosomal recessive RP (Barhoum *et al.*, 2008; Rivas & Vecino, 2009; Arroba *et al.*, 2011; Roche *et al.*, 2016; Campochiaro & Mir, 2017). Results from this study thus far demonstrate that Norgestrel, through bFGF, utilises ROS signalling to promote neuronal survival of the 661W cell line. Therefore, we next sought to evaluate the contribution of ROS to Norgestrel-mediated protection of photoreceptors in the retina. Using retinas from rd10 mice we prepared single-cell suspensions as previously described, to be used for FACS analysis (Byrne *et al.*, 2016b). In order to confirm the photoreceptor population, we compared a P30 C57 wild-type retinal preparation to that of a P30 rd10. The rd10 retina has undergone extensive photoreceptor loss by P30 (Roche *et al.*, 2016). Thus, since the C57 wild-type retina presented a population of cells that were absent from the rd10 retina (Figure 3.3.12), we understood this population to represent photoreceptors. This method of gating rd10 photoreceptors has previously been published (Byrne *et al.*, 2016b).

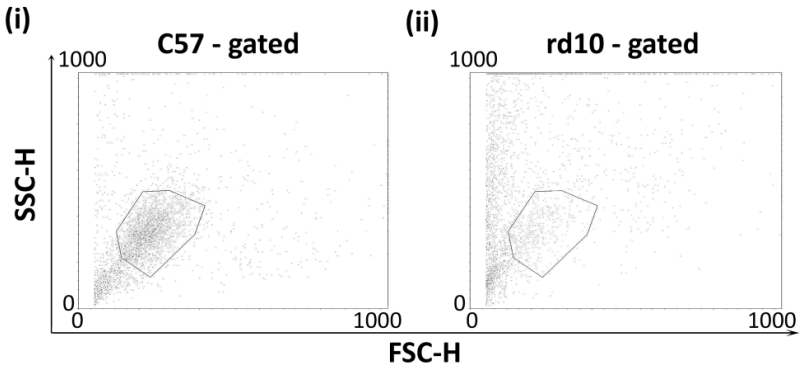


Figure 3.3.12. Gated population used in the experiments performed by FACS. Photoreceptor population was gated using P30 C57BL/6 wild type **(i)** and rd10 retinas **(ii)**, as this population was absent from rd10 retinas.

We subsequently evaluated the effects of Norgestrel on single-cell suspensions from P15 rd10 retinas, a suitable time point as photoreceptors have initiated cell death mechanisms but photoreceptor loss is not yet apparent (Roche *et al.*, 2016; Roche & Wyse-Jackson *et al.*, 2016). Retinal explants were treated with either 20 µm Norgestrel or DMSO (vehicle) over 3 hours and analysed for changes in photoreceptor ROS production. The population of photoreceptors from rd10 retinas treated with Norgestrel showed an increase in ROS production compared to those treated with vehicle control, at 30 min and 1 h (independent samples *t*-test, 30 min: $t_{22} = 2.486$, $P = 0.021$; 1 h: $t_{22} = 3.723$, $P = 0.0013$; $n = 4$ per group; Figure 3.3.13).

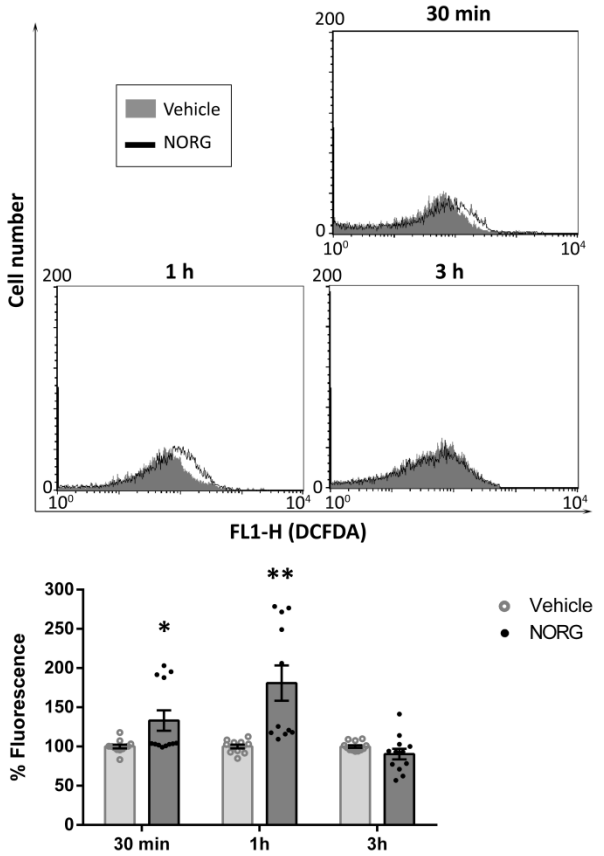


Figure 3.3.13. Norgestrel increases ROS production in photoreceptors *ex vivo*. FACS analysis of photoreceptors using single cell suspensions from P15 rd10 retinas. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed and individual Norgestrel treatments were compared to their timed DMSO vehicle control (*t*-test comparing individual treatments). Norgestrel up-regulated ROS levels at 30 min and up to 1 h. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$).

Retinal explants have been used in the past to study Norgestrel effects on cell death over 24 h assessed by TUNEL assay (Doonan *et al.*, 2011; Roche *et al.*, 2017a, 2017b; Wyse Jackson & Cotter, 2016; Wyse Jackson *et al.*, 2016a). However, shorter time points to elucidate Norgestrel effects have not been utilised yet. Interestingly, Norgestrel-mediated reduction in photoreceptor cell death was only evident at 1 h, as assessed by the TUNEL assay (independent samples *t*-test, independent samples *t*-test, 30 min: $t_{20} = 0.1706$, $P = 0.8663$; 1 h: $t_{18} = 3.211$, $P = 0.0068$; $n = 3$ per group; Figure 3.3.14). Therefore, we observed an increase in ROS prior to the Norgestrel-mediated protection of photoreceptors.

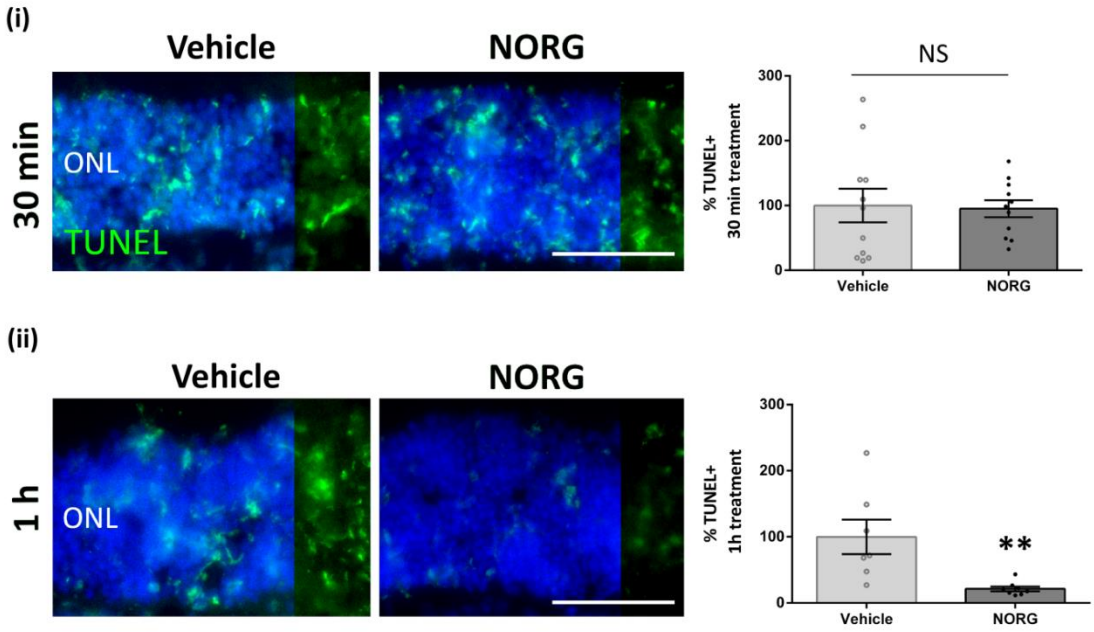


Figure 3.3.14. Norgestrel reduces photoreceptor cell death in retinal explants. Apoptosis of retinal cells in the outer nuclear layer (ONL) was detected by TUNEL (green) at 30 min **(i)** and at 1 h **(ii)**. Norgestrel reduced TUNEL-positive staining in the central rd10 retina after 1 h treatment, but not at 30 min compared with the vehicle control (*t*-test comparing individual treatments). Scale bar 50 µm. Hoechst staining reveals cell nuclei. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant, ** $P < 0.01$).

In order to determine if this increase in photoreceptor ROS is necessary for Norgestrel-mediated protection in rd10 explants, we utilised the antioxidant Tiron. C57 explants pre-treated with 1mM Tiron showed a significant decrease in their basal levels of ROS compared to H_2O vehicle control, as indicated by flow cytometry. This suggests that 1 mM Tiron is a

suitable method by which to abrogate ROS production in retinal explants (independent samples *t*-test $t_{16} = 3.337$, $P = 0.0042$; $n = 3$ per group; Figure 3.3.15A). P15 rd10 explants were consequently pre-treated for 1 h with 1 mM Tiron before 1 h treatment with Norgestrel or DMSO (vehicle). TUNEL assay revealed that Norgestrel could no longer protect rd10 explants from photoreceptor cell death over 1 h, when ROS production was inhibited through Tiron antioxidant (independent samples *t*-test $t_{29} = 0.06121$, $P = 0.9516$; $n = 4$ per group; Figure 3.3.15B). This supports our findings in 661Ws that Norgestrel initiates a pro-survival burst of ROS, assisting in the survival of retinal photoreceptor cells.

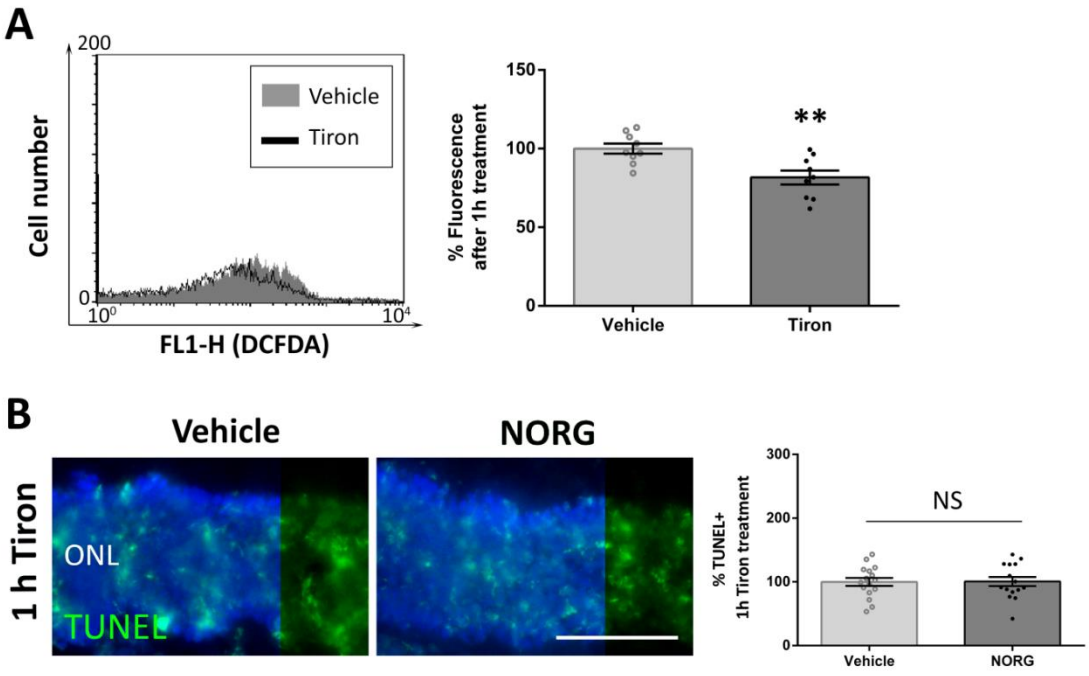


Figure 3.3.15. Norgestrel induces a pro-survival production of ROS in photoreceptors *ex vivo*. **(A)** FACS analysis of photoreceptors from C57BL/6 retinas. Treatment with antioxidant Tiron for 1 h reduced significantly ROS levels. **(B)** TUNEL assay in ONL of P15 rd10 retinas. Norgestrel was not able to reduce TUNEL-positive staining after 1 h treatment in the presence of Tiron. Scale bar 50 µm. Hoechst staining reveals cell nuclei. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant, ** $P < 0.01$).

3.4. Discussion

Studies investigating the role of reactive oxygen species in cell survival have uncovered contrasting findings (Burdon, 1995; Rhee, 2006; Trachootham *et al.*, 2008; Gough & Cotter, 2011; Ray *et al.*, 2012; Schieber & Chandel, 2014; Leveillard & Sahel, 2016; Moloney & Cotter, 2017). These molecules are thought to be in part responsible for the death of cones after the loss of rods in RP (Shen *et al.*, 2005; Komeima *et al.*, 2006, 2008; Barhoum *et al.*, 2008; Usui *et al.*, 2009; Kim *et al.*, 2015; Campochiaro & Mir, 2017). On the other hand, their role as signalling molecules to enhance cell survival has also been well studied during the last decades (Remacle *et al.*, 1995; Abid *et al.*, 2000; Lee, Yu, *et al.*, 2003; Rhee, 2006; D'Autréaux & Toledano, 2007; Trachootham *et al.*, 2008; Bae *et al.*, 2011; Finkel, 2011; Gough & Cotter, 2011; Ray *et al.*, 2012; Marinho *et al.*, 2014; Schieber & Chandel, 2014; Brandes *et al.*, 2014b; Tao, Geng, *et al.*, 2016; Battistelli *et al.*, 2016; Brand, 2016; Harel *et al.*, 2017). In the retina, our group has previously documented both protective and detrimental properties of ROS signalling (Carmody *et al.*, 1999; Sanvicens *et al.*, 2004; Sanvicens & Cotter, 2006; Carmody & Cotter, 2007; Mackey *et al.*, 2008; O'Driscoll *et al.*, 2008; Groeger *et al.*, 2009a; Bhatt *et al.*, 2010; Farrell *et al.*, 2011; O 'Driscoll *et al.*, 2011; Groeger *et al.*, 2012; Byrne *et al.*, 2016b).

Under normal conditions, cells maintain a delicate balance between ROS production and elimination through many mechanisms. Thus, any alteration that disrupts this redox homeostasis can cause an increase of intracellular ROS and thus, oxidative stress. In this way, ROS become cytotoxic under specific conditions. For instance if an increased production occurs at the wrong time or place, if the signal is too strong or if it persists for long periods of time (Nathan & Cunningham-Bussel, 2013; Trachootham *et al.*, 2008). Several therapeutic strategies of the retina have utilised this system: increasing antioxidant machinery in order to reduce oxidative stress and increase survival (Komeima *et al.*, 2006,

2007; Lu *et al.*, 2009; Taherian *et al.*, 2014). Komeima *et al.* proved that injections in rd1 and rd10 mice, both models of RP, with a cocktail of antioxidant substances between P18 and P35, significantly reduced oxidative damage markers which resulted in an increase in a higher cone density and improved their functionality in the retina (Komeima *et al.*, 2006, 2007). Indeed, studies from our group establish that Norgestrel can reduce the damaging ROS levels produced after 24-48 h light exposure. This enhances neuroprotection in the balb/c induced light damage mouse model of retinal degeneration, and involves the up-regulation of the antioxidant transcription factor NF-E2-related factor-2 (Nrf2) (Byrne *et al.*, 2016b). Norgestrel, therefore, is able to act as an antioxidant when ROS levels become toxic to the retina after the damage produced by a long exposure to light.

Nevertheless, to state that these molecules are always damaging would be an oversimplification, for ROS can have opposing effects under different physiological conditions. In fact, under normal physiological conditions, moderate levels of ROS participate in many biological processes as second messengers or intermediates (Holmström & Finkel, 2014; Remacle *et al.*, 1995; Rhee, 2006). For this reason, the implication of ROS in survival responses has gained attention. Previous studies from our laboratory have shown that hydrogen peroxide acts as a second messenger in intracellular signalling pathways inducing cell survival in 661W cells and C57BL/6 retinal explants (Groeger *et al.*, 2009a; 2009b; Mackey *et al.*, 2008). The current study also reveals how these molecules have a survival nature in the first 30 min of 661W serum deprivation (Figure 3.3.3). In light of previous and current findings, it therefore appears that ROS signalling can play a protective or detrimental role depending on the intensity, cellular location and period of the event.

In 2009, the synthetic progesterone Norgestrel was found by this group to possess neuroprotective effects due to a study in the cone photoreceptor 661W cell line (Doonan *et*

al., 2009, 2011). Since then, Norgestrel-mediated neuroprotection has been demonstrated in different neuronal/retinal degeneration models (Doonan *et al.*, 2011; Byrne *et al.*, 2016a, 2016b; Roche & Wyse-Jackson *et al.*, 2016; Wyse Jackson & Cotter, 2016; Wyse Jackson *et al.*, 2016a, 2016b; Roche *et al.*, 2017a, 2017b). In those studies, Norgestrel has been involved in many responses in order to enhance cell survival. Such responses mainly consist of the up-regulation of some neurotrophic factors (Doonan *et al.*, 2011; Byrne *et al.*, 2016a; Wyse Jackson & Cotter, 2016), the attenuation of inflammation and gliosis processes produced by RP in the rd10 retina (Roche & Wyse-Jackson *et al.*, 2016; Roche *et al.*, 2017a, 2017b) and the reduction of harmful ROS levels in a light damage model (Byrne *et al.*, 2016b).

However, its mechanism of action at earlier time points is not well understood yet. The importance of the current study, therefore, was to comprehend the role that ROS play in an earlier response following Norgestrel treatment. Norgestrel, acting through the progesterone receptor PGRMC1 (Figure 3.3.8) (Wyse Jackson *et al.*, 2016a), increases some neurotrophic factors expression such as bFGF (Figure 3.3.11) (Wyse Jackson & Cotter, 2016) and the leukemia inhibitory factor (LIF) (Byrne *et al.*, 2016a), resulting in an early up-regulation of ROS levels in 661W cells (Figure 3.3.6 and 3.3.7). In fact, the relationship between progesterone-mediated neuroprotection and the stimulation of growth factors production is not surprising. Progesterone has been stablished to be protective due to the up-regulation of growth factors such as bFGF or the brain-derived neurotrophic factor (BDNF) in multiple neurodegenerative models (Campochiaro *et al.*, 1996; Peluso & Pappalardo, 1999; Gonzalez Deniselle *et al.*, 2003; Peluso, 2003; Gonzalez *et al.*, 2005; Yu *et al.*, 2010; Espinosa-García *et al.*, 2014; Qin *et al.*, 2015).

Decreasing ROS production using two different antioxidants (Tiron and NAC) revealed the survival nature of these molecules (Figure 3.3.5 and 3.3.6). Tiron, a vitamin E analog which acts as a superoxide scavenger, and NAC, being a precursor of the reduced glutathione, have been previously used as antioxidant compounds (Curtin *et al.*, 2002; Groeger, Mackey, *et al.*, 2009; Taherian *et al.*, 2014). This result highlights the importance of moderate levels of ROS as intermediate molecules in the Norgestrel-mediated signalling. Future studies will investigate the downstream pathways stimulated by ROS up-regulation, leading to enhanced survival.

Results from the 661W photoreceptor-like cell line so far (Figure 3.3.1-3.3.11) suggested that Norgestrel's neuroprotective role in the retina involved an up-regulation of pro-survival ROS. In order to substantiate this claim, we next employed the rd10 mouse model of retinitis pigmentosa. The rd10 mouse model represents a very useful tool when it comes to study retinal neurodegeneration, allowing for development of potential therapeutics (Pittler & Baehr, 1991; Barhoum *et al.*, 2008; Samardzija *et al.*, 2012; Han *et al.*, 2013; Cuenca *et al.*, 2014; Roche *et al.*, 2016). Through the use of rd10 mice, our group has discovered the mechanisms behind the Norgestrel-mediated neuroprotection *ex vivo* and *in vivo* (Doonan *et al.*, 2011; Roche & Wyse-Jackson *et al.*, 2016; Roche *et al.*, 2017a, 2017b). Roche & Wyse-Jackson *et al.* showed that mice who were given a Norgestrel-supplemented diet showed significant retinal protection compared with the control diet. This was in part achieved by the anti-inflammatory properties of Norgestrel, which attenuates microglial activity in the diseased retina (Roche & Wyse-Jackson *et al.*, 2016) and reduces the gliosis produced by the crosstalk between microglia and Müller glial cells (Roche *et al.*, 2017b). These studies demonstrate the effectiveness of Norgestrel and its capacity to produce a wide response within the retina to enhance neuroprotection. However, there is little information on the role of ROS in the Norgestrel-mediated response in the rd10 retina.

Using single cell suspensions of rd10 retinal explants for FACS analysis, we investigated the response of photoreceptors to Norgestrel, in terms of ROS levels. Given that the aim of the study was to analyse the response in the photoreceptor layer, we harvested retinas at P15, prior to the loss of photoreceptors which takes place from P18~P21 in the rd10 retina (Barhoum *et al.*, 2008; Samardzija *et al.*, 2012; Roche *et al.*, 2016). The time course of the retinal degeneration is slower in the rd10 model compared with others, such as the rd1, in which rods begin to die around P8~P10 (Chang *et al.*, 2007; Barhoum *et al.*, 2008). Norgestrel produced a similar early increase in ROS levels from 30 min in photoreceptors of the rd10 retina (Figure 3.3.13). We hypothesise that this peak in the production of ROS might be in some part responsible for the proceeding decrease in TUNEL-positive staining observed at 1 h following Norgestrel treatment, in light of our findings in 661W cells and rd10 explants pre-treated with antioxidants (Figure 3.3.14 and 3.3.15). In support of this, ROS have been implicated in survival mechanisms in the retina (Groeger *et al.*, 2009a, 2012) and in other systems (Mackenzie *et al.*, 2013; Shafique *et al.*, 2013; Trachootham *et al.*, 2008).

This study presents an important aspect to Norgestrel's neuroprotective properties, up-regulating ROS production as an immediate response, through the activation of PGRMC1 and increased production of bFGF (Figure 3.4.1). This early burst of ROS is an essential step, as Norgestrel's neuroprotective effects are prevented in the presence of antioxidants. Although this early burst of ROS is short-lived, the protective effects of Norgestrel persist for several weeks in a mouse model of RP (Roche & Wyse-Jackson *et al.*, 2016), with Norgestrel affecting various cellular and molecular targets throughout the course of degeneration (Byrne *et al.*, 2016a, 2016b; Roche & Wyse Jackson *et al.*, 2016; Wyse Jackson & Cotter, 2016; Roche *et al.*, 2017a, 2017b). The current study therefore reinforces the prospect of Norgestrel as a promising therapeutic for retinal degeneration, utilising ROS signalling in the early stages of injury to promote survival.

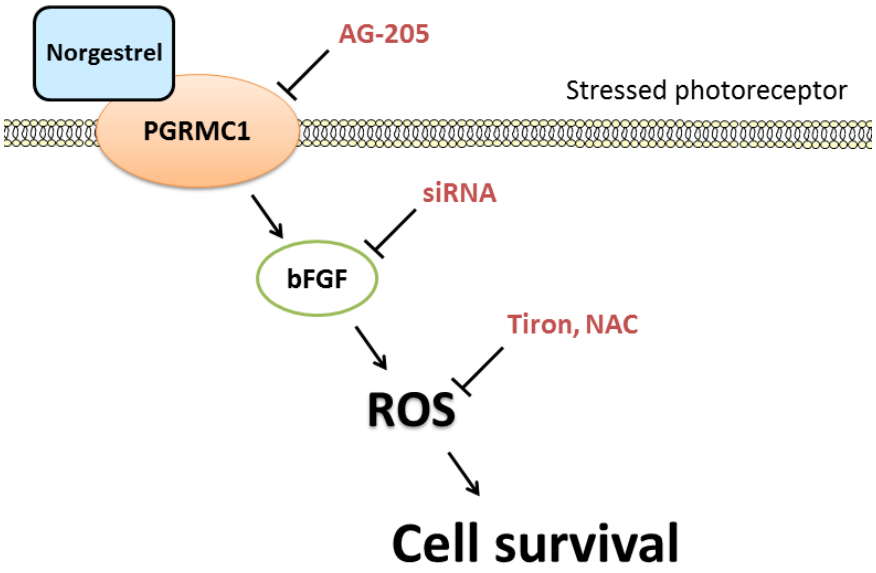


Figure 3.4.1. Schematic representation of Norgestrel-mediated pro-survival redox signalling. This shows the proposed pathway Norgestrel uses to promote neuroprotection in serum-starved 661W cells, including the compounds used to inhibit each step.

Chapter 4: Progesterone analogue ‘Norgestrel’ stimulates NOX4 to induce ROS-mediated protection in stressed photoreceptor cells

4.1. Abstract

Reactive oxygen species (ROS) have been related not only to cellular damage, but also to survival responses in recent years. Our laboratory has demonstrated that these molecules participate in protecting both the 661W photoreceptor-like cell line and the rd10 retina, a model of retinitis pigmentosa (RP), following the treatment with the progesterone analogue, Norgestrel. We already know this compound involves a great variety of responses to induce neuroprotection in the diseased retina. One of such effects is that Norgestrel uses the neurotrophic factor basic fibroblast growth factor (bFGF) to stimulate intracellular ROS production in early stages under stress conditions. However, the mechanism by which ROS levels are increased remains unclear. This study is therefore focused on delineating the source of the Norgestrel-mediated redox signalling. Through the use of specific inhibitors in the photoreceptor-derived 661W cell line, we confirm that this increase in pro-survival ROS levels stimulated as a consequence of the treatment with Norgestrel, is produced by NADPH oxidases (NOX), specifically NOX4. Furthermore, we also demonstrate that mitochondria and cyclooxygenases (COX), which represent other sources of ROS production within the cell, do not play a role in this signalling pathway. Therefore, this study demonstrates how Norgestrel stimulates NOX4 in order to induce cell survival in stressed 661W cells.

4.2. Introduction

The potential sources of reactive oxygen species (ROS) within the cell are numerous. These molecules can be formed as by-products, as a consequence of biological reactions. One of the most widely recognised sources is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family of proteins, which was the first source identified to have an exclusive role in producing ROS rather than generate them as a by-product (Bedard & Krause, 2007; Finkel, 2011; Brandes *et al.*, 2014a). In fact, these enzymes have been demonstrated to participate in several distinct mechanisms such as cellular proliferation, survival and death (Abid *et al.*, 2000; Clark & Valente, 2004; Bedard & Krause, 2007; Brown & Griendling, 2009; Bhatt *et al.*, 2010; Brandes *et al.*, 2014b; Moloney *et al.*, 2017a, 2017b; Moloney & Cotter, 2017). In support of this, our group has previously highlighted a role of these enzymes in the production of ROS leading to cell survival (Groeger *et al.*, 2009a). The NOX family consists of seven transmembrane members (NOX1-5, DUOX1-2), differing mainly in their organ-specific expression, control of their activity and the type of ROS they release (Brandes *et al.*, 2014a; Moloney & Cotter, 2017). NOX1-4 are known to interact with the small transmembrane protein p22 phagocyte oxidase ($p22^{phox}$) whereas activation of NOX1-3 require the formation of a complex with other cytosolic proteins (Ambasta *et al.*, 2004; Bae *et al.*, 2011; Brandes *et al.*, 2014a; Moloney & Cotter, 2017).

Mitochondria are also a major site of free radical production, given that this organelle generates adenosine triphosphate (ATP) in an oxygen-dependent manner (Harman, 1956; Jarrett *et al.*, 2008; Murphy, 2009; Liochev, 2013; Lefevere *et al.*, 2017). Superoxide anion ($O_2^{\bullet -}$) is the primary ROS produced by mitochondria, which is not diffusible (Andreyev *et al.*, 2005; Liochev, 2013). Thus, it is rapidly converted to hydrogen peroxide (H_2O_2) to cross biological membranes and act in both pro-survival and pro-death cellular responses (Rhee, 2006; Groeger *et al.*, 2009b; Finkel, 2011; Bordt & Polster, 2014; Holmström & Finkel,

2014; Marinho *et al.*, 2014; Dan Dunn *et al.*, 2015; Antunes & Brito, 2017; Moloney & Cotter, 2017; Sies, 2017). These molecules can be generated in at least eleven different mitochondrial sites, mainly at complexes I and III of the electron transport chain (Andreyev *et al.*, 2005; Murphy, 2009; Brand, 2016). In addition to the mitochondria and NADPH oxidases, other sources of ROS production include cyclooxygenases (COX), peroxisomes, lysosomes, cytochrome p450 enzymes in the endoplasmic reticulum (ER), xanthine oxidases, nitric oxide synthases and lipoxygenases (Trachootham *et al.*, 2008; Finkel, 2011; Birben *et al.*, 2012; Holmström & Finkel, 2014). ROS produced by these other sources have been linked mainly with cellular damage (Di Meo *et al.*, 2016).

Over the last decade, we have demonstrated the neuroprotective effects of Norgestrel, a compound commonly used in the female oral contraceptive pill. This has been demonstrated in a variety of models and involves multiple mechanisms (Doonan *et al.*, 2011; Byrne *et al.*, 2016a; 2016b; Roche & Wyse-Jackson *et al.*, 2016; Wyse Jackson & Cotter, 2016; Wyse Jackson *et al.*, 2016a; 2016b; Roche *et al.*, 2017a, 2017b). For example, we have recently discovered the role of ROS in survival responses produced by Norgestrel in both the 661W cell line and the rd10 retina (Chapter 3; Ruiz-Lopez *et al.*, 2017). This was shown by the use of antioxidants to decrease ROS production alongside Norgestrel administration, which prevented Norgestrel from providing neuroprotection. However, the mechanism responsible for this increase in ROS levels has not been elucidated yet. Therefore, the aim of this study is to investigate the possible sources of pro-survival ROS in the Norgestrel-mediated response.

4.3. Results

4.3.1. Inhibition of NOX abrogates the Norgestrel-induced increase in ROS in stressed 661W cells

Previous studies from this group have documented the production of pro-survival ROS following Norgestrel treatment for 30 min under serum-deprived conditions (Chapter 3; Ruiz-Lopez *et al.*, 2017). In the current study we utilised inhibitors to establish the possible source of this increase in endogenous ROS levels. Diphenyleneiodonium (DPI) is commonly used to block NOX activity (Cross & Jones, 1986; Greene *et al.*, 2000; Clark & Valente, 2004; Abid *et al.*, 2007; Shi *et al.*, 2015; Demelash *et al.*, 2017; Prieto-Bermejo & Hernández-Hernández, 2017). However, its specificity has been widely debated over the years. It is thought to be a nonspecific inhibitor of flavoenzymes such as NOX proteins (Maghzal *et al.*, 2012) due to its effects on mitochondrial ROS production (Majanders *et al.*, 1994; Li & Trush, 1998; Murphy, 2009; Sena & Chandel, 2012; Prieto-Bermejo & Hernández-Hernández, 2017).

In light of this, the effects of DPI on NOX enzymes in this study were corroborated using the specific NOX inhibitor 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-tria-zolo[4,5-d]pyrimidine (VAS2870) (ten Freyhaus *et al.*, 2006; Wingler *et al.*, 2011, 2012; Gatto *et al.*, 2013; Altenhö *et al.*, 2015; Ma *et al.*, 2017; Rastogi *et al.*, 2017). Wingler *et al.* showed that VAS2870 is able to block NOX1 and NOX2 (Wingler *et al.*, 2011). In addition, they demonstrated that NOX inhibition by VAS2870 in wild-type mice produced the same effect that NOX4 delection in NOX4 knockout mice (Wingler *et al.*, 2012), demonstrating its specificity is not relevant for any NOX isoform. In the current study, a range of concentrations was tested for its effects on cellular viability as measured by MTS assay and on ROS production as measured by flow cytometry. 1 µM of DPI caused a decrease in cell

viability compared with the vehicle, whereas VAS2870 did not affect cell viability (independent samples *t*-test, DPI: $t_{22} = 3.324$, $P = 0.0031$; VAS2870: $t_{22} = 0.7210$, $P = 0.4785$; $n = 4$ per group; Figure 4.3.1A) and both inhibitors at this concentration significantly decreased ROS levels (independent samples *t*-test, DPI: $t_{16} = 6.808$, $P < 0.0001$; VAS2870: $t_{16} = 7.006$, $P < 0.0001$, $n = 3$ per group; Figure 4.3.1B).

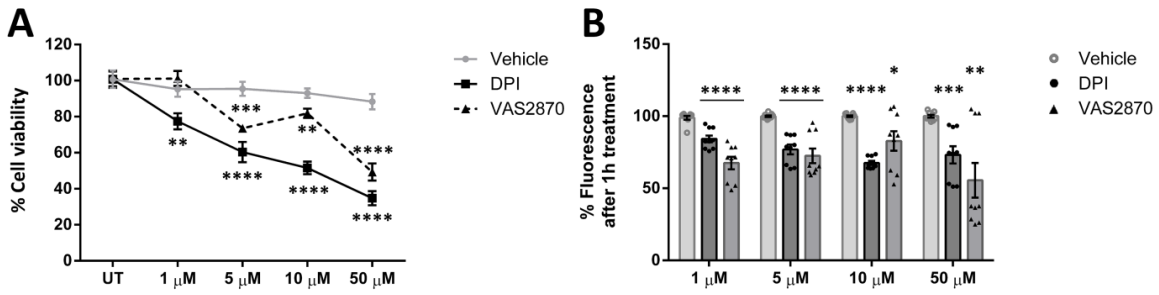


Figure 4.3.1. NOX inhibition produced by DPI and VAS2870 in 661W cells. Dose-response curve measuring cell viability **(A)** and DCFDA fluorescence **(B)** of 661W cells treated with increasing concentrations of inhibitors against NOX proteins, DPI and VAS2870 (*t*-test comparing individual treatments). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Cells were then pre-treated with either DPI or VAS2870 at a concentration 1 μM for 1 h in complete media prior to 20 μM Norgestrel treatment in serum-free media for 30 minutes. The burst of ROS produced by Norgestrel after 30 min (independent samples *t*-test, $t_{15} = 3.006$, $P = 0.0089$; $n = 3$-4 per group; Figure 4.3.2A) was abrogated when NOX proteins were inhibited using either DPI or VAS2870 as detected by decreased DCFDA fluorescence intensity (DPI: $t_{18} = 4.082$, $P = 0.0007$; VAS2870: $t_{15} = 3.626$, $P = 0.0005$; $n = 3$-4 per group). According to these results, although DPI is considered a less specific inhibitor of NOX proteins than VAS2870, both inhibitors had a similar effect, demonstrating that this response is NOX-dependent.

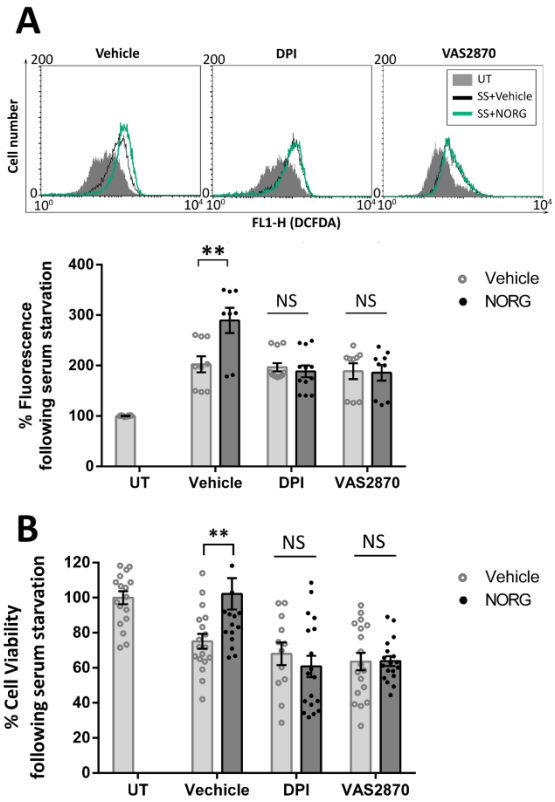


Figure 4.3.2. Pro-survival ROS-induced increase in stressed 661W cells by Norgestrel is NOX-dependent. (A) FACS analysis of 661W cells pre-treated with 1 µM DPI or VAS2870 prior to Norgestrel treatment. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual treatments). Norgestrel-mediated ROS up-regulation only took place in the absence of inhibitors (vehicle control). **(B)** Changes in cell viability were measured by the MTS assay and graphed as a percentage of 100% viable UT control (*t*-test comparing individual treatments). NOX inhibition by DPI and VAS2870 abolish Norgestrel-mediated protection at 30 min following serum starvation. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant, ** $P < 0.01$).

According to previous studies, the increase in ROS produced by Norgestrel has been proved to be pro-survival (Ruiz-Lopez *et al.*, 2017). In order to corroborate this, cell viability was assessed by MTS assay following NOX inhibition. We found that protection enhanced by Norgestrel at 30 min (independent samples *t*-test, $t_{28} = 3.498$, $P = 0.0016$; $n = 3$ per group; Figure 4.3.2B) was abolished in the when NOX were blocked by DPI and VAS2870 (DPI: $t_{28} = 0.7924$, $P = 0.4348$; VAS2870: $t_{28} = 0.04007$, $P = 0.9683$; $n = 3$ per group). This confirms that NOX-induced ROS production is pro-survival as shown before.

Given that some studies also use knockdowns/knockouts to verify the results obtained by the use of inhibitors (Block *et al.*, 2009; Wingler *et al.*, 2012; Dikalov *et al.*, 2014; Demelash *et al.*, 2017), we sought to investigate whether the lack of NOX activity would affect 661W cells. Incubation of these NOX inhibitors, DPI and VAS2870, for 24 h produced a dramatic decrease in cell viability in all concentrations studied (independent samples *t*-test, 1 µM: $t_{22} = 6.014$, $P < 0.0001$; 5 µM: $t_{22} = 7.376$, $P < 0.0001$; 10 µM: $t_{22} = 5.820$, $P < 0.0001$; 50 µM: $t_{22} = 21.62$, $P < 0.0001$; $n = 3$ per group; Figure 4.3.3), indicating other approaches such as the use of knockdowns are unsuitable to block NOX activity in 661W cells.

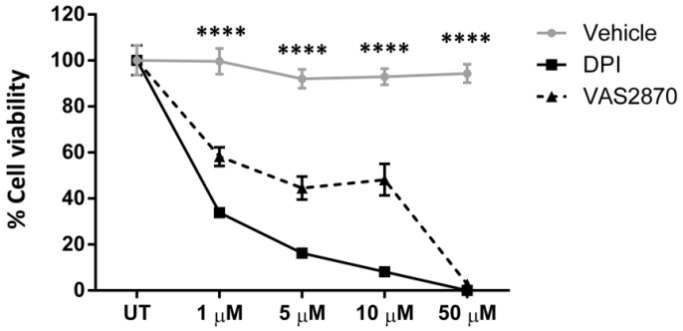


Figure 4.3.3. DPI and VAS2870 decrease cell viability after 24 h treatment in 661W cells. Dose-response curve measuring cell viability of 661W cells treated with increasing concentrations of inhibitors against NOX proteins, DPI and VAS2870 (*t*-test comparing individual treatments to their vehicle control). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (**** $P < 0.0001$).

4.3.2. NOX4 is up-regulated by Norgestrel in serum-deprived 661W cells

Next, we sought to identify which NOX isoforms may be responsible for this protection. It has been previously shown that only two members of the NOX family are expressed in 661W cells: as two isoforms, NOX2 and NOX4 (Groeger *et al.*, 2009a). Interestingly, some studies have suggested a link between these two isoforms, which have been described to signal to kinases/phosphatases in a similar manner (Brown & Griendling, 2009). Furthermore, it has been demonstrated that both NOX2 and NOX4 are involved in the pro-survival production of ROS in endothelial cells (Petri *et al*., 2006).

Therefore, both NOX2 and NOX4 mRNA levels were analysed using RT-qPCR. NOX4 was up-regulated at 30 min (independent samples *t*-test, $t_{12} = 2.604$, $P = 0.023$; $n = 3$ per group; Figure 4.3.4) followed by NOX2 which showed an increase at 1 h following Norgestrel treatment ($t_{14} = 2.337$, $P = 0.0348$; $n = 4$ per group).

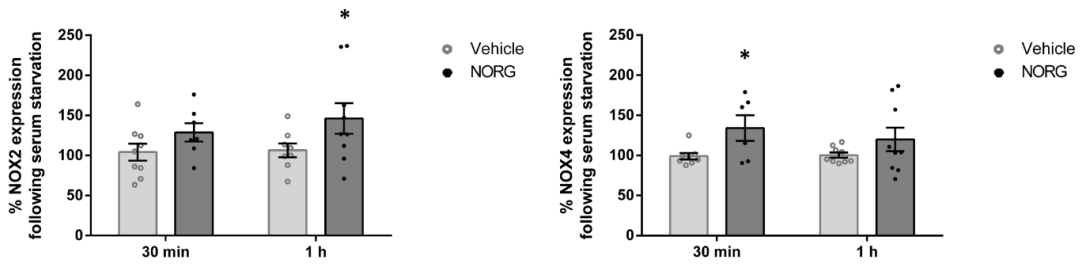


Figure 4.3.4. Norgestrel increases NOX2 and NOX4 expression in stressed 661W cells. NOX2 and NOX4 mRNA levels were measured in stressed 661W cells following Norgestrel treatment (*t*-test comparing individual Norgestrel treatments to their timed vehicle control). Norgestrel stimulates NOX4 and NOX2 expression at 30 min and at 1 h, respectively. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$).

We subsequently studied protein levels by western blotting. Norgestrel significantly increased NOX4 at 30 min (independent samples *t*-test, $t_7 = 4.335$, $P = 0.0034$; $n = 3$ per group; Figure 4.3.5), supporting the mRNA results and predicting an important role of NOX4 in this survival response. In addition, NOX2 was up-regulated at 1 h ($t_7 = 2.752$, $P = 0.0284$; $n = 3$ per group), suggesting that this protein might not be involved in the early Norgestrel-mediated response at 30 min.

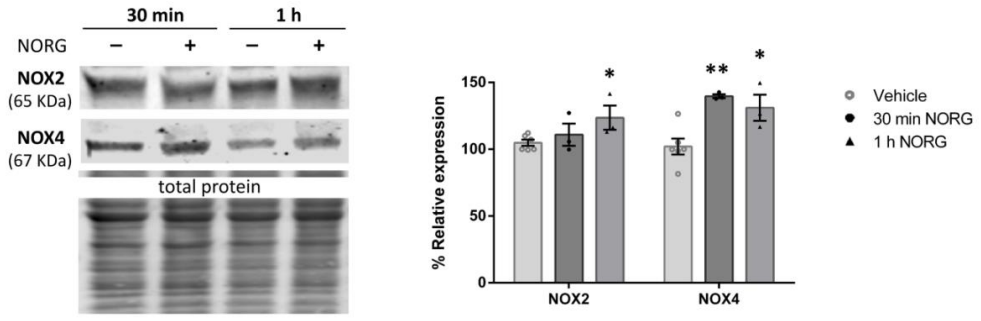


Figure 4.3.5. Norgestrel increases NOX2 and NOX4 protein levels in stressed 661W cells. Western blotting confirmed an increase in NOX2 and NOX4 (*t*-test comparing individual Norgestrel treatments to their timed vehicle control). Equal loading of protein was demonstrated by REVERT total protein stain. Blots are representative of n=3. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$).

To corroborate the increase of NOX4 in stressed 661W cells, protein levels were also analysed by immunofluorescence. We confirmed that Norgestrel stimulated both NOX4 and the subunit $p22^{phox}$ at 30 min compared with the vehicle control (Figure 4.3.6). This result supports other studies in which NOX4 has been related with survival responses in the eye and other systems (Mahadev *et al.*, 2004; Groeger, MacKey, *et al.*, 2009; Ghatak *et al.*, 2017; Harel *et al.*, 2017; Moloney *et al.*, 2017a, 2017b).

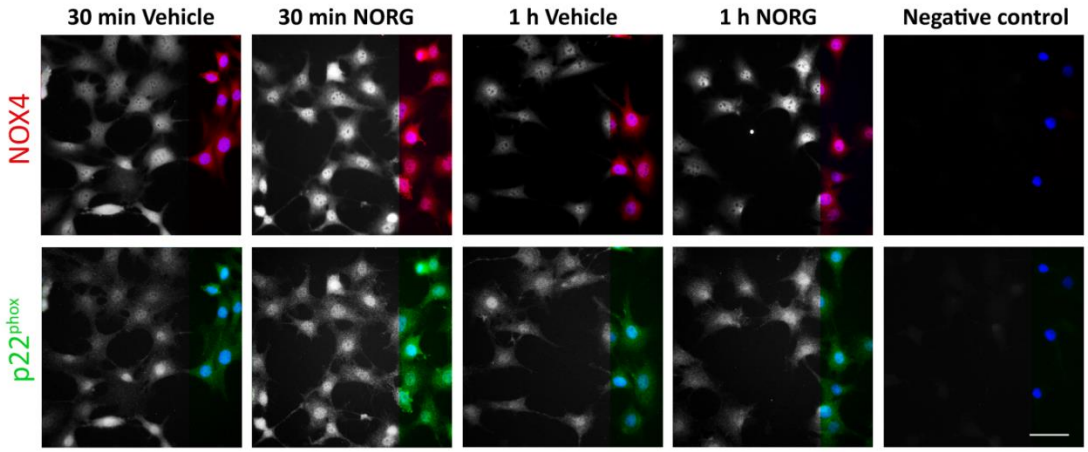


Figure 4.3.6. Norgestrel increases NOX4 and $p22^{phox}$ subunit levels in 661W cells following 30 min serum starvation. Fluorescent microscopic images confirmed that Norgestrel stimulates both NOX4 and $p22^{phox}$ at 30 min following serum starvation. For a better interpretation of the results, colours were replaced with black & white in the left region. Secondary antibody only was added as negative control. Images are representative of n=3. Scale bar 30 µm. Hoechst staining reveals cell nuclei.

In order to demonstrate Norgestrel-mediated neuroprotection is NOX4-dependent, we sought to generate a NOX4 knockout (KO) cell line using the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) gene editing system. Since the field of genome engineering was discovered, the CRISPR/Cas9 technology has gained considerable attention in the last years. This complex is part of the prokaryotic immune system of archaea and bacteria to defence against invading viruses (Mojica *et al.*, 2005). It consists of repeating sequences separated by variable short DNA sequences termed as 'spacers' which are remnants of the genetic code from past invaders (Mojica *et al.*, 2005; Mojica & Rodriguez-Valera, 2016; Khan *et al.*, 2018). This system, therefore, serves as a

genetic memory that helps the cell to detect and destroy invaders when they return. Using modified versions of Cas9, we can now target a specific sequence and modulate gene expression (Schaeffer & Nakata, 2015; Khan *et al.*, 2018).

In this study, we used the CRISPR/Cas9 gene editing system to generate a NOX4 KO cell line using 661W cells. To do so, we use the double nickase technique which differs to the traditional CRISPR/Cas9 engineering. In the latter one, the guide RNA (gRNA) binds to one target sequence and Cas9 produces a double-strand DNA break, whereas with the double nickase, a modified Cas9 interacts with gRNAs targeted to two sites, one that binds upstream and another that binds downstream of the target sequence, which results in single-strand cleavage activity as represented in figure 4.3.7, increasing its specificity (Schaeffer & Nakata, 2015; Gopalappa *et al.*, 2018).

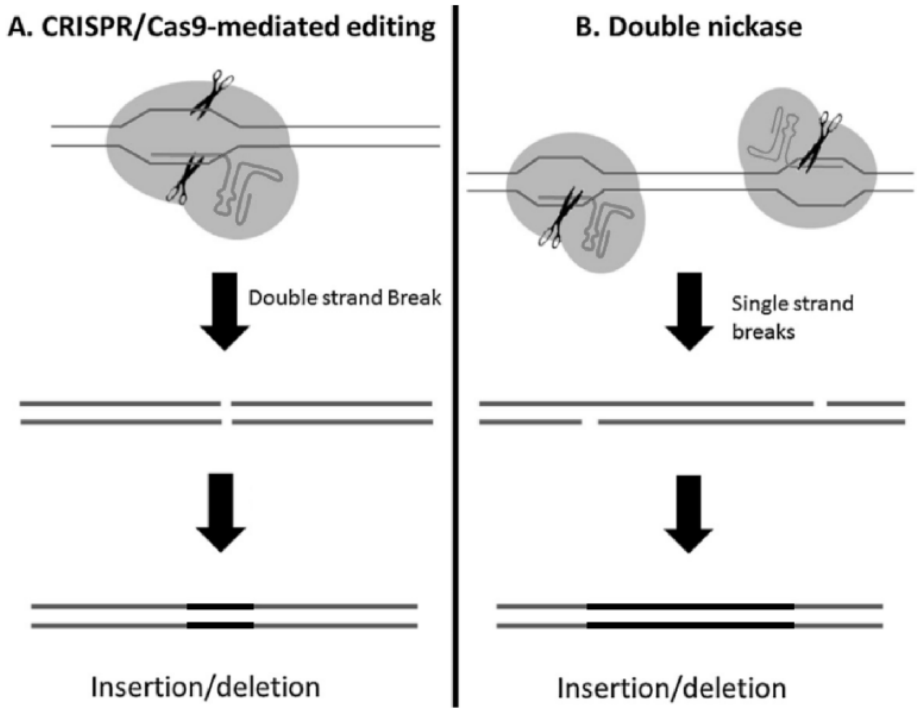


Figure 4.3.7. CRISPR/Cas9-mediated gene editing technologies. (A) Traditional CRISPR/Cas9 engineering where gRNA specifically binds to target sequence in genome. Cas9 introduces a double-strand cleavage to target DNA. It is during this repair process that a small insertion or deletion events occurs. **(B)** Double nickase where modified Cas9 interacts with gRNAs targeted to two sites. Modifications to Cas9 result in only single-strand cleavage activity. Modified from Schaeffer & Nakata, 2015.

Given that the results obtained in figure 4.3.3 suggest that the blockage of NOX proteins during 24 h has detrimental consequences on cell viability of 661W cells, we next sought to determine the effect of NOX4 KO following 48 h plasmids transfection. Cells treated with transfection reagent alone, which were used as a negative control (Mock), showed a decrease in cellular viability compared with the untreated (UT) control (one-way ANOVA, transfection effect, $F_{2,51} = 209.3$, $P < 0.0001$; Tukey's HDS *post hoc* test, $P < 0.0001$; $n = 3$ per group; Figure 4.3.8). Cell survival and proliferation were severely compromised in cells transfected with the NOX4 Double Nickase Plasmid compared with both UT and Mock controls (Tukey's HDS *post hoc* test, $P < 0.0001$), confirming the pro-survival nature of NOX4. In 2017, Jafari *et al.* demonstrated that CRISPR/Cas9-mediated NOX4 KO inhibits cell proliferation in HeLa cells (Jafari *et al.*, 2017).

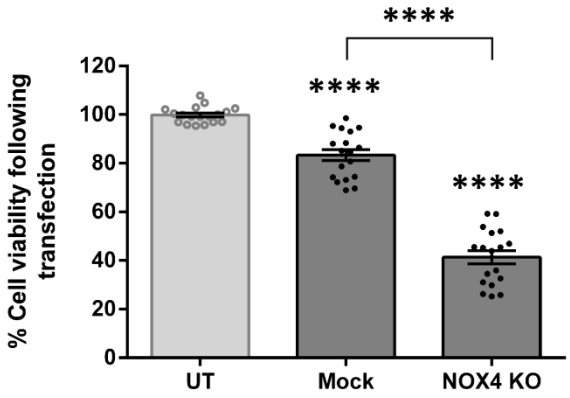


Figure 4.3.8. NOX4 is essential for cellular survival and proliferation in 661W cells. Changes in cell viability and proliferation were measured by the MTS assay and graphed as a percentage of 100% viable UT control (Tukey's HDS *post hoc* test for multiple comparisons). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (**** $P < 0.0001$).

After transfection, cells were screened by immunofluorescence for green fluorescent protein (GFP)-positive cells. We found that GFP+ cells were enucleated cells as revealed by Hoechst staining, suggesting they die in the absence of NOX4 expression (Figure 4.3.9). UT and Mock (transfection reagent alone) confirmed no GFP+ cells as expected. Enucleation has

been previously described as a mechanism of cell death (Paunescu *et al.*, 2014), corroborating our results in 661W cells.

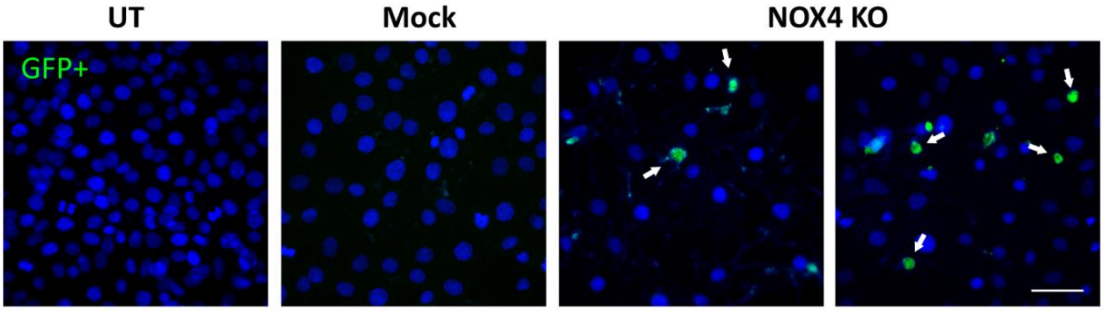


Figure 4.3.9. NOX4 KO produces cell death in 661W cells. GFP-positive cells screening by immunofluorescence. GFP+ cells were enucleated cells and hence, dying cells (white arrows). Images are representative of *n*=2. Scale bar 30 µm. Hoechst staining reveals cell nuclei.

In order to select NOX4 KO colonies, if any, cells were treated with 1 µg/ml puromycin antibiotic for 6 days. This concentration was selected among other two for being the lowest concentration that killed 100% cells of non-transfected cells in 6 days (one-way ANOVA, puromycin treatment, $F_{3,68} = 893.4$, $P < 0.0001$; Dunnett's *post hoc* test, 1 µg/ml: $P < 0.0001$; 5 µg/ml: $P < 0.0001$; 10 µg/ml: $P < 0.0001$; $n = 3$ per group; Figure 4.3.10).

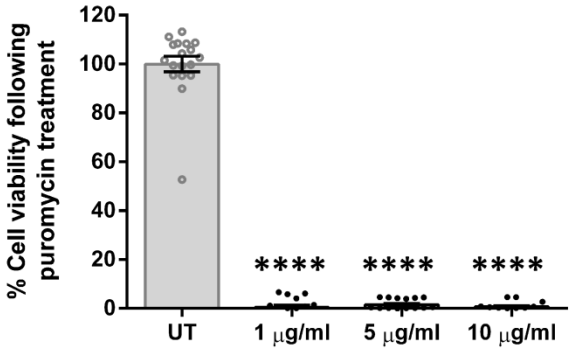


Figure 4.3.10. Puromycin treatment optimisation in 661W cells. Changes in cell viability were measured by the MTS assay and graphed as a percentage of 100% viable UT control (Dunnett's post hoc test, comparing all concentrations to the UT control). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (**** $P < 0.0001$).

Immunofluorescence studies demonstrated that untreated cells die after puromycin treatment (Figure 4.3.11A). We observed a few cells in the NOX4 KO samples, however, these cells expressed NOX4 and hence, were non-transfected cells that survived to both the transfection process and puromycin treatment. It is possible that these cells survived due to a partial,

rather than a total, deletion of the NOX4 gene. Such cells were grown in completed media for two weeks to reach ~80% confluence in order to confirm NOX4 expression by western blotting (Figure 4.3.11B). KO cells expressed same levels of NOX4 than the UT control and therefore, it suggests that GFP+ cells (NOX4 KO) observed in figure 4.3.9 died and were lost during the subsequent washes performed in the protocol.

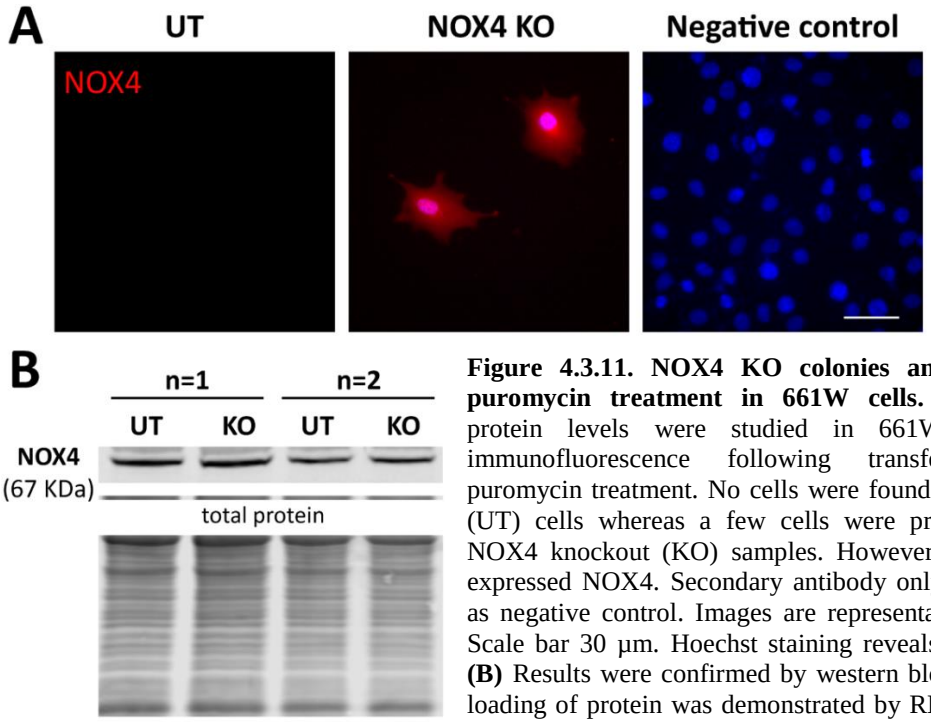


Figure 4.3.11. NOX4 KO colonies analysis after puromycin treatment in 661W cells. (A) NOX4 protein levels were studied in 661W cells by immunofluorescence following transfection and puromycin treatment. No cells were found in untreated (UT) cells whereas a few cells were present in the NOX4 knockout (KO) samples. However, these cells expressed NOX4. Secondary antibody only was added as negative control. Images are representative of *n*=2. Scale bar 30 µm. Hoechst staining reveals cell nuclei. **(B)** Results were confirmed by western blotting. Equal loading of protein was demonstrated by REVERT total protein stain.

We concluded that NOX4 has an important pro-survival role within the cell, taking into account its complete abrogation using the CRISPR/Cas9 system produces the death of transfected cells. Since several evidences are found in the literature describing the pro-survival role of NOX4 protein (Brown & Griendling, 2009; Takac *et al.*, 2012; Moloney & Cotter, 2017; Moloney *et al.*, 2017a, 2017b), these results are not surprising. We hypothesise this occurs given that 661W cells only express two NOX isoforms: NOX2 and NOX4, as described before. In the absence of NOX4, NOX2 might not be able to assume NOX4 role, leading to cell death of NOX4 KO 661W cells. This suggests that the use of inhibitors during shorter time points would be a better approach to study NOX proteins in 661W cells.

4.3.3. Mitochondrial ROS production is induced by the mitochondrial inhibitor Rotenone in 661W cells

Given that mitochondria are another main producer of ROS within the cell, we asked whether they participate in the Norgestrel-mediated pro-survival response at 30 min. We treated cells with various concentrations of Rotenone, a widely used mitochondrial inhibitor that acts by supressing complex I superoxide production (Barrientos & Moraes, 1999; Isenberg & Klaunig, 2000; Chauvin *et al.*, 2001; Curtin *et al.*, 2002; Fato *et al.*, 2009). We found 20 µM to be the lowest concentration that effectively reduced ROS production, resulting in a decrease in DCFDA fluorescence intensity (independent samples *t*-test, $t_{20} = 15.23$, $P < 0.0001$; $n = 4$ per group; Figure 4.3.12B), although it did result in cell death ($t_{16} = 6.752$, $P < 0.0001$; $n = 3$ per group; Figure 4.3.12A). This effect on cellular viability has been documented so far in several *in vitro* and *in vivo* systems (Abdo *et al.*, 1988; Cunningham *et al.*, 1995; Isenberg & Klaunig, 2000; Chauvin *et al.*, 2001; Li *et al.*, 2003; Heinz *et al.*, 2017).

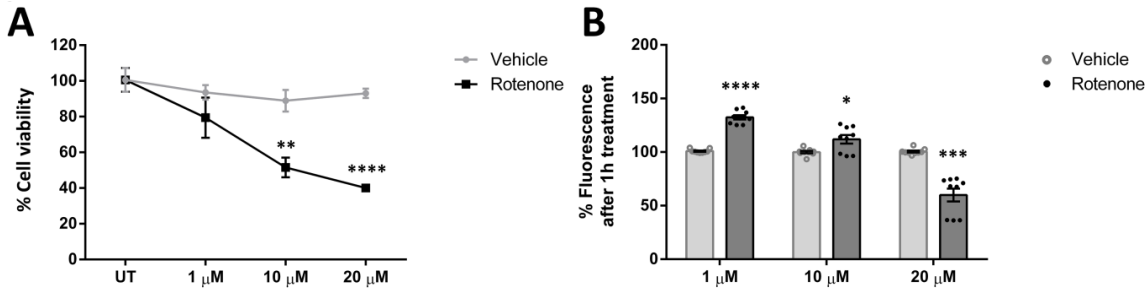


Figure 4.3.12. Rotenone treatment only decreases intracellular ROS levels at 20 µM. Dose-response curve measuring cell viability **(A)**, DCFDA fluorescence **(B)** of 661W cells treated with increasing concentrations of Rotenone, an inhibitor of mitochondria (*t*-test comparing individual treatments). Treatment with 20 µM effectively attenuates intracellular ROS production measured by DCFDA. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

In order to corroborate mitochondrial inhibition produced by Rotenone, mitochondrial ROS were studied by flow cytometry using the probe MitoSOX (Figure 4.3.13). MitoSOX is a derivative from dihydroethidium (DHE) that detects specifically $O_2^{\bullet-}$ produced by

mitochondria from the one-electron reduction of O_2 (Murphy, 2009) and it has been widely used in fluorescence/confocal microscopy and flow cytometry studies in live cells (Robinson *et al.*, 2006; Mukhopadhyay *et al.*, 2007a, 2007b; Pehar *et al.*, 2007; Zimmerman *et al.*, 2007).

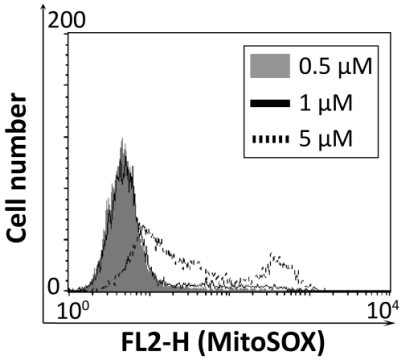


Figure 4.3.13. MitoSOX was used to detect mitochondrial superoxide levels. Different concentrations were tested in order to find an optimal peak of fluorescence. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. 0.5 µM was used in all subsequent experiments.

Surprisingly, mitochondrial ROS production was increased in the presence of Rotenone in all the concentrations used in the current study (independent samples *t*-test, 1 µM: $t_{16} = 10.98$, $P < 0.0001$; 10 µM: $t_{16} = 3.117$, $P = 0.0109$; 20 µM: $t_{16} = 26.14$, $P < 0.0001$; $n = 3$ per group; Figure 4.3.14). We suggest this increase may be explained by a severe impairment in mitochondrial activity shown by a decrease in cellular viability in cells treated with Rotenone, demonstrating this inhibitor it is not an accurate way to study mitochondrial implication in this signalling pathway.

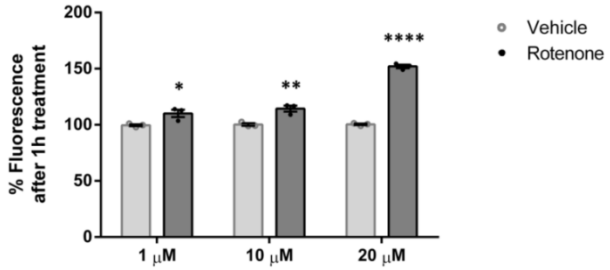


Figure 4.3.14. Mitochondrial inhibition by Rotenone increases the production of mitochondrial ROS in 661W cells. Dose-response curve measuring MitoSOX fluorescence of 661W cells treated with increasing concentrations of Rotenone, an inhibitor of mitochondria (*t*-test comparing individual treatments). None of the concentrations studied showed a decrease in mitochondrial ROS levels as shown by MitoSOX fluorescence. This suggests the effects of Rotenone on mitochondria are not specific enough to block mitochondrial participation in ROS production in 661W cells. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

According to this, Li *et al.* confirmed that treatment with Rotenone leads to cytochrome *c* release, activation of caspase 3 activity and DNA fragmentation, all biomarkers of apoptosis (Li *et al.*, 2003). This Rotenone-induced ROS production has also been related with an inhibition of cell proliferation given that it binds to tubulin, affecting therefore the microtubule assembly (Srivastava & Panda, 2007).

4.3.4. Mitochondria do not participate in the pro-survival production of ROS in 661W cells

In light of the results obtained with Rotenone, we decided to use an alternative compound to target mitochondrial production of ROS without affecting cell viability. As a result, (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)2oxoethyl)triphenylphosphoniumchloride monohydrate (MitoTEMPO), a mitochondria-targeted antioxidant, was used due to its specific effects on mitochondrial ROS (Dikalova *et al.*, 2010; Woolley *et al.*, 2013; Bordt & Polster, 2014; McCarthy & Kenny, 2016; Zhang *et al.*, 2017).

A range of concentrations was tested for its effects on cellular viability as measured by MTS assay and on ROS production as measured by flow cytometry. 5 μM resulted in similar levels of cell viability compared with the vehicle (independent samples *t*-test, $t_{34} = 1.775$, $P = 0.0848$; $n = 3$ per group; Figure 4.3.15A), whilst significantly decreasing both intracellular (independent samples *t*-test, $t_{16} = 5.311$, $P < 0.0001$; $n = 3$ per group; Figure 4.3.15B) and mitochondrial ROS levels ($t_{16} = 4.701$, $P < 0.0001$; $n = 3$ per group; Figure 4.3.15B) measured by DCFDA and MitoSOX, respectively.

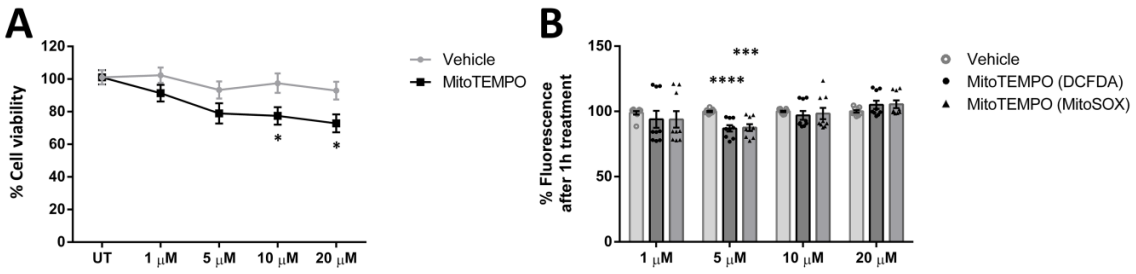


Figure 4.3.15. MitoTEMPO reduces ROS levels in 661W cells. Dose-response curve measuring cell viability **(A)**, DCFDA fluorescence and MitoSOX fluorescence **(B)** of 661W cells treated with increasing concentrations of MitoTEMPO, a mitochondria-targeted antioxidant (*t*-test comparing individual treatments). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$).

Thus, cells were pre-treated with 5 µM MitoTEMPO for 1 h in complete media prior to 20 µM Norgestrel treatment in serum-free media for 30 minutes. Use of MitoTEMPO induced a slight decrease of ROS levels compared with the vehicle control, which confirms that it is inhibiting production of ROS (Figure 4.3.16). Norgestrel could still produce an increase in ROS at 30 min in the absence or presence of the antioxidant as detected by DCFDA fluorescence intensity, suggesting that mitochondrial-derived ROS are not involved in this response (independent samples *t*-test, vehicle: $t_{16} = 7.878$, $P < 0.0001$; MitoTEMPO: $t_{16} = 4.903$, $P = 0.0002$; $n = 3$ per group; Figure 4.3.16).

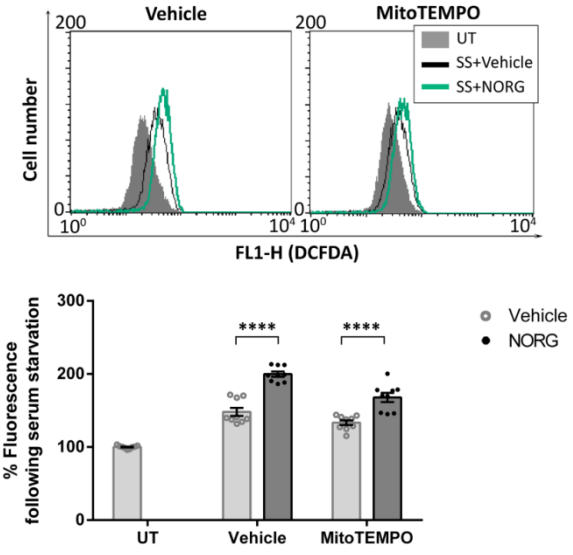


Figure 4.3.46. Mitochondria are not involved in the Norgestrel-mediated production of ROS in serum-starved 661W cells. FACS analysis of 661W cells pre-treated with 5 µM MitoTEMPO prior to Norgestrel treatment. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual treatments). Norgestrel was able to increase ROS levels in the presence or absence of the mitochondria-targeted antioxidant MitoTEMPO, suggesting this response is not mitochondria-dependent. Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (**** $P <$ 0.0001).

In support of this, repetition of this experiment but using MitoSOX rather than DCFDA demonstrated that Norgestrel does not increase mitochondrial superoxide levels in the absence or presence of MitoTEMPO (independent samples *t*-test, vehicle: $t_{16} = 0.3778$, $P = 0.7105$; MitoTEMPO: $t_{16} = 0.6440$, $P = 0.5287$; $n = 3$ per group; Figure 4.3.17). MitoTEMPO also decreased MitoSOX fluorescence corroborating its effect on reducing mitochondrial ROS production as previously described (Dikalova *et al.*, 2010; McCarthy & Kenny, 2016; Zhang, Wang, *et al.*, 2017). All together suggests that ROS production stimulated by Norgestrel is not mitochondria-dependent.

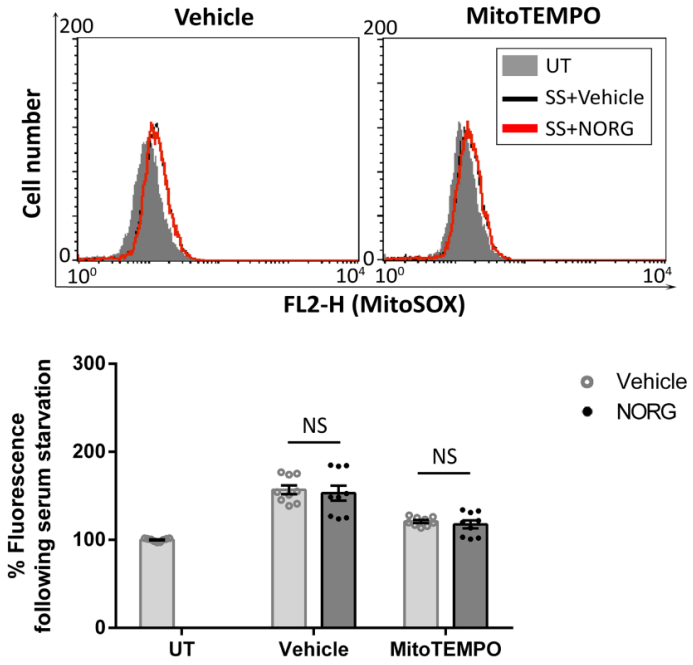


Figure 4.3.17. Mitochondrial superoxide levels are not affected by Norgestrel in stressed 661W cells. FACS analysis of 661W cells pre-treated with 5 µM MitoTEMPO prior to Norgestrel treatment. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of MitoSOX fluorescence and hence mitochondrial superoxide levels (*t*-test comparing individual treatments). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant).

4.3.5. COX proteins are not involved in the response induced by Norgestrel in stressed 661W cells

Cyclooxygenase (COX) enzymes, also known as prostaglandin synthase, have been described to produce ROS as a result of their peroxidase activity (Pepicelli *et al.*, 2002; Nathan & Cunningham-Bussel, 2013; Holmström & Finkel, 2014; Di Meo *et al.*, 2016). Such ROS production is suggested to contribute to an increase in inflammation and cellular damage in different models, including Alzheimer's and cardiovascular diseases (Nogawa *et al.*, 1997; Pasinetti, 1998; Pepicelli *et al.*, 2002; Virdis *et al.*, 2005; Hernanz *et al.*, 2014; Hee Kim *et al.*, 2015; Yin *et al.*, 2017).

During years there was little, if any, evidence in the literature about the implication of COX-induced ROS production in survival signalling. In fact, little is known about how these enzymes produce ROS. Hernanz *et al.* suggested that ROS can be generated both during the process of prostaglandin synthesis itself and through the effects on other ROS sources such as NOX proteins (Hernanz *et al.*, 2014), which has also been shown before (Martínez-Revelles *et al.*, 2012). Since studies in this group have suggested that COX enzymes are implicated in pro-survival ROS production downstream of bFGF (Farrell *et al.*, 2011), we sought to investigate whether these proteins play a role in the Norgestrel-mediated response. Cells were treated with four different concentrations of Diclofenac sodium salt, a well-known inhibitor of COX activity (Gan, 2010; Moloney *et al.*, 2017a; Ulubay *et al.*, 2017). 1 µM did not affect cell viability (independent samples *t*-test, $t_{28} = 0.1587$, $P = 0.875$; $n = 3$-4 per group; Figure 4.3.18A) and it significantly decreased ROS levels ($t_{16} = 11.58$, $P < 0.0001$; $n = 4$ per group; Figure 4.3.18B).

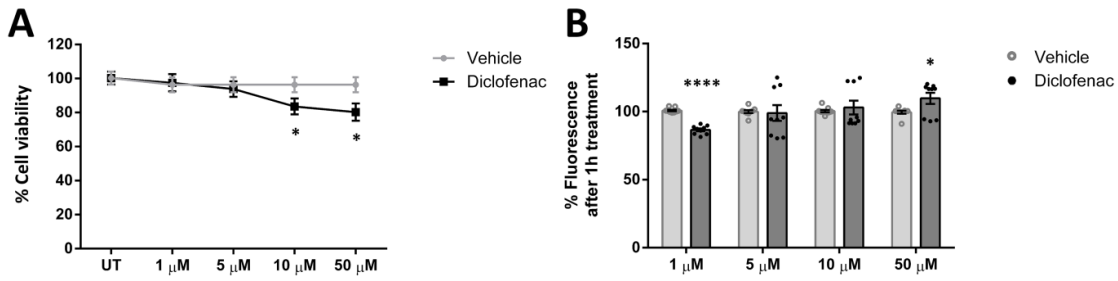


Figure 4.3.18. COX inhibition produced by Diclofenac in 661W cells. Dose-response curve measuring cell viability **(A)** and DCFDA fluorescence **(B)** of 661W cells treated with increasing concentrations of Diclofenac which inhibits COX enzymes (*t*-test comparing individual Norgestrel treatments to their timed vehicle control). Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (* $P < 0.05$, **** $P < 0.0001$).

Therefore, cells were pre-treated with 1 µM Diclofenac for 1 h before Norgestrel treatment for 30 min. Norgestrel treatment produced a burst of ROS at 30 min even in the presence of inhibitor (independent samples *t*-test, vehicle: $t_{15} = 3.006$, $P = 0.0089$; Diclofenac: $t_{11} = 2.431$, $P = 0.0333$; $n = 3$ per group; Figure 4.3.19), suggesting that COX are not essential in this response.

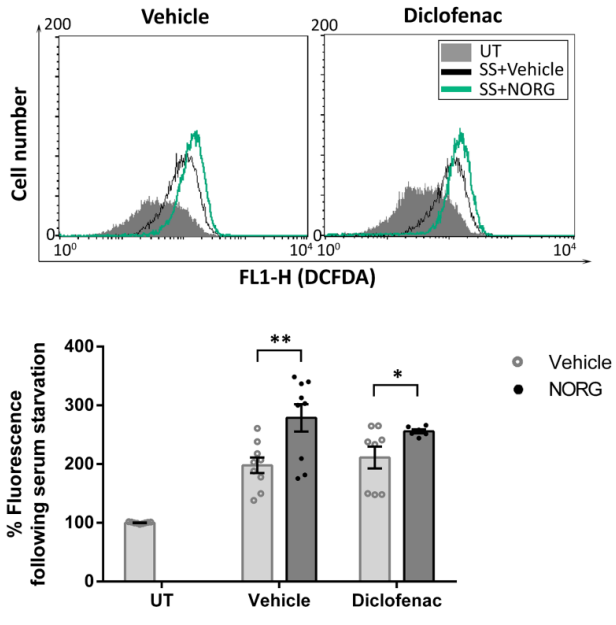


Figure 4.3.19. Norgestrel-mediated response is not COX-dependent in stressed 661W cells. FACS analysis of 661W cells pre-treated with 1 µM diclofenac prior Norgestrel treatment. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual treatments). Norgestrel increased ROS production in the absence or presence of COX inhibitor.Results are presented as mean ± SEM from three independent experiments. Asterisks indicate significant difference (NS not significant, * $P < 0.05$, **** $P < 0.0001$).

4.4. Discussion

Interest in the hormone progesterone was initiated by Atella *et al.* in 1987, who observed that this hormone was able to improve functionality in pseudopregnant rats compared with the normal-cycling females, both suffering from traumatic brain injury (TBI) (Attella *et al.*, 1987). Some years later, it was confirmed that progesterone administration was beneficial in both male and female rats (Roof *et al.*, 1992, 1994) and mice presenting TBI (Hua *et al.*, 2011). Since then, experimental and clinical evidences indicating the neuroprotective properties of progesterone are numerous in the literature (Gonzalez Deniselle *et al.*, 2003; Gonzalez *et al.*, 2005; Garay *et al.*, 2009, 2011, 2012; De Nicola *et al.*, 2013; Singh & Su, 2013; Schumacher *et al.*, 2016; Espinosa-Garcia *et al.*, 2017; Fabres *et al.*, 2018).

Studies on neurodegeneration and Norgestrel-mediated neuroprotection have also been previously described in various models of photoreceptor cell death, through *in vitro*, *in vivo* and *ex vivo* preparations (Doonan *et al.*, 2011; Byrne *et al.*, 2016a; 2016b; Roche *et al.*, 2016, 2017a, 2017b; Roche & Wyse-Jackson *et al.*, 2016; Wyse Jackson & Cotter, 2016; Wyse Jackson *et al.*, 2016a; 2016b; Ruiz-Lopez *et al.*, 2017). Norgestrel elicits neuroprotective effects by binding to the progesterone receptor PGRMC1, increasing the expression of the neurotrophic factor bFGF to promote cell survival using redox-signalling as shown in the previous chapter (Wyse Jackson & Cotter, 2016; Wyse Jackson *et al.*, 2016a; Ruiz-Lopez *et al.*, 2017).

Furthermore, the relationship between ROS generation and growth factors, especially in cases of induced stress, has been well documented so far (Lieberthal *et al.*, 1998; Tammariello *et al.*, 2000; Curtin *et al.*, 2002; Bae *et al.*, 2011; Dikalov *et al.*, 2014; Ghatak *et al.*, 2017). Several growth factors including bFGF appear to modulate ROS formation

within the cell (Sundaresan *et al.*, 1995; Tammariello *et al.*, 2000; Kirkland *et al.*, 2007; Bai *et al.*, 2014; Ghatak *et al.*, 2017). Kirkland *et al.*, showed that nerve growth factor (NGF) withdrawal induces an increase in oxidative stress, leading to apoptosis in mice neurons. They also showed that readdition of NGF suppresses the up-regulation of ROS levels and inhibits the release of cytochrome *c* (Kirkland *et al.*, 2007). According to this, Ghatak *et al.* and Bai *et al.* presented an important role of the transforming growth factor β1 (TGFβ1) in the NOX4-induced ROS production (Bai *et al.*, 2014; Ghatak *et al.*, 2017), elucidating the importance of neurotrophic factors in the production and modulation of ROS.

In fact, studies in our laboratory have demonstrated how ROS production induces cellular survival (Mackey *et al.*, 2008; Groeger *et al.*, 2009) downstream of bFGF in 661W cells and retinal explants from both wild-type and diseased mice (Farrell *et al.*, 2011; Groeger *et al.*, 2012; Ruiz-Lopez *et al.*, 2017). Nevertheless, this pro-survival nature of redox state should be always considered as a short event that happens in a specific moment in the cell, essentially as a first mechanism to respond against any stressful stimuli and always in a regulated manner (Remacle *et al.*, 1995; Rhee, 2006; Ray *et al.*, 2012; Reczek & Chandel, 2015; Prieto-Bermejo & Hernández-Hernández, 2017).

The importance of NOX enzymes participating in cellular signalling is not surprising, as the only known function of which is ROS production (Lambeth, 2004; Bedard & Krause, 2007; Brown & Griendling, 2009; Wingler *et al.*, 2011; Takac *et al.*, 2012; Brandes *et al.*, 2014b; Moloney & Cotter, 2017). This has been demonstrated by previous studies from our group in stressed 661W cells (Groeger *et al.*, 2009a). The current study also validates the role of NOX proteins, mainly NOX4, as ROS generators at an early stage in serum-starved 661W cells, but also as a result of Norgestrel treatment, which had not been shown previously (Figure 4.3.2). In fact, the role of NOX proteins in diseases such as RP is not well understood. Some

studies have suggested that the elevated levels of oxygen in the retina when rods die produce the participation of NOX proteins, increasing the generation of ROS molecules (Usui *et al.*, 2009). However, which NOX isoform is responsible for such effects is still unknown. In the current study, NOX4 expression, both mRNA and protein levels, were increased by Norgestrel at 30 min following stress (Figure 4.3.4-6). Interestingly, this protein has been shown to be constitutively active and therefore, its activity depends on its expression level (Bae *et al.*, 2011; Brandes *et al.*, 2014a; Nisimoto *et al.*, 2014). This is not the first time NOX4 has been implicated in redox signalling (Crestani *et al.*, 2011; Nisimoto *et al.*, 2014; Moloney *et al.*, 2017b). Thus, NOX4 produces hydrogen peroxide in the signalling of different growth factors such as insulin (Mahadev *et al.*, 2004), bFGF (Lo & Cruz, 1995) and TGFβ1 (Peshavariya *et al.*, 2014; Hakami *et al.*, 2015).

On the other hand, ROS derived from mitochondria have traditionally been associated with aging and other mechanisms related with cell damage (Choksi *et al.*, 2008; Nakamura *et al.*, 2012; Ni *et al.*, 2016; Sanz & Bernardi, 2016; Jelinek *et al.*, 2018). However, our understanding of these processes is changing. Mitochondrial involvement in redox signalling has been gaining attention in recent years (Zhang & Gutterman, 2006; Sena & Chandel, 2012; Harel *et al.*, 2017). Several mitochondrial-targeted inhibitors have been widely utilised to study mitochondrial role in multiple responses, with one of the most commonly used being Rotenone given its action on complex I of the mitochondrial respiratory chain (Curtin *et al.*, 2002; Brand, 2016; Heinz *et al.*, 2017).

The effects of Rotenone have been tested in this study using different concentrations in 661W cells. Despite the fact that 20 μM Rotenone decreased intracellular ROS production measured by DCFDA, none of the concentrations used reduced mitochondrial $O_2^{\bullet-}$ as measured by MitoSOX fluorescence (Figure 4.3.14). A loss of ROS by DCFDA fluorescence

could be linked to the detrimental effects on cell viability (Figure 4.3.12). Hence, use of Rotenone was an unsuitable method to demonstrate mitochondrial participation in this signalling pathway, in support of other studies (Abdo *et al.*, 1988; Cunningham *et al.*, 1995; Isenberg & Klaunig, 2000; Li *et al.*, 2003; Beretta *et al.*, 2006; Srivastava & Panda, 2007; Fato *et al.*, 2009; Karlsson *et al.*, 2016; Heinz *et al.*, 2017). In the mentioned studies, Rotenone resulted toxic due to its ability to induce cell death in a variety of cells. This compound has been shown to elevate the production of ROS, which results lethal for cells. In addition to this, Rotenone has been linked with the prevention of microtubules assembly as mentioned in the results section (Srivastava & Panda, 2007). Srivastava & Panda showed that treatment with Rotenone depolymerised spindle microtubules of two different cell lines. As a result, Rotenone treatment inhibited cell proliferation (Srivastava & Panda, 2007). In 2012, this process was also confirmed by Hongo *et al.*, who demonstrated that Rotenone reduces the amount of polymerised tubulin, which indicates and induction of microtubule desestabilization (Hongo *et al.*, 2012).

The use of a more specific approach in the study of mitochondrial-ROS was employed through the use of the mitochondria-targeted antioxidant MitoTEMPO. MitoTEMPO is known to act specifically in mitochondria given that it accumulates in the mitochondrial matrix, acting as a superoxide dismutase mimetic (Dikalova *et al.*, 2010; Dikalov, 2011; McCarthy & Kenny, 2016; Zhang, Wang, *et al.*, 2017). Using MitoTEMPO to reduce mitochondrial ROS activity, the current study indicates that these organelles are not involved in the pro-survival Norgestrel-mediated response at 30 min following serum starvation (Figure 4.3.16 and 4.3.17).

Given that COX enzymes were responsible for the bFGF-mediated response in 661W cells shown by our lab in 2011 (Farrell *et al.*, 2011), we next employed the COX inhibitor

Diclofenac in order to investigate whether these proteins participate in the up-regulation of ROS levels by Norgestrel. Diclofenac is an anti-inflammatory drug widely used as analgesic due to its effects on COX proteins, blocking the prostaglandin synthesis (Euchenhofer *et al.*, 1998; Gan, 2010; Van Den Brandhof & Montforts, 2010; Ulubay *et al.*, 2017; Fontes *et al.*, 2018), and making it a suitable approach to elucidate COX implication in cellular processes. In the current study, inhibition of COX enzymes did not prevent Norgestrel-induced ROS increase in 661W cells (Figure 4.3.19), demonstrating that these enzymes are not implicated in this process.

As a conclusion, this study clarifies the pro-survival mechanism behind Norgestrel-mediated response (Ruiz-Lopez *et al.*, 2017), identifying NOX4 as the responsible source for increasing pro-survival ROS levels within the first 30 min following the stress caused by serum starvation in 661W cells (Figure 4.4.1).

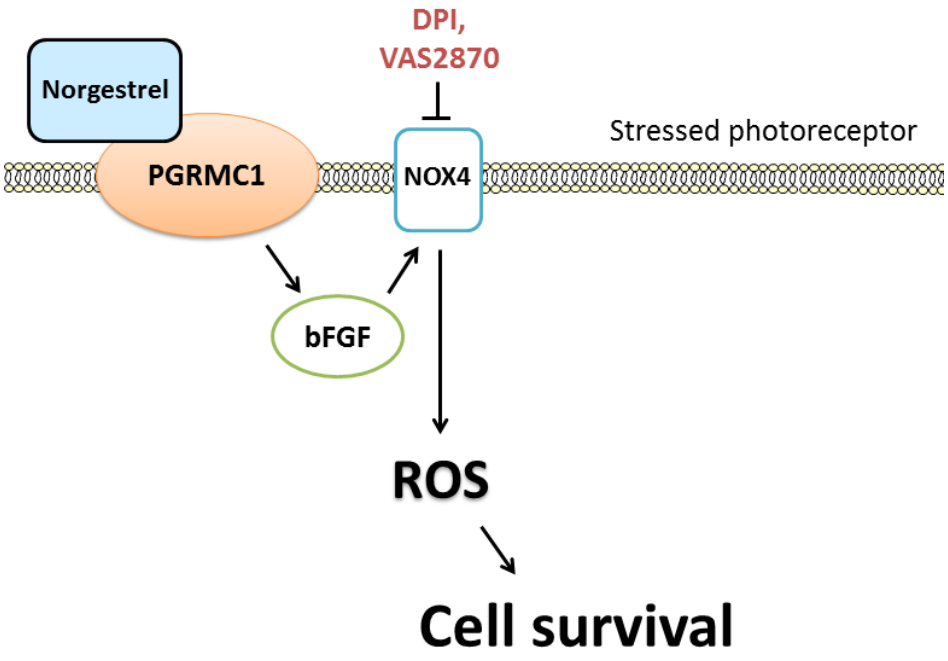


Figure 4.4.1. Proposed pathway of Norgestrel-mediated response. It has been previously shown that Norgestrel acts through the progesterone receptor membrane complex I (PGRMC1) to produce ROS downstream of bFGF (Ruiz-Lopez *et al.*, 2017). This schematic represents how Norgestrel uses NOX4 to induce ROS production and promote neuroprotection in serum-starved 661W cells, including the compounds used to inhibit each step.

Chapter 5: The synthetic progesterone ‘Norgestrel’ mediates an antioxidant response to long term stress in the rd10 mouse model of retinitis pigmentosa

5.1. Abstract

Norgestrel, a progesterone analogue, has shown promising results for the potential treatment of retinal diseases like retinitis pigmentosa (RP). Our laboratory has demonstrated a wide variety of pro-survival responses produced by this compound in both the 661W photoreceptor-like cell line and the rd10 retina, a model of RP. This neuroprotective response is explained in part by the utilization of neurotrophic factors such as the basic fibroblast growth factor (bFGF), anti-inflammatory properties through alleviating detrimental microglial activity and gliosis, and the modulation of reactive oxygen species (ROS). Moderate levels of ROS are known to participate as second messengers in survival responses. For instance, our group has recently demonstrated that Norgestrel up-regulates pro-survival ROS levels at very early time points following stress in both 661W cells and rd10 photoreceptors. At sustained high levels, these molecules can become toxic for cells, leading to the worsening of different diseases including RP. To counteract this, cells possess two mechanisms: reducing the production of ROS or increasing the antioxidant defence. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family of proteins and superoxide dismutases enzymes (SOD) constitute the major ROS generators and ROS scavengers within the cell, respectively. This study was designed to investigate Norgestrel's ability to reduce toxic levels of ROS in the diseased retina. Through the analysis of intracellular ROS levels, mRNA and protein levels, we conclude that Norgestrel decreases ROS levels in the rd10 retina *via* activation of the transcription factor nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) and one of its effector antioxidant enzymes, SOD2.

5.2. Introduction

Retinitis pigmentosa (RP), among other retinopathies, is characterised by the progressive loss of photoreceptors leading to morphological and functional damage of the retina (Cuenca *et al.*, 2014; Roche *et al.*, 2016). This disease firstly affects rods with the subsequent degeneration of cones (Hamel, 2006; Punzo *et al.*, 2012; Daiger *et al.*, 2013; Tao *et al.*, 2016; Athanasiou *et al.*, 2017). The complex nature of developing a treatment for RP is based on the heterogeneity of genetic mutations, which disrupts a host of mechanisms, including the phototransduction cascade, RNA splicing machinery and retinal metabolism (Soest *et al.*, 1999; Dias *et al.*, 2017). Neurotrophic factors have been studied as a possible strategy to treat this disease given their role in stimulating cell survival. However, their short half-lives have come as a limitation for treatment so far (Buch *et al.*, 2007; Guadagni *et al.*, 2015; Dias *et al.*, 2017). Therefore, a therapeutic approach that stimulates endogenous production of neurotrophic factors is desirable.

In this context, the synthetic progesterone Norgestrel, which is used in some oral contraceptives, was found by our laboratory to be neuroprotective in the retina in 2011 (Doonan *et al.*, 2011; Doonan & Cotter, 2012). Since then, we have elucidated some components of its mechanism of action in the retina, including the up-regulation of neurotrophic factors such as basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) (Byrne *et al.*, 2016a; Wyse Jackson & Cotter, 2016). In addition, neuroprotective effects of this compound include anti-inflammatory and anti-oxidative properties within the diseased or injured retina (Byrne *et al.*, 2016b; Roche & Wyse-Jackson *et al.*, 2016; Wyse Jackson *et al.*, 2016a, 2016b; Roche *et al.*, 2017a, 2017b; Ruiz-Lopez *et al.*, 2017).

Futhermore, reactive oxygen species (ROS) have been linked with RP, given that they represent one of the main contributing factors to cone photoreceptor cell death (Shen *et al.*, 2005; Komeima *et al.*, 2006, 2007, 2008; Usui *et al.*, 2009). Implication of ROS in RP and other retinal diseases is not surprising due to the fact that the retina has a high demand of oxygen (Ames, 2000; Cuenca *et al.*, 2014). This largest use of oxygen renders the retina very sensitive tissue to oxidative damage (Cingolani *et al.*, 2005; Shen *et al.*, 2005; Martínez-Fernández de la Cámara *et al.*, 2013). It is known that ROS can participate as signalling molecules when levels are low as described in chapters 3 and 4 (Rhee, 2006; Groeger *et al.*, 2009b; Ruiz-Lopez *et al.*, 2017).

However, once ROS levels become pathological, the cell attempts to eliminate them, either by reducing the production of ROS or by increasing the amount of antioxidants present in the cell (Cadenas, 1997; Valko *et al.*, 2007; Moloney & Cotter, 2017). The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family of proteins and mitochondria constitute the major ROS generators whilst the endogenous antioxidant machinery mainly consists of enzymes: superoxide dismutases (SOD), glutathione peroxidases, superoxide reductases, glutathione reductase, peroxiredoxin, thioredoxin and catalases (Usui, Oveson, *et al.*, 2009; Martínez-Fernández de la Cámara *et al.*, 2013; Moloney & Cotter, 2017). As a result, biological and non-biological antioxidants have gained attention in the treatment of diseases such as RP (Guadagni *et al.*, 2015). Norgestrel's antioxidant properties have been demonstrated in the balb/c induced light damage mouse model by activating the transcription factor nuclear factor erythroid 2 (NF-E2)-related factor-2 (Nrf2) which induces an increase in SOD2 activity when ROS become toxic (Byrne *et al.*, 2016b). Our study investigates the relevance of this mechanism in the inherited rd10 mouse model of retinitis pigmentosa.

5.3. Results

5.3.1. Exogenous H_2O_2 addition does not affect serum-starved 661W cells

In RP the death of rods, which represent >90% of the photoreceptor population in the retina and thus, consume the majority of oxygen, causes a sharp increase in the oxygen level (hyperoxia) of cones and their subsequent death (Curtin *et al.*, 2002; Punzo *et al.*, 2012; Tao, Chen, *et al.*, 2016). Another cause of cone cell death after rods die is the cessation of trophic support (Komeima *et al.*, 2006, 2008; Finnegan *et al.*, 2010; Athanasiou *et al.*, 2018). Studies in mouse models of RP have shown how hyperoxia, caused after the death of rods, results in the narrowing of retinal blood vessels and the subsequent down-regulation of several growth factors (Komeima *et al.*, 2006). Komeima *et al.* demonstrated that vascular endothelial growth factor (VEGF) expresion was reduced as a consequence of such effect produced by hyperoxia in rd1 mice (Komeima *et al.*, 2006).

In order to mimic the stress suffered by the cones in such circumstances, cells were serum-starved and treated with 20 μM Norgestrel or vehicle (DMSO) and different concentrations of a hydrogen peroxide (H_2O_2) solution for 24 h to increase oxidative stress. Exogenous addition of H_2O_2 has been widely used as a signalling molecule to induce cellular survival at short time points (Carmody & Cotter, 2007; Forman, 2007; Mackey *et al.*, 2008; Wang *et al.*, 2018). However, it is thought to produce oxidative damage and cell death when it becomes toxic after a long exposure (Wang *et al.*, 2018). In the current study, a range of concentrations was tested for its effects on cellular viability as measured by MTS assay. Norgestrel was only significantly protective in the presence of 1 μM H_2O_2 (independent samples *t*-test, $t_{30} = 2.695$, $P = 0.0114$; $n = 3$ per group; Figure 5.3.1).

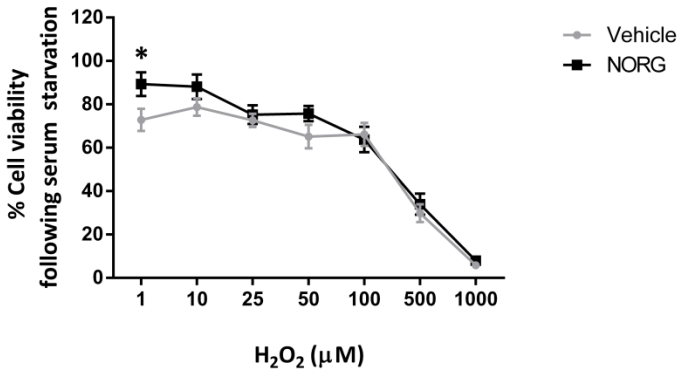


Figure 5.3.1. Norgestrel enhances cell survival at a sublethal dose of H_2O_2 in serum-starved 661W cells. Dose-response curve measuring cell viability of 661W cells treated with increasing concentrations of hydrogen peroxide (H_2O_2) solution following serum starvation (*t*-test comparing individual treatments). Norgestrel was only able to enhance cell survival at 1 µM H_2O_2 compared with the vehicle control. Asterisks indicate significant difference (* $P < 0.05$).

We subsequently aimed to understand the effects produced by the addition of H_2O_2 compared with serum starvation (SS) itself in order to find a valid model to study cell death caused by oxidative damage. 661W cells were treated with the same H_2O_2 concentrations used in figure 5.3.1 in serum-free media for 24 h, and cell viability was measured by MTS assay. Vehicle (DMSO) concentrations from figure 5.3.1 were not considered in this experiment given that DMSO might affect the results due to its anti-inflammatory properties (Elisia *et al.*, 2016).

We did not find differences in cellular viability between H_2O_2 treatment and SS only at any concentration used, except from 500-1,000 µM, which resulted in a dramatic reduction of cell survival (one-way ANOVA, H_2O_2 effect, $F_{7,132} = 34.38$, $P < 0.0001$; Dunnett's *post hoc* test, 500 and 1000 µM: $P < 0.0001$; $n = 3$ per group; Figure 5.3.2). This increase in cell death at higher H_2O_2 concentrations has been already reported in 661W cells (Mackey *et al.*, 2008).

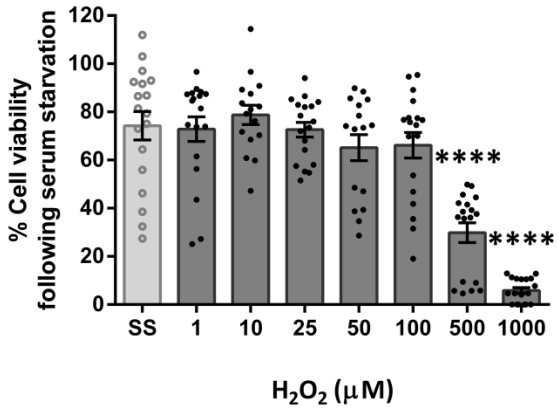


Figure 5.3.2. H_2O_2 addition has the same effect on cell viability as SS in 661W cells. Dose-response curve measuring cell viability of 661W cells treated with increasing concentrations of hydrogen peroxide (H_2O_2) solution following serum starvation (Dunnett's post hoc test, comparing all concentrations to the SS only control). Asterisks indicate significant difference (**** $P < 0.0001$).

Once confirmed that SS and H_2O_2 have the same effect on 661W cells, we sought to investigate whether Norgestrel-mediated protection was the same in both cases. Thus, cells were treated with 20 µM Norgestrel or vehicle with the addition or not of 1 µM H_2O_2 in serum-free media for 24 h, and cell viability was measured by MTS assay. Protection induced by Norgestrel compared with the vehicle control with or without the addition of H_2O_2 presented similar values in 661W cells (independent samples *t*-test, SS: $t_{42} = 3.537$, $P = 0.001$; SS+H_2O_2: $t_{30} = 2.695$, $P = 0.0114$; n = 3-4 per group; Figure 5.3.3).

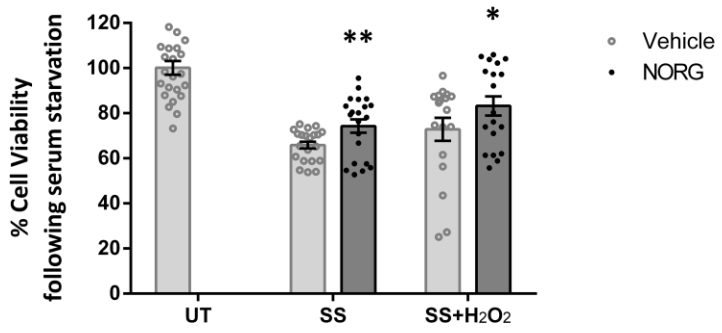


Figure 5.3.3. SS and H_2O_2 present similar effects in Norgestrel-mediated protection on 661W cells. Changes in cell viability were measured by MTS assay and graphed as a percentage of 100% viable UT control. Norgestrel-mediated protection was similar in the presence or absence of H_2O_2 (*t*-test comparing individual treatments). Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$).

In order to verify H_2O_2 addition had no different effects as SS on 661W photoreceptor cells, we use β-actin to stain the cytoskeleton and therefore, to study changes in cellular morphology by immunofluorescence (Figure 5.3.4). Untreated cells (UT) showed an expanded cytoplasm which was widely connected with surrounding cells, whilst cells of both vehicle controls (SS and SS+H_2O_2) presented a retracted cytoplasm and affected cell-cell connections (white arrows). Norgestrel partially restored UT morphology.

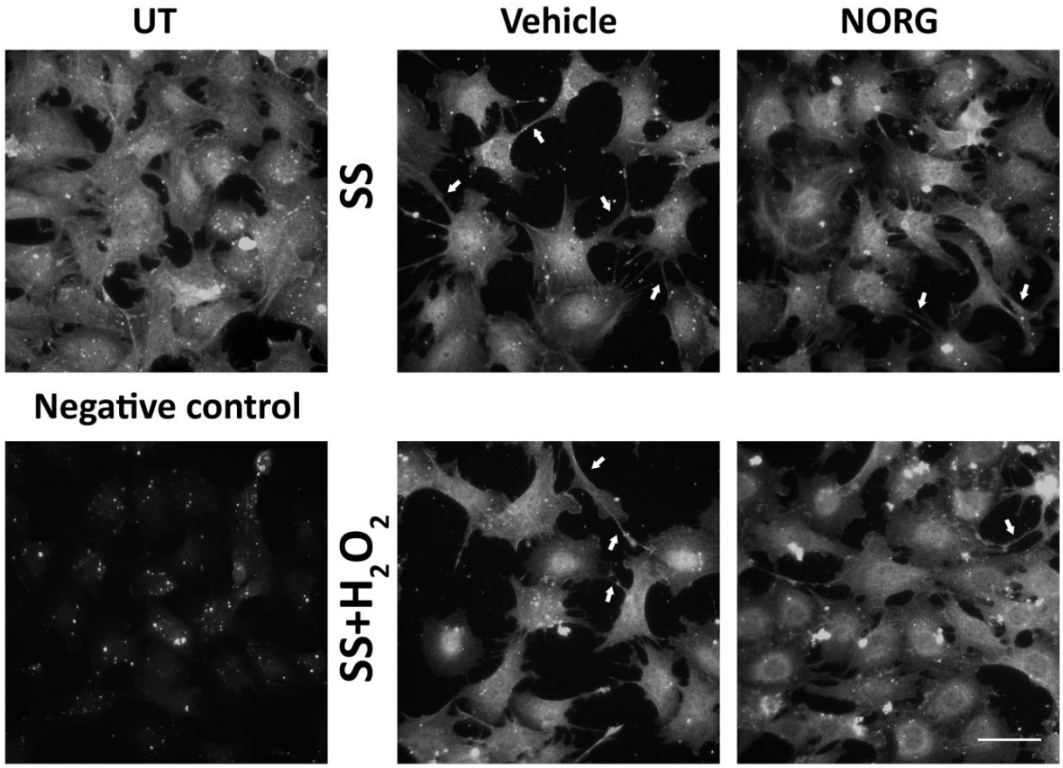


Figure 5.3.4. SS and H_2O_2 present similar effects on 661W cells morphology. Fluorescent microscopic images showed that both SS and H_2O_2 addition retracted the cytoplasm and cell-cell connections (white arrows) of 661W cells. Norgestrel partially rescued untreated (UT) morphology in both cases. Secondary antibody only was added as negative control. Scale bar 30 µm.

According with the results obtained, we decided to use SS as a valid model to study oxidative damage, given that exogenous addition of H_2O_2, a well-known oxidative molecule, had the same effect on the 661W cone photoreceptor-like cell line.

5.3.2. Norgestrel decreases ROS in order to enhance cell survival in long term stressed 661W cells

In addition to the above results, SS has been demonstrated to produce ROS as a consequence of the lack of growth factors support (Gómez-Vicente *et al.*, 2005; Mackey *et al.*, 2008; Groeger *et al.*, 2009a; Kuznetsov *et al.*, 2011; Li *et al.*, 2013; Ruiz-Lopez *et al.*, 2017) and is therefore, a valid model to study photoreceptors cell death. In chapters 3 and 4, we have shown how Norgestrel is able to up-regulate ROS levels at early time points under stress in order to enhance cell survival in both 661W cells and rd10 mice. Nevertheless, we also demonstrated that Norgestrel reduces ROS levels at later time points in the balb/c induced light damage mouse model (Byrne *et al.*, 2016b). However, this response has not been demonstrated in 661W cells.

In the current study, cells were treated with 20 μM Norgestrel or vehicle (DMSO) in serum-free media for 24 h and levels of ROS were studied by flow cytometry using the probe DCFDA. Norgestrel treatment decreased intracellular ROS levels produced by the oxidative stress induced by serum deprivation (independent samples *t*-test, $t_{30} = 11.56$, $P < 0.0001$; n = 4-5 per group; Figure 5.3.5A). To investigate the effect of reducing ROS levels on cell survival, the MTS assay was used to analyse cellular viability and proliferation with Norgestrel at 24 h following SS. Norgestrel significantly increased cell survival in stressed 661W cells (independent samples *t*-test, $t_{42} = 3.537$, $P = 0.001$; $n = 4$ per group; Figure 5.3.5B). This increase in the survival coinciding with a decrease in the ROS production suggests that Norgestrel enhances cellular survival by reducing the damaging ROS produced by a long exposure to stress in 661W cells.

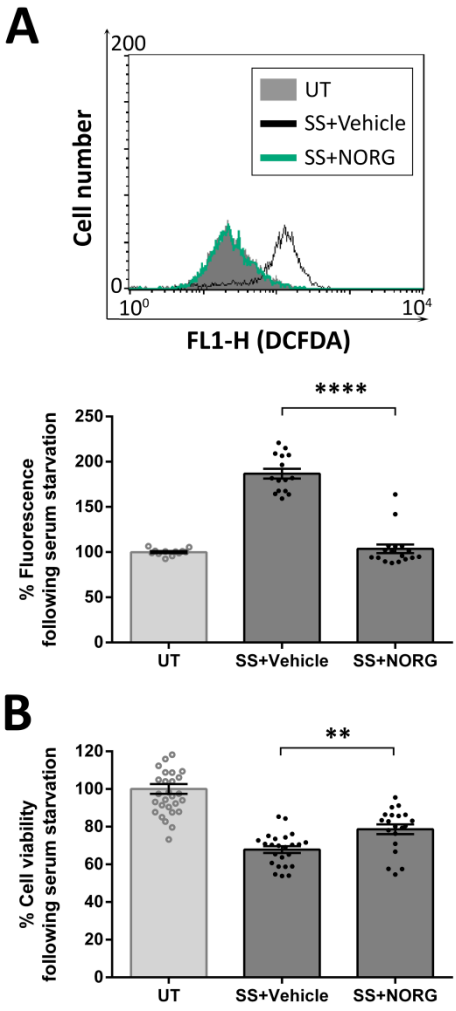


Figure 5.3.5. Norgestrel decreases damaging ROS levels in stressed 661W cells. (A) DCFDA probe was used to detect ROS levels by flow cytometry and plotted on a histogram. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual treatments). ROS levels produced by serum starvation (SS) were abolished by Norgestrel at 24 h. **(B)** Changes in cell viability were measured by the MTS assay and graphed as a percentage of 100% viable UT control (*t*-test comparing individual treatments). Norgestrel induced cellular survival at 24 h compared with the vehicle. Asterisks indicate significant difference (** $P < 0.01$, **** $P < 0.0001$).

We subsequently repeated the experiment using 48 h serum deprivation in order to investigate whether Norgestrel effects last over time. By means of flow cytometry analyses, Norgestrel treatment significantly decreased intracellular ROS levels at 48 h (independent samples *t*-test, $t_{31} = 2.483$, $P = 0.0186$; $n = 4$-5 per group; Figure 5.3.6A), although this reduction was smaller than the decrease seen at 24 h (Figure 5.3.5A). In contrast with results

obtained at 24 h, Norgestrel was no longer protective in stressed 661W cells at 48 h (independent samples *t*-test, $t_{46} = 1.364$, $P = 0.1792$; $n = 4$ per group; Figure 5.3.6B).

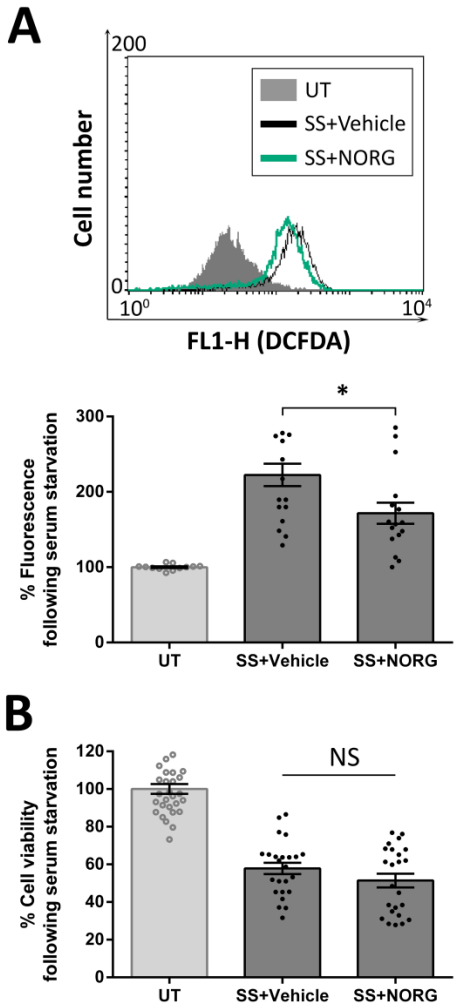


Figure 5.3.6. Norgestrel cannot longer protect 661W cells following 48 h following SS. (A) DCFDA probe was used to detect ROS levels by flow cytometry and plotted on a histogram. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed compared to UT cells (*t*-test comparing individual treatments). Norgestrel significantly reduced ROS levels produced by serum starvation (SS) at 48 h compared with the vehicle. **(B)** Changes in cell viability were measured by the MTS assay and graphed as a percentage of 100% viable UT control (*t*-test comparing individual treatments). Norgestrel was no longer protective at 48 h following SS in 661W cells. Asterisks indicate significant difference (NS not significant, * $P < 0.05$).

These results suggest that the exposure of 661W cells to stress for 48 h cause an irreparable damage that Norgestrel is not able to counteract. Thus, all subsequent experiments conducted in the 661W cell line were performed using 24 h serum withdrawal.

5.3.3. Norgestrel does not modulate NOX-mediated ROS production in long term stressed 661W cells

Under physiological conditions, cells have two main mechanisms to maintain a reduction-oxidation (redox) homeostasis and avoid oxidative damage: decreasing ROS production and increasing the amount of scavenging enzymes. NOX proteins are implicated in endogenous ROS production. The only known function of NOX family members is as ROS generators within the cell (Bedard & Krause, 2007; Finkel, 2011; Moloney & Cotter, 2017). Despite the fact that NOX isoforms have been demonstrated to participate in survival signalling in 661W cells at early time points after serum starvation (Groeger *et al.*, 2009), these proteins have also been related with the worsening of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Ma *et al.*, 2017) and with cone cell death in RP (Usui, Oveson, *et al.*, 2009). We also have demonstrated that Norgestrel utilises NOX4 to increase ROS levels to act as signalling molecules at early time points (Chapter 4). However, the role of these proteins following Norgestrel treatment after a longer exposure of stress has not been elucidated yet.

Given that only NOX2 and NOX4 are expressed in 661W cells (Groeger *et al.*, 2009), mRNA levels of both proteins were analysed using RT-qPCR. Neither NOX2 nor NOX4 expression were affected by Norgestrel treatment at 24 h following SS (independent samples *t*-test, NOX2: $t_{14} = 0.01506$, $P = 0.9882$; NOX4: $t_{16} = 0.6402$, $P = 0.5311$; $n = 3$ per group; Figure 5.3.7A). We subsequently studied protein levels by western blotting, confirming that there were no changes in either NOX isoform following serum deprivation and Norgestrel treatment for 24 h (independent samples *t*-test, NOX2: $t_4 = 0.2094$, $P = 0.8444$; NOX4: $t_4 = 0.4322$, $P = 0.6879$; $n = 3$ per group; Figure 5.3.7B). These results suggest that Norgestrel does not alter NOX activity as a mechanism to reduce intracellular ROS levels in 661W cells.

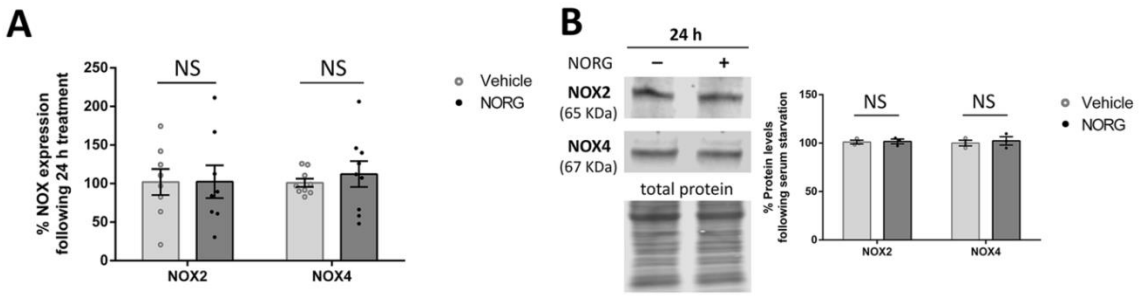


Figure 5.3.7. Norgestrel does not decrease ROS production at 24 h in serum-starved 661W cells. (A) NOX2 and NOX4 mRNA levels were measured by RT-qPCR in stressed 661W cells following Norgestrel treatment (*t*-test comparing individual treatments). Neither NOX2 nor NOX4 expression were changed by Norgestrel at 24 h following serum starvation. **(B)** Western blotting confirmed no changes in NOX2 and NOX4 protein levels (*t*-test comparing individual treatments). Equal loading of protein was demonstrated by REVERT total protein stain. Blots are representative of *n*=3. Asterisks indicate significant difference (NS not significant).

5.3.4. Norgestrel increases the expression of pNrf2 and SOD2, but not SOD1, in long term serum-starved 661W cells

As presented in figure 5.3.7, Norgestrel does not combat ROS levels through a decrease in ROS production. Thus, we next investigated a second mechanism known to decrease intracellular ROS levels. Given that oxidative stress contributes to many neurological diseases including RP, cells have integrated antioxidant systems, which consist of enzymatic and non-enzymatic antioxidants to maintain redox homeostasis and counteract the harmful effects of ROS (Birben *et al.*, 2012; Holmström & Finkel, 2014; Moloney & Cotter, 2017). This system is very effective but it can become overwhelmed under pathological conditions, leading to the progression of many diseases (Birben *et al.*, 2012; Ray *et al.*, 2012; Pisoschi & Pop, 2015) as happens during cone cell death in RP (Usui, Oveson, *et al.*, 2009).

This antioxidant machinery includes glutathione, flavonoids and vitamins (A, C and E) as non-enzymatic antioxidants and different enzymes including SODs, glutathione peroxidases and catalases (Poprac *et al.*, 2017). Although these enzymes are expressed in all cells, they are subject to regulation by transcription factors such as the nuclear factor kappa B (NF-κB)

or Nrf2 (Ray *et al.*, 2012; Xiong *et al.*, 2015; Marengo *et al.*, 2016). Interestingly, some of those components of the antioxidant machinery have been shown to be up-regulated by Norgestrel treatment in the balb/c induced light damage model, including the transcription factor Nrf2 and its antioxidant effector SOD2 (Byrne *et al.*, 2016b). Hence, expression levels of SOD proteins and Nrf2 were analysed by RT-qPCR. Norgestrel significantly increased SOD2 but not SOD1 or Nrf2 mRNA levels compared with the vehicle control (independent samples *t*-test, SOD1: $t_{16} = 0.7189$, $P = 0.4826$; SOD2: $t_{15} = 2.453$, $P = 0.0269$; Nrf2: $t_{14} = 1.408$, $P = 0.1827$; $n = 3$ per group; Figure 5.3.8A).

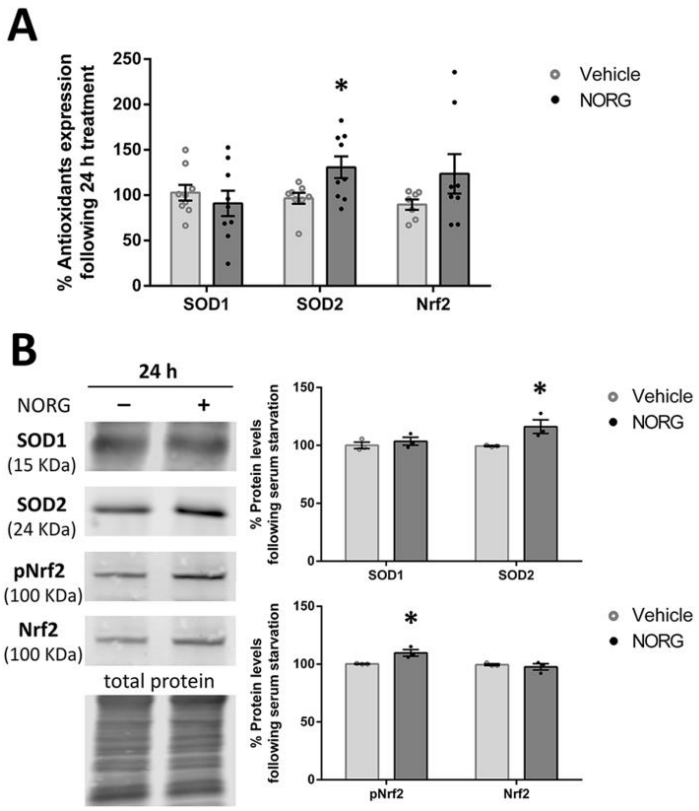


Figure 5.3.8. Norgestrel utilises antioxidants to reduce ROS levels in stressed 661W cells. (A) SOD1, SOD2 and Nrf2 mRNA levels were measured by RT-qPCR in serum-starved 661W treated with Norgestrel for 24 h (*t*-test comparing individual treatments). SOD2 expression was significantly increased by Norgestrel. **(B)** Protein levels were studied by western blotting (*t*-test comparing individual treatments). Norgestrel produced a significant increase in SOD2 and the phosphorylated form of Nrf2 (pNrf2) in 661W following 24 h serum starvation. Equal loading of protein was demonstrated by REVERT total protein stain. Blots are representative of n=3. Asterisks indicate significant difference (* $P < 0.05$).

Protein levels were subsequently studied by western blotting. We confirmed the up-regulation of SOD2 with Norgestrel treatment. We also found that phosphorylation levels at serine 40 (S40) of Nrf2 (pNrf2) were significantly increased at 24 h following serum starvation (independent samples *t*-test, SOD1: $t_4 = 0.7924$, $P = 0.4725$; SOD2: $t_4 = 2.845$, $P = 0.0466$; pNrf2: $t_4 = 3.011$, $P = 0.0395$; Nrf2: $t_4 = 0.6478$, $P = 0.5524$; $n = 3$ per group; Figure 5.3.8B). All together, we suggest that Norgestrel reduces intracellular ROS levels by

increasing antioxidant defences rather than by decreasing ROS production in stressed 661W cells. These results are not surprising, given that SOD enzymes have been documented to be up-regulated by progesterone treatment (Unfer *et al.*, 2006, 2015).

5.3.5. Norgestrel decreases ROS in order to enhance cell survival in long term stressed photoreceptors *ex vivo*

Above results in 661W cells demonstrate that Norgestrel acts as an antioxidant, decreasing ROS levels following 24 h serum deprivation. Therefore, we next sought to validate our results in photoreceptors using retinas from the rd10 model of RP. Retinal explants were treated with either 20 µM Norgestrel or vehicle (DMSO) for 24 h and analysed for changes in photoreceptor ROS production through the preparation of single-cell suspensions to be used for FACS analysis (Byrne *et al.*, 2016; Ruiz-Lopez *et al.*, 2017). P15 rd10 retinas were used given that photoreceptors have initiated cell death mechanisms but photoreceptor loss is not yet apparent (Roche *et al.*, 2016). Photoreceptor populations were identified by the comparison between a P30 C57 wild-type retinal preparation to that of a P30 rd10, where all photoreceptors are absent in the latter, as previously described (Ruiz-Lopez *et al.*, 2017). Norgestrel was able to successfully reduce intracellular ROS production in the photoreceptors from rd10 retinas compared with those treated with the vehicle control, as indicated by flow cytometry (independent samples *t*-test, $t_{22} = 4.462$, $P = 0.0002$; $n = 4$ per group; Figure 5.3.9A).

In order to determine if this decrease in photoreceptor ROS levels is necessary for Norgestrel-mediated protection in rd10 explants, we used TUNEL assay to analyse cell death in the photoreceptor layer/outer nuclear layer (ONL). We found that Norgestrel significantly protected rd10 explants from photoreceptor cell death (independent samples *t*-test, $t_9 =$

4.570, P = 0.0013; n = 5-6 per group; Figure 5.3.9B). This suggests that Norgestrel reduces damaging ROS levels to enhance photoreceptor survival in rd10 retinal explants.

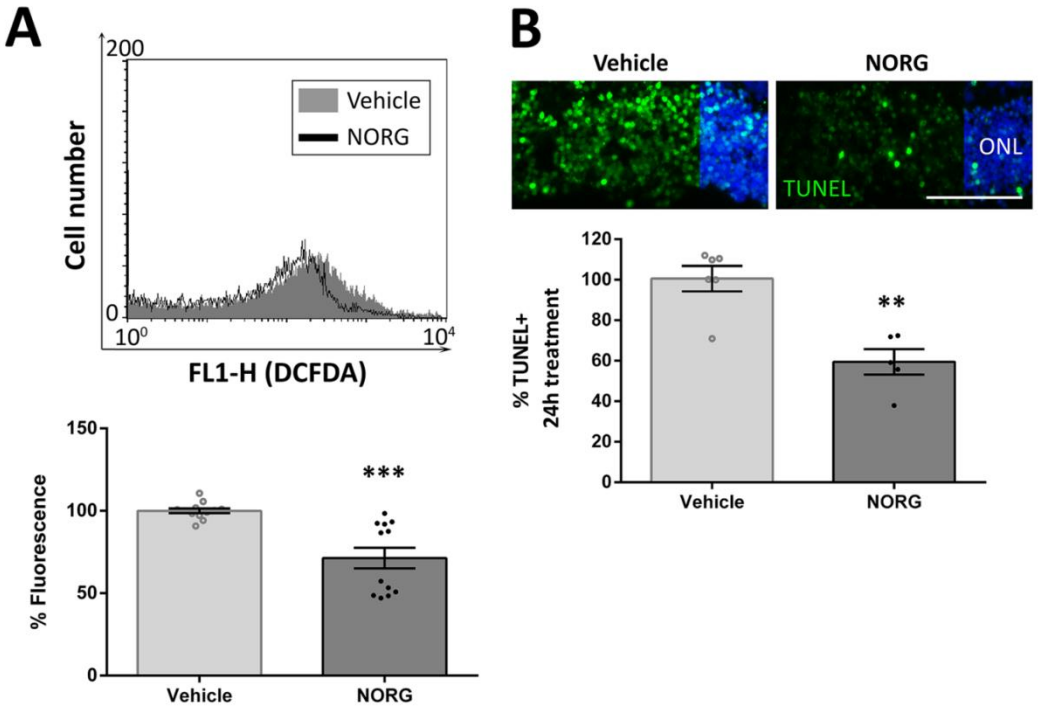


Figure 5.3.9. Norgestrel decreases damaging ROS levels in diseased photoreceptors *ex vivo*. (A) FACS analysis of photoreceptors using single cell suspensions from P15 rd10 retinas. *Y*-axis represents cell counts, *x*-axis (logarithmic scale) measures the level of fluorescence. A shift to the right along the *x*-axis represents an increase in DCFDA fluorescence and hence ROS levels. Geomeans were graphed and Norgestrel treatment was compared to its DMSO vehicle control (*t*-test). ROS production was significantly decreased by Norgestrel in rd10 photorreceptors. **(B)** Apoptosis of retinal cells in the outer nuclear layer (ONL) was detected by TUNEL (green) at 24 h. Norgestrel reduced TUNEL-positive staining in the central rd10 retina after 24 h treatment compared with the vehicle control (*t*-test). Images are representative of n=3. Scale bar 50 µm. Hoechst staining reveals cell nuclei. Asterisks indicate significant difference (** $P < 0.01$, *** $P < 0.001$).

5.3.6. Norgestrel decreases ROS levels by increasing the antioxidants SOD2 and pNrf2 *ex vivo*

Using the 661W cell line, we suggest that Norgestrel-mediated reduction of ROS levels occurs through an increase in antioxidants rather than by decreasing their production from NOX proteins (Figures 5.3.7 and 5.3.8). To substantiate our hypothesis, P15 rd10 retinal explants were treated with either Norgestrel or vehicle for 24 h and mRNA levels of NOX2 and NOX4 were studied by RT-qPCR. Due to the fact that NOX1 and NOX3 are not

expressed in the retina at this stage (Groeger *et al.*, 2009) and NOX5 is not found in rodents (Lambeth *et al.*, 2007), only NOX2 and NOX4 were analysed. Neither NOX2 nor NOX4 were affected by treatment with Norgestrel (independent samples *t*-test, NOX2: t_{10} = 0.06509, P = 0.9494; NOX4: t_{10} = 0.01837, P = 0.9857; n = 2 per group; Figure 5.3.10A). In order to confirm this, protein levels were analysed by western blotting. We confirmed that there were no changes in either NOX2 or NOX4 after Norgestrel treatment in rd10 retinal explants (independent samples *t*-test, NOX2: t_4 = 1.260, P = 0.2762; NOX4: t_4 = 0.6788, P = 0.5345; n = 3 per group; Figure 5.3.10B).

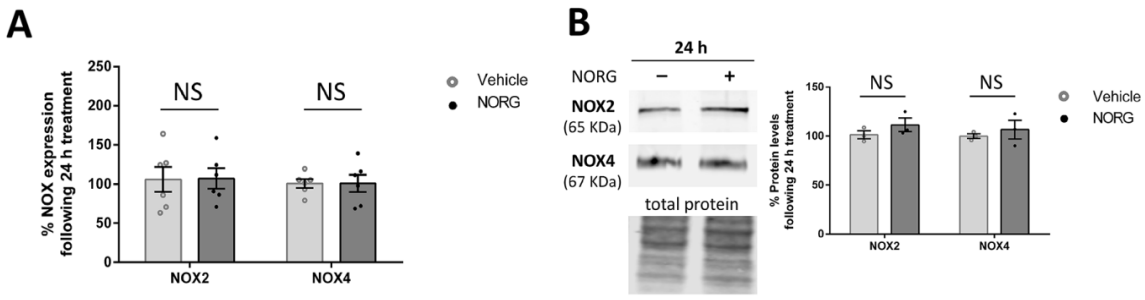


Figure 5.3.10. Norgestrel does not decrease ROS production at 24 h in rd10 retinal explants. NOX2 and NOX4 were studied by RT-qPCR **(A)** and western blotting **(B)** of rd10 retinal explants treated with Norgestrel for 24 h. Norgestrel did not change either of them (*t*-test comparing individual treatments). Equal loading of protein was demonstrated by REVERT total protein stain. Blots are representative of n=3. Asterisks indicate significant difference (NS not significant).

We subsequently sought to investigate whether Norgestrel treatment affects antioxidant expression in the rd10 retina. Thus, SOD1, SOD2 and Nrf2 were analysed by RT-qPCR. Norgestrel did not change SOD1 expression (independent samples *t*-test, SOD1: t_{10} = 0.2106, P = 0.8374; n = 2 per group; Figure 5.3.11A), whereas SOD2 and Nrf2 were increased at 24 h (SOD2: t_{10} = 4.402, P = 0.0013; Nrf2: t_{10} = 2.670, P = 0.0235; n = 2 per group). In order to corroborate these results, western blotting was used to study changes in protein levels. Levels of SOD1 were not affected by Norgestrel treatment at 24 h, supporting the mRNA results (independent samples *t*-test, SOD1: t_4 = 0.4912, P = 0.649; n = 3 per group; Figure 5.3.11B). In addition, SOD2 and the active form of Nrf2 (pNrf2) were significantly increased by Norgestrel as previously seen by RT-qPCR (SOD2: t_4 = 3.021, P

= 0.0391; pNrf2: $t_4 = 3.308$, $P = 0.0297$; Nrf2: $t_4 = 0.03685$, $P = 0.9724$; $n = 3$ per group). This supports our findings in 661Ws that Norgestrel increases the antioxidant defenses in order to reduce the production of ROS when they become toxic in photoreceptor cells.

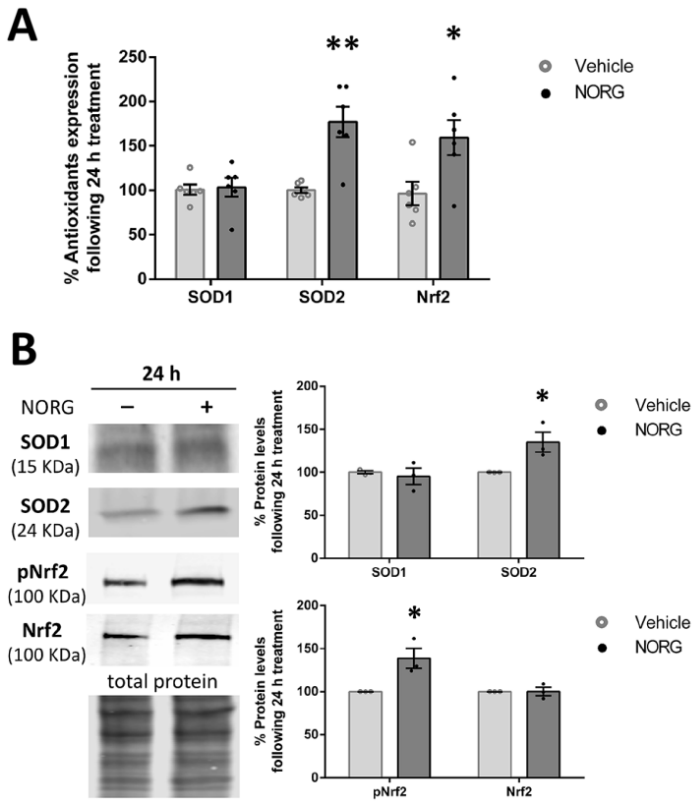


Figure 5.3.11. Norgestrel reduces damaging ROS by increasing antioxidants in rd10 retinal explants. SOD1, SOD2 and Nrf2 were subsequently analysed by RT-qPCR **(A)**. SOD2 and Nrf2 expression was up-regulated by Norgestrel at 24 h (*t*-test comparing individual treatments), which was confirmed by western blotting **(B)**. Both SOD2 and phosphorylated Nrf2 (pNrf2) protein levels were increased by Norgestrel treatment compared with the vehicle (*t*-test) Equal loading of protein was demonstrated by REVERT total protein stain. Blots are representative of *n*=3. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$).

5.3.7. Norgestrel decreases ROS levels by increasing the antioxidants SOD2 and pNrf2 *in vivo*

Norgestrel has been demonstrated to reduce ROS *in vivo* in a balb/c induced light damage mouse model (Byrne *et al.*, 2016b). Importantly, the current study confirms that this effect is evident in the inherited disease model *ex vivo*. We have demonstrated the role of antioxidants in this response, specifically SOD2 and the active form of Nrf2. To test our hypothesis *in vivo*, we administered a Norgestrel-supplemented diet to rd10 mice from P10. In the rd10 mouse, rod degeneration begins ~P15 and peaks at P25 (Chang *et al.*, 2002; Gargini *et al.*, 2007; Barhoum *et al.*, 2008; Samardzija *et al.*, 2012; Roche *et al.*, 2016). We have demonstrated significant neuroprotective effects of Norgestrel in the rd10 retina (Roche & Wyse-Jackson *et al.*, 2016; Roche *et al.*, 2017a, 2017b).

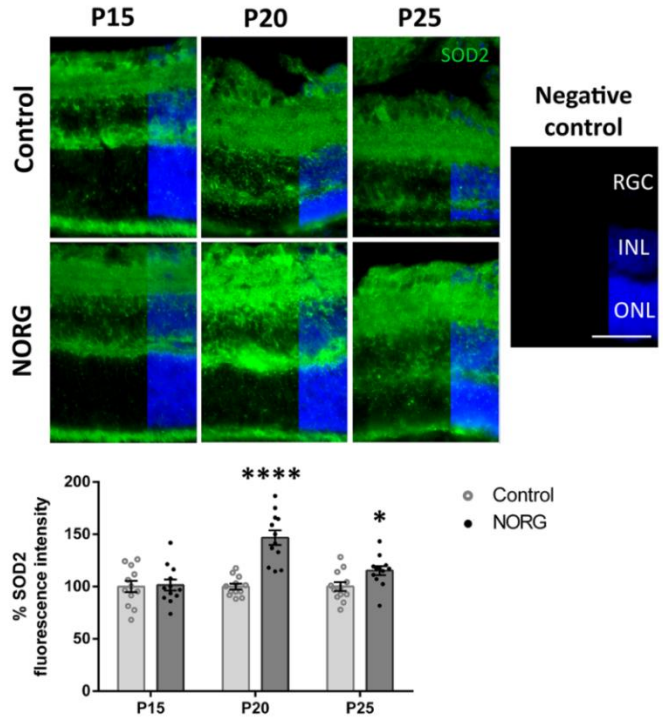


Figure 5.3.12. Norgestrel increases antioxidants SOD2 in the diseased retina *in vivo*. SOD2 protein levels were studied by immunofluorescence in rd10 retinas from P15 to P25 Norgestrel-fed or control-fed mice. SOD2 was significantly up-regulated by Norgestrel at P20 and P25 (*t*-test comparing individual treatments). Images are representative of n=3. Scale bar 50 µm. Hoechst staining reveals cell nuclei. RGC: retinal ganglion cells, INL: inner nuclear layer, ONL: outer nuclear layer. Asterisks indicate significant difference (* $P < 0.05$, **** $P < 0.0001$).

This is not the first time progesterone treatment has been associated with a reduction in oxidative stress (Webster *et al.*, 2015; Yousuf *et al.*, 2016) and with increased levels of antioxidants (Unfer *et al.*, 2015; Byrne, Ruiz-Lopez, *et al.*, 2016; Zhang, Wu, *et al.*, 2017). In order to establish whether the antioxidant proteins, SOD2 and Nrf2, play a role in the Norgestrel-mediated protection in the diseased rd10 retina, immunohistochemical analysis was performed at P15, P20 and P25. We found that Norgestrel significantly increased SOD2 and pNrf2 protein levels at P20 and P25 in the diseased retina (independent samples *t*-test, P20 SOD2: $t_{22} = 6.240$, $P < 0.0001$; P25 SOD2: $t_{22} = 2.533$, $P = 0.019$; $n = 3$ per group; Figure 5.3.12; P20 pNrf2: $t_{20} = 8.255$, $P < 0.0001$; P25 pNrf2: $t_{20} = 5.344$, $P = 0.019$; $n = 3$ per group; Figure 5.3.13).

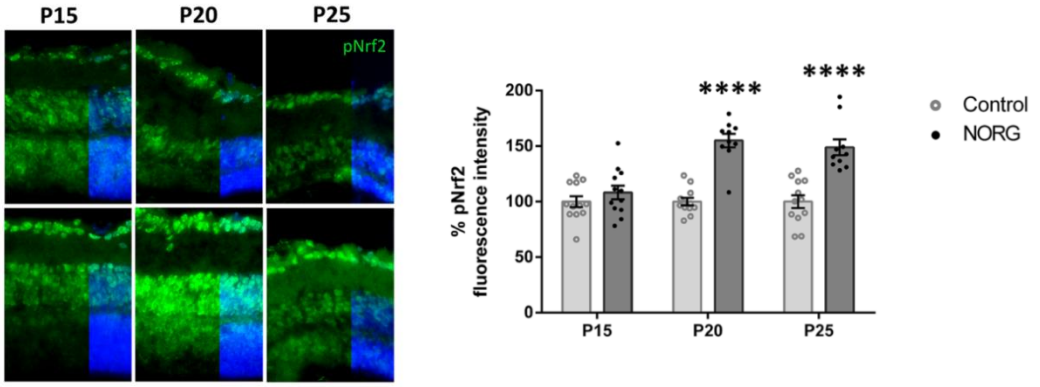


Figure 5.3.13. Norgestrel increases antioxidants SOD2 in the diseased retina *in vivo*. Active Nrf2 (pNrf2) protein levels were studied by immunofluorescence in rd10 retinas from P15 to P25 Norgestrel-fed or control-fed mice. pNrf2 was significantly up-regulated by Norgestrel at P20 and P25 (*t*-test comparing individual treatments). Images are representative of *n*=3. Hoechst staining reveals cell nuclei. RGC: retinal ganglion cells, INL: inner nuclear layer, ONL: outer nuclear layer. Asterisks indicate significant difference (**** $P < 0.0001$).

The progression of RP was clearly identified by measuring ONL thickness. Norgestrel enhanced the preservation of photoreceptors in the three time points studied compared with the control diet as previously shown (Roche & Wyse-Jackson *et al.*, 2016) (independent samples *t*-test, P15: $t_{22} = 0.06578$, $P = 0.9481$; P20: $t_{22} = 16.79$, $P < 0.0001$; P25: $t_{22} = 16.02$, $P < 0.0001$; $n = 3$ per group; Figure 5.3.14). Norgestrel-mediated protection through the preservation of the ONL became clearer when RP progression was obvious from P20 in the rd10 control-fed mice.

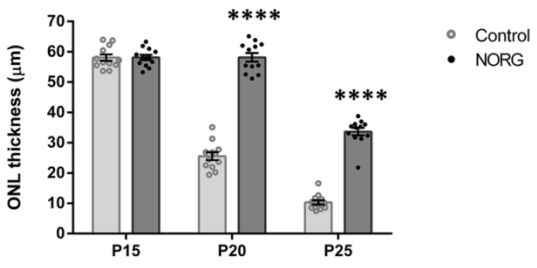


Figure 5.3.14. Norgestrel delays photoreceptor degeneration in rd10 mice. Photoreceptor layer/outer nuclear layer (ONL) thickness was measured and represented in a histogram. Norgestrel delays photoreceptor cell death and preserves ONL thickness in rd10 retinas (*t*-test comparing individual treatments). Asterisks indicate significant difference (**** $P < 0.0001$).

Norgestrel therefore, acts through the modulation of antioxidants in order to reduce harmful ROS levels in the rd10 retina *in vivo*. These results are supported by other *in vivo* studies, which have shown similar findings. In 2008, Lu *et al.* demonstrated that progesterone treatment offers retinal protection against ischemia-reperfusion injury by increasing SOD activity in rats (Lu *et al.*, 2008).

5.4. Discussion

Previous studies from our laboratory have demonstrated that Norgestrel stimulates ROS production in order to enhance cell survival at 30 min following serum starvation (Ruiz-Lopez *et al.*, 2017). This response is just one among a great variety of mechanisms Norgestrel is able to produce in *in vitro, in vivo* and *ex vivo* models (Doonan *et al.*, 2011; Byrne *et al.*, 2016a; 2016b; Roche & Wyse-Jackson *et al.*, 2016; Wyse Jackson & Cotter, 2016; Wyse Jackson *et al.*, 2016a; 2016b; Roche *et al.*, 2017a; 2017b).

Low or moderate levels of ROS have been discovered to constitute an important part of signalling pathways within the cell during the first minutes after any cell damage (Remacle *et al.*, 1995; Rhee, 2006; D'Autréaux & Toledano, 2007; Mackey *et al.*, 2008; Finkel, 2011; Ray *et al.*, 2012; Moloney & Cotter, 2017; Hashad *et al.*, 2018). Nevertheless, an increase in the amount of ROS present in the cell or a longer exposure to them can cause the cell to die (Yakes & Houten, 1997; Kim *et al.*, 2015; Fam *et al.*, 2018). This disruption of the redox homeostasis is known as 'oxidative stress'. Shokolenko *et al.* demonstrated that oxidative stress results in the degradation of mitochondrial DNA (Shokolenko *et al.*, 2009), which is one of the main causes of mutations and their subsequent effects, such as aging or some diseases (Fam *et al.*, 2018). In the current study, hydrogen peroxide, together with serum starvation, was used in order to find a valid model to study oxidative stress in the 661W cone photoreceptor cell line. Despite the fact that this molecule has been shown to be toxic after a long exposure and induce oxidative stress (Wang *et al.*, 2018), we found that its effects are very similar to serum withdrawal itself in 661W cells (Figure 5.3.1-5.3.4) and therefore, serum deprivation represents a valid model of oxidative stress.

Fortunately, cells house defence mechanisms which involve enzymatic antioxidants to counteract the damaging effects of ROS (Cadenas, 1997; Benzie, 2000; Valko *et al.*, 2007; Pisoschi & Pop, 2015; Moloney & Cotter, 2017).

However, this system cannot cope with sustained high levels of ROS. Diseases such as RP are characterised by an increase in the oxidative stress when rods first die, resulting in the subsequent death of the other type of photoreceptors: the cones (Cuenca *et al.*, 2014; Elachouri *et al.*, 2015; Campochiaro & Mir, 2017). For this reason, several therapeutic strategies target antioxidant machinery or incorporate exogenous antioxidant addition in order to reduce oxidative stress and enhance survival (Komeima *et al.*, 2006, 2007; Cuenca *et al.*, 2014; Taherian *et al.*, 2014; Tao *et al.*, 2016; Poprac *et al.*, 2017). Tao *et al.* hypothesised that exogenous addition of antioxidants might be useful in the treatment of RP when endogenous antioxidant machinery becomes insuficient for maintaining retinal homeostasis or optimal visual function. They suggest that such approach not only benefits photoreceptors, but also helps subjacent cells types to maintain visual signal transduction (Tao *et al.*, 2016). The molecule proposed to accomplish such function is hydrogen given its low molecular weight and lipid solubility to easily penetrate the blood-retina barrier. However, hydrogen therapy needs to be optimised to help RP patients. Even though not all the clinical trials employing antioxidant supplementation have successfully worked, antioxidant therapies are still very promising. Some of the possible explanations regarding their failure might be related with finding an optimal dose, the use of several antioxidants to work in combination or the stage of the disease in which the therapy starts (Poprac *et al.*, 2017). For these reasons, a compound that could stimulate endogenous intracellular antioxidant mechanisms, rather than exogenous addition, would be ideal given that cells could autoregulate these pathways.

We show in the current study that Norgestrel enhances cell survival through the decrease of damaging ROS produced at 24 h following serum starvation in 661W photoreceptor cells (Figure 5.3.5). However, we found that Norgestrel is no longer protective at 48 h following SS (Figure 5.3.6). We hypothesise that the stress suffered at this time point is so severe that Norgestrel is not able to face such damage. Studies on other cell lines confirm this hypothesis (Tavaluc *et al.*, 2007; Chen *et al.*, 2012). In fact, Chen *et al.* also described a remarkable decrease in cell survival upon 48 h SS (Chen *et al.*, 2012). As previously mentioned, the reduction in ROS levels can be either the result of a decrease in the production by intracellular sources, or because of an increase in the antioxidant defence (Moloney & Cotter, 2017). In this study, we have shown that Norgestrel does not change levels of NOX proteins (Figure 5.3.7), which are known to be one of the major generators of ROS molecules within the cell. These proteins were of interest in this study due to the fact that they have been associated with cone cell death occurring in RP (Usui *et al.*, 2009; Zeng *et al.*, 2014). Usui *et al.* proved that NOX-mediated ROS production, and not other sources, is responsible of such cell death in the rd1 mouse model of RP.

On the other hand, Norgestrel was able to increase levels of the antioxidant SOD2 and phosphorylation levels of the transcription factor Nrf2 (pNrf2) in order to reduce ROS produced by suppression of trophic supplementation in 661W photoreceptor-like cells (Figure 5.3.8). In fact, Chen *et al.* demonstrated in 2017 that exposure to blue light for 24 h increases Nrf2 levels in 661W cells (Chen *et al.*, 2017). Our findings were more profound in retinal explants from rd10 mice (Figure 5.3.9-5.3.11) and in retinas of rd10 mice fed with a Norgestrel-supplemented diet (Figure 5.3.12-5.3.14), strengthening our hypothesis that Norgestrel utilises antioxidants to decrease harmful ROS in the diseased retina. Moreover, increased levels of SOD2 but not SOD1 have been observed in stressed 661W cells and rd10 retinal explants treated with another neuroprotective compound, Rosiglitazone, during 24 h

(Doonan *et al.*, 2009). Our results reinforce the use of 661W cells and retinal explants as valid approaches to study photoreceptor degeneration occurring in RP.

Nrf2 is a basic-leucine-zipper protein that once activated by phosphorylation acts as a transcription factor in the nucleus, modulating the expression of different genes, including antioxidant enzymes such as SOD proteins (Gwarzo, 2009; Kaspar *et al.*, 2009; Niture *et al.*, 2013). Thus, Nrf2, which is ubiquitously expressed in a wide range of tissues and cell types, serves as a sensor of oxidative stress in the cell and is regarded as a critical component of the antioxidant defence (Dhakshinamoorthy *et al.*, 2001; Jaiswal, 2004). Several studies support the idea that Nrf2 plays an important role in cell survival (Lee, Calkins, *et al.*, 2003; Manandhar *et al.*, 2007; Dong *et al.*, 2008; Park *et al.*, 2018). In addition, Nrf2 has been linked with neuroprotection in neurodegenerative diseases (Chen *et al.*, 2009; Dinkova-Kostova *et al.*, 2018) and protection in the eye (Himori *et al.*, 2013; Xu *et al.*, 2014; Xiong *et al.*, 2015; Nakagami, 2016) as a result of progesterone treatment (Zhang *et al.*, 2017). Indeed, Norgestrel has been demonstrated to modulate activation of Nrf2 to reduce damaging ROS in an induced light damage mouse model (Byrne *et al.*, 2016b). Therefore, Norgestrel-mediated activation of this important cellular component of the protective response against oxidative damage in the rd10 retina is not surprising.

SOD metalloproteins, which contain cooper, zinc or manganese in their catalytic centre, are the most important antioxidants in the defence system against ROS (Fukai & Ushio-Fukai, 2011). CuZn-SOD (SOD1) and Mn-SOD (SOD2) are located in the cytoplasm and mitochondria, respectively (Zelko *et al.*, 2002), and are key effectors of Nrf2. They were of interest in this study given that there is evidence in the literature showing that they can be up-regulated by progesterone (Unfer *et al.*, 2006, 2015; Byrne *et al*,. 2016b). Unfer *et al.* demonstrated not only that progesterone treatment prevented the decrease in blood SOD1

and SOD2 levels that occurs in postmenopausal women, but also an increase in both enzymes activity to increase antioxidant capacity (Unfer *et al.*, 2006, 2015).

In summary, this study presents an important function in the Norgestrel-mediated neuroprotection in the diseased retina through the utilization of antioxidants to reduce damaging ROS (Fig. 5.4.1).

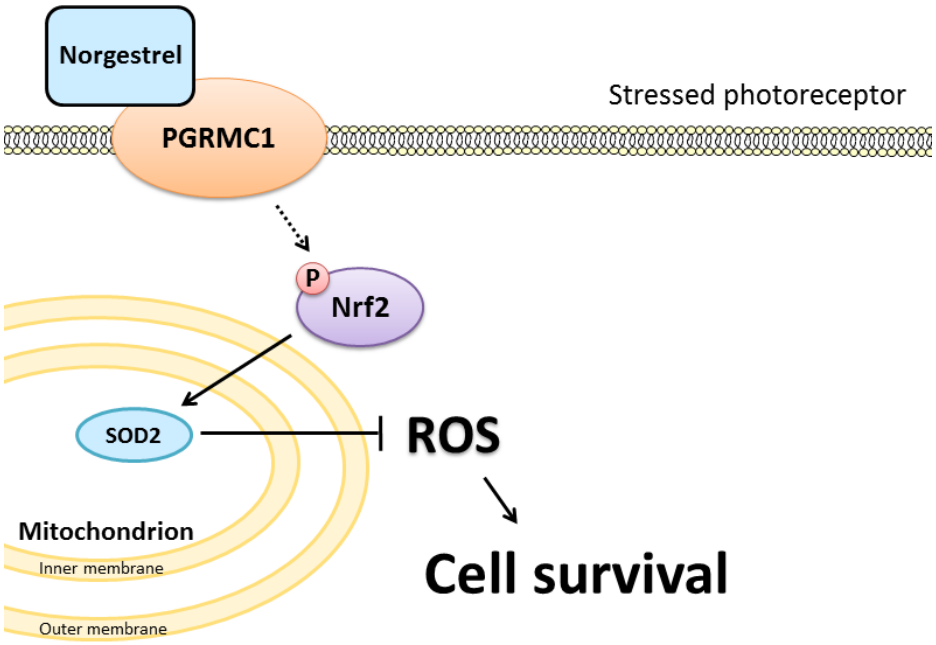


Figure 5.4.1. Proposed pathway of Norgestrel-mediated response in diseased photoreceptors. It has been previously shown that Norgestrel acts through the progesterone receptor membrane complex I (PGRMC1) to modulate ROS signalling (Ruiz-Lopez *et al.*, 2017). This schematic represents how Norgestrel induces Nrf2 phosphorylation (pNrf2) and increases mitochondrial SOD2 to reduce intracellular ROS levels and promote neuroprotection in diseased photoreceptor cells.

Chapter 6: General Discussion

The study of molecules such as reactive oxygen species (ROS) has completely changed in the last decades. Traditionally believed to induce cellular damage, attempts have been made to find mechanisms to reduce their production in order to prevent cell death. Throughout this thesis, we have shown a dual nature of these molecules, being also able to participate as signalling messengers to enhance photoreceptor survival (Chapter 3 and 4). Their persistence during time convert ROS into toxic molecules that subsequently induce cell death (Chapter 5). Numerous studies from this group (Carmody *et al.*, 1999; Sanvicens *et al.*, 2004; Sanvicens & Cotter, 2006; England *et al.*, 2006; Carmody & Cotter, 2007; Mackey *et al.*, 2008; Groeger *et al.*, 2009a, 2009b; Bhatt *et al.*, 2010; Farrell *et al.*, 2011; Gough & Cotter, 2011; Groeger *et al.*, 2012; Stanicka *et al.*, 2015; Byrne *et al.*, 2016b; Moloney *et al.*, 2017a, 2017b) and others (Herrlich & Böhmer, 2000; Imlay, 2003; Lee *et al.*, 2003; Andreyev *et al.*, 2005; Rhee, 2006; Lee *et al.*, 2007; Bae *et al.*, 2011; Benitez-Alfonso *et al.*, 2011; Finkel, 2011; Burgoyne *et al.*, 2013; Schieber & Chandel, 2014; Brandes *et al.*, 2014b; Kim *et al.*, 2015; Reczek & Chandel, 2015; Battistelli *et al.*, 2016; Di Meo *et al.*, 2016; Antunes & Brito, 2017; Sies, 2017) have described this double nature of ROS so far.

This thesis highlights a novel and important property of Norgestrel: the modulation of intracellular ROS levels in retinal degeneration (Chapter 3, 4 and 5). This is the first time that Norgestrel has been demonstrated to utilise ROS as second messengers as part of its mechanism of action in photoreceptors (Chapter 3; Ruiz-Lopez *et al.*, 2017). Furthermore, we have also demonstrated that such increases in ROS levels at early time points are the consequence of the Norgestrel-mediated modulation of intracellular sources, as is the case of NOX4 (Chapter 4).

The importance of such findings relies on the fact that even though Norgestrel is an exogenous compound, its binding to the transmembrane progesterone receptor PGRMC1 (Figure 3.3.8) (Wyse Jackson *et al.*, 2016a; Ruiz-Lopez *et al.*, 2017) causes an intracellular signalling cascade that cells can easily autoregulate. Hence, despite both bFGF (Figure 3.3.9) and ROS (Figure 3.3.6), being up-regulated as the consequence of Norgestrel's effects, we believe their levels are most probably controlled by photoreceptors depending on the duration of the signalling response. According to this, we observed that bFGF and ROS levels go back to normal once the signalling response has finished.

Similarly, Norgestrel modulates NOX4 expression as the main source of pro-survival ROS in photoreceptor cells. We have shown that Norgestrel is no longer protective in the absence of NOX enzymes (Chapter 4). Other studies have also demonstrated that these proteins, especially NOX4, are important in the stimulation of survival mechanisms in a wide range of cell types (Abid *et al.*, 2007; Brown & Griendling, 2009; Groeger *et al.*, 2009a; Takac *et al.*, 2012; Brandes *et al.*, 2014b; Accetta *et al.*, 2016; Moon *et al.*, 2016; Prieto-Bermejo & Hernández-Hernández, 2017). Abid *et al.* demonstrated that NOX-derived ROS are required for the VEGF-mediated activation of the Akt pathway and subsequent proliferation of endothelial cells (Abid *et al.*, 2007). Moreover, ROS produced by NOX enzymes are thought to play a role in the vascular and inmune systems, hormone synthesis and the physiology of the brain as reviewed by Brown & Griendling (Brown & Griendling, 2009), Brandes *et al.* (Brandes *et al.*, 2014b) and Prieto-Bermejo & Hernández-Hernández (Prieto-Bermejo & Hernández-Hernández, 2017). This is not surprising given that the only known function of NOX proteins is the production of ROS (Bedard & Krause, 2007; Takac *et al.*, 2012; Brandes *et al.*, 2014a), suggesting they are easier to regulate than other intracellular sources.

Both mitochondria, the other major ROS generators, and COX proteins, which have been related with bFGF-mediated ROS response in 661W cells (Farrell *et al.*, 2011), were found not to play a role in the Norgestrel-mediated signalling response (Figure 4.3.16-4.3.19). The fact that NOX4 was responsible for such mission was not surprising (Figure 4.3.4-4.3.11) given that NOX4 appears to be constitutively active (von Löhneysen *et al.*, 2012). Our results therefore, strengthen our hypothesis that Norgestrel uses NOX4 to produce pro-survival quantities of ROS, provided that it might be a source that cells can effectively autoregulate.

Although this autoregulated system seems to be infallible, a prolonged exposure to stress, such as serum starvation, inevitably leads to cell death. We have widely discussed throughout this thesis that the presence of high amounts of ROS can be lethal (Chance, 1979; Gorman *et al.*, 1997; Curtin *et al.*, 2002; Rhee, 2006; Battistelli *et al.*, 2016). In fact, some types of cell death produced in diseases such as retinitis pigmentosa have been linked with oxidative damage (Shen *et al.*, 2005; Carmody & Cotter, 2007; Punzo *et al.*, 2012; Campochiaro *et al.*, 2015). Thus, an effective antioxidant defence machinery is required in order to balance ROS levels and avoid oxidative stress. This system has widely changed during the evolution of the atmosphere and living organisms. Due to a hydrogen-rich atmosphere, procaryotic organisms used hydrogen as an electron donor and released oxygen as a waste product. However, once atmospheric O_2 levels began to rise, more complex and compartmentalised eukaryotic forms appeared to use oxygen as a final electron acceptor. Consequently, antioxidant defence mechanisms evolved to combat oxidative damage produced by the toxic intermediates of oxygen, such as $O_2^{\bullet-}$ and OH• (Benzie, 2000). "Living with the risk of oxidative stress is a price that aerobic organisms must pay for more efficient bioenergetics" (quoted from Skulachev (Skulachev, 1996)).

Given that superoxide is the first free radical formed as the consequence of the partial reduction of oxygen, its elimination represents a major step in the antioxidant response. Such task is performed by superoxide dismutases (SODs), which therefore, constitute the first line of defence against ROS. CuZn-SOD (SOD1), Mn-SOD (SOD2) and SOD3 are the three isoforms of this family of proteins. SOD3 has not been considered in this thesis taking into account its extracellular location as mentioned in chapter 1. Overall, SOD1 (cytoplasmic) and SOD2 (mitochondrial) are responsible for the intracellular conversion of $O_2^{\bullet-}$ to H_2O_2, and SOD2, the most important member of the SOD family providing superoxide is mainly generated in the mitochondria (Murphy, 2009). These enzymes are regulated by transcription factors such as Nrf2 (Gwarzo, 2009; Kaspar *et al.*, 2009; Niture *et al.*, 2013). In 2009, Gwarzo showed a 50% reduction in the brain levels of SOD2 in the Nrf2 knockout mice, $Nrf2^{(-/-)}$ (Gwarzo, 2009), confirming the link between these proteins.

In 2016, we demonstrated that Norgestrel was able to increase the transcription factor Nrf2 and its antioxidant effector, SOD2, in order to reduce damaging ROS in the balb/c induced light damage mouse model (Byrne *et al.*, 2016b). Despite the fact that this mouse model is an invaluable tool to study cell death within the stressed retina, it is important to notice that the photoreceptor cell death occuring in the balb/c mouse is light-induced and therefore, is not a model of any genetic disease, including retinitis pigmentosa. In this context, we designed this study in order to elucidate such response in the genetic rd10 mouse model of RP. Interestingly, we could confirm such results, being the first time that Norgestrel's effects on harmful ROS levels have been studied in the diseased retina (Chapter 5). Norgestrel decreases the amount of ROS when they become pathological *in vitro* (Figure 5.3.5) and in the photoreceptors of the rd10 retina (Figure 5.3.9) through the up-regulation of Nrf2 and its antioxidant effector SOD2 as described before (Byrne *et al.*, 2016b).

These results are very promising in the treatment of late stages of RP, given that the majority of RP cases are detected when rods degeneration has already started and preservation of the remaining photoreceptor cells is essential. Taking into that account subsequent cone cell death happens due to oxidative damage (Shen *et al.*, 2005; Carmody & Cotter, 2007; Punzo *et al.*, 2012; Campochiaro *et al.*, 2015), the use of antioxidants as therapeutic molecules for retinitis pigmentosa is desirable. Numerous studies have demonstrated that the exogenous addition of natural and/or synthetic antioxidants successfully prevents cell death (Jarrett *et al.*, 2006; Komeima *et al.*, 2006, 2007; Valko *et al.*, 2007; Dong *et al.*, 2008; Trachootham *et al.*, 2008; Lu *et al.*, 2009; Usui *et al.*, 2009, 2011; Cuenca *et al.*, 2014). In fact, antioxidant supplementation is able to slow the progression of the disease in both the rd1 and the rd10 mice model of RP (Komeima *et al.*, 2006, 2007).

Unfortunately, the majority of these antioxidant strategies have proved inefficient (Nickel *et al.*, 2014; Sanz & Bernardi, 2016), although the reasons are not very clear. One explanation is that the exogenous addition of antioxidants in order to abolish the total production of ROS might be erroneous. The importance of moderate and controlled quantities of ROS acting as signalling molecules has been demonstrated throughout this thesis. We believe the total abolishment of such action would not only be inefficient, but also prejudicial for cells as suggested by other authors (Bickham & Smolen, 1994; Sheu *et al.*, 2006; Nickel *et al.*, 2014). Nickel *et al.* suggested that the blockage of NOX4-derived ROS might have detrimental results due to its protective roles, and proposed that an antioxidant therapy directed to specific sources, such as mitochondria, would improve this strategie (Nickel *et al.*, 2014). Therefore, mitochondria-targeted drugs are being developed for such purposes (Sheu *et al.*, 2006; McCarthy & Kenny, 2016), as is the case of MitoTEMPO (Ni *et al.*, 2016; Zhang *et al.*, 2017), which has been used in the chapter 4 of this thesis.

Nevertheless, some studies suggest that mitochondrial ROS can also participate in cell signalling (Dan Dunn *et al.*, 2015; Diebold & Chandel, 2016; Sanz & Bernardi, 2016). Interestingly, some authors have demonstrated the existence of a crosstalk between mitochondria and NOX proteins to produce pro-survival ROS (Kimura *et al.*, 2005; Daiber, 2010; Dikalov, 2011; Bordt & Polster, 2014; Dikalov *et al.*, 2014; Harel *et al.*, 2017; Kim *et al.*, 2017; Zinkevich *et al.*, 2017). This system represents a feed-forward vicious cycle of ROS production that might serve to amplify the signal within the cell as reviewed by Dikalov (Dikalov, 2011). If true, mitochondrial-targeted drugs might be inefficient as well.

We believe that a compound that could stimulate the intracellular antioxidant machinery when needed would be a more efficient approach. In this context, Norgestrel has been demonstrated to be a perfect candidate to do so. Rosiglitazone, another FDA-approved compound that has been mentioned in this thesis, also shows such ability through the up-regulation of the intracellular levels of the antioxidant SOD2 (Doonan *et al.*, 2009). Nonetheless, the level of neuroprotection of this compound was not as important as the one observed following Norgestrel treatment. We hypothesise that the reason for such difference relies on the broad spectrum of mechanisms that Norgestrel is able to modulate in order to consolidate a neuroprotective environment to enhance cell survival.

Despite the fact that progesterone has been used to regulate pro-survival pathways in the CNS (De Nicola, 1993; Joëls, 1997; Azcoitia *et al.*, 1999; McEwen, 1999; Schumacher *et al.*, 2000; Petersen *et al.*, 2013), retinal studies have failed to show beneficial effects of progesterone (Káldi & Berta, 2004). Doonan *et al.* however, achieved retinal neuroprotection using Norgestrel in the balb/c induced light damage mouse model and in the genetic rd10 mouse (Doonan *et al.*, 2011). This protection could be produced given that synthetic progestins, such as Norgestrel, are rapidly absorbed, have a longer half-life and maintain

stable levels in the blood in comparison to natural progesterone, which means that higher concentrations of Norgestrel last for longer in the body (Doonan & Cotter, 2012).

Since 2011, our laboratory has focussed on elucidating Norgestrel's mechanism of action. All this work has greatly improved since a Norgestrel-supplemented diet was developed by Testdiet (Middlesex, UK), which prevents adverse reactions of invasive techniques, such as intraperitoneal injections. The level of success of this non-invasive techinque in the rd10 retina has been demonstrated so far (Roche & Wyse-Jackson *et al.*, 2016; Roche *et al.*, 2017a, 2017b), being the first time that such a great protection has been seen in this model of RP.

Clearly, our understanding of how this progesterone-analogue works to prevent cell death is expanding. This thesis elucidates another important aspect of its mechanism of action, demonstrating that redox biology is another part of the multi-faceted action of Norgestrel within the diseased retina. Reactive oxygen species are both up- (Chapter 3 and 4) and down-regulated (Chapter 5) by Norgestrel according to the needs of photoreceptors. We believe these results, together with Norgestrel's ability to modulate neurotrophic factors expression (Doonan *et al.*, 2011; Byrne *et al.*, 2016a; Wyse Jackson & Cotter, 2016; Ruiz-Lopez *et al.*, 2017) and to regulate inflammation and gliosis (Roche & Wyse-Jackson *et al.*, 2016; Roche *et al.*, 2017a, 2017b), are the reasons why Norgestrel is a particularly interesting neuroprotectant.

Mutations in the *Pde6b* gene, which result in both the rd1 and rd10 mouse models, represent only a 4-5% of autosomal recesive RP cases as discussed in chapter 1. Other types of retinitis pigmentosa also display a rod-cone dystrophy caused by the first lost of rods and subsequent

death of cones, as is the case of autosomal dominant RP produced by mutations in the rhodopsin (*RHO*) gene (Athanasiou *et al.*, 2018). Since rhodopsin is also part of the phototransduction cascade and therefore, photoreceptors cell death might be produced in a similar way as the one seen in rd1 and rd10 mice, we believe that Norgestrel could also enhance neuroprotection in this and other types of RP. In fact, some therapies used in the treatment of RP produced by rhodopsin mutations include the use of neurotrophic factors (Mendes *et al.*, 2005), which have been demonstrated to be up-regulated by Norgestrel (Doonan *et al.*, 2011; Byrne *et al.*, 2016a; Wyse Jackson & Cotter, 2016; Ruiz-Lopez *et al.*, 2017).

Furthermore, other diseases in which inflammation, gliosis and reactive oxygen species are in part responsible for irreversible blindness (Jarrett *et al.*, 2008; Dogru *et al.*, 2009; Schrier & Falk, 2011; Kruk *et al.*, 2015), such as glaucoma and age-related macular degeneration (AMD), can also be considered for the study of the Norgestrel-mediated neuroprotection. Hormone therapy used for the patients with glaucoma has shown promising results (Nakazawa *et al.*, 2006; Kolomeyer & Zarbin, 2014), indicating that Norgestrel could be a suitable candidate to be taken into account. Thus, we hope to bring our work to the point that this treatment is ready for clinical trials, closer to patients suffering from retinal degeneration.

Chapter 7: Bibliography

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