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Pro-survival redox signalling in progesterone-mediated retinal neuroprotection

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Abstract

Retinitis pigmentosa (RP) is a group of hereditary retinal diseases, 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 hypothesized 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.

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). 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), a group of neurodegenerative diseases of the retina, apoptosis has also been related with a high production of ROS (Carmody & Cotter, 2000; Tao, 2016; Usui *et al.*, 2009). 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-

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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). 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). 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 basic fibroblast growth factor (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 *et al.*, 2016, 2017; Roche & Wyse-Jackson *et al.*, 2016, Wyse-Jackson & Cotter, 2016). 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 hypothesized 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.

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Materials and methods

Drugs, reagents, primers and antibodies

All reagents were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise stated.

Cell culture

The 661W retinal cone photoreceptor-like cell line was generously provided by Dr Muayyad Al-Ubaidi (University of Oklahoma, Norman, USA). Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, D6429), supplemented with 10% foetal bovine serum (FBS, F7524) and 1% penicillin/streptomycin (P0781). 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) or the equivalent dimethyl sulfoxide (DMSO, D2650) in complete (for untreated cells) or serum-free medium. In the case of antioxidant treatments, cells were pre-treated for 1 h with the superoxide scavenger Tiron (4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt, #172553) at a concentration 1mM or with 100 µM N-acetyl-L-cysteine (NAC, A7250) which is known to act both as a direct and indirect ROS scavenger since it is a precursor of reduced glutathione (Taherian *et al.*, 2014). In order to block PGRMC1, cells were pre-treated for 10 min with 1 µM AG-205 (A1487) previously optimized (Wyse Jackson *et al.*, 2016b), before Norgestrel treatment. DMSO, which has been widely used and verified not to affect the viability of cells (Hebling *et al.*, 2015) and H_2O (for Tiron and NAC) were used as vehicle controls. After incubation at indicated times, cells were washed in PBS and detached using Accutase solution (A6964).

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Detection of ROS and flow cytometry

Intracellular ROS production was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, #35845), a general oxidative stress indicator. This compound is converted to DCF which is detectable at FL-1 (530 nm) with excitation at 488 nm by flow cytometry. 50 µM DCFDA was added to cells and incubated for 30 min at 37°C before analysis on 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.

MTS assay for cell viability

A colorimetric tetrazolium (MTS) assay (G3580; Promega, Madison, WI, USA) was used to determine the number of viable cells. 2,000 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 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 control and all treatments were compared to 100% viable control. All samples were carried out in 6 replicates in a total of at least three independent experiments.

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Small interfering RNA (siRNA) silencing

661W cells (300,000) were seeded in a T75 flask the day before transfection. Both 25 nM FGF2 siRNA (GS14173, Qiagen) and AllStars Negative Control siRNA (Qiagen) were transfected and incubated for 48 h using HiPerFect Transfection Reagent (Qiagen). Mock transfected cells and cells treated with HiPerFect Transfection Reagent alone were included as a siRNA negative control. 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.

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

Total RNA isolation from 661W cells was performed using an RNeasy Mini Kit (Qiagen, West Sussex, UK) following manufacturer's protocol, including DNase treatment. 1 µg of cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen) to perform RT-qPCR with QuantiTect Primer Assays (β-Actin, QT00095242; Gapdh, QT00199388; Hprt, QT00166768; Fgf2, QT00128135) 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 normalized 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).

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% paraformaldehyde (PFA) for 20 min at room temperature (RT), followed by blocking for 30 min at RT with 5% donkey serum (containing 0.1%

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Triton X-100 for permeabilization). Coverslips were subsequently incubated with purified mouse anti-bFGF antibody (#610073; BD Biosciences Europe, Oxford, UK) at a dilution 1:250 in 5% donkey serum overnight at 4°C. After washes, conjugated secondary antibody (1:5000; Alexa Fluor 488 donkey anti-mouse, 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).

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$^{rd10/J}$) and C57BL/6 mice (C57) were used as controls. Mice were supplied by the Biological Services Unit, University College Cork and were humanely euthanized by cervical dislocation.

FACS analysis in retinal explants

Eyes were removed and retinas explanted as previously described (Wyse Jackson *et al.*, 2016a), using a total number of 4 mice per treatment. Retinal explants were treated with 20 μM Norgestrel or the equivalent DMSO (vehicle) at the time points indicated. In the case of treatment with an antioxidant, cells were treated with 1mM Tiron or H_2O (vehicle) for 1 h. Explants were then transferred to a trypsin-EDTA solution 0.25% (T4049) containing 50 μL deoxyribonuclease II from bovine spleen (10,000 units/ml; D8764) 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.

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TUNEL staining

Localization of dying cells within retinal explants was assessed with the terminal dUTP nick end-labelling (TUNEL) assay on fixed tissue as previously described (Roche *et al.*, 2016). Frozen retinal sections were permeabilized 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.

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 (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 (untreated, Scrambled). Differences were considered significant if $P < 0.05$ indicated on the graphs with asterisks (*).

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Results

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

To confirm that serum starvation produces ROS as previously reported (Mackey *et al.*, 2008), cells were incubated with 2',7'-dichlorofluorescin diacetate (DCFDA), one of the most commonly used probes for detecting intracellular ROS levels (Eruslanov & Kusmartsev, 2010; Karlsson *et al.*, 2010). Untreated 661W cells (UT) were used to gate the population analysed in all subsequent experiments (Fig. 1A). A significant increase in ROS was observed 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; Fig. 1B).

To establish whether this burst of ROS is beneficial to 661W cells, 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) (independent samples *t*-test, Tiron: $t_{16} = 7.313$, $P < 0.0001$; NAC: $t_{16} = 2.324$, $P = 0.0336$, $n = 3$ per group; Fig. 1C(i)). 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; Fig. 1C(ii)). 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: $P = 0.0411$).

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

Previous studies from this group have documented the protective effects of Norgestrel in the stressed 661W photoreceptor-like cell line (Doonan *et al.*, 2011; Wyse Jackson & Cotter, 2016). Here, we sought to understand the role that ROS play in the Norgestrel-mediated response. By means of flow

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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; Fig. 2A). The use of DMSO as vehicle also increases ROS levels in all time points compared with the UT cells (Fig. 2A), possibly through its anti-inflammatory effects. For this reason, these serum starved samples are not comparable with samples in Fig. 1B. 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; Fig. 2B), 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; Fig. 2C). 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.

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). To examine the effects of Norgestrel on this early pro-survival burst of ROS, 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; Fig. 3A) 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; Fig. 3B) but no difference in response was detected beyond this point. These

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data show that Norgestrel enhances the early pro-survival response of increased ROS levels in serum-starved 661W cells.

Norgestrel signals through progesterone receptor membrane component 1 (PGRMC1) to mediate redox signalling

Previous studies have shown that PGRMC1, a membrane-localised progesterone receptor, is responsible for the neuroprotective actions of Norgestrel (Wyse Jackson *et al.*, 2016a, 2016b). 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. 4) was abrogated when PGRMC1 was inhibited as detected by decreased DCFDA fluorescence intensity ($t_{16} = 6.185$, $P < 0.0001$; $n = 3$ per group), suggesting the importance of the receptor in this response.

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 (Farrell *et al.*, 2011). We therefore sought to establish if Norgestrel-induced increases in ROS were dependent on bFGF activity. Firstly, we show 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; Fig. 5A(i)). However, when treated with Norgestrel, 661W cells displayed increased expression of bFGF following 30 min of serum-starvation (independent samples *t*-test, $t_{21} = 2.844$, $P = 0.0097$; $n = 4$ per group; Fig. 5A(ii)). 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

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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; Fig. 5B(i)) and by immunofluorescence (Fig. 5B(ii)). 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, $t_{37} = 2.198$, $P = 0.0343$; $n = 5$ per group; Fig. 5C).

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

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 the rd10 model of retinitis pigmentosa (Roche *et al.*, 2016) 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 (Fig. 6A), we understood this population to represent photoreceptors. This method of gating rd10 photoreceptors has previously been published (Byrne *et al.*, 2016b). We subsequently evaluated the effects of Norgestrel on single cell suspensions from P15 rd10 retinas, a suitable timepoint 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 cell suspensions 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; Fig. 6B). 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*-

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test, 30 min: $t_{20} = 0.1706$, $P = 0.8663$; 1 h: $t_{18} = 3.211$, $P = 0.0068$; $n = 3$ per group; Fig. 6C). Therefore, we observed an increase in ROS prior to the Norgestrel-mediated protection of photoreceptors.

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; Fig. 6D(i)). 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; Fig. 6D(ii)). This supports our findings in 661Ws that Norgestrel initiates a pro-survival burst of ROS, assisting in the survival of retinal photoreceptor cells.

Discussion

Studies investigating the role of ROS in cell survival have uncovered contrasting findings (Rhee, 2006). In the retina, we have previously documented both protective and detrimental properties of ROS signalling (Groeger *et al.*, 2009b; 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; Taherian *et al.*, 2014). Indeed,

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studies from our group demonstrate that Norgestrel can reduce the damaging ROS produced after 24-48 h light exposure. This enhances neuroprotection in the balb/c light damage mouse model of retinal degeneration, and involves the up-regulation of the antioxidant transcription factor Nrf2 (Byrne *et al.*, 2016).

Nevertheless, to state that these molecules are always damaging would be an oversimplification, for ROS can have opposing effect since 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 lab have shown that hydrogen peroxide (H_2O_2) 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 (Fig. 1). 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.

Norgestrel-mediated neuroprotection has been demonstrated in different neuronal/retinal degeneration models (Byrne *et al.*, 2016; Roche *et al.*, 2016; Wyse Jackson & Cotter, 2016). The importance of the current study was to understand the role that ROS play in this response. Norgestrel, acting through the progesterone receptor PGRMC1 (Fig. 4) (Wyse Jackson *et al.*, 2016a), increases bFGF expression (Fig. 5) (Wyse Jackson & Cotter, 2016) resulting in an early up-regulation of ROS levels in 661W cells (Fig. 2 and 3). Decreasing ROS production using two different antioxidants (Tiron and NAC) revealed the survival nature of these molecules (Fig. 1 and 2). Future studies will investigate the downstream pathways stimulated by ROS upregulation, leading to enhanced survival.

Results from the 661W photoreceptor-like cell line (Fig. 1-5) suggested that Norgestrel's neuroprotective role in the retina involved an up-regulation of pro-survival ROS. In order to

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substantiate this claim, we next employed the rd10 mouse model of retinitis pigmentosa. Using single cell suspensions of 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 postnatal day (P) 15, prior to the loss of photoreceptors which takes place from P18~P21 in the rd10 retina (Barhoum *et al.*, 2008; Roche *et al.*, 2016). Norgestrel produced a similar early increase in ROS levels from 30 min in photoreceptors of the rd10 retina (Fig. 6). We hypothesize 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 (Fig. 6). 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).

In summary, 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 (Fig. 7). 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 *et al.*, 2016, 2017; Roche & Wyse Jackson *et al.*, 2016; Wyse Jackson & Cotter, 2016). The current study therefore reinforces the prospect of Norgestrel as a therapeutic for retinal degeneration, utilising ROS signalling in the early stages of injury to promote survival.

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Figure Legends

Figure 1. Serum deprivation induces a pro-survival production of ROS in 661W cells. (A) A healthy population of untreated (UT) cells was gated for all samples. **(B)** 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. **(C) (i)** Antioxidants Tiron and NAC decreased the ROS production in 661W cells (*t*-test comparing individual treatments to their vehicle control). **(ii)** 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 decreased cell viability at 30 min. Stressed cells pre-treated with antioxidants showed a further decrease in survival compared with the vehicle. Asterisks indicate significant difference **(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).**

Figure 2. 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. Asterisks indicate significant difference **(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).**

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Figure 3. 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. Asterisks indicate significant difference **(* *P* < 0.05, ** *P* < 0.01, **** *P* < 0.0001).**

Figure 4. 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. Asterisks indicate significant difference **(NS not significant, **** *P* < 0.0001).**

Figure 5. Inhibition of bFGF abrogates Norgestrel-induced increases in ROS in serum-starved 661W cells. (A) 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. **(B)** bFGF levels detected by RT-PCR **(i)** and

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immunofluorescence **(ii)** in 661W cells transfected with siRNA targeted against bFGF. Scale bar 30 µm. **(C)** 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). Asterisks indicate significant difference **(NS not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).**

Figure 6. Norgestrel induces an increase in ROS production in photoreceptors *ex vivo*. (A) Photoreceptor population was gated using P30 C57BL/6 wild type and rd10 retinas, as this population was absent from rd10 retinas. **(B)** 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). Norgestrel up-regulated ROS levels at 30 min and up to 1 h. **(C)** 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. **(D) (i)** FACS analysis of photoreceptors from C57BL/6 retinas. Treatment with antioxidant Tiron for 1 h reduced significantly ROS levels. **(ii)** 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. Asterisks indicate significant difference **(NS not significant, * $P < 0.05$, ** $P < 0.01$).**

Figure 7. 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.

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Data Accessibility

The authors confirm that all data underlying the findings are fully available without restriction and will be shared with the research community upon request. All relevant data are within the paper.

Conflict of interest

The authors have no conflict of interest to declare.

Author Contribution

Conceived and designed the experiments: AMRL, SLR, ACWJ, TGC

Funding acquisition: TGC

Performed the experiments: AMRL, SLR

Analysis data and writing (original draft): AMRL

Review & editing: AMRL, SLR, ACWJ, JNM, AMB, TGC

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Abbreviations

bFGF: basic fibroblast growth factor

P: postnatal day

PGRMC1: progesterone receptor membrane component 1

ROS: reactive oxygen species

RP: retinitis pigmentosa

SS: serum starvation

TUNEL: terminal dUTP nick-end labelling

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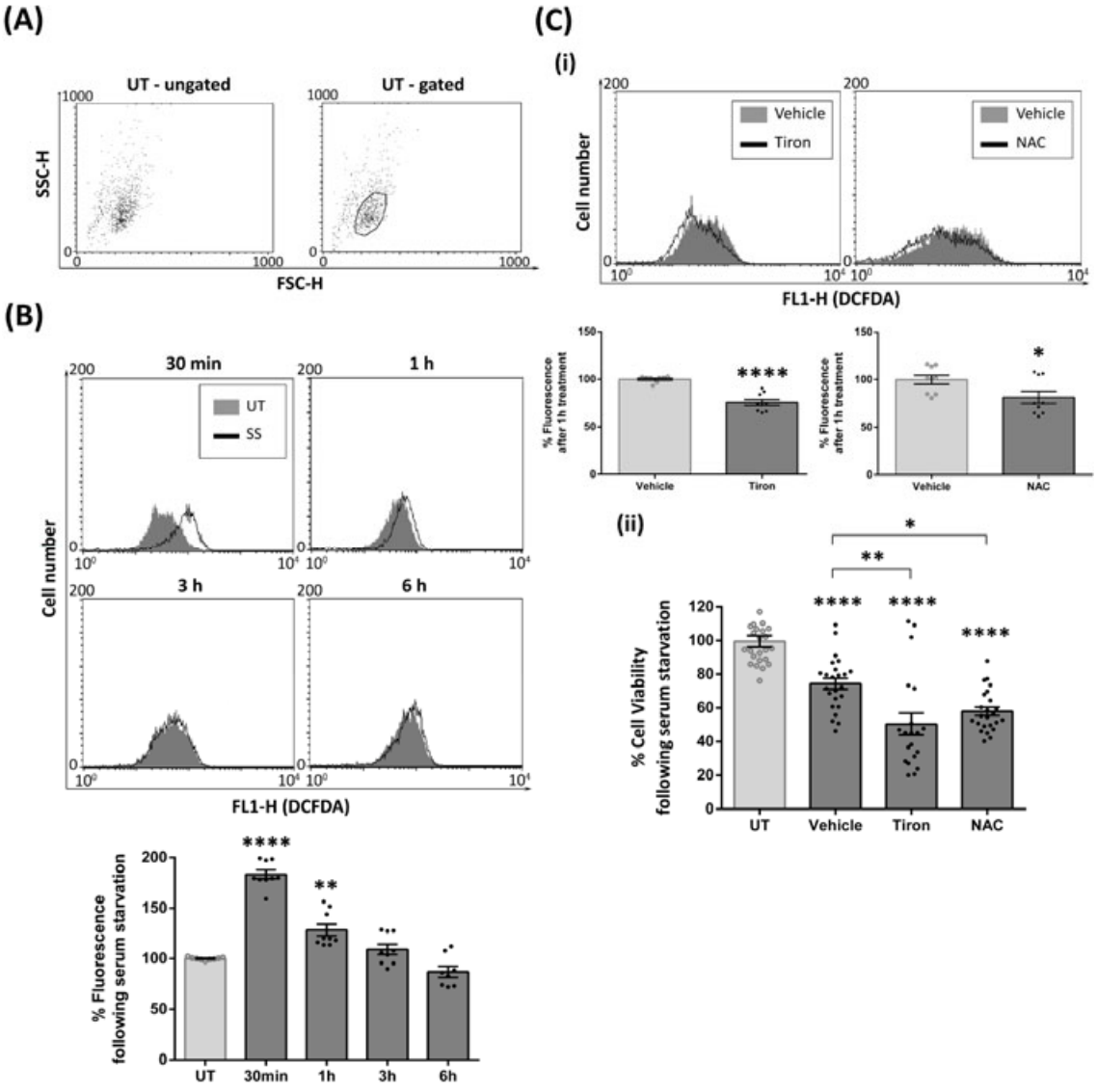
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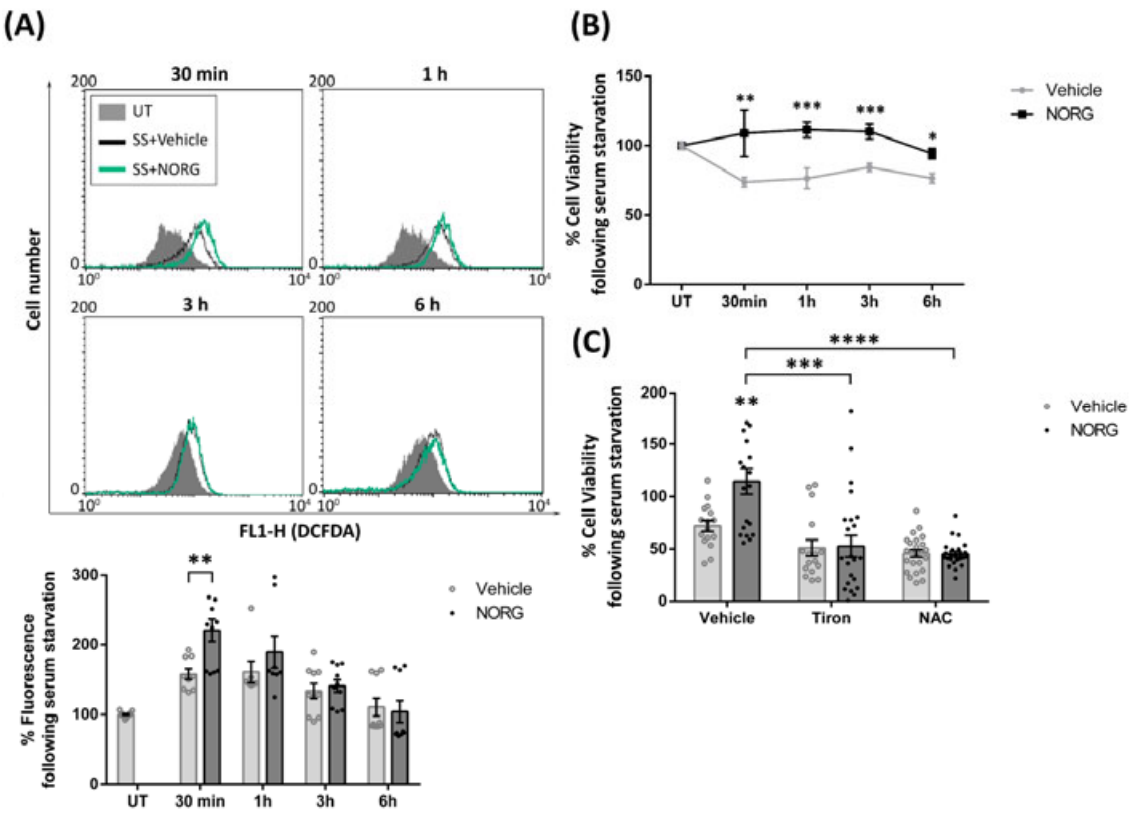
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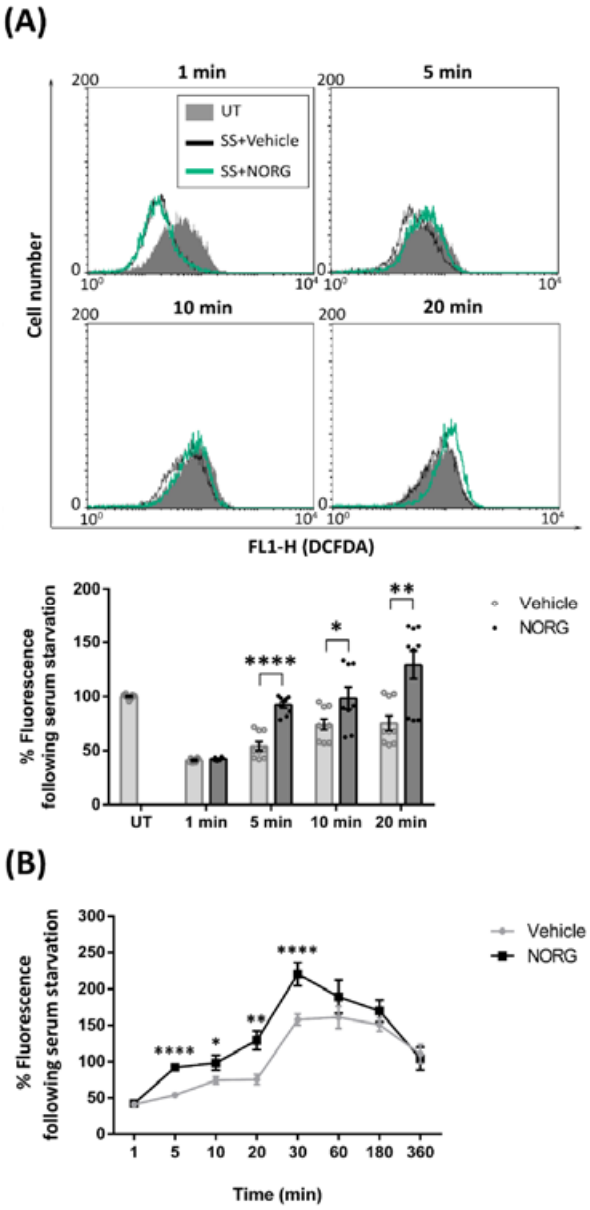
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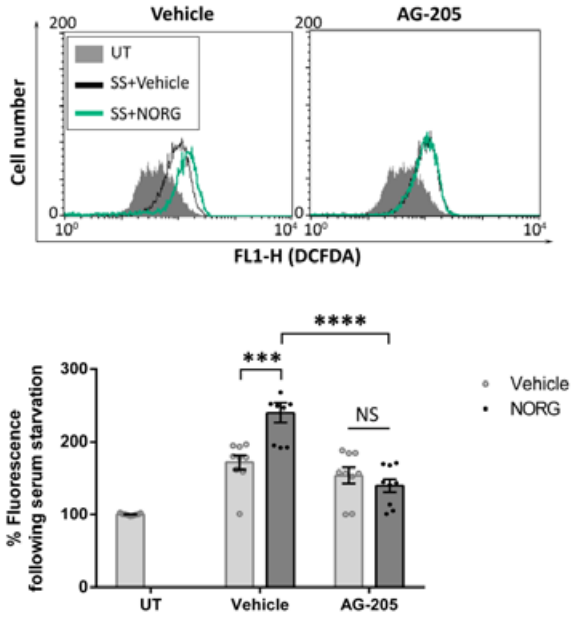
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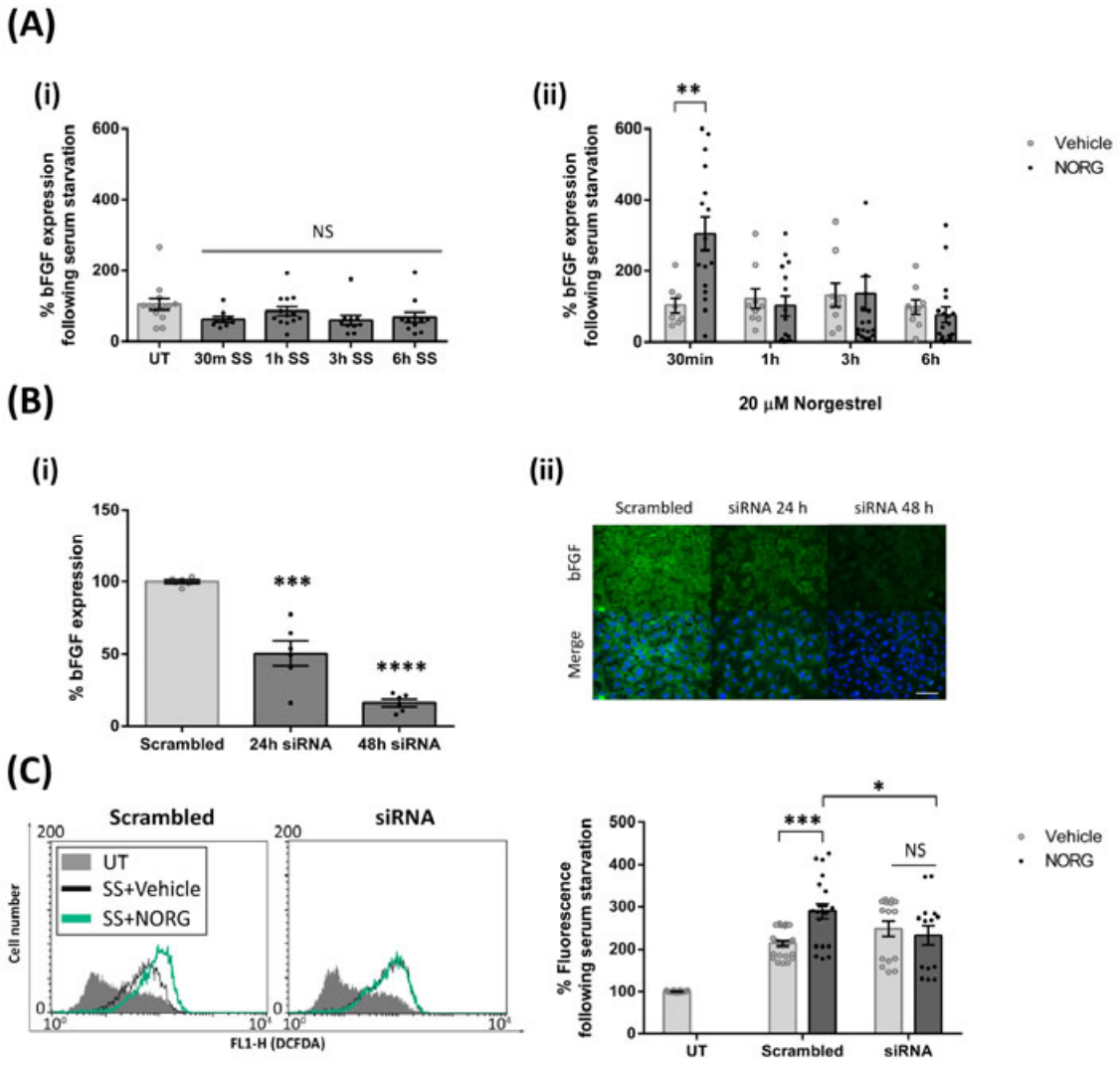
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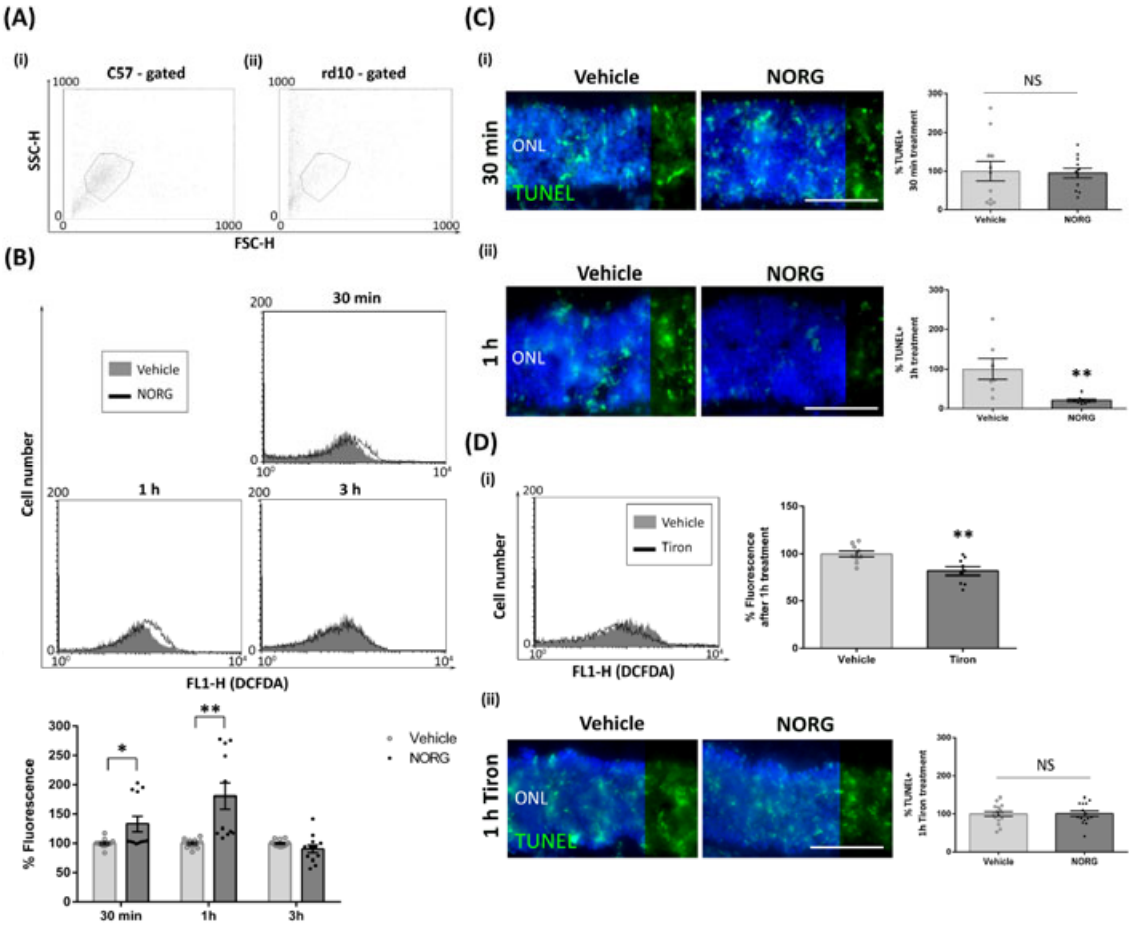
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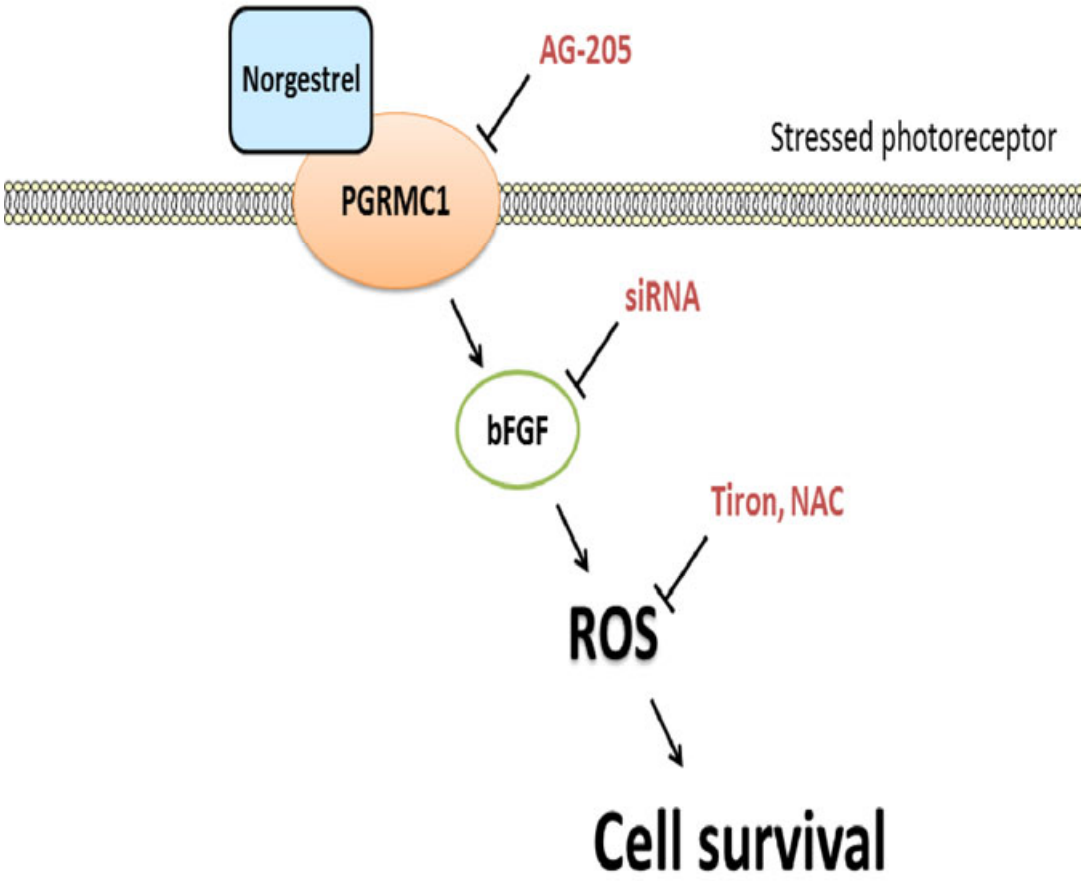
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