Photobiomodulation and Histamine H4 receptors in Müller glial cells: Dual approach for treatment of Diabetic Retinopathy

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Photobiomodulation and Histamine H_4 receptors in Müller glial cells: Dual approach for treatment of Diabetic Retinopathy

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Abstract

Diabetic retinopathy is one of the leading causes of blindness in diabetic patients. Müller cells are the primary glial cells of the retina and they are pivotal for the normal functioning of the retina. Histamine is involved in the blood-retinal barrier (BRB) breakdown that is associated with the pathogenesis of diabetic retinopathy, and recent evidence has specifically implicated an important role of histamine H_4 receptor in vascular permeability. Photobiomodulation (PBM) refers to low-intensity infra-red light therapy which has shown promise in retinopathies. We have demonstrated, for the first time, the presence of the histamine H4 receptor on murine Müller cells, particularly on the end feet of these cells. The principle aims of this investigation were to confirm the presence and characterize the Histamine H_4 receptor on human Müller cells and to determine whether H_4 receptor antagonists or PBM near infrared (NIR) wavelength 1068 nm has any therapeutic potential against diabetic retinopathy. Immunofluorescence (IF) analysis showed the presence of Histamine H_4 receptor in the human Müller cells of the retina (Mio-1 cells), and this expression was upregulated in a high (4500 mg/L), versus low (3151 mg/L), glucose environment. IF analysis has also hinted at the presence of a modest nascent histamine pool in the retina through the presence of Histidine decarboxylase (HDC). Further pharmacological characterization of the receptor included the release of calcium from intracellular pools upon receptor activation with selective H_4 receptor agonists, VUF8430 and ST-1006 of concentrations ranging from 10 nM – 100 µM. On testing the protective properties of H_4 receptor agonist, VUF8430 (10 µM) and antagonist, JNJ7777120 (10 µM) against H_2O_2 induced (400 µM) oxidative and glyoxal-induced (2 mM) inflammatory damage in human Müller cells, they both showed no significant protection. However, pilot data showed that VUF8430 exacerbated an inflammatory response. Single PBM treatment at 10 mW/sq cm^2 power density against oxidative stress showed modest protection, but double PBM treatment of the cells both pre- and post-oxidative stress displayed complete glioprotection, and an anti-inflammatory effect upon TNF-alpha gliosecretion. In conclusion, this is the first demonstration that histamine H_4 receptors are functionally expressed on human Müller cells, which indicates the therapeutic potential of neutral H_4 receptor antagonists in diabetic retinopathy. The study also provides evidence for a new non-invasive therapeutic approach (antioxidant and anti-inflammatory) for diabetic retinopathy based on a PBM NIR1068 nm protocol.

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Candidate Declaration

I confirm that no part of the materials presented has previously been submitted for a degree in this or any other University. If materials have been generated through joint work, my independent contribution has been clearly indicated. In all other cases, materials from the work of others has been clearly indicated, acknowledged and quotations and paragraphs indicated.

Abbreviations

Adenylyl Cyclase (AC)

Advanced Glycation End-products (AGE)

Age-related Macular Degeneration (AMD)

Ammonium Peroxodisulphate (APS)

Adenosine Tri-Phosphate (ATP)

Blood-Retina Barrier (BRB)

Cath.-a-differentiated (CAD)

cAMP Responsive Element-Binding protein (CREB)

Central Nervous System (CNS)

cyclic Adenosine Monophosphate (cAMP)

Diabetic Retinopathy (DR)

DiAcylGlycerol (DAG)

DiAmine Oxidase (DAO)

Dithiothreitol (DTT):

ElectroMagnetic (EM)

Electron Transport Chain (ETC)

Enzyme-Linked Immunosorbent Assay (ELISA)

Extracellular signal Regulated Kinase (ERK)

Fetal Bovine Serum (FBS)

Gamma-Aminobutyric Acid (GABA)

Glutamate Aspartate Transporter (GLAST)

Glutamine Synthetase (GS)

G-Protein Coupled Receptors (GPCR)

Histidine Decarboxylase (HDC)

Hydrogen Peroxide (H_2O_2)

inducible Nitric Oxide Synthase (iNOS)

InfraRed (IR)

Inner Limiting Membrane (ILM)

Inner Nuclear Layer (INL)

Inner Plexiform Layer (IPL)

Inositol 1,4,5-trisPhosphate (IP3)

Inositol-1, 4, 5, -triPhosphate (InsP3)

Lactate DeHydrogenase (LDH)

Light Emitting Diodes (LED)

Mitochondrial Membrane Potential (MMP)

Mitogen Activated Protein Kinase (MAPK)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Nitric Oxide (NO)

Non-Obese Diabetic (NOD)

Outer Limiting Membrane (OLM)

Outer Nuclear Layer (ONL)

Outer Plexiform Layer (OPL)

Phosphatidyl-4,5-bisPhosphate (IP2)

Phosphoinositide 3 Kinase (PI3K)

Phospholipase C (PLC)

PhotoBioModulation (PBM)

Pigment Epithelium-Derived Factor (PEDF)

Protein Kinase A (PKA)

Reactive Oxygen Species (ROS)

Retinopathy Of Prematurity (ROP)

RNA-Binding Protein with Multiple Splicing (RBPMS)

Standard Error of the Mean (SEM)

Streptozotocin (STZ)

Ultraviolet (UV)

Vascular Endothelial Growth Factor (VEGF)

World Health Organization (WHO)

Zonula Occludens (ZO)

Contents

Chapter 1

Introduction

1.1 Diabetes

Diabetes is a group of metabolic diseases that affects and induces long term damage to organs such as eyes, heart, kidneys, blood vessels and nerves due to defects in insulin secretion and insulin action (figure 1). Diabetes is characterised by hyperglycaemia, which is defined as having high blood glucose levels in the blood (higher than 1.26 g/l).

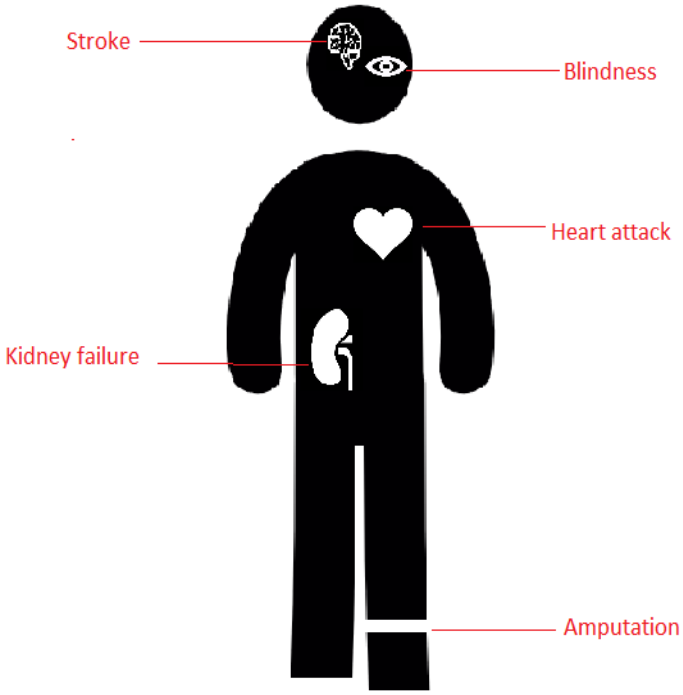

Figure 1: **Schematic diagram of the consequences of diabetes**. Figure shows various complications in many parts of the body that can be caused by diabetes.

Diabetes falls into two categories: Type 1, which is characterised by insulin producing beta cell destruction in the pancreas and type 2, the most common form of diabetes, is characterised by both abnormalities in insulin action and secretion (Alberti and Zimmet, 1998).

Diabetic patients have abnormalities in metabolism of protein, carbohydrate and fat because of the defective functioning of insulin. Insulin may not function properly if there is lack of insulin secretion or if tissues become less responsive to insulin, and this problem can coexist in most patients. Some symptoms of acute hyperglycaemia include weight loss, polyuria, polyphagia, polydipsia and blurred vision. Other symptoms can include susceptibility to infections and growth impairment. Severe symptoms due to long term hyperglycaemia include retinopathy (leads to vision loss), peripheral neuropathy (increased risk of amputations, foot ulcers and Charcot joints), autonomic neuropathy (increased incidence of cardiovascular and peripheral arterial disease) and nephropathy (leads to renal failure) (Diagnosis and Classification of Diabetes Mellitus, 2010).

The global prevalence of diabetes was at 8.3% in 2014 and this is estimated to increase by 55% in 2030 (Shaw et al., 2014); thus, posing significant burden for healthcare costs worldwide. Diabetic patients have a high risk of developing long-term complications like cardiovascular and microvascular diseases and current antidiabetic drugs are only effective in reducing the risk of acute complications but are not effective in reversing its progression (Murphy, 1998). Therefore, a better understanding of the underlying mechanism of this disease is needed for more effective treatments.

1.2 Diabetic Retinopathy

According to World Health Organization (WHO), diabetic complications can be divided into two broad categories, macrovascular complications and microvascular complications. Macrovascular complications include stroke, peripheral arterial disease and coronary artery disease. Microvascular complications include diabetic retinopathy, neuropathy and nephropathy.

Of the millions of people with diabetes, about a third of them have diabetic retinopathy, from which a third of them are at risk of loss of vision due to severe retinopathy called macular oedema (Saaddine, 2008). Diabetic retinopathy is also the most common cause of preventable blindness in working-age adults (Mohamed, Gillies and Wong, 2007). Even though developing countries have a low prevalence of diabetic retinopathy (Raman et al., 2009), this will change due to increasing population numbers and longer lifespans for people with diabetes around the world (figure 2).

Symptoms of diabetic retinopathy can be divided into non-proliferative and proliferative. Non-proliferative diabetic retinopathy is defined by haemorrhages, microaneurysms, venous abnormalities and hard exudates. Proliferative retinopathy is characterized by pre-retinal or vitreous haemorrhages, neovascularization and fibrovascular proliferation (Cheung, Mitchell and Wong, 2010) (figure 3). All these can eventually lead to retinal detachment, glaucoma and vision loss (Cai and McGinnis, 2016).

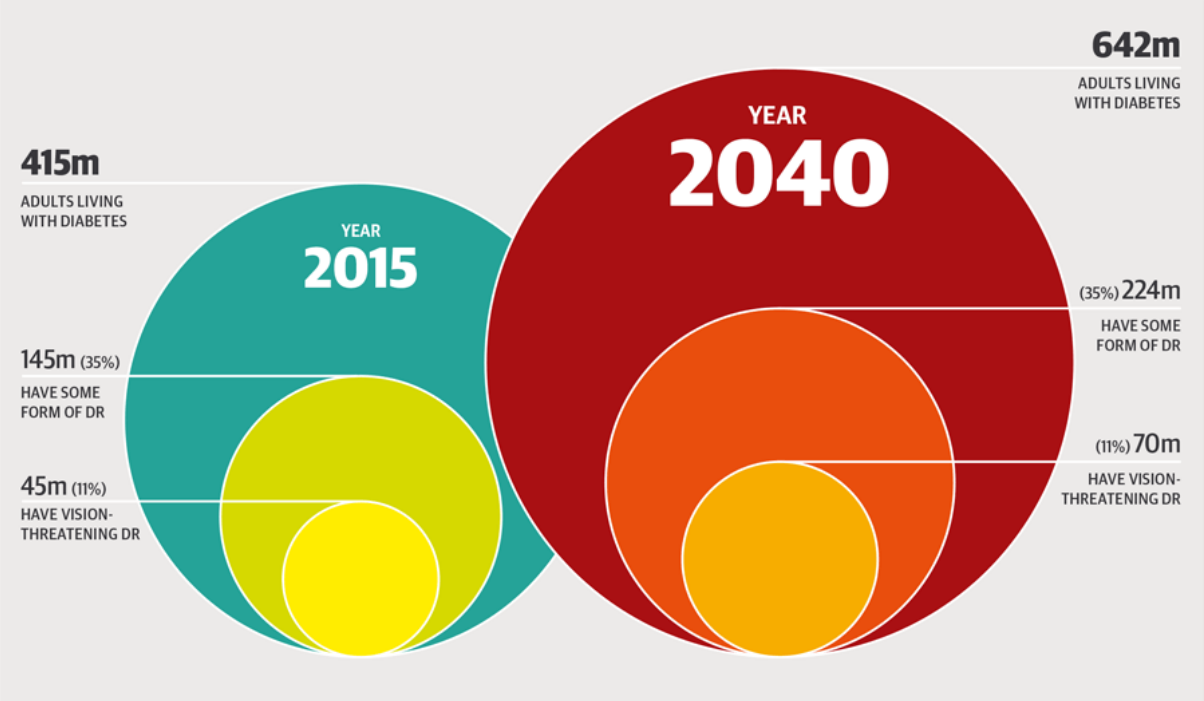

Figure 2: The global prevalence of people with diabetes and diabetic retinopathy. Chart highlighting the increased prevalence of diabetes and diabetic retinopathy (DR) due to

increasing population and lifespan. Source: http://atlas.iapb.org/vision-trends/diabetic-retinopathy/ (Accessed on 11th February 2019)

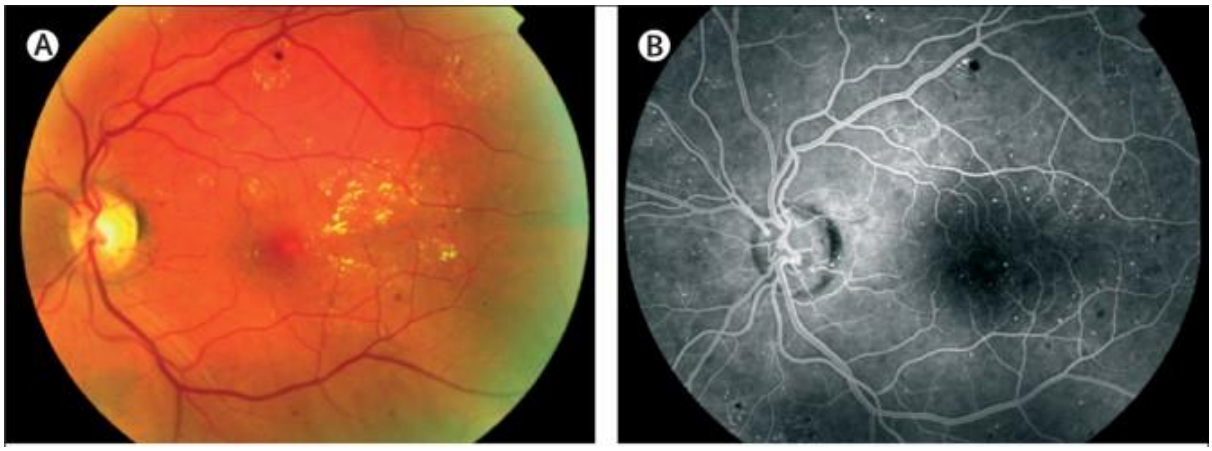

Figure 3. Signs of non-proliferative diabetic retinopathy. Some clinical features of diabetic retinopathy include microaneurysms, hard exudates and hemorrhages (A and B) (Cheung, Mitchell and Wong, 2010).

1.2.1 Molecular mechanisms of diabetic retinopathy

Our knowledge of the underlying molecular mechanisms for the development of diabetic retinopathy is still evolving. What we know so far is that chronic exposure to hyperglycemia and other causal risk factors leads to a cascade of biochemical and physiological changes that ultimately causes retinal dysfunction and microvascular damage. The biochemical changes proposed include effects on growth factors, signaling and cellular metabolism (reviewed by Cheung, Mitchell and Wong, 2010). Some of these pathways include accumulation of advanced glycation end-products (AGE), protein kinase C activation, oxidative stress, inflammation and upregulation of vascular endothelial growth factor (VEGF) and renin-angiotensin system. Growing evidence points to a prominent role of inflammation in the pathogenesis of diabetic retinopathy (Antonetti et al., 2006), (Xu, Chen and Forrester, 2009). A vast array of inflammatory mediators is upregulated in response to hyperglycemia and other stresses, which then triggers para inflammatory responses that causes abnormal leucocyte-endothelial interactions, and eventually leads to retinal microvascular

damage. Results from new experimental studies suggest that diabetes affects the entire neurosensory retina through altered metabolism of neuro-retinal supporting cells and accelerated neuronal activation and apoptosis (Antonetti et al., 2006). Understanding of this effect of diabetes on the neural retina can potentially lead to new treatments through neuroprotective agents.

1.2.2 Current treatments

Current guidelines for eye care in diabetic patients include blood pressure control and tight glycemic control, along with laser therapy when needed (Mohamed, Gillies and Wong, 2007). Several new therapies are coming up based on our understanding of the underlying molecular mechanisms of diabetic retinopathy. One of these is the drug ruboxistaurin, which is a selective protein kinase C inhibitor. Protein kinase C is activated by hyperglycemia, which in turn increases retinal neovascularization and vascular permeability. Thus, inhibition of protein kinase C can lead to reduced risk of progression of macular oedema (Schwartz, Flynn and Aiello, 2009). Another drug, pimagedine, an aminoguanidine, inhibits the formation of AGE in the retina (Bolton et al., 2004). The accumulation of AGE in the retina increases due to hyperglycemia and the level of AGE is correlated to retinopathy progression. Another drug includes rosiglitazone, which is an insulin-sensitising agent, can delay onset of proliferative retinopathy in type-2 diabetes (Genuth et al., 2005). Another therapeutic target for diabetic retinopathy includes VEGF inhibitors. VEGF is a potent mediator for abnormal retinal vessel growth and leakage (Wirostko, Wong and Simo, 2008) and has also been shown to be correlated to hypoxia and active neovascularization (Aiello et al., 1994).

Laser photocoagulation is still the main ophthalmic therapy for vision threatening diabetic retinopathy due to its effectiveness in prevention of vision loss, but this treatment method is still destructive and can lead to significant ocular side-effects. Surgical treatments available for retinopathy include vitrectomy, which is effective against persistent vitreous hemorrhage and tractional retinal detachment, but it increases the risk of cataract formation and neovascularization (reviewed by Cheung, Mitchell and Wong, 2010). There is an urgent need for new therapeutic strategies that prevents and reverses ocular oedema without tissue destruction. Treatments

that target several pathways underlying retinal oedema and neovascularization has shown the most potential so far going forward.

1.3 The retina

The retina lies at the back of the eye and is approximately 0.5 mm thick. A simplistic model of the retina is composed of sensory photoreceptors and ganglion cells connected by few interneurons packed in the central part of the retina (figure 4).

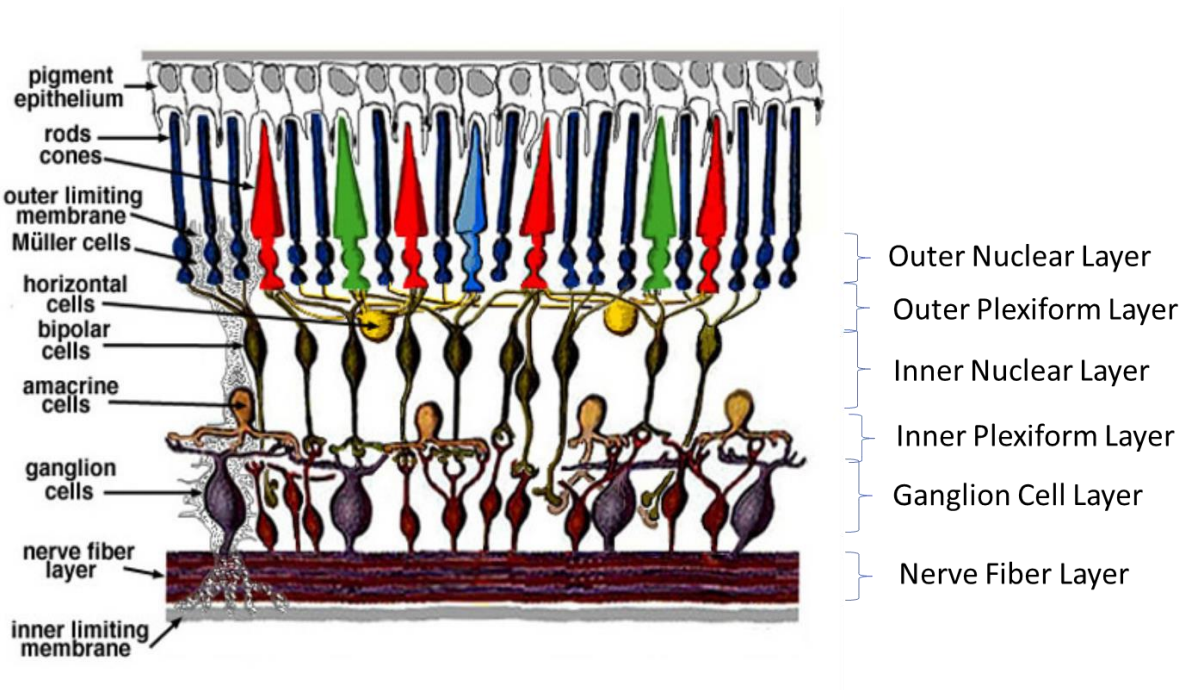

Figure 4: **Simple anatomical structure of the retina** (adapted from Kolb, Fernandez and Nelson, 1995).

Vertebrate retinas contain three layers of nerve cell bodies and two layers of synapses. The nerve cell layers are composed of outer nuclear layer (ONL), containing cell bodies of rods and cones, and the inner nuclear layer (INL), which contains cell bodies of amacrine, bipolar and horizontal cells, and finally the ganglion cell layer, consisting of cell bodies of ganglion cells and displaced amacrine cells. These three nerve cell layers are divided by two neurophils where synaptic contact occurs. The outer plexiform layer (OPL) is where connections between rods and cones, bipolar cells and horizontal cells occur. Inner plexiform layer (IPL) acts as a relay station for bipolar cells and ganglion cells. The outer limiting membrane (OLM) is composed of adherens junctions between Müller cells and photoreceptor cell

inner segments, which forms a barrier between the subretinal space and the inner and outer segments of the photoreceptors project into this subretinal space to be in close contact with the pigment epithelial layer. Inner limiting membrane (ILM) is made up of Müller cell endfeet and associated basement membrane constituents and it lies at the inner surface of the retina bordering the vitreous humor, thus forming a diffusion barrier between vitreous humor and neural retina.

The retina is supplied through capillaries running through all parts of the retina, and the delicate photoreceptor layer is nourished by choriocapillaris found behind the pigment epithelium layer.

Since the retina is a delicate organisation of glia, neurons and blood vessels, in most eye diseases, the retina gets damaged or compromised (figure 5), and eventually leads to serious damages to the nerve cells carrying vital signals to the brain, and thus causing blindness.

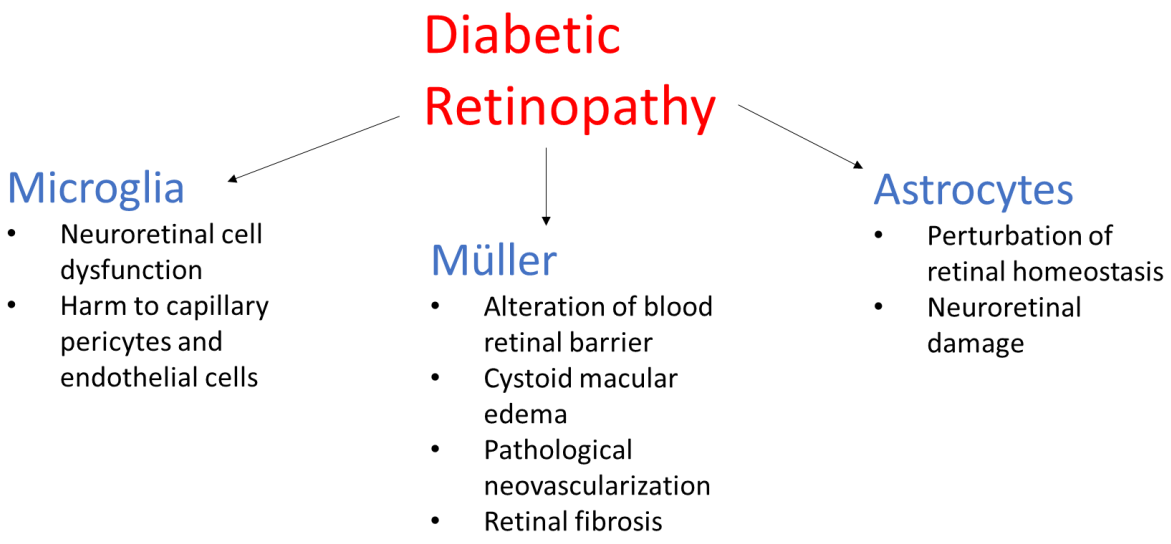

Figure 5: Damaging effects in the retina during the course of diabetic retinopathy. Diagram illustrates the glial cells in the retina that are affected by diabetic retinopathy and the adverse effects that are associated with damage to each of these cells. Müller cells play an important role in diabetic retinopathy as damage to these cells caused the most adverse symptoms associated with the disease.

1.3.1 Müller cells

Müller cells are one of the primary glial cell types found in the retina, and since they are the only cells that span the entire width of the retina and have contact with both retinal blood vessels and retinal neurons, they are uniquely positioned to perform a variety of functions to maintain retinal function and homeostasis.

Müller cells have three important functions in the healthy retina:

1 Uptake and recycling of neurotransmitters, retinoic acids, and ions

Because of the extensive contact of Müller cells with retinal neurons, they can actively participate in neurotransmission. They do this by rapidly taking up and clearing glutamate and gamma-aminobutyric acid (GABA) in the inner plexiform layer (IPL) (Barbour, Brew, & Attwell, 1988; Biedermann, Bringmann, & Reichenbach, 2002; Derouiche & Rauen, 1995). The extracellular glutamate is taken up the Glutamate Aspartate Transporter (GLAST) and its removal through conversion by glutamine synthetase into glutamine, prevents neurotoxicity in the retina and provides substrate for neurotransmitter synthesis in the neurons (Harada et al., 1998; Rauen, Taylor, Kuhlbrodt, & Wiessner, 1998).

Through a process called ''potassium spatial buffering'', Müller cells normalize and redistribute K^+ in the surrounding environment to prevent prolonged accumulation of K^+ (Orkand, Nicholls, and Kuffler, 1966). They do this by taking up K^+ from the inner and outer plexiform layers through Kir2.1 potassium channels and releasing the K^+ into vitreous humor through Kir4.1 channels in the Müller cell processes (Bringmann, Reichenbach, & Wiedemann, 2004; Nagelhus et al., 1999).

Finally, Müller cells participate in the retinoid cycle through cone photoreceptors by taking up all-trans retinol from the subretinal space, converted to 11-cis retinol by all-trans retinol isomerase, and released into the extracellular space to be taken up by the cone photoreceptors to restart the visual cycle (Edwards, Adler, Dev, & Claycomb, 1992; Kanan et al., 2008).

2 Supply of nutrients and control of metabolism in the retina

Müller cells primarily produce ATP through glycolysis rather than oxidative phosphorylation (Winkler, Arnold, Brassell, & Puro, 2000) so that other cell types and retinal neurons can use oxygen through oxidative phosphorylation to produce ATP. Müller cells are also the primary site of glycogen storage in the retina (Kuwabara & Cogan, 1961; Winkler et al., 2000), which means that they can provide metabolites to other cells when nutrient supplies are low. Furthermore, the lactate that Müller cells produce through glycolysis can be transported to photoreceptors in case of need to be used as an alternative source of energy (Poitry-Yamate, Poitry, & Tsacopoulos, 1995; Winkler et al., 2000).

3 Regulation of blood flow and maintenance of blood retinal barrier

In terms of a healthy retina, one of the most important functions of Müller cells is their ability to regulate retinal blood flow and maintain the integrity of the blood retinal barrier, to prevent leakage of blood and pathogens from entering the retinal tissue. Evidence of this role of Müller cells includes the study where conditional ablation of the cells resulted in severe blood retinal barrier breakdown (Shen et al., 2012). The mechanism of contribution of Müller cells to the blood retinal barrier includes secretion of pigment epithelium-derived factor (PEDF) and thrombospondin-1, which increase the tightness of the endothelial barrier due to their anti-angiogenic properties (Bringmann et al., 2006); though this mechanism is still highly debated.

1.4.1 Müller cells in disease

Since Müller cells have intricate contact with other cell types in the retina, it is easy to see why any disturbances to these cells can affect function, viability and cross-talk in neurons. As stated above, Müller cell ablation in an experiment resulted in vascular leakage, photoreceptor degeneration and intraretinal neovascularization, which suggests that these cells are important for neuronal and vascular function (Shen et al., 2012, 2014). When Müller cell environment is changed through

hyperglycemia, it was observed that functional interaction with pericytes was altered in these cells (Muto et al., 2014). It was suggested that the breakdown of the blood retinal barrier is caused by improper localization of the dystrophin-Dp71 protein as deletion of the protein causes the protein to localize in the endfeet of Müller cells, thus causing extensive vascular leakage and edema in the mouse retina (Vacca et al., 2016).

1.4 Histamine

Histamine [2-(4-imidazolyl)-ethylamine] is an endogenous autacoid that is found in high concentrations in vertebrate tissues like skin, lungs and gastrointestinal tract. It is synthesized from L-histidine through catalytic activity of histidine decarboxylase (HDC) (Bodmer, 1999) (figure 6).

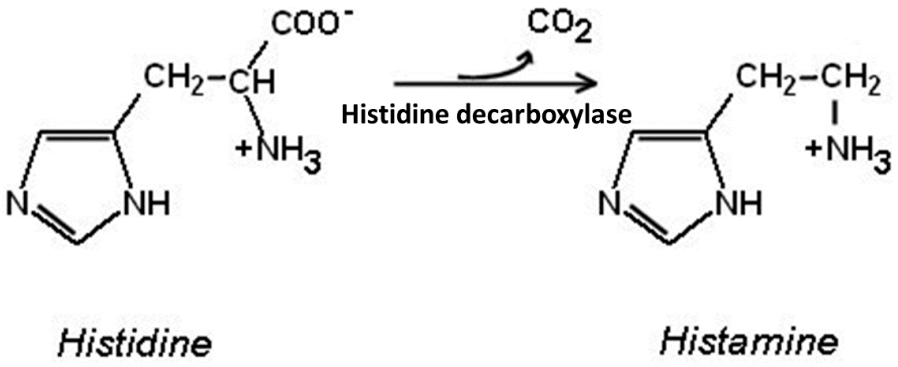

Figure 6: Chemical reaction showing the formation of Histamine from L-histidine through catalytic activity of HDC.

Histamine is responsible for a range of physiological processes such as dilation of capillaries, smooth muscle contraction, gastric acid secretion, inflammation and neurotransmission. This autacoid acts through binding to four distinct G-protein coupled receptors (GPCR), H1R, H2R, H3R and H4R, in chronological order of discovery. Homology between the receptors is very low, except H_3 and H_4 receptors (Leurs et al., 2012). A common feature in all four receptors is the negatively charged aspartate residue in transmembrane 3, which is the binding site for the amine function of histamine. Histamine is most commonly found in cytostolic granules in

basophils and mast cells and released in response to immunological and non-immunological responses through exocytosis (Kakavas S, 2006).

1.4.1 Histamine H1 receptor

The H1 receptor is expressed on various cell types, such as hepatocytes, endothelial cells, chondrocytes, nerve cells (Lo, 1987), dendritic cells (Ohtani, 2003) and neutrophils (Taniguchi, 1991). The receptor is linked to Gq/11 protein, and on activation of the receptor, the Gq/11 protein activates phospholipase C (PLC), which then catalyzes the hydrolysis of phosphatidyl-4,5-bisphosphate (IP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). When the role of histamine was discovered in allergic reactions, the first anti-histamines targeted the H1 receptor by antagonizing the effects of histamine on the receptor (Hill et al., 1997, Simons and Simons, 2011).

1.4.2 Histamine H2 receptor

The histamine H2 receptor was first discovered to modulate smooth muscle and gastric acid secretion in the periphery (Hill et al., 1997). In terms of its mechanism of action, it is constitutively active, and upon activation, it activates adenylyl cyclase through Gs-proteins (Dy and Schneider, 2004).

1.4.3 Histamine H_3 receptor

Histamine H_3 receptor was first found as a pre-synaptic auto-receptor on histaminergic neurons in the brain (Arrang et al., 1983), but we know that it is present on both histaminergic and non-histaminergic neurons. H_3 receptor is mostly expressed in central nervous system (CNS) tissues, but it is also detected in the periphery such as mast cells and the gut (Imamura, 1995, Coruzzi, 2010). The receptor is constitutively active, and it acts through linked Gi/o proteins (Morisset, 2000). On activation, H_3 receptor can inhibit the release of histamine and other neurotransmitters such as dopamine, glutamate, serotonin, noradrenalin, norepinephrine and γ-amino butyric acid (GABA) (Leurs, 1998). H_3 receptor is

different from H_1 and H_2 receptors pharmacologically as activation of the receptor inhibits cAMP production through adenylyl cyclase.

H_3 receptor is known for its critical role in homeostatic regulatory functions such as food intake and bodyweight (Hancock et. al, 2005). Recently, this receptor has been shown to be present in pancreatic alpha and beta cells, and activation of the receptor with Imetit inhibits insulin secretion in MIN6 cells (Nakamura et. al, 2014); thus, showing a potential diabeto-genic effect of H_3 receptor in the pancreas.

1.4.4 Histamine H_4 receptor

H_4 receptor is mostly expressed on cells from the haematopoietic lineage e.g. T-cells, basophils, mast cells, eosinophils and dendritic cells (van Rijn et al., 2008, Dijkstra et al., 2007, Dijkstra et al., 2008, Oda T., 2001). Apart from cells of haematopoietic lineage, H_4 receptors have also been found on neurons of dorsal root ganglia, lamina I and II of the lumbar spinal cord (Lethbridge and Chazot, 2010b, Connelly et al., 2009, Strakhova et al., 2009) and on nerves of human nasal mucosa (Nakayama et al., 2004).

1.4.4.1 Biochemistry

Once H_4 receptor is activated, its Gα and Gβγ subunits dissociate from the receptor, and the Gβγ subunits activate phospholipase C (PLC), resulting in the production of diacyglycerol (DAG) and inositol-1, 4, 5, -triphosphate (InsP3). InsP3 further releases calcium stored in intracellular calcium vesicle stores. The Gα subunit once activated, negatively regulates the activity of adenylyl cyclase (AC), which is responsible for the formation of cAMP from ATP. cAMP is involved in activating protein kinase A (PKA), which then phosphorylates cAMP responsive element-binding protein (CREB). The reduction in PKA activity results in increase in mitogen activated protein kinase (MAPK) activity (Van Rijn, 2006). This mechanism of H_4 receptor signaling is shown in figure 7. Activation of H_4 receptor also inhibits forskolin induced cyclic adenosine monophosphate (cAMP) production (Lovenberg, 1999). Interestingly, it has also been shown that H_4 receptor exhibits constitutive activity, as illustrated by increased basal level of MAP kinase phosphorylation and [35S] GTPγS binding in H_4 receptor expressing cells (Morse et al., 2001).

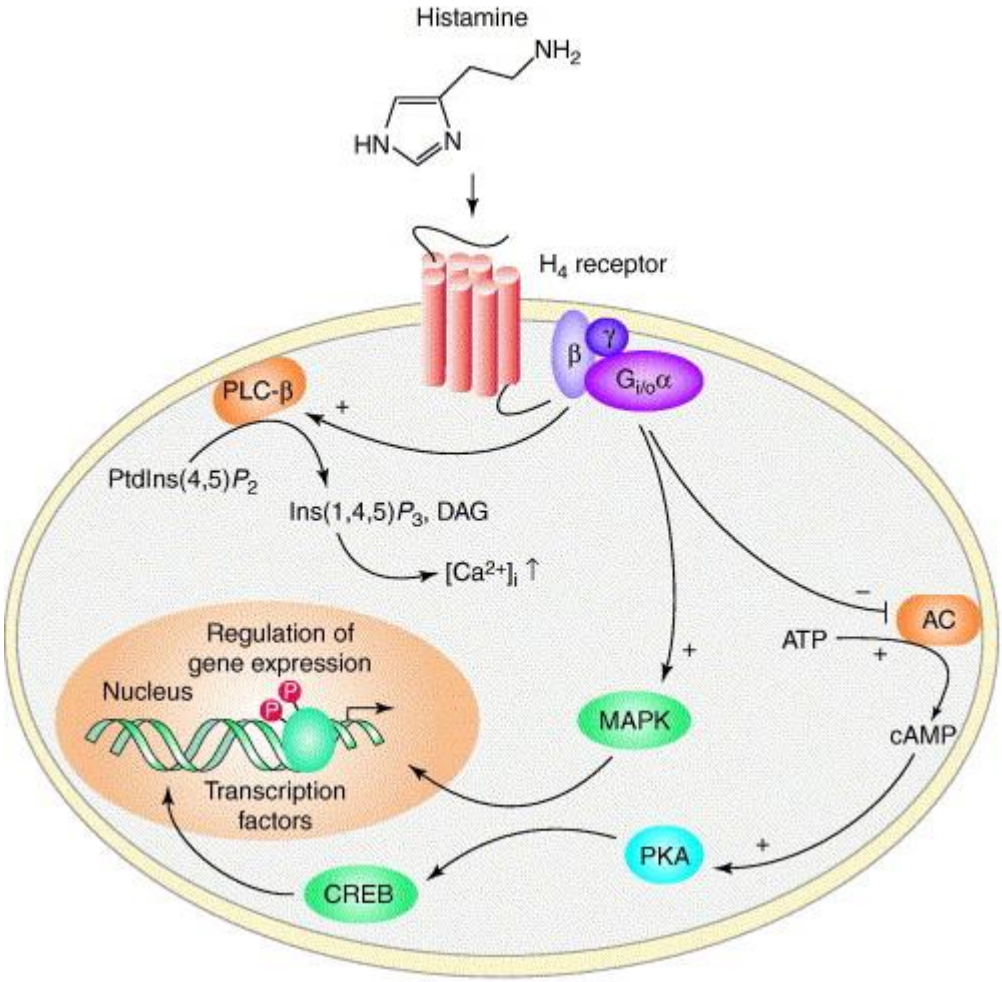

Figure 7: An overview of H_4 receptor signal transduction (Iwan, 2005). On H_4 receptor activation, both Gα and Gβγ subunits take part in intracellular signaling. Gβγ subunit activation leads to production of DAG and InsP3. InsP3 further releases calcium stored in intracellular calcium vesicle stores. The Gα subunit once activated, negatively regulates the activity of adenylyl cyclase (AC), which results in decreased levels of cAMP and subsequent downregulation of the transcription factor CREB. This in turn results in increase in mitogen activated protein kinase (MAPK) activity.

1.4.4.2 Agonists

Early pharmacological studies of the H_4 receptor was carried out using ligands designed for the homologous H_3 receptor as there was a lack of specific H_4 ligands. OUP-16 (figure 8A) was the first ligand selective for H_4 receptor, with a 40-fold selectivity over the H_3 receptor (Hashimoto et al., 2003). 4-methylhistamine (figure 8B) is another ligand with high affinity for the H_4 receptor and displays a 100-fold

selectivity over the other histamine receptors (Lim et al., 2005). Another important agonist is VUF 8430 (figure 8C), which has full agonistic activity and a 30-fold selectivity over the H_3 receptor (Lim et al., 2009). 4-methylhistamine and VUF 8430 together can be used as powerful tools to study H_4 receptor pharmacology. Another agonist utilized in this study includes the aminopyrimidine ligand ST-1006, which is a partial H_4 receptor ligand (Panula et al., 2015).

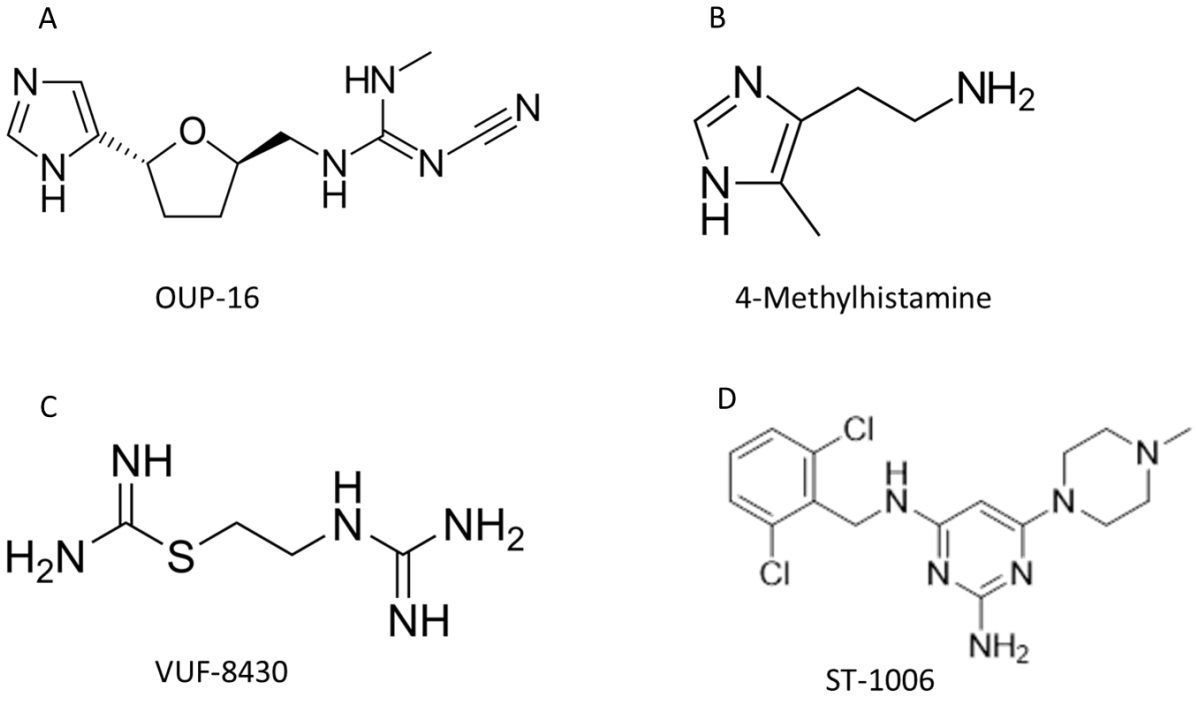

Figure 8: H_4 receptor agonists.

1.4.4.3 Antagonists

The first H_4 receptor antagonists used to characterize the receptor were imidazole-containing dual H_3 and H_4 receptor compounds (Gbahou et al., 2006) such as thioperamide (figure 9A), which is a H_3 antagonist and H_4 receptor inverse antagonist (Buckland et al., 2003, Hofstra et al., 2003). The most important human H_4 receptor antagonist so far is JNJ 7777120 (figure 9B), as it has high affinity for H_4 receptor (Ki, 4 nM) and 1000-fold selectivity over the other receptors (Fung-Leung et al., 2004). This H_4 antagonist has been used extensively to elucidate the role of H_4 receptor in various inflammatory and allergic processes (Coruzzi et al., 2012), and its anti-

inflammatory properties suggests that H_4 receptor antagonists can possibly be used as anti-inflammatory drugs.

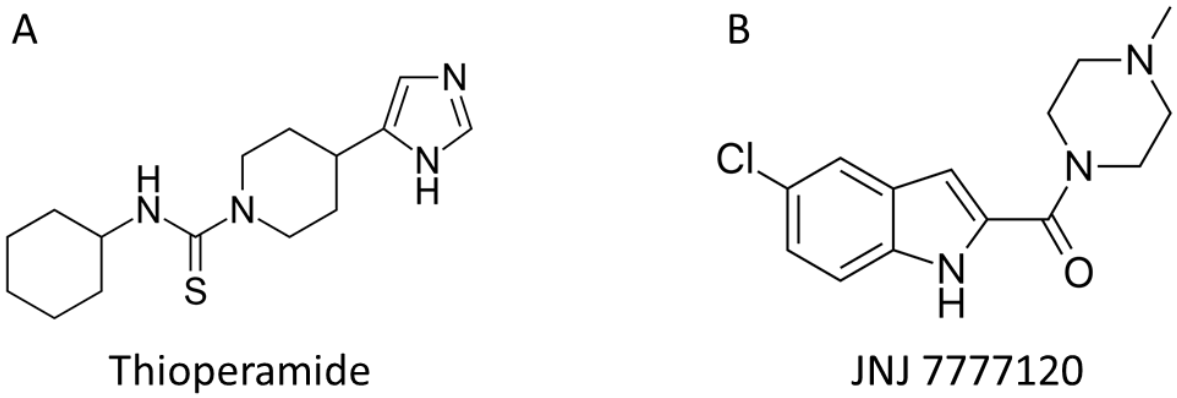

Figure 9: H_4 receptor antagonists.

1.4.5 Role of Histamine in diabetes

A lot of current evidence points to a significant role of histamine in diabetes. The first evidence for the role of histamine in diabetes came from the work of Gill and colleagues who showed that there is an increase in histamine content in plasma and leucocyte of patients with diabetes which they concluded resulted in pathogenesis of increased endothelial permeability in diabetic patients (Gill et al., 1989). Other evidence includes the activation of mast cells by advanced glycation end-products (AGEs), which results in release of histamine from mast cells and as a result causes the low-grade inflammation which is typical of diabetes (Sick et al., 2010). Work in rodents has also contributed considerable evidence; this includes increased histamine level in the kidney, lung, brain, plasma, heart, intestine and pancreas of diabetic rats (Fogel et al., 1990) and when insulin is administered to these rats, it was reported to lower histamine production in plasma and aorta (Orlidge and Hollis, 1982, Hollis et al., 1985).

Further evidence of this include the report of an increase in pancreatic islet histamine content streptozotocin (STZ)-induced diabetic rats (Azevedo et al., 1990). Due to its well-known vascular actions, histamine has been suggested to be a key triggering stimulus for microvascular diseases of diabetes, such as diabetic retinopathy (El-Asrar et al., 2004). The role of histamine in this microvascular disease

has been implicated using antihistamines, such as astemizole, diphenhydramine and ranitidine, which have been shown to reduce leakage of retinal vessels in diabetic rats and human (Hollis et al., 1992, Gardner et al., 1995) and also reduce permeability of blood-brain barrier (Patnaik et al., 2000). Thus, these data suggest that histamine plays a role in the neural cell contribution in diabetes-induced vascular leakage.

The observation that there is reduced hyperglycemia in $HDC^{-/-}$ NOD mice (Alkan et al., 2015), which is the animal model for spontaneous type 1 diabetes, suggests that there is nascent histamine pool in various tissues. This is backed up by the observation that there is a simultaneous reduction in intestinal diamine oxidase (DAO) activity and increase in histidine decarboxylase (HDC) activity in tissues (Gill, Thompson and Dandona, 1990).

In terms of the role of histamine in diabetic retinopathy, it has been reported that there is reduced zonula occludens (ZO)-1 expression in cultured retinal microvascular endothelial cells in the presence of histamine (Gardner et al., 1996) and the same effect is observed in the presence of high glucose and low insulin (Gardner et al., 1995). These data suggest that histamine induces BRB (blood-retina barrier) dysfunction in diabetes and consequently vascular leakage, which contributes to the pathogenesis in diabetic retinopathy. Interestingly, these effects are similar to those observed for VEGF on permeability leakage (Leal et al., 2005). More evidence that points to a direct role of histamine in diabetic retinopathy is the study where an increase in histamine synthesis was observed in the retinas of diabetic rats (Hollis et al., 1992, Carroll et al., 1988) due to an over-expression of HDC in retinal neurons and glia (Gastinger et al., 2001) and the histamine overproduction was reduced when HDC or insulin was administered (Carroll et al., 1988). This is important because of the current acknowledgment that in diabetic retinopathy, molecular, cellular and functional changes occur in all the retinal cellular compartments (Barber et al., 1998, Abu-El-Asrar et al., 2004).

1.4.6 Importance of H_3 and H_4 receptors in Diabetes

The histamine receptors that will be the focus of this study will be H_3 receptor and H_4 receptor. H_3 receptor is primarily located in the CNS, where it acts as both an auto- and hetero-receptor to control the release of histamine and other neurotransmitters. Apart from CNS, H_3 receptor has also been found in sensory neurons and dorsal root ganglia (Cannon et al., 2007). Like H_3 receptor, H_4 receptor is also found in human CNS (Coge et al., 2001), but recently it has been found to be also expressed in endothelial cells, which indicates its potential role in vascular permeability (Karlstedt et al., 2013). It is well known that there are different isoforms of histamine receptors, and our group has shown that three of the common H_3 receptor isoforms (hH3 329, hH3 365, hH3 445) are expressed in the human brain (Lethbridge, 2011). This is important as human H_3 receptor homomeric isoforms and heteromeric subtypes display differential pharmacological properties.

Recent data also points to the involvement of peripheral H_3 receptor in the insulin-histamine loop (Pini et al., 2015). The presence of functional H_3 receptor was also reported in diabetic pancreas, showing the involvement of H_3 receptor in diabetes for the first time, where the authors showed that the activation of the receptor with Imetit inhibits insulin secretion in a high glucose environment (Nakamura et al., 2014). So far, this has been tested by a synthesized H_3 receptor antagonist in diabetic animal models, where the drug was effective in reducing non-fasting glucose levels by blocking the increase of HbA1 (Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes, 2012). On the contrary, it's reported that the activation of H_3 receptors in DiO mice increases energy expenditure and decreases food intake, while chronic exposure of the mice to H_3 receptor agonist reduces fat mass, body weight, hyperinsulinemia and hyperleptinemia. In contrast, it was observed that the H_3 receptor agonist Proxyfan improves glucose exclusion in mice, increasing their plasma insulin levels without any changes in plasma glucagon levels (Henry et al., 2011). In addition to this, H_3 receptor deficient mice, which are mildly obese, demonstrate insulin and leptin resistance along with defective glucose tolerance (Yoshimoto et al., 2006). In the kidney, it's also shown there is a profound increase in H_3 receptor and H_4 receptor

expression in diabetic animals (Pini et al., 2015, Rosa et al., 2013). These studies raise the possibility of contradictory effects mediated by central and peripheral H_3 receptor. These contradictory observations could also be attributed to the different animal models used, the variance in doses, selectivity of the compounds and the administration route, which could all differentially affect the peripheral and central histaminergic system (Pini et al., 2018).

1.5 Photobiomodulation

Electromagnetic (EM) waves are photons of light that vibrate at different rates. They display both magnetic and electrical properties and due to the different vibration rates of the photons, EM waves have different wavelengths and photon energy (Enwemeka, 2004). The EM spectrum consists of infrared (IR) radiation, ultraviolet rays, gamma rays, x-rays, radio waves and cosmic rays (figure 10). Due to the varying energy of the photons, different wavelengths of the spectrum have varying effects in cells. For example, at the lower end of the spectrum, with shorter wavelengths and higher energy, gamma rays, x-rays and UV rays can produce damaging effects in cells. Whereas at the other end of the spectrum, lower energy and longer wavelengths like radio waves are harmless to cells. Visible light and near-IR fall between the two extremes and these wavelengths have been shown to have therapeutic effects in cells.

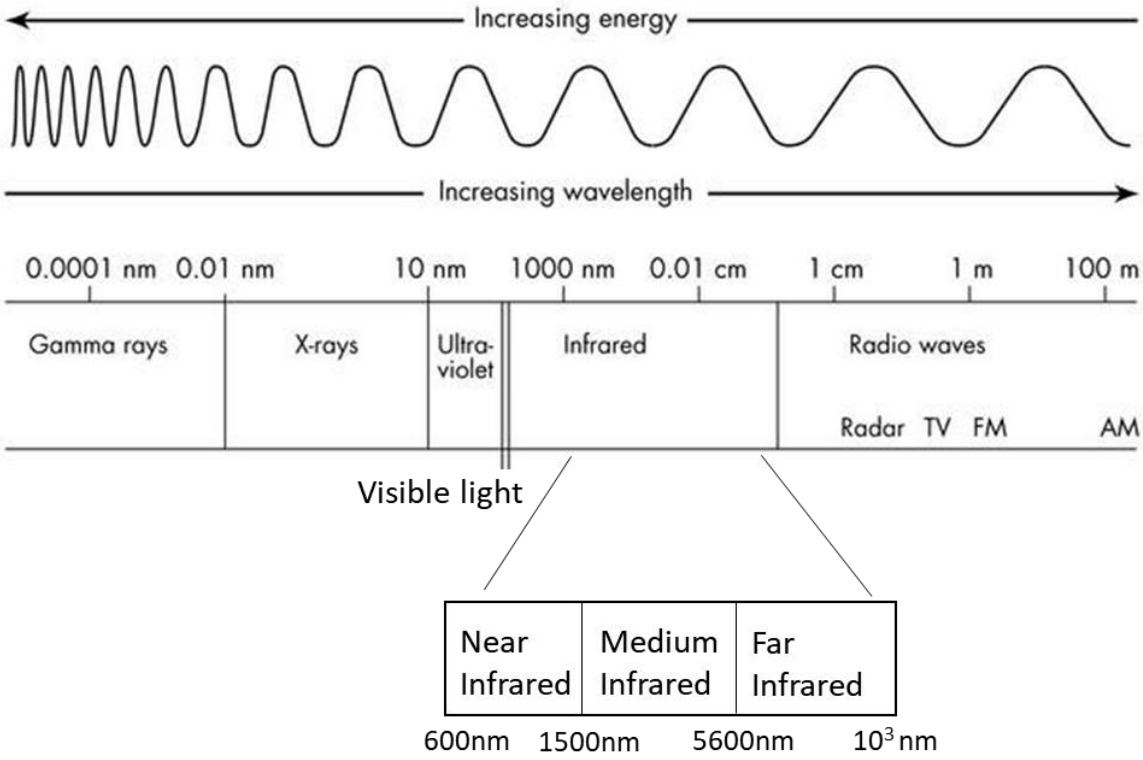

Figure 10: The electromagnetic spectrum. Figure illustrating the different wavelength of the electromagnetic spectrum and the subcategories that infrared light is split into.

1.5.1 Use of IR and near-IR light as a therapeutic tool

The use of lasers in a clinical setting is impractical for various reasons: limitations in altering wavelengths, bulky equipment and excessive hardware and the risk of damage to tissues and the retina from the laser light (Whelan et al., 2001; Eells et al., 2004; Brawn and Kwong-Hing, 2007). Light Emitting Diodes (LED) have become a suitable alternative to lasers as they can produce varying wavelengths, have less cumbersome hardware and have reduced risk of tissue and eye damage due to lower production of heat compared to lasers (Whelan et al., 2001; Eells et al., 2004; Brawn and Kwong-Hing, 2007). On top of this, experimental evidence at the cellular level has shown that Laser and LED at the same wavelength, irradiation time and intensity have similar biological effects. (Karu, 2003).

Photobiomodulation (PBM) refers to the use of red or near-infrared light (630-1100 nm) to heal, stimulate and protect tissues which are injured or is at risk of dying. PBM was accidentally discovered by Endre Mester in 1967, when while repeating an experiment, he used a low-powered laser to destroy a cancerous tumor implanted

onto a rat. He noticed that instead of destroying the tumor, the light had caused the wounded skin of the heat to heal quicker compared to the control rats. He followed up this observation with further experiments on burns, skin defects, ulcers, wounds and bedsores (Enwemeka, 2004).

A common effect observed with PBM is increased cellular proliferation. This effect has been observed at different wavelengths for various cell types like fibroblasts (Vinck et al., 2003), osteoblast cells (Kreisler et al., 2003a; Ueda and Shimizu, 2003; Pires Oliveira et al., 2008), olfactory ensheathing cells (Byrnes et al., 2005b), human larynx carcinoma cells (Kreisler et al., 2003b) and rabbit aortic endothelial cells (Ricci et al., 2009). Thus, from these observations, it can be inferred that tissue repair seen with PBM treatment may be due to increased cellular proliferation.

In animal models, PBM has been shown to reduce oxidative stress associated with diabetes (Henshel et al., 2009, Tsai et al., 2001). In in-vitro models, treatment of visual cortical neurons with 670 nm NIR light before exposing the neurons to potassium cyanide reduced apoptosis of the cells by up to 50% (Liang et al., 2006). Eells et al., (2003) further showed that brief exposures of NIR light at 670 nm can reduce the toxicity of methanol derived formate in mitochondria. Research has also focused on Müller cells as these cells offer protection to photoreceptors in the retina. Albarracin and Valter, (2012) showed that treating albino rats with 670 nm light before exposing them to damaging light to induce retinal degeneration results in a reduction of light damage induced changes in Müller cells.

1.5.2 Photoreceptors for red to near-IR light

All photoreceptors discovered so far have a common feature: a protein with one or several chromophores covalently or non-covalently bound to the protein (Batschauer, 2003) and thus photoreceptors for red to near-IR light should display similar characteristics. Most of the current evidence points to cytochrome C oxidase as the primary photoacceptor for light in the far to near-IR range (Karu, 1999; Eells et al., 2004; Barolet, 2008; Gao and Xing, 2009). Cytochrome C oxidase is the terminal enzyme of the electron transport chain (ETC). Other photoacceptors that have been suggested including Complex II and IV of the ETC (Silveira et al., 2007), tyrosine

protein kinase receptor (Shefer et al., 2001) and the mobile electron transporters in ETC, cytochrome C, and Ubiquinone.

1.5.3 Primary molecular mechanisms of PBM

Mitochondria are important organelles in any cell to maintain its homeostasis through processes such as energy production, signal transduction, apoptosis and metabolism of amino acids, nucleotides, lipids and steroids. As a result, any biochemical changes in the mitochondria can have significant effect in cellular processes (Chinnery and Schon, 2003).

The primary role of mitochondria in respiring cells are the aerobic catabolism of dietary intermediates such as fatty acids and amino acids into pyruvate (Duchen, 2004; Burroughs and French, 2007). The pyruvate is eventually converted to carbon dioxide, water and ATP via the ETC. Cells such as neurons and cardiomyocytes, which are energetically active cells, require efficient ATP supply, and thus this can only be provided through mitochondrial oxidative phosphorylation via ETC (Duchen, 2004; Burroughs and French, 2007).

It has been shown that mitochondria are sensitive to NIR light and it can absorb as much as 50% of the light through its chromophores, such as Cytochrome C oxidase (Beauvoit et al., 1995, Beauvoit et al., 1994).

Cytochrome C oxidase is a large multi-component membrane protein (complex IV) of the ETC and it has been shown to have absorption bands in the red to near-IR spectrum (Huang et al., 2009). Its center consists of two heme moieties (heme a and heme a3), one zinc, one magnesium and two redox-active copper sites (CuA and CuB). All of these components are possible chromophores for red to near-IR light (Eells et al., 2004; Gao and Xing, 2009). Examples include increased electron transfer with 660-680 nm wavelength (Gordon and Surrey, 1960; Pastore et al., 2000) and upregulation of cytochrome oxidase activity in cultured neuronal cells (Wong-Riley et al., 2001). An Investigation by Karu et al. (1995) showed that the reduced form of CuA accepts light of 620nm wavelength, oxidized CuB accepts 680nm, reduced CuB accepts 760nm and oxidized CuA accepts 825nm. However, they also showed that cytochrome C is not the primary photoacceptor when it is in a fully oxidized or fully

reduced state (Karu et al., 1995; Karu and Kolyakov, 2005), therefore it is not clear which of the intermediates of cytochrome C oxidase is the primary photo acceptor (Gao and Xing, 2009).

NO and N3 are reported to have considerable influence over cytochrome c oxidase activity and thus they are regarded as important intra-mitochondrial signaling regulators. These two gas molecules competitively bind cytochrome c oxidase by dislodging oxygen and as a result inhibit respiration in stressed cell to some degree (Reviewed - Brookes et al., 2002, Karu, 2008, Karu et al., 2004). NIR at 670 nm has been shown to reverse this process of cytochrome c oxidase inhibition by NO (Trimmer et al., 2009, Zhang et al., 2009).

Mitochondrial retrograde signaling process is the communication between the mitochondria and nucleus of a cell which affects several cellular activities (Karu, 1999; Gao and Xing, 2009) and this process can be initiated by the changes in ATP production, pH and mitochondrial membrane potential (MMP) following PBM treatment (Passarella et al., 1984). Subsequently, mitochondrial retrograde signaling is thought to initiate several pathways such as Mitogen-activated protein kinase (MAPK) cascade, extracellular signal regulated kinase (ERK) signaling, phosphoinositide 3 kinase (PI3K) and ATP-related signaling following PBM treatment (Gao and Xing, 2009).

Mitochondria also play an important role in calcium storage and release. For example, mitochondria accumulate Ca^{2+} to protect the cell when cytosolic Ca^{2+} levels rise to critical levels. Following PBM treatment in hepatocytes and mast cells, it has been reported that there is an increased intracellular Ca^{2+} level and three to eight-fold increase in intramitochondrial Ca^{2+} levels (Reviewed - Butow and Avadhani, 2004, Karu, 2008, Scheffler, 2001). If the increased level of Ca^{2+} is sustained in the mitochondria then this can have a direct impact on the Krebs cycle, mitochondrial membrane potential and indirectly on the ATP/ADP availability (Wan et al., 1989). Disruption of the mitochondrial membrane potential releases Ca^{2+} in the cytosol, thus increasing intracellular Ca^{2+} concentration, which then enhances the activity of nuclear factor KB (NF-KB), several kinases and other signaling pathways to ultimately activate several nuclear genes. Hence Ca^{2+} is thought to be one of the primary

effectors of the mitochondrial-retrograde signaling mechanisms (Butow and Avadhani, 2004, Karu, 2008, Nicholls and Budd, 2000).

Absorption of NIR light by mitochondria increases its membrane potential, ATP production and reactive oxygen species (ROS) production; all resulting in induction of signaling pathways and increase in energy availability (Reviewed - Gao and Xing, 2009) (figure 11). Some of the cellular processes modulated by ROS include nucleic acid production and protein synthesis. They have also been shown to have effects on transcription factors such as NF-kB, AP-1, Ref-1, CREB and p53 (Reviewed – Hamblin and Demidova, 2006, Huang et al., 2009).

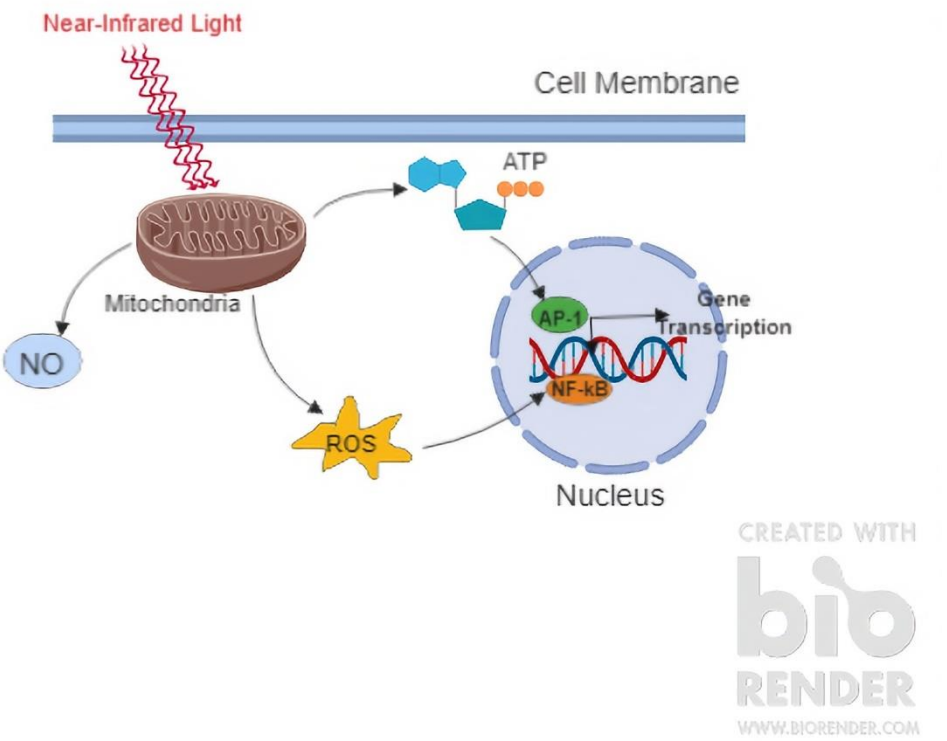

Figure 11: Cellular signaling mechanisms of PBM. Schematic diagram showing NIR light absorption by mitochondria, which results in increased ATP production by the mitochondria, and also reactive oxygen species (ROS) and other signaling molecules like nitric oxide (NO). Increased ATP and ROS production can lead to transcriptional changes in the cell through activation of transcription factors like NF- ƙB and AP1 (figure adapted from Chung et al., 2012 using Biorender.com).

1.5.4 Secondary signalling

In other studies, NIR light has been shown to affect the expression of a range of genes critical to cellular functions, such as trafficking and degradation driving proteins, DNA repair proteins, antioxidant enzymes, molecular chaperones and proteins that are essential for cell growth and maintenance (Desmet et al., 2006). In a similar study, a cDNA microarray analysis of gene expression profile in human fibroblast cells after red light treatment showed increased expression of proteins that comprise the mitochondrial respiratory chain and anti-oxidant genes and a downregulation of genes that are involved in apoptosis and stress response.

1.5.5 Importance of the 1068 nm wavelength

Most of the work published for PBM has primarily focused on the 600nm-900nm wavelength. One of the major components of light penetration in tissues is its transmission through water, and narrow IR wavelength of 1068 nm is an optimum wavelength for transmission through water (Dougal and Kelly, 2001). Previous work with this wavelength has already shown its therapeutic potential such as improving the healing time of Herpes labialis (Dougal and Kelly, 2001), protecting against UVA toxicity in lymphocytes (Bradford et al., 2005) and improved acquisition of working memory in CD-1 mice (Michalikova et al., 2008). Bradford et al. (2005) have also shown that cell viability in human lymphocytes is the highest when treated with 1068 nm wavelength compared to wavelengths of 660nm, 880nm, 950nm, 1267nm and untreated controls. Therefore, further experiments with IR light at a wavelength of 1068 nm is essential to further establish the therapeutic potential of this wavelength.

Near infrared (NIR) light (600-1500 nm) will be the focus of this study as it has been shown to have a range of biological effects and therapeutic benefits. NIR range of 630-800 nm has high tissue penetrability as they have been shown to be able to penetrate up to 23 cm through the skin into muscle tissue. This range can also vary depending on the wavelength, tissue type and fluence; in addition, NIR absorption properties of tissues can vary depending on their constituents, such as water, fat and collagen (Hamblin and Demidova, 2006, Huang et al., 2009, Karu, 1999, Tsai et al., 2001), along with the temperature and ion content and concentration (Tsai et al.,

2001). One downside of this NIR range is that it is strongly absorbed by water, which limits its infiltration into deeper tissues. An alternative for this is NIR light at 1068 nm, which requires less energy and intensity to enter biological matter, and can pass through water molecules (Bradford et al., 2005, Burroughs, 2010, Tsai et al., 2001).

There have been numerous studies by our group that have implicated the importance of IR 1068 nm wavelength in various biological processes. Bradford et al., 2005, showed a cytoprotective effect of IR 1068 nm against UVA toxicity on human lymphocytes. Burroughs, 2010, found IR 1068 nm exposure provided concentration-dependent protection of rat primary cultures against glutamate-induced excitotoxicity and most recently Duggett, 2013 established that IR 1068 nm can protect CAD neuronal cell line against Alzheimer's disease-related insults such as hydrogen peroxide and β-amyloid (Grillo et al., 2013).

1.6 Primary Aims and Hypotheses

The aim of this project was to explore the possibility of a combination of H_4 receptor ligands and photobiomodulation (1068 nm) as a synergistic modulation/cytoprotection strategy against oxidative stress/inflammation using an *in vitro* human retina glioculture model.

The four primary hypotheses this project addressed were:

- **H_3 and H_4 receptors are differentially expressed in mouse and human retinal cells.**
- **H_4 receptor antagonists protect human Müller cells against oxidative stress-induced toxicity and inflammatory stress.**
- **Functional H_4 receptors exist on human histaminergic Müller cells.**
- **PBM using NIR1068 nm protects human Müller cells against oxidative stress-induced toxicity and inflammatory stress.**

Chapter 2

Materials and Methods

2.1 Frequently used medias, buffers and reagents

Stacking gel buffer:

0.5 M Tris-glycine, pH 6.8 containing 8 mM EDTA and 0.4% (w/v) SDS.

Resolving gel buffer:

50 mM Tris, 384 mM glycine, 1.8 mM EDTA, pH 8.8 and 0.1% (w/v) SDS.

Stock acrylamide:

40% (v/v) acrylamide and N,N'-methylenebisacrylamide.

Electrode buffer:

50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS pH 8.8

Sample buffer:

30 mM sodium hydrogen phosphate, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 7.5% (w/v) SDS.

Pre-stained molecular weight markers:

Pre-stained molecular weight marker stored in sample buffer

Ammonium Peroxodisulphate (APS):

1 mM in dH_2O

Dithiothreitol (DTT):

200 mM in dH_2O

Transfer buffer:

25 mM Tris, pH 8.4, 192 mM glycine and 20% (v/v) methanol.

Tris buffered saline:

50 mM Tris-HCl, 0.9% NaCl pH 7.4.

Tris/EDTA (TE) buffer:

10 mM Tris, 1 mM EDTA, pH 8.0.

Homogenisation buffer:

50 mM Tris-HCl pH 7.4, containing 5 mM EDTA and 5 mM EGTA.

H_4 receptor binding buffer:

50 mM Tris-HCl, pH 7.4

Immunoblotting blocking buffer:

5% marvel (w/v), 0.2% Tween 20 (v/v) and TBS pH 7.4

Immunoblotting incubation buffer:

2.5% marvel (w/v), TBS pH 7.4

Immunoblotting wash buffer:

2.5% (w/v), 0.2% (v/v) Tween 20, TBS pH 7.4

ECL Developing solution A:

1 ml 250 mM luminal, 0.44 ml 90 mM coumaric acid, 10 ml 1 M Tris-base pH8.5.

Make up to 100 ml with dH_2O

ECL Developing solution B:

64 µl 30% H_2O_2, 10 ml 1 M Tris-base, pH 8.5. Make up to 100 ml with dH_2O

Kodak developing solution:

100 ml stock developer and 400 ml dH_2O.

Kodak fixative solution:

125 ml stock fixer and 375 ml dH_2O.

2.2 Lab techniques

2.2.1 MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) uses mitochondrial dehydrogenase activity as a measure of cell viability. MTT solution was made up at a concentration of 25 mg/ml in sterile PBS and 80 µl of the MTT solution was added to each well and incubated for 2.5 hours at 37°C and at 5% CO_2. After this time, all the solution along with the formazan crystals from the wells was transferred into Eppendorf tubes and centrifuged at 1000x g for 10 minutes. The supernatant was discarded and 250 µl of isopropanol added to each Eppendorf. After the Eppendorfs were vortexed, 100 µl of the solution from each Eppendorf was transferred to corresponding wells in a 96-welled (flat bottom) plate and the density of each well was measured spectrophotometrically at 595nm using a Multiskan Ascent Plate Reader, Version 2.6. Background readings (blank wells with cell medium and MTT) were subtracted from the average absorbance readings of the treated wells to get adjusted absorbance readings for cell viability of treated cells.

2.2.3 Immunofluorescence

In a fume hood, a sterile 13 mm glass coverslip was added to each well of a 6-well plate using sterile tweezers. Following steps for cell culture, the cells were transferred to the well-plate for final dilution of 1:2. The well-plates were placed in the incubator at 37°C, 5% CO_2 overnight. After cells had reached around 70% confluency, media was removed from the wells and coverslips washed with 2 ml of PBS^{++} (1 mM $CaCl_2$, 0.5 mM $MgCl_2$ in PBS) for five minutes, twice. Cells were fixed in 4% paraformaldehyde (10 ml paraformaldehyde in 30 ml PBS^{++}) for 15 minutes and then washed twice, as stated previously. Free aldehyde groups were removed with 50 mM NH_4Cl for 15 minutes and then washed thrice as stated previously. Cells were then permeabilised using 0.1% (v/v) Triton X-100 in PBS^{++} for 10 minutes and washed a further time. Cells were then blocked using 0.2% BSA in PBS^{++} for 15 minutes, after which the primary antibody was diluted in 0.2% BSA according to the supplier's recommendation, and cells were incubated with the primary antibody (0.5 µg/ml) for 90 minutes at room temperature. Cells without any primary antibodies were used

as a control. Cells were then washed three times with PBS^{++}, each for 5 minutes and then incubated with the secondary antibody 1:500 anti-rabbit Alexafluorophore 488 in 0.2% BSA for 25 minutes. After the incubation period, cells were washed twice with PBS^{++} for 5 minutes each and incubated again with 1:1000 anti-DAPI in 0.2% BSA for 10 minutes and coverslips were cleaned by dipping in dH_2O after the incubation period. The coverslips were mounted on microscope slides using Mowiol (Calbiochem, UK). Cells were inspected using a Carl Zeiss Apotome microscope equipped with CO_2 and temperature controls. Images were captured using Axiovision software.

2.3 Light assays

The apparatus for PBM treatments of cell cultures consists of LED arrays which emit infra-red light at 1068 nm. The LED arrays are grouped together and held above the cell culture plate so that all the cells in the plate are irradiated. Two different power outputs of LED were used for experiments, 3 mW/sq cm and 10 mW/sq cm. The temperature of the LED lights was kept at room temperature by cooling fans attached to the apparatus to prevent thermal drift of the peak wavelength. This apparatus for PBM experiments was supplied by Virulite Distribution Limited, UK and was validated by Dr. Gordon Dougal (Virulite Distribution Ltd, UK). All the PBM assays was carried out in 24-well plates, with three of the columns used for treatment and the other three used as sham (figure 12)

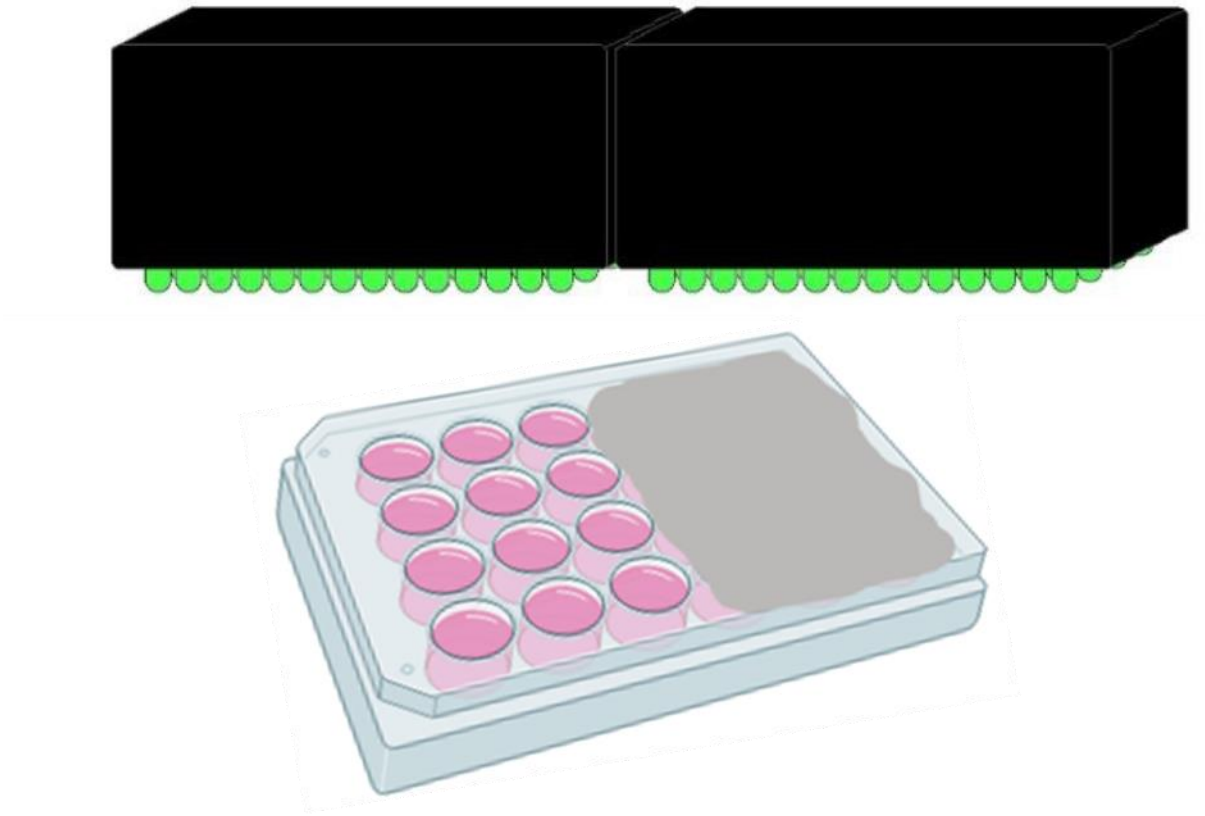

Figure 12: NIR1068 nm light treatment apparatus. All PBM experiments were carried out in 24 well plates, with first 3 columns used for light treatment and the last 3 columns covered in foil for sham treatment.

2.4 Cell cultures

2.4.1 CAD cell culture

CAD (Cath.-a-differentiated) cells were grown at 37°C and at 5% CO_2 in 75 cm^2 tissue culture flasks (Sarstedt, Newton, NC) in Dulbecco's modified eagles' medium DMEM / F-12 Media - GlutaMAX™-I (GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO). CAD cells can differentiate to form neuronal processes in serum free media. To induce this morphological differentiation, cells were switched to same medium without serum supplementation after 24h.

Splitting the CAD cell line

The cells were passaged every 2-3 days at around 80% confluency at a 1:4 dilution. Original medium from the T75 flask was removed. 10 ml of DMEM / F-12 Media -

GlutaMAX™-I with 10% FBS was added to the flask and the cells are detached from the bottom through several pipetting steps. 10 ml of the cell mixtures were transferred to a 15 ml sterile Falcon tube and centrifuged at 100x g for 5 minutes. The supernatant was removed and 5 ml complete medium was added to resuspend the pellet. The cells were divided 1:4 into 75 cm^2 flasks, with 10 ml total medium in each flask.

Cryopreservation and storage of the CAD Cell line

Sub-confluent cultures (70-80%) were dislodged by gentle pipetting; transferred to 15 ml falcon tube and centrifuged at 200x g for 5 minutes at 4 ^oC. The pellet was resuspended in DMEM／F-12 Media - GlutaMAX™-I supplemented with 10% FBS and 10% DMSO. The cell suspension was immediately divided into cryovials with 1 ml volume in each and stored at –80 ^oC for 24 hours and then transferred to liquid nitrogen.

Resuscitation of frozen CAD and Müller Cell Line

A cryogenic vial of cells from liquid nitrogen storage was collected and then quickly transferred to a 37°C water bath. It is important to thaw rapidly to minimise any damage to the cell membranes. The vial was then wiped with a tissue soaked in 70% alcohol prior to opening. The whole content of the vial was pipetted into 15 ml sterile falcon tube. Pre-warmed medium was added to reach a total volume of 10 ml, the tube was then centrifuged at 100x g for 5 min. Supernatant was removed and the CAD cell pellet was resuspended in DMEM/F-12 Media - GlutaMAX™-I supplemented with 10% FBS. Müller cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The resuspended cells were grown at 37°C and at 5% CO_2 in 75 cm^2 tissue culture flasks.

2.4.2 Müller glial cell culture

Müller (MIO-M1) cells were grown at 37°C and at 5% CO_2 in 75 cm^2 tissue culture flasks (Sarstedt, Newton, NC) in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose (Cat number 61965-026,

Invitrogen UK) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO).

Splitting the Müller cell line

The cells were passaged every 3-4 days at around 80% confluency at a 1:5 dilution. Original medium from the T75 flask was removed. 3 ml of TryplE ('trypsin') (TryplE TM Express Enzyme (1X), no phenol red. Cat number 12604013) was added per T75 flask, ensuring all cells are covered by the enzyme. The flask was then incubated at 37°C and at 5% CO_2 for approx. 3 min, until cells are rounded and floating. 10 ml of complete medium was added to the flask and 10 ml of the cell mixtures were transferred to a 15 ml sterile Falcon tube and centrifuged at 100x g for 5 minutes. The supernatant was removed, and 1 ml complete medium was added to resuspend the pellet. The cells were divided 1:5 into 75 cm^2 flasks, with 10 ml total medium in each flask.

Cryopreservation and storage of the Müller Cell line

Sub-confluent cultures (70-80%) were dislodged by adding 3 ml of TryplE per T75 flask; incubated at 37°C and at 5% CO_2 for approx. 3 min, until cells are rounded and floating. 10 ml of complete medium was added to the flask and 10 ml of the cell mixtures were transferred to a 15 ml sterile falcon tube and centrifuged at 200x g for 5 minutes at 4 ^oC. The pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% fetal bovine serum and 10% DMSO. The cell suspension was immediately divided into cryovials with 1 ml volume in each and stored at –80 ^oC for 24 hours and then transferred to liquid nitrogen.

2.4.3 Calcium Imaging

Müller cells were grown in Ibidi 35 mm µ-dishes as mentioned in section 2.4.2 at 1:6 dilution 24 hours prior to the start of the experiment.

On the day of the imaging experiments, the growth media from µ-dishes was removed and the cells were washed once with 1 ml of physiological buffer (150

mM NaCl, 1 mM MgCl2, 10 mM HEPES, 2 mM CaCl2, 5 mM KCl). The cells were then incubated for 40 minutes in 1 ml of 1µM dye solution (Fluo-4 AM (Life Technologies, Invitrogen™) at 37°C and at 5% CO_2 in the dark. After the incubation period, the cells were washed once again gently with the physiological buffer in the dark and then kept in the physiological buffer and submitted immediately to the imaging experiments.

For imaging, the µ-dish was placed in a chamber on the stage of the microscope at 37°C and at 5% CO_2. The field of cells was monitored by observing epifluorescence after excitation at 488 nm and images were acquired every 1 second, by programming the Zen 2™ (black edition) software to automatically do so. Cells were initially exposed to control (physiological buffer), followed by incubation with different concentration of drugs at 100 seconds interval.

2.5 Statistical analysis

All data were analysed using Microsoft Excel 2007 and GraphPad Prism version 8, GraphPad Software Inc. All column data are displayed as mean ± standard error of the mean (SEM). For comparing sham and IR1068 conditions, data were subjected to an unpaired two-tailed Student's T-test and the other data were subjected to one-way ANOVA analysis of variance, with a Bonferroni post-hoc test.

Chapter 3

Histamine Receptors H_4 in mouse and human retinal Müller cells

3.1 Objectives

The aim of this chapter was to determine the presence of H_4 receptor in Müller cells under different culture conditions. Another aim was to also probe for the presence of histamine decarboxylase, HDC, in the Müller cells. The next aim was to perform a series of pharmacological experiments to determine the functional expression of H_4 receptors in Müller cells and test any therapeutic potential of the receptor.

3.2 Introduction

There is an increased histaminergic tone associated with diabetes, and emerging evidence also points to a role of histamine in the BRB breakdown that is associated with the pathogenesis of diabetic retinopathy (Leal et.al, 2005). This role of histamine in diabetic retinopathy is still not deeply investigated given the neurodegenerative nature of the disease and its effect on other components of the retina such as glial cells and neurons are considered. Also, since a lot of the investigations on the role of histamine in diabetes were before the discovery of the newest histamine receptors H_3 and H_4 (Panula et. al, 2015), there is lot of potential for new insights into the role of these receptors in diabetes and opportunity to develop new drugs and therapies.

Histamine H_4 receptor is a member of the GPCR superfamily like the other histamine receptors (Alexander et al., 2013), and it has a distinct pharmacological profile compared to the other receptors (De Esch et al., 2005). Recent evidence in the literature has implicated an important role of H_4 receptor in vascular permeability as it has been shown to be expressed in rat brain endothelial cells. Based on this, we hypothesized an important role of H_4 receptor in diabetic retinopathy. To test this, a

pilot immunological study was performed by our group previously on fixed mouse (C57 strain) retinal slices, which revealed a differential topological expression pattern. Results have shown that the histamine H_3 and H_4 receptors display a differential protein expression pattern. The H_3 receptor labels all nuclear bodies in the photoreceptor layer (ONL) and some bipolar cells (INL) but little in the plexiform layers or ganglion cell layer (figures 13 and 14). There are some processes labelled in the IPL, we think these are from histaminergic amacrine cells (figure 13). For the H_4 receptor, these are mainly labelling the plexiform layers (IPL and OPL) (figures 13 and 15). These might be in the Müller glial cells, and their endfeet are strongly labelled. As can be seen, there are faint stratifications in the IPL, and these are the ON and OFF sublamina divisions (figure 15).

From these initial results observed in the mouse retina, where there was positive evidence for H_4 receptor expression on the Müller cells, and the important role of these cells in diabetic retinopathy, we decided to focus on characterizing this receptor in-vitro in this chapter.

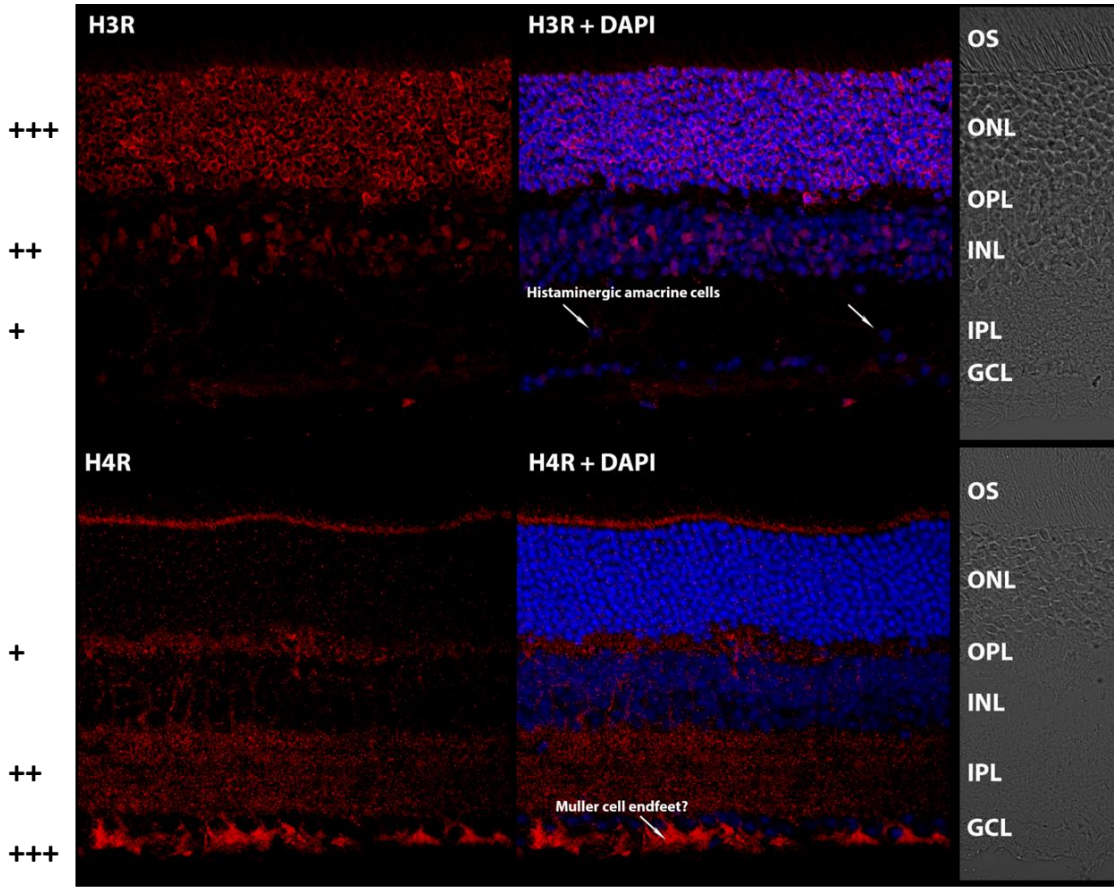

Figure 13: **Immunohistochemistry of mouse (C57 strain) retinal slices.** Cell were probed with DAPI (blue) and H_3, H_4 receptor specific antibodies (Abcam anti-HRH3 and anti-HRH4 commercial antibodies) (red) showing differential H_3 & H_4 receptor topology in the mouse retina.

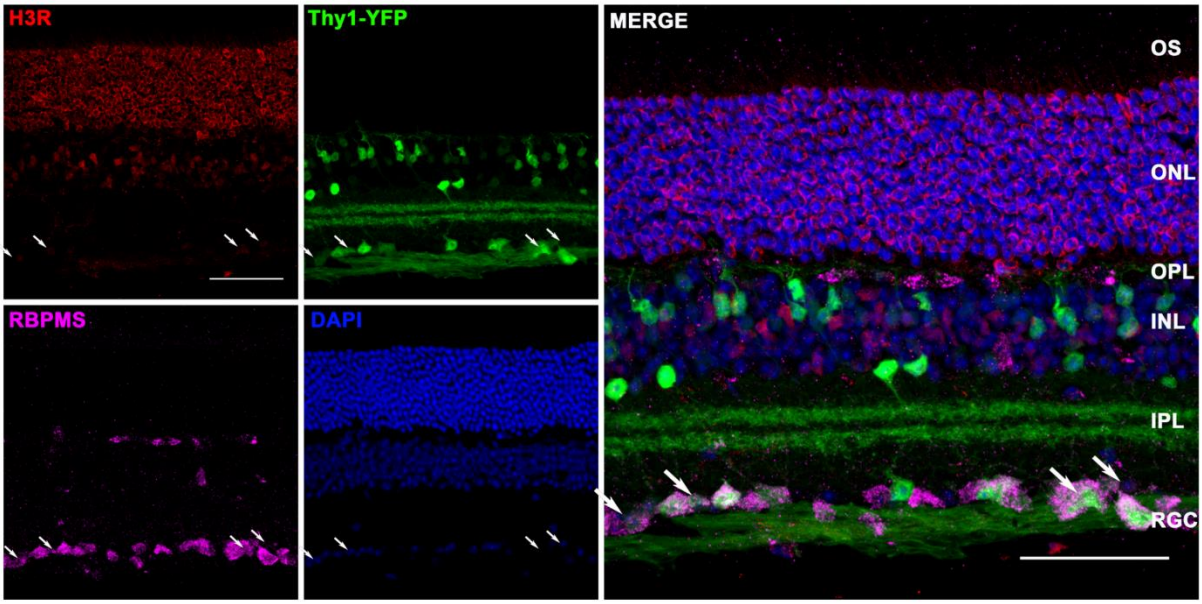

Figure 14: **Immunohistochemistry of C57 strain retinal slices.** Cells were probed with DAPI (blue), H_3 receptor specific antibody (Abcam anti-HRH3 commercial antibody) (red), Thy1-YFP (green) and RNA-binding protein with multiple splicing (RBPMS) (purple). H_3 receptor is expressed on photoreceptors, some bipolar cells & Thy-1-ve/RBPMS +ve ganglion neurons

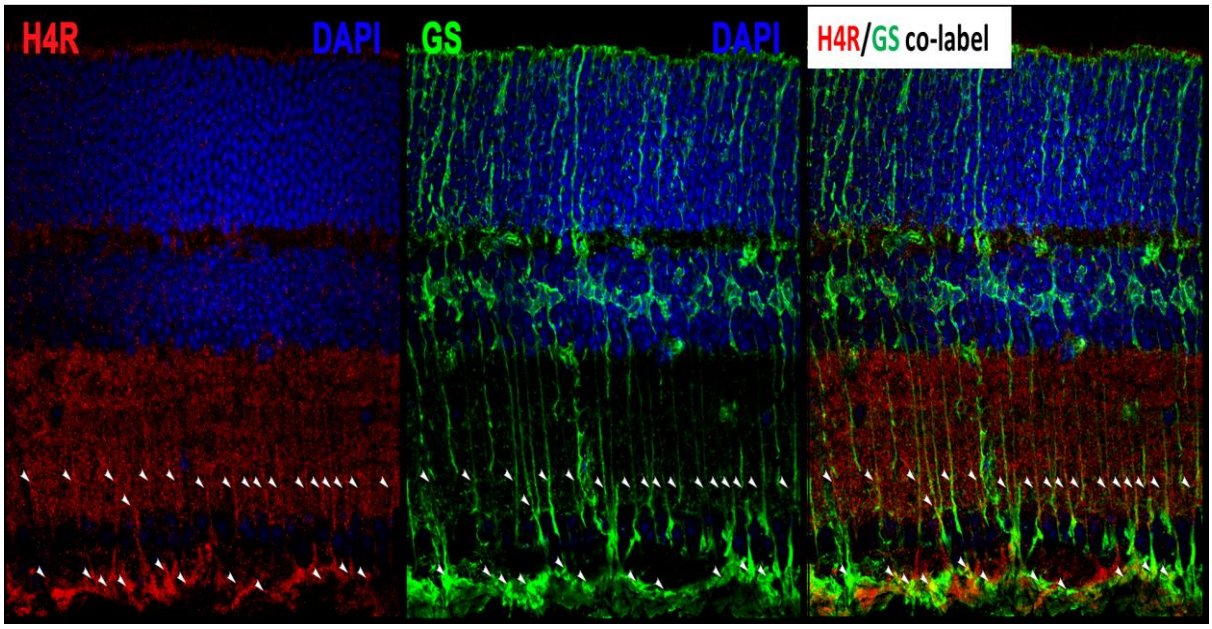

Figure 15: **Immunohistochemistry of C57 strain retinal slices.** Cells were probed with DAPI (blue), H_4 receptor specific antibody (Abcam anti-HRH4 commercial antibody) (red) and Glutamine Synthetase (GS) (green). H_4 receptors are expressed on mouse plexiform layers & retinal Müller cells.

3.3 Methods

3.3.1 Immunofluorescence for anatomical characterisation of histamine H_4 receptor and HDC

IF on Müller cells was carried out as mentioned in section 2.2.3. For Müller cells exposed to high glucose environment, the cells were grown in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose (Cat number 61965-026, Invitrogen UK) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO). This experiment was repeated 4 times, and the images with the best resolution were selected.

For low glucose environment, the cells were grown in Dulbecco's modified eagles' medium DMEM / F-12 Media - GlutaMAX™-I (GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO). This experiment was repeated 3 times, and the images with the best resolution were selected.

3.3.2 Calcium^{2+} imaging of Müller cells stimulated with the H_4 receptor agonists, VUF 8430 and ST-1006

One of the downstream effects in the cell when H_4 receptor is activated is the release of calcium from the intracellular storage vesicles through interaction with $InsP_3$ receptor. Thus, to determine the functional expression of H_4 receptors in Müller cells, changes in the intracellular calcium concentration [Ca^{2+}] of single Müller cells were measured by super resolution digital microscopy, using a Zeiss LSM 880 with Airyscan. Müller cells were grown in Ibidi 35 mm µ-dishes as mentioned in section 2.4.2 at 1:6 dilution 24 hours prior to the start of the experiment.

On the day of the imaging experiments, the growth media from µ-dishes was removed and the cells were washed once with 1 ml of physiological buffer (150 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM $CaCl_2$, 5 mM KCl). The cells were then incubated for 40 minutes in 1 ml of 1 µM dye solution (Fluo-4 AM (Life Technologies,

Invitrogen™) at 37°C and at 5% CO_2 in the dark. After the incubation period, the cells were washed once again gently with the physiological buffer in the dark and then kept in the physiological buffer and submitted immediately to the imaging experiments.

For imaging, the µ-dish was placed in a chamber on the stage of the microscope at 37°C and at 5% CO_2. The field of cells was monitored by observing epifluorescence after excitation at 488 nm and images were acquired every 1 second, by programming the Zen 2™ (black edition) software to automatically do so. Cells were initially exposed to control (physiological buffer), followed by incubation with different concentration of drugs at 100 seconds interval.

All the test drugs were dissolved in the physiological buffer as stock solutions 10 times more concentrated than the final desired concentration for the experiments.

Any background reading was deducted from the cell fluorescence intensity, and the final fluorescence intensity was calculated by dividing the intensity of the cell with its area. The average fluorescence intensity of 5 cells were taken for graphical representation.

3.3.3 Oxidative stress – dose determination in Müller cells

Hydrogen peroxide (H_2O_2) is commonly used to elicit oxidative stress in cells. Usually, production of reactive oxygen species (ROS) such as H_2O_2, are a natural by-product of oxidative phosphorylation in the cell, and in low concentrations, they can act as signaling molecules for various cellular processes. However, high concentrations of H_2O_2 is detrimental to cells, where the ROS damages mitochondrial membrane potential, disrupting the ETC and thus ultimately results in the release of Cytochrome C and apoptosis (Beere, 2005, Tabner et al., 2005). Therefore, hydrogen peroxide was chosen to induce retinal damage to model this aspect of Diabetic Retinopathy.

H_2O_2 induced insult with a series of different concentrations of H_2O_2 was carried out to determine the dose-dependent effects on Müller cell survival. A stock solution of H_2O_2 (2500 µM) in PBS was prepared and a series of different H_2O_2 concentrations were then prepared using Dulbecco's Modified Eagle Medium 1X with GlutaMAX,

without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% fetal bovine serum (FBS) to achieve final concentrations of 10 µM, 50 µM, 100 µM, 200 µM, 250 µM, 300 µM, 400 µM and 500 µM. Sub-confluent cultures (70-80%) were dislodged as described in section 2.4.2 and transferred to a 15 ml falcon tube and centrifuged at 100x g for 5 minutes. The cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The cell suspension was then plated in 24-well plates and incubated at 37°C and at 5% CO_2. The media was replaced after 24 hours with the different H_2O_2 concentrations prepared and grown in culture for 4-hour and 24-hour time periods, after which an MTT assay was performed to determine cell viability (section 2.2.1).

3.3.4 Glyoxal Treatment of Müller cells

It is well known that diabetic retinopathy is a chronic, low-grade inflammatory disease, and several biochemical pathways have been implicated in this pathogenesis. One such pathway is the formation of AGEs, which irreversibly modify the chemical properties and functions of various key molecules (Wautier and Guillausseau, 2001). An intermediate of AGEs, glyoxal, has been shown in several studies of diabetes-associated retinal abnormalities to be an important and powerful inducer of cell apoptosis in the retina (Shen et. al, 2009; Kniep et. al, 2006). Glyoxal was also reported to cause inflammatory damage in human vascular endothelial cells (Yamawaki and Hara, 2008). More importantly, there is accumulating evidence that inflammatory factors contribute to the BRB breakdown associated with diabetic retinopathy (Joussen et. al, 2002; Carmo et. al, 2000; Funatsu et. al, 2001; Qaum et. al, 2001) and Müller cells have been demonstrated to be involved in the inflammatory process in-vitro (Walker and Steinle, 2007; Shelton et. al, 2007). A more recent study has demonstrated that glyoxal can induce inflammatory response in Müller cells in-vitro (Lei et. al, 2011). Therefore, in this study we have used glyoxal to induce inflammatory response in Müller cells and have checked whether it can be regulated by H_4 receptor ligands under the condition of diabetic retinopathy.

Sub-confluent cultures (70-80%) of Müller cells were dislodged as described in section 2.4.2 and transferred to a 15 ml falcon tube and centrifuged at 100x g for 5

minutes. The cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The cell suspension was then plated in 24-well plates and incubated at 37°C and at 5% CO_2 until cells were 70-80% confluent in each well. Stock solutions (1 mM) of H_4 receptor agonist VUF 8430 and antagonist JNJ 7777120 was freshly made up in dH_2O. The H_4 receptor ligand stock solutions were then added to the cells in the 24-well plate (except control) to give a final concentration of 10 µM for both the agonist and the antagonist. The cells were then incubated for 1 hour at 37°C and at 5% CO_2. After the one-hour incubation period, stock glyoxal solution (2 M) was added to the wells (except control) to give final glyoxal concentration of 2 mM and the cells were further incubated for 13 hours at 37°C and at 5% CO_2 before extracting cell supernatant for TNF-alpha analysis and carrying out MTT assay (section 2.2.1) to determine the cytotoxic effects of glyoxal.

Concentration of TNF-alpha was measured with Abcam's TNF alpha Human in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit (ab 46087) according to the instructions of the kit. The values were compared with the standard curve of the cytokine and normalized to total protein concentrations.

3.3.5 Neuroprotective assay using H_4 receptor ligands in Müller cells

Once an optimal dose of H_2O_2 was determined that elicited significant loss of cell viability, an assay was designed to check the neuroprotective effects of H_4 receptor ligands in Müller cells.

Sub-confluent cultures (70-80%) of Müller cells were dislodged as described in section 2.4.2 and transferred to a 15 ml falcon tube and centrifuged at 100x g for 5 minutes. The cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The cell suspension was then plated in 24-well plates and incubated at 37°C and at 5% CO_2 until cells were 70-80% confluent in each well. Stock solutions (1 mM) of H_4 receptor agonist VUF 8430 and antagonist JNJ 7777120 was freshly made up in dH_2O. The H_4 receptor ligand stock solutions were then added to the cells in the 24-well plate (except control) to give a final concentration of 10 µM for both the agonist

and the antagonist and incubated for 1 hour at 37°C and at 5% CO_2. After the one-hour period, H_2O_2 was added to the corresponding wells from a stock solution of H_2O_2 (2500 µM) in PBS to give final concentration of 400 µM in the corresponding wells. The cells were kept in incubator for 4 hours, after which an MTT assay was performed to determine cell viability (section 2.2.1).

3.4 Results

Due to positive results seen for the presence of H_4 receptor in-vivo as discussed previously, the focus was on characterizing the H_4 receptor in-vitro using Müller cells to understand the important role of these cells in diabetic retinopathy

The presence of H_4 receptor and HDC was probed in Müller cells under different culture conditions using immunofluorescence. Once the presence of the receptor and enzyme was confirmed, a series of pharmacological experiments was performed to determine the functional expression of H_4 receptors in Müller cells and test any therapeutic potential of the receptor.

3.4.1 IF shows the presence of H_4 receptor in human Müller cells grown in different culture conditions.

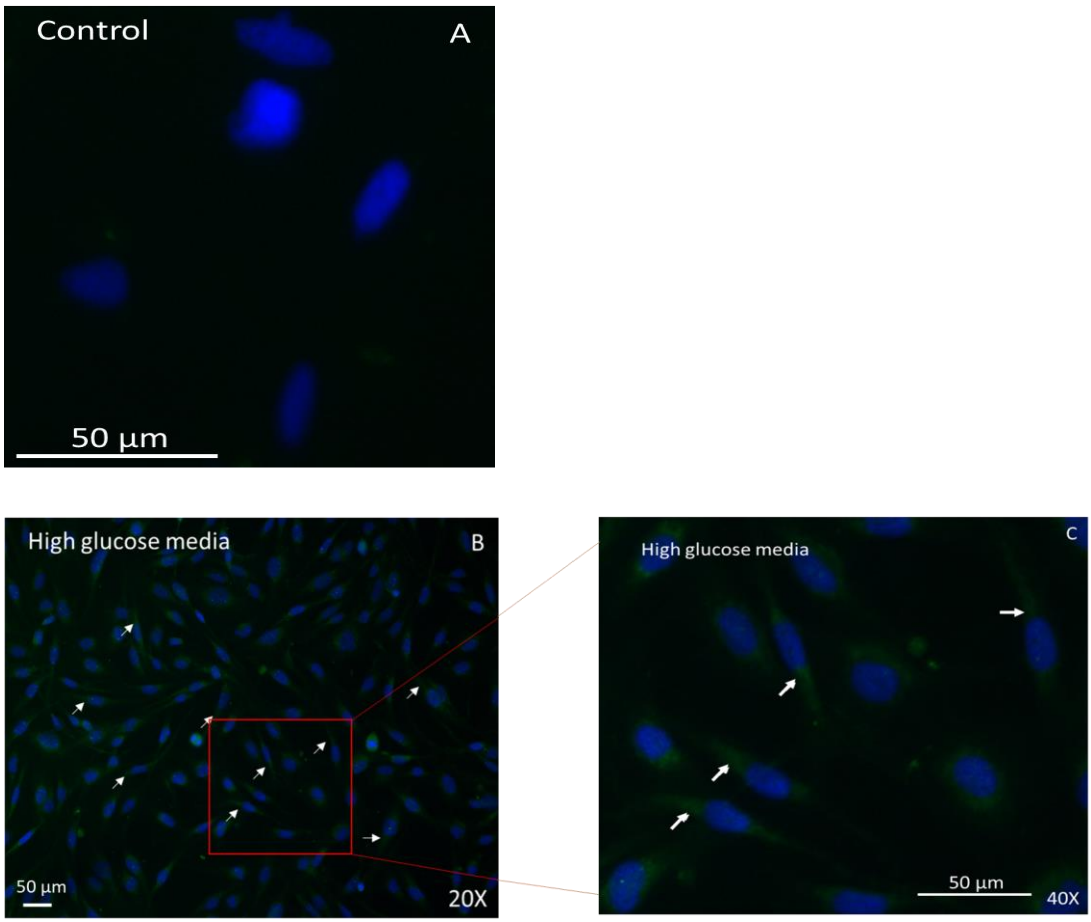

Figure 16: Immunofluorescence labelling for H_4 receptor in human Müller cells grown in high glucose media. Cells were grown in High glucose media and probed with anti-H_4R antibody (0.5 µg/ml) (In house anti-HRH4 commercial antibody (van Rijn, Chazot et al., 2006) (green). Nuclei were stained with DAPI (blue). All the slides were examined at x20 (B) and x40 (C) magnification using Zeiss Apotome microscope and shows H_4 receptor expression in the Müller cell processes (arrow heads) compared to control (A). Cells without any primary antibodies were used as a control.

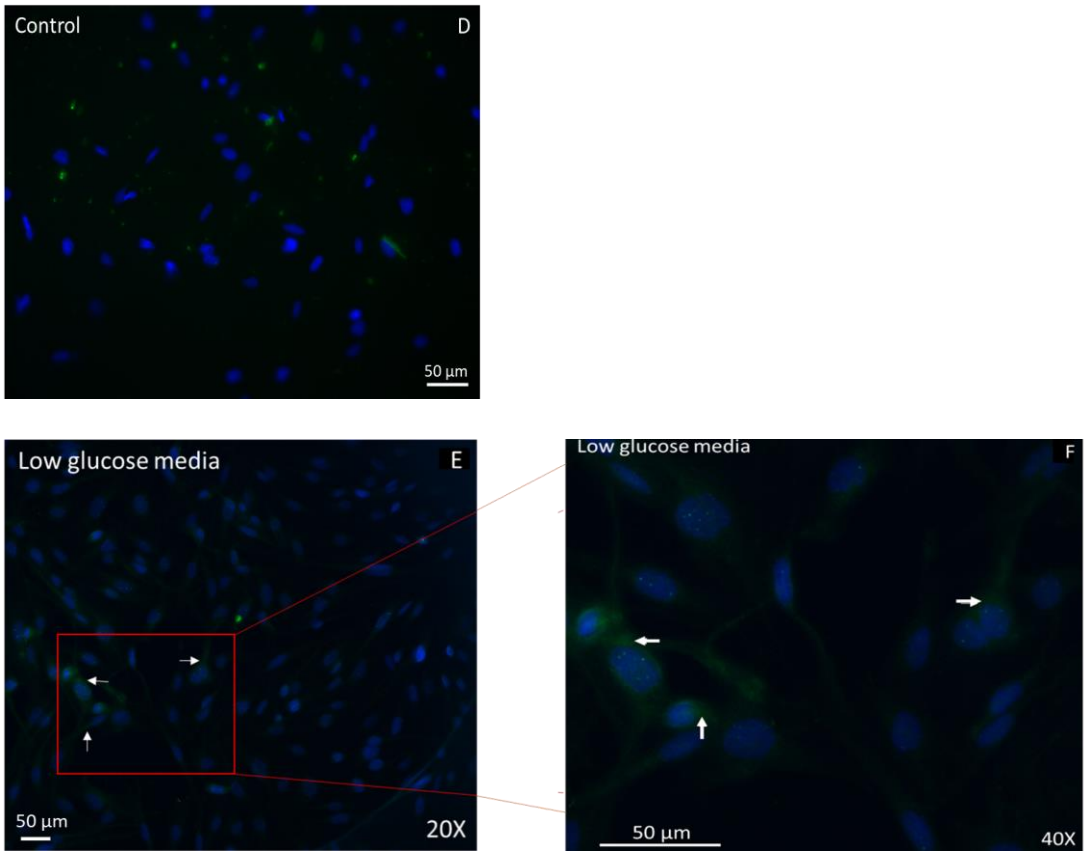

Figure 17: Immunofluorescence labelling for H_4 receptor in human Müller cells grown in low glucose media. Cells were grown in low glucose media and probed with anti-H_4R antibody (0.5 µg/ml) (In house anti-HRH4 commercial antibody (van Rijn, Chazot et al., 2006) (green). Nuclei were stained with DAPI (blue). All the slides were examined at x20 (E) and x40 (F) magnification using Zeiss Apotome microscope and shows H_4 receptor expression in the Müller cell processes (arrow heads) compared to control (D). Cells without any primary antibodies were used as a control.

Figure 16 and 17 shows Immunofluorescence labelling of human Müller cells. H_4 immunoreactivity was observed on the Müller cell processes (figure 16B-C and figure 17E-F) compared to controls (figure 16A and 17D), and this expression seems to be higher – shown by higher immunoreactivity - in Müller cells when the cells were grown in high glucose media (figure 16B-C) compared to when they were grown in low glucose media (figure 17E-F).

3.4.2 IF shows the presence of HDC in in human Müller cells

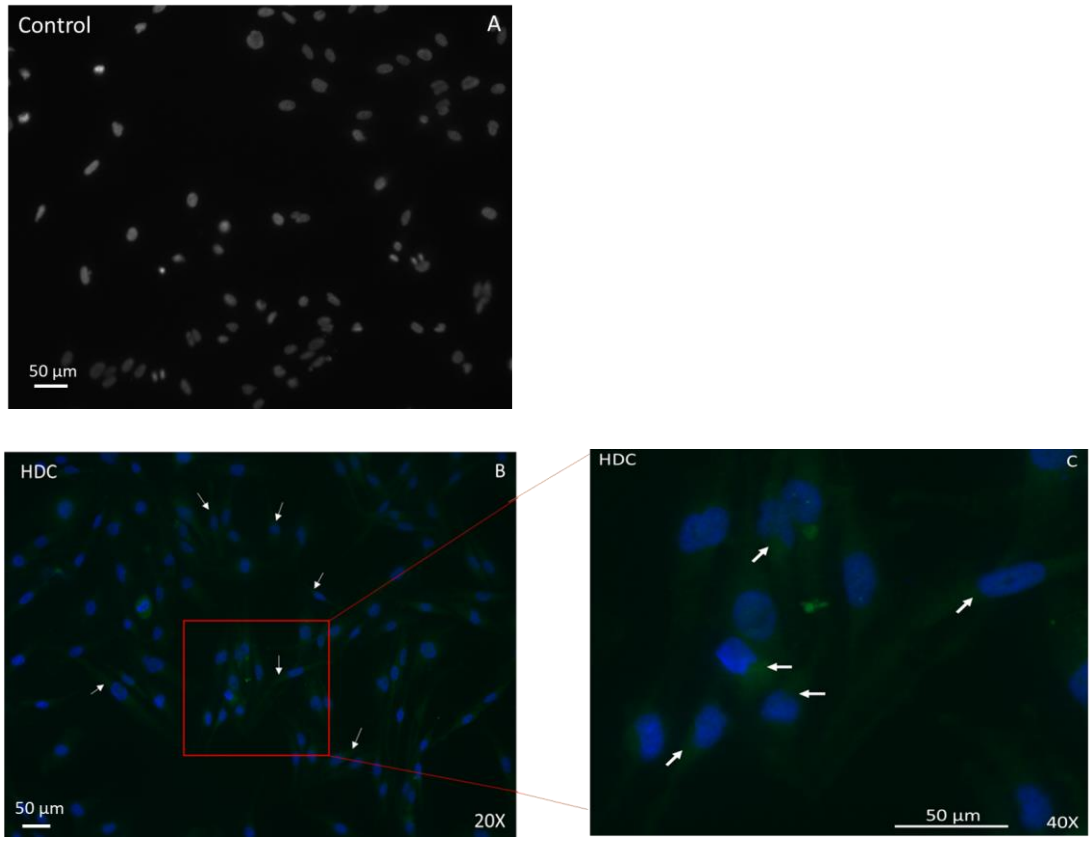

Figure 18: Immunofluorescence labelling for HDC expression in human Müller cells. Cells were grown in high glucose media and probed with HDC specific antibody (0.5 µg/ml) (Abcam anti-HDC commercial antibody) (green). Nuclei were stained with DAPI (blue). All the slides were examined at x20 (B) and x40 (C) magnification using Zeiss Apotome microscope, and shows HDC expression in the Müller cell processes (arrow heads) compared to control (A). Cells without any primary HDC antibodies were used as a control.

Figure 18 shows Immunofluorescence labelling of human Müller cells. Histamine synthesis enzyme, HDC, immunoreactivity was observed on the Müller cell processes (figure 18B-C) compared to control (figure 18A).

3.4.3 Functional expression of histamine H_4 receptor in Human Müller cells using selective H_4 receptor agonists VUF 8430 and ST-1006

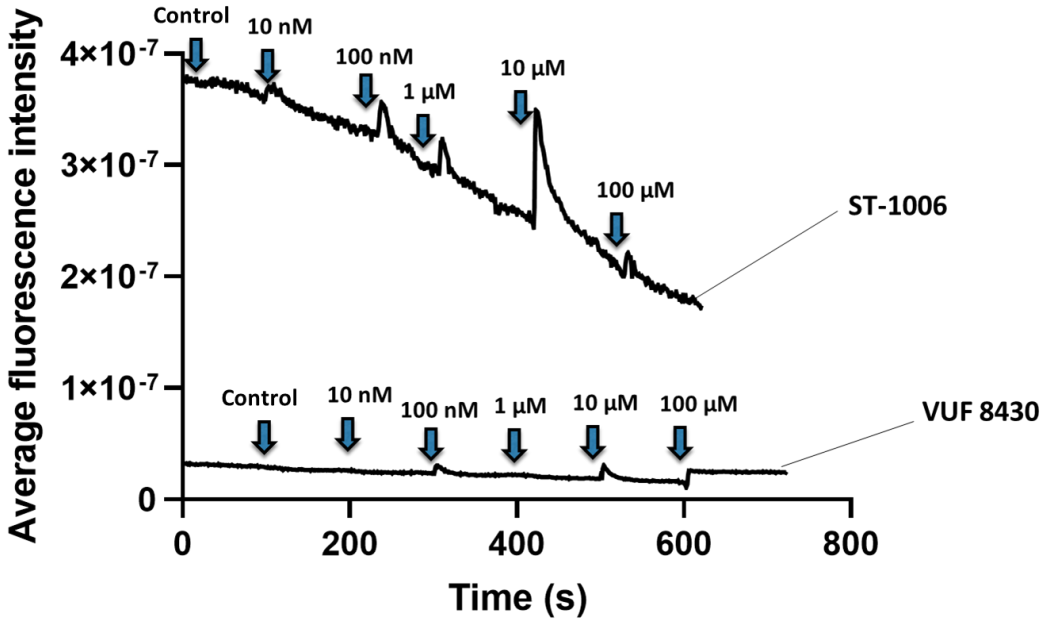

Figure 19: Dose response in Müller cells for H_4 receptor agonists VUF8430 and ST-1006. Changes in fluorescent intensity of Müller cells in response to control and 10 nM, 100 nM, 1 µM, 10 µM and 100 µM concentrations of H_4 receptor ligands. Following injection with control (PBS) for 100 seconds, the different concentrations of the drugs were injected at 100 second intervals. The Ca^{2+} release induced fluorescent intensity of 5 different Müller cells from three independent experiments were measured and averaged for graphical representation**.** Experiments for both agonists were done separately and represented on the same scale. Arrows indicate the time points at which the different treatments were added to the cells.

Figure 19 shows that following administration of the selective H_4 receptor agonists VUF8430 and ST-1006, there was an increase in the fluorescence intensity observed in the cells. The increase in fluorescence was higher for ST-1006 treatment relative to VUF 8430 treatment and for both treatments, the max fluorescence was seen when the cells are treated with a dose concentration of 10 µM, which is consistent with similar experiments in the literature. The fluorescent intensity was used to

measure the H_4 receptor activity through intracellular calcium mobilization from intracellular stores. This is consistent with an increase in the release of intracellular calcium from the cells upon H_4 receptor activation.

3.4.4 Effects of concentration-dependent hydrogen peroxide insult (24 and 4 hours)

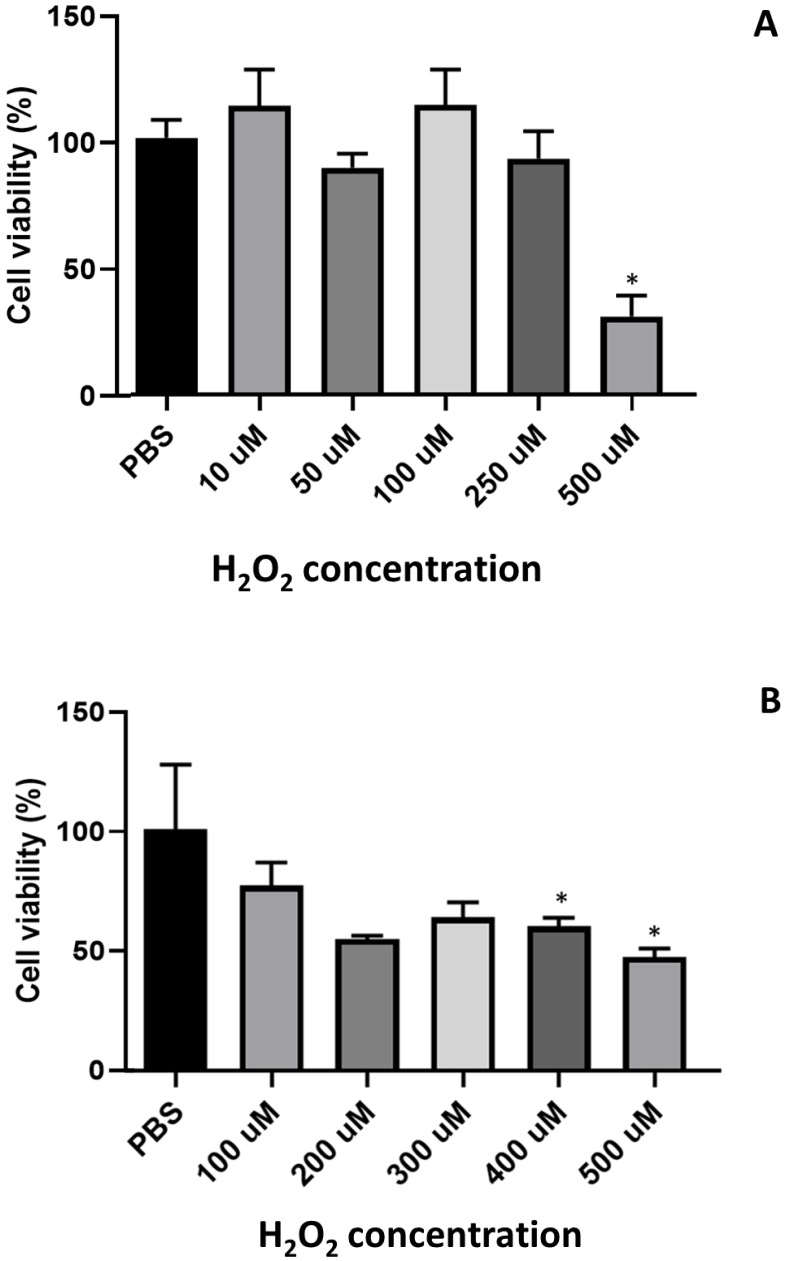

Figure 20: **24-hour and 4-hour dose-dependent effect on Müller cell survival using H_2O_2.** 24 (A) and 4 hours (B) insult of Müller cell cultures with varying hydrogen peroxide concentrations compared to cells treated with control (PBS). Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicate. * p <0.05 Vs control.

Figure 20 shows concentration dependent H_2O_2 insult in Müller cells of varying concentration and incubated for 24 hours (A) and 4 hours (B). This was to determine a concentration of H_2O_2 that would illicit a significant neurotoxic effect in Müller cells. With 24 hours incubation, only a concentration of 500 µM induced a significant loss of cell viability (67%) compared to control but this loss is too high for neuroprotective experiments. The protocol was then changed with higher starting concentrations of H_2O_2 and shorter incubation period of 4 hours. With this protocol, optimal loss of cell viability was achieved with 400 µM (50%) and 500 µM (60%). For neuroprotective experiments, H_2O_2 concentration of 400 µM and incubation period of 4 hours was selected.

3.4.5 Lack of protection with H_4 ligands Vs oxidative stress

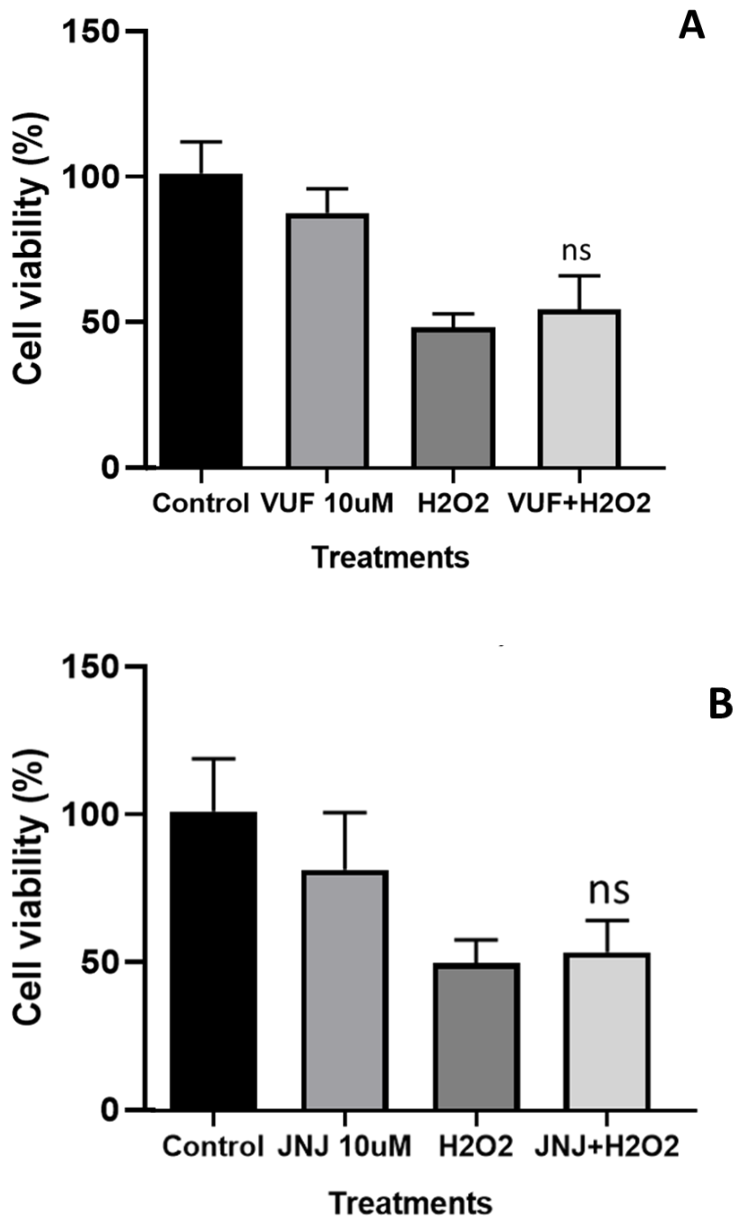

Figure 21. Cell viability assay in Müller cells using H_4 receptor antagonists VUF8430 and JNJ 7777120 against oxidative insult. Müller cells were preconditioned with 10 µM of H_4 receptor antagonists VUF8430 (A) and JNJ 7777120 (B) for 1 hour before 4 hours oxidative insult with 400 µM H_2O_2. Control was treatment with PBS only for 1 hour. Cell viability was measured using MTT assay. Results are mean ± SD for two independent experiments performed in quadruplicates. 'ns' was compared to H_2O_2 treatment.

Figure 21 shows that there was no neuroprotection with H_4 receptor agonist or antagonist when Müller cells are subjected to oxidative stress using H_2O_2. For treatment with VUF 8430 and JNJ 7777120, there was no significant difference in cell viability in comparison to 400 µM H_2O_2 treated cells. VUF 8430 and JNJ 7777120 alone, also doesn't seem to illicit a toxic effect in the Müller cells as there was no significant difference in cell viability when the cells are treated with agonist or antagonist only in comparison control cells.

3.4.6 H_4 receptor ligands worsen cell viability during inflammatory stress

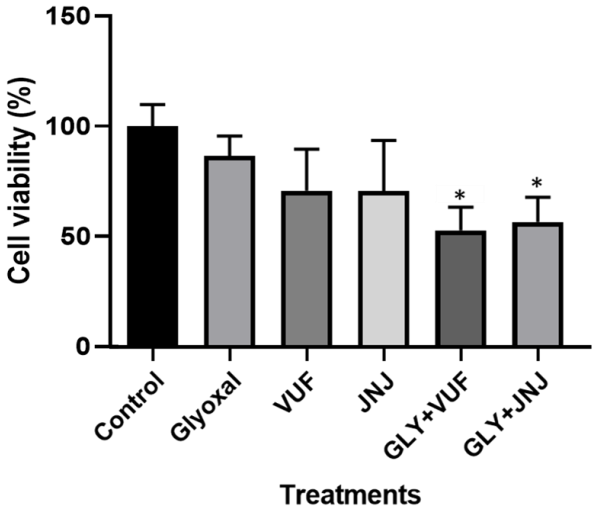

Figure 22: Cell viability assay in Müller cells using H_4 receptor ligands against inflammatory stress. Müller cells were preconditioned with 10 µM of H_4 receptor antagonist JNJ 7777120 or agonist VUF8430 for 1 hour before 13 hours inflammatory insult with 2 mM glyoxal. Control was treatment with PBS only for 1 hour. Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicate. * p <0.05 Vs control or Glyoxal alone.

As seen from figure 22 that interestingly, when cells are preconditioned with H_4 receptor ligands for one hour and then stressed with 2 mM glyoxal for 13 hours, the cell viability decreases significantly compared to control or when cells are treated with glyoxal alone.

Next, the supernatant of these cells was analysed for cytokine (TNF-alpha) expression levels (figure 23). This time, for cells preconditioned with VUF 8430 and then treated with glyoxal, TNF-alpha expression level increases significantly compared to control or when cells are treated with glyoxal only. Interestingly though, when the Müller cells are treated with the agonist alone, the cytokine expression level seems to be reduced compared to control or when cells are treated with glyoxal only.

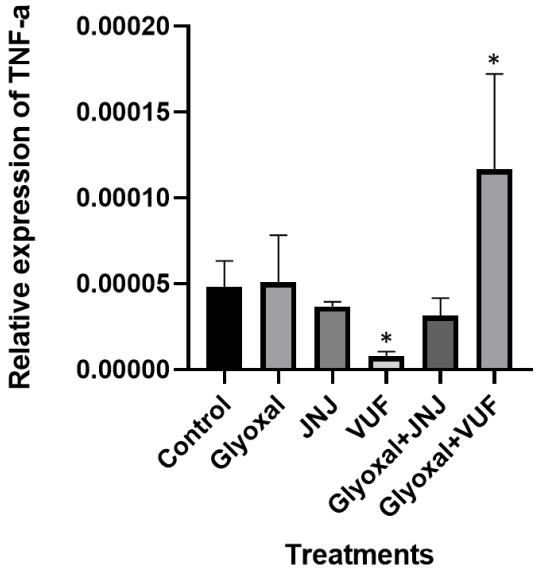

Figure 23: Effects of H_4 receptor ligands on TNF-alpha expression when Müller cells are subjected to inflammatory stress. Müller cells were preconditioned with 10 µM of H_4 receptor antagonist JNJ 7777120 or agonist VUF8430 for 1 hour before 13 hours inflammatory insult with 2 mM glyoxal. Control was treatment with PBS only for 1 hour. Expression levels of the cytokine was measured using ELISA assay. Results are mean ± SD for one independent experiment performed in quadruplicate. * $p < 0.05$ Vs control or Glyoxal alone. Expression levels of TNF-a shown in g/ml.

3.5 Discussion

Initial immunological study performed by our group on fixed mouse (C57 strain) retinal slices showed positive evidence for H_4 receptor expression in Müller cells in the plexiform layers. H_3 receptor expression was only detected in the photoreceptor layers and in some bipolar cells but was absent in the plexiform layers. Thus, we decided to characterize the expression of H_4 receptor in Müller cells in-vitro because of their importance in diabetic retinopathy.

This chapter characterized the presence of Histamine H_4 receptor in the Müller cells of the retina for the first time through Immunohistochemical and pharmacological studies. The chapter also explored the possibility of using H_4 receptor ligands as a new therapeutic approach to treat diseases where the cells are under oxidative or inflammatory stress.

3.5.1 Characterisation of Histamine H_4 receptor in Müller cells

Immunological studies using H_4 receptor-specific antibody in Müller cells confirmed the presence of the receptor in the cells, specifically in the projections of the cells. This fits well with the observation in the mouse retina where H_4 receptor labelling was seen in the end-feet of the Müller cells. When the cells were grown in high glucose media, this expression of the receptor appeared more prominent compared to when the cells were grown in low glucose media. This is consistent with several studies that has implicated the role of histamine in diabetic conditions through upregulation of histamine in different organs in diabetic patients and rats (Gill et al., 1989; Fogel et al., 1990), and upregulation of the histamine H_4 receptor (Pini et al., 2018; Rosa et al., 2013)

Histamine H_4 receptor shares some homology with the H_3 receptor (Leurs et al., 2012), and H_3 receptor has been widely accepted to act as both an auto- and hetero-receptor to control the release of histamine and other neurotransmitters in the CNS; hence we further investigated this property in H_4 receptor by investigating for the presence of HDC enzyme through immunofluorescence. HDC was also found to be present in the Müller cell processes; which suggests that there is nascent histamine pool in these glial cells. This observation further supports the studies in the retinas

of diabetic rats where there is an over expression of HDC and increased histamine synthesis (Hollis et al., 1992, Carroll et al., 1988; Gastinger et al., 2001).

To further Illustrate the presence of H_4 receptor, the immunostaining experiments need to be repeated with HDC knockout Müller Cell lines to demonstrate the specificity of the H_4 receptor antibody for histamine cells in the retina. This experiment could then also be followed up with double staining of the Müller cells against H4 receptor and an antibody against HDC.

On functional characterization of the H_4 receptor in Müller cells, both the agonists, VUF 8430 and ST-1006, induced Ca^{2+} release from the intracellular calcium store in the cells. The agonist ST-1006 surprisingly induced a higher response in the cells as this H_4-specific agonist is characterized as the most potent to date (Gschwandtner et al., 2013) but it is only a partial H_4 receptor agonist.

3.5.2 High tolerance for oxidative insult in Müller cells

Müller cells seem to have a high tolerance for oxidative insults as illustrated by the high concentrations of H_2O_2 required to induce a significant loss of cell viability. This contrasts with other studies in the laboratory, when the same protocol was carried out in differentiated CAD (Cath.a differentiated) murine CNS catecholaminergic neuronal cell line (figure 24) where lower doses of H_2O_2 resulted in a large loss of cell viability. The ability of Müller cells to tolerate high levels of oxidative stress hints at intrinsic compensatory mechanisms in these cells to cope with oxidative stress as shown by Toft-Kehler et al., 2016, where they have shown that Müller cells with intact energy supply are only affected by oxidative stress at high concentrations. These results also emphasize the important protective and supportive role of Müller cells in the retina.

To confirm the high tolerance of Müller cells to H_2O_2, the experiment needs to be repeated and quantified against a H_2O_2 sensitive cell line such as 293T-Cells.

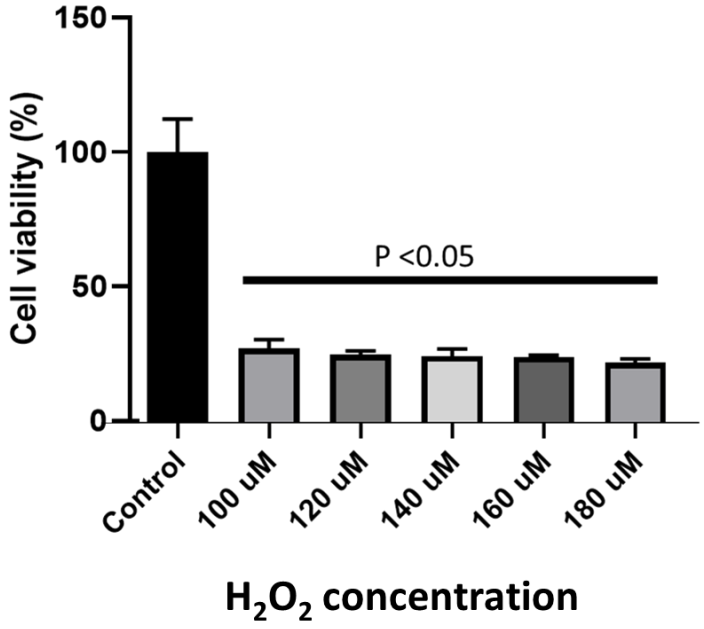

Figure 24: **4-hour dose-dependent effect on CAD cell survival using H_2O_2.** 4 hours insult of CAD cell cultures with hydrogen peroxide of concentrations of 100 µM, 120 µM, 140 µM, 160 µM and 180 µM compared to cells treated with control (PBS). Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicate. * p <0.05 Vs control.

3.5.3 Müller cell H_4 receptor as a potential drug target against oxidative and inflammatory stress

Because of the widespread expression of H_4 receptor in different tissues types and its role in diabetes (Pini et al., 2015, Rosa et al., 2013) and vascular permeability (Karlstedt et al., 2013), the H_4 receptor is a potential target for therapeutic interventions in diabetic retinopathy. On testing the H_4 receptor agonist VUF 8430 and antagonist JNJ 7777120 against oxidative stress, there was no significant difference in cell viability and the ligands were not effective in protecting against oxidative insults. When the same protocol was carried out against glyoxal-induced inflammatory insult, both the ligands seem to worsen the toxicity and on analysis of the cytokine TNF-alpha secretion in the treated cells, pretreatment of the cells with the agonist before inflammatory stress seems to increase the level of TNF-alpha

secretion significantly and when the cells are treated alone with the agonist, it has the opposite effect of reducing TNF-alpha level. This result suggests that H_4 receptor agonist is effective in blocking production of basal level TNF-alpha in Müller cells as also demonstrated in a study by Gschwandtner et al.,2013, who showed that H_4 receptor agonist VUF 8430 reduces the cytokine IL-12 production in human monocytes and this production is only partially blocked by the antagonist JNJ 7777120. There is evidence in the literature where JNJ 7777120 acts as a partial H_4 receptor agonist in some species (Wifling et al., 2015) and this might explain the partial blockage of IL-12 production by JNJ7777120 observed by Gschwandtner et al.,2013 in human monocytes. But the observation that treatment of Müller cells with VUF8430 or JNJ777120 worsens inflammatory stress-induced cell viability hints that during inflammatory stress, activation of the H_4 receptor somehow worsens the toxicity induced by glyoxal. It will be interesting to use a neutral antagonist in the same experiment to compare to the effects of JNJ7777120 and determine whether JNJ7777120 was acting as a partial agonist in these experiments. A good candidate for another antagonist to use would be VUF-10214 or VUF-10148, which both have good H_4 receptor potency and have also demonstrated anti-inflammatory properties in rat model of carrageenan-induced paw edema (Smits et al., 2008); therefore, if JNJ7777120 was substituted for a specific neutral antagonist in this experiment, then we might see inhibition of the negative effects associated with inflammation.

3.5.4 Chapter Supplement and Support

The histamine H_4 receptor is the most recently identified histamine receptor (Nakamura et al., 2000; Oda et al., 2000) and it is particularly expressed in the tissues of the haematopoetic system. It was thought that H_4 receptor is mainly involved in inflammatory diseases (Engelhardt et al., 2009; Gutzmer et al., 2011), but newer studies suggest that the receptor is also involved in other diseases such as neuropathic pain, cancer, vestibular disorders and type 2 diabetes (Gutzmer et al., 2011; Kiss and Keseru, 2012). Because of this widespread role of the H_4 receptor, its function needs to be thoroughly characterized for therapeutic interventions.

The results from this chapter supplements and supports the initial characterization and distribution of the H_4 receptor in the murine retina by our group. Further

pharmacological characterization of the receptor compliments other similar studies in the literature such as the intrinsic compensatory mechanisms in Müller cells to cope with high levels of oxidative stress (Toft-Kehler et al., 2016) and the ability of the H_4 receptor agonist VUF8430 to block the cytokine IL-12 production in Müller cells (Gschwandtner et al.,2013).

This chapter provided an anatomical and functional basis for H_4 receptor in Müller cells and diabetic retinopathy. The next chapter will investigate whether an alternative method, PBM, rather than synthetic drug approach, with a specific wavelength, previously shown to be neuroprotective and immunomodulatory, has a positive effect against oxidative and inflammatory stress in retinal Müller glial cells. This will explore whether this device-based approach has potential as a therapeutic intervention against diabetic retinopathy.

Chapter 4

Photobiomodulation in Müller Cells Against Oxidative and Inflammatory Stress

4.1 Objectives

The aim of this chapter was to determine whether PBM at NIR wavelength 1068 nm has any therapeutic potential against diabetic retinopathy. This will be investigated using human Müller cells of the retina, and diabetic conditions will be mimicked using H_2O_2 as oxidative insult and glyoxal to induce inflammatory stress. Since this was a novel procedure, different protocols were explored to determine optimal conditions for PBM treatment of Müller cells.

4.2 Introduction

The therapeutic benefits of light therapy have been known for many decades since its discovery by Endre Mester in 1967. Commonly PBM therapy is applied to wound healing, reduction in neurological pain, healing after peripheral nerve injury, heart attacks and stroke. But recently, PBM has been used as an innovative and non-invasive therapeutic approach various sight-threatening retinal conditions such as age-related macular degeneration (AMD), diabetic retinopathy, retinopathy of prematurity (ROP) and methanol-induced retinal damage. There is shift away from traditional laser treatments for retinal conditions as devices containing LED arrays are available at only a fraction of the cost for lasers.

It is widely acknowledged that mitochondria are the primary subcellular target of PBM and that cytochrome C oxidase in mitochondria serves as the primary photoacceptor. Stimulation of cytochrome C oxidase by PBM leads to an increase in energy production by mitochondria and also increases the metabolic rate and cell

proliferation (Hamblin and Demidova, 2006; Lovschall and Arenholt-Bindslev, 1998). Studies in the mouse retina have already shown that PBM leads to decrease in diabetes-induced inflammation on the retinal vessels (Tang et al., 2013). Since both mitochondrial health and the inflammatory state are affected by PBM, we hypothesized that PBM can treat diabetic retinopathy.

4.3 Methods

4.3.1 IR1068 nm apparatus and PBM assays

The apparatus for PBM experiments was set up as discussed in section 2.3. In the PBM assays, H_2O_2 was used to illicit oxidative stress in the Müller cells and glyoxal was used to induce inflammatory stress as described in the previous chapter. Previous PBM work by our group (Bradford et al., 2005) involved testing a range of NIR treatment protocols on cell viability in human lymphocytes, and it was found that the protocol where cells were irradiated with NIR1068 nm using five 3 minute light exposures showed significantly higher cell viability compared to cells irradiated with other NIR wavelengths using the same protocol; hence the protocol for PBM assays in Müller cells was devised based upon this previous work. Data were analysed using a one-way ANOVA followed by Bonferroni post-test. Data represent mean ± SEM.

4.3.1.1 Müller light treatment pre H_2O_2 injection

Sub-confluent cultures (70-80%) were dislodged as described in section 2.4.2 and transferred to a 15 ml falcon tube and centrifuged at 100x g for 5 minutes. The cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The cell suspension was then plated in 24-well plates and incubated at 37°C and at 5% CO_2 for 72 hours before use.

Cells were exposed to five sets of either high power (10 mW/sq cm) or low power (3 mW/sq cm) three-minute treatments of IR1068 nm or sham treatments, with 20 minutes between each exposure. A stock solution of H_2O_2 (2500 µM) in PBS was prepared and different H_2O_2 concentrations were then prepared using Dulbecco's

Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% fetal bovine serum (FBS) to achieve final concentrations of 300 µM and 400 µM. Media from the 24-well plate light-treated cells was replaced with the different H_2O_2 concentrations prepared and grown in culture for 4-hour period, after which an MTT assay was performed to determine cell viability (section 2.2.1).

4.3.1.2 Müller light treatment post H_2O_2 injection

A stock solution of H_2O_2 (2500 µM) in PBS was prepared and different H_2O_2 concentrations were then prepared using Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% fetal bovine serum (FBS) to achieve final concentrations of 300 µM and 400 µM. Sub-confluent Müller cell cultures (70-80%) were dislodged as described in section 2.4.2 and transferred to a 15 ml falcon tube and centrifuged at 100x g for 5 minutes. The cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The cell suspension was then plated in 24-well plates and incubated at 37°C and at 5% CO_2. The media was replaced after 24 hours with the different H_2O_2 concentrations prepared and grown in culture for 4-hour period.

Cells were exposed to five sets of either high power or low power three-minute treatments of IR1068 nm or sham treatments, with 20 minutes between each exposure, after which an MTT assay was performed to determine cell viability (section 2.2.1).

4.3.1.3 Müller light treatment pre and post H_2O_2 injection

Sub-confluent cultures (70-80%) were dislodged as described in section 2.4.2 and transferred to a 15 ml falcon tube and centrifuged at 100x g for 5 minutes. The cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The cell suspension was then plated in 24-well plates and incubated at 37°C and at 5% CO_2 for 72 hours before use.

Cells were exposed to five sets of high power three-minute treatments of IR1068 nm or sham treatments, with 20 minutes between each exposure. A stock solution of H_2O_2 (2500 µM) in PBS was prepared and different H_2O_2 concentrations were then prepared using Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% fetal bovine serum (FBS) to achieve final concentrations of 300 µM and 400 µM. Media from the 24-well plate light-treated cells was replaced with the different H_2O_2 concentrations prepared and grown in culture for 4-hour period.

After the 4-hour period, the H_2O_2 media in the 24-well plate was replaced with Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS.

The cells were again exposed to five sets of high power three-minute treatments of IR1068 nm or sham treatments, with 20 minutes between each exposure, after which an MTT assay was performed to determine cell viability (section 2.2.1).

4.3.1.4 Müller light treatment pre and post Glyoxal injection

Sub-confluent cultures (70-80%) were dislodged as described in section 2.4.2 and transferred to a 15 ml falcon tube and centrifuged at 100x g for 5 minutes. The cell pellet was resuspended in Dulbecco's Modified Eagle Medium 1X with GlutaMAX, without sodium pyruvate, 4500 mg/L D-Glucose supplemented with 10% FBS. The cell suspension was then plated in 24-well plates and incubated at 37°C and at 5% CO_2 for 72 hours before use.

Cells were exposed to five sets of high power three-minute treatments of IR1068 nm or sham treatments, with 20 minutes between each exposure.

After the light treatment, stock glyoxal solution (2 M) was added to the wells (except control) to give final glyoxal concentration of 2 mM and the cells were further incubated for 13 hours at 37°C and at 5% CO_2.

The cells were again exposed to five sets of high power three-minute treatments of IR1068 nm or sham treatments, with 20 minutes between each exposure, after

which cell supernatant was extracted for TNF-alpha analysis and MTT assay was carried out (section 2.2.1) to determine the cytotoxic effects of glyoxal.

4.4 Results

Due to a previous study showing the beneficial effect of PBM against diabetes-induced inflammation on the retinal vessels (Tang et al., 2013), the focus was to determine whether PBM at NIR wavelength 1068 nm has any therapeutic potential against diabetic retinopathy.

The positive effects of PBM was investigated using human Müller cells of the retina, and diabetic conditions was mimicked using H_2O_2 as oxidative insult and glyoxal to induce inflammatory stress. Since there are no standard protocols for PBM experiments, different protocols were explored to determine optimal conditions for PBM treatment of Müller cells.

4.4.1 Low level light therapy does not protect Müller cells against oxidative stress

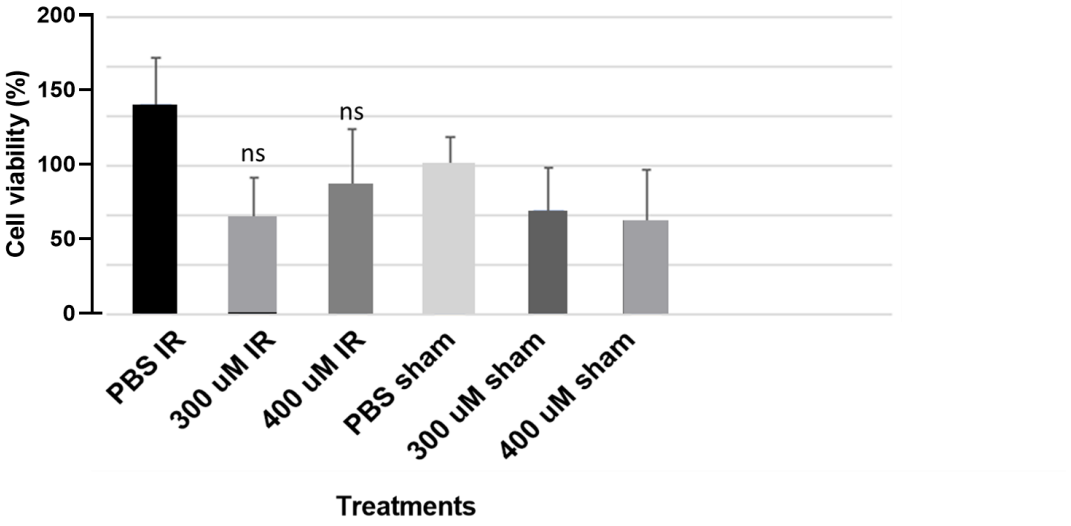

Figure 25: Preconditioning of Müller cells with Low power NIR1068 nm treatment against oxidative insult. PBM treatment of Müller cells (3 mW/sq cm) was carried prior to 4 hours oxidative insult with 300 µM and 400 µM H_2O_2. Control was exposure to PBS with light (IR) and without light (sham). Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicates. 'ns' was compared to sham treatments.

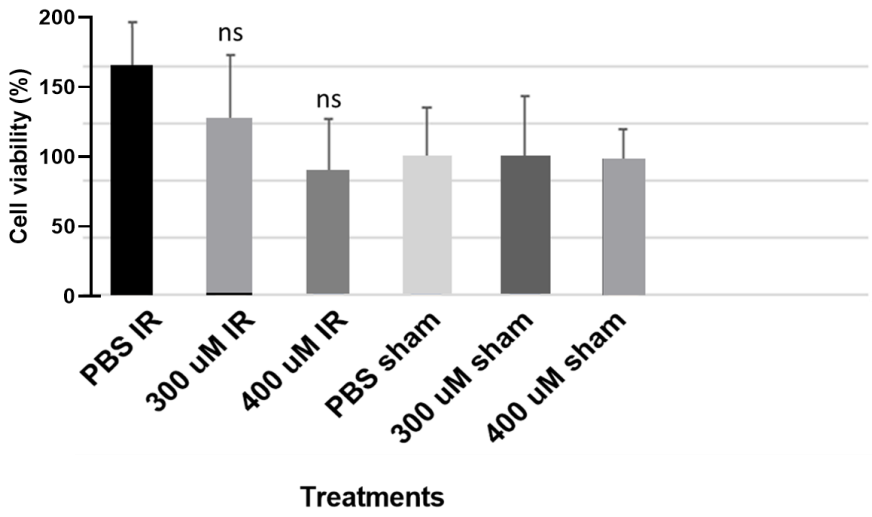

Figure 26. Low power NIR1068 nm treatment of Müller cells post oxidative insult. Low power (3 mW/sq cm) NIR1068 nm treatment of Müller cells after 4 hours oxidative insult with 300 µM and 400 µM H_2O_2. Control was exposure to PBS with light (IR) and without light (sham). Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicates. 'ns' was compared to sham treatments.

When Müller cells were preconditioned with low powered NIR1068 nm treatment before stressing them with two different concentrations of H_2O_2, there was no significant difference in cell viability compared to the cells which were treated with H_2O_2 but not exposed to any light treatment (figure 25). Thus, the next step was to alter the protocol and see if there is any increase in cell viability when the cells undergo PBM after exposure to H_2O_2. Again, figure 26 shows that low powered NIR1068 nm treatment of the cells post oxidative stress does not have any protective effect as there was no significant difference between the treatments and the sham experiment.

Since low level NIR1068 nm treatment did not protect Müller cells against oxidative stress, the next step was to test the PBM apparatus with higher energy output.

4.4.2 High level light therapy has modest protection in Müller cells against oxidative stress

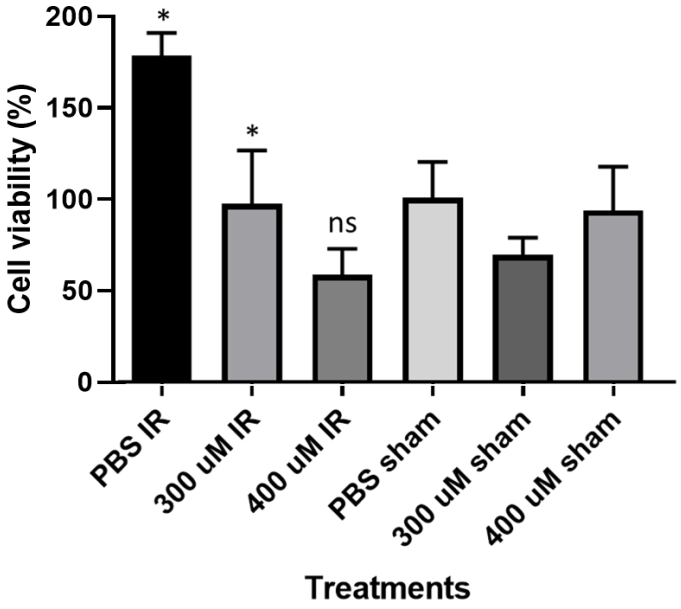

Figure 27: High power NIR1068 nm treatment of Müller cells post oxidative insult. High power (10 mW/sq cm) NIR1068 nm treatment of Müller cells **after** 4 hours oxidative insult with 300 µM and 400 µM H_2O_2. Control was exposure to PBS with light (IR) and without light (sham). Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicates. * $p < 0.05$ Vs 300 µM sham. 'ns' was compared to 400 µM sham

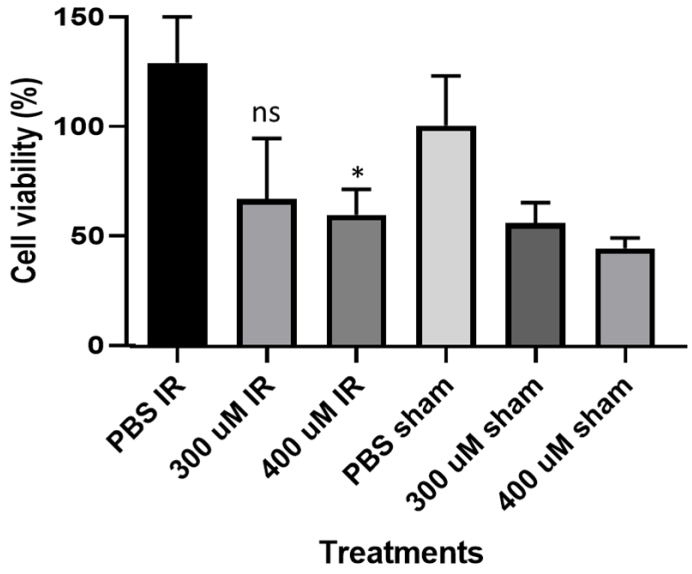

Figure 28: Preconditioning of Müller cells with high power NIR1068 nm treatment against oxidative insult. High power (10 mW/sq cm) NIR1068 nm treatment of Müller cells **before** 4 hours oxidative insult with 300 µM and 400 µM H_2O_2. Control was exposure to PBS with light (IR) and without light (sham). Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicates. * p <0.05 Vs 400 µM sham. 'ns' was compared to 300 µM sham.

When the same protocol as the low powered NIR1068 nm light treatment were carried out with higher powered NIR 1068 nm light, both pre (figure 28) and post oxidative stress (figure 27) light treatment showed modest improvements in cell viability against oxidative stress.

When the cells were preconditioned with light treatment before exposure to oxidative stress, the light treatment seems to improve cell viability when they were stressed with higher H_2O_2 concentration (400 µM) compared to cells stressed with 300 µM H_2O_2, but the data variance for light treatment at 300 µM H_2O_2 treatment was too high compared the control and this needs further investigation.

The result was opposite when the cells were treated with light post H_2O_2 exposure, as cell viability seems to improve for the lower H_2O_2 concentration compared to

control. Interestingly, for this method, there seems to be a significantly higher cell viability in the controls treated with light compared to controls under sham conditions (figure 27).

4.4.3 Double treatment of Müller cells with high level light reduces oxidative stress

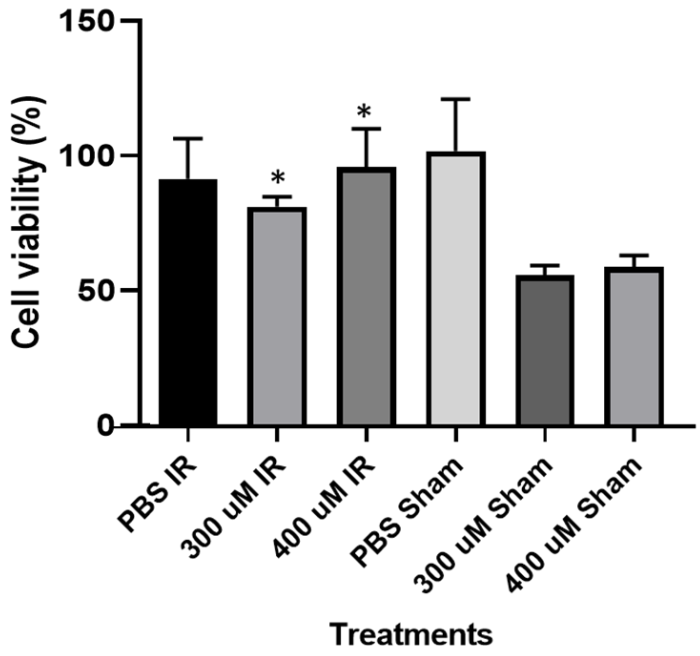

Figure 29: High power NIR1068 nm treatment of Müller cells before and after oxidative insult. High power (10 mW/sq cm) NIR1068 nm treatment of Müller cells before and after 24-hour oxidative stress with 300 µM and 400 µM H_2O_2. Control was exposure to PBS with light (IR) and without light (sham). Cell viability was measured using MTT assay. Results are mean ± SD for one independent experiment performed in quadruplicates. * p <0.01 Vs 300 µM or 400 µM sham.

On obtaining positive results with high powered NIR1068 nm treatment of Müller cells against oxidative stress, we decided to test another protocol where the cells would undergo light treatment twice, once before oxidative insult and once after.

This protocol has a much better protective effect and as can be seen from figure 29 that there was significant improvement in cell viability (up to almost 50% increase) for both 300 µM and 400 µM concentration of H_2O_2 compared to sham treatments, to yield complete protection.

Next, this same protocol was tested against inflammatory stress in Müller cells to see if there was any similar improvement in cell viability.

4.4.4 Double treatment of Müller cells with high level light has no protective effect against inflammatory stress

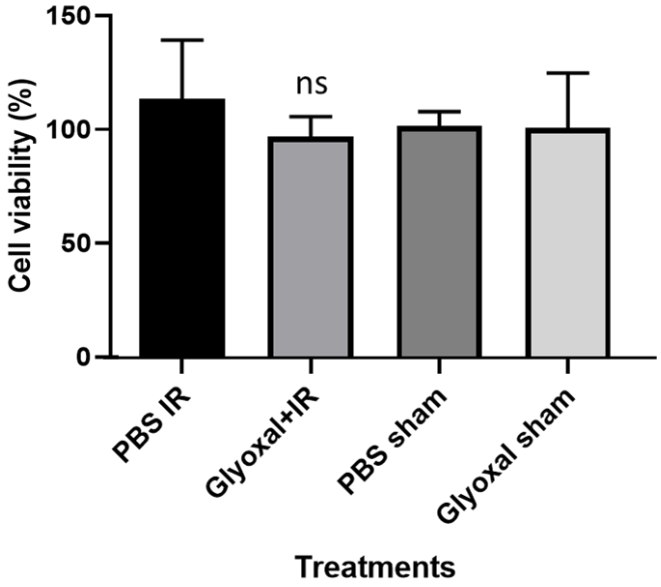

Figure 30: High power NIR1068 nm treatment of Müller cells before and after inflammatory insult. High power (10 mW/sq cm) NIR1068 nm treatment of Müller cells before and after 13-hour inflammatory stress with 2 mM glyoxal. Control was exposure to PBS with light (IR) and without light (sham). Results are mean ± SD for two independent experiment performed in quadruplicates. 'ns' was compared to glyoxal sham treatment.

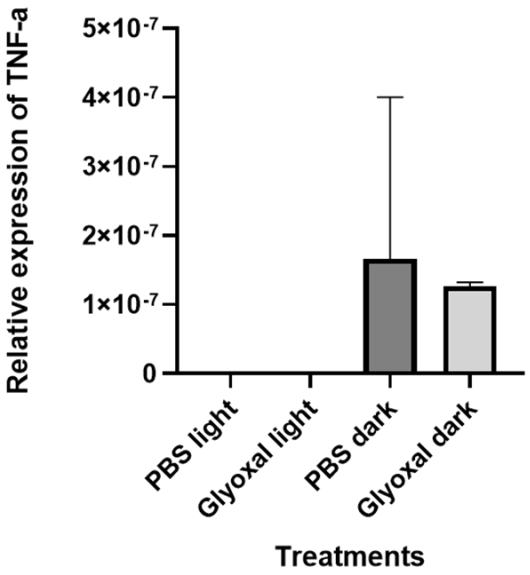

Figure 31: TNF-alpha expression levels on high power NIR1068 nm treatment of Müller cells before and after Müller cells are subjected to inflammatory stress. High power (10 mW/sq cm) NIR1068 nm treatment of Müller cells before and after 13-hour inflammatory stress with 2 mM glyoxal. Expression levels of the cytokine was measured using ELISA assay. Results are mean ± SD for two independent experiments performed in quadruplicates. * p <0.05 Vs Glyoxal dark. Expression levels of TNF-a shown in g/ml.

When the double light exposure protocol was tested against inflammatory stress induced by glyoxal, there was no significant improvement in cell viability compared to the sham treatment (figure 30), although this treatment did not cause any significant cytotoxicity. The cell supernatant from this treatment was further analysed for TNF-alpha expression and interestingly it looks like there was complete protection from glyoxal-induced TNF-alpha secretion when the cells are subjected to the double light treatment protocol but the value was in the negative so it was not conclusive (figure 31). It will be of interest to repeat this and also check for the expression of other cytokines such as IL-1beta, IL-6 and VEGF using real-time PCR along with ELISA.

4.5 Discussion

This chapter explored the therapeutic potential of using PBM at NIR wavelength 1068 nm against both oxidative and inflammatory insults in Müller cells. According to the outcome of each experiment, the protocol was tweaked slightly for different experiments to obtain optimal conditions for protection against gliotoxicity caused by oxidative stress, and cytokine release induced in inflammatory stimulus both noted in retinal conditions such as diabetic retinopathy.

4.5.1 Low powered NIR1068 nm treatment of Müller cells have no beneficial effect against oxidative stress

When NIR1068 nm light apparatus with a power output of 3 mW/sq cm^2 and energy density (energy per unit mass) of 0.6 J/cm^2 was used to treat Müller cells against oxidative stress, there was no beneficial effect in cell viability compared to the sham treatments. This is not surprising as evidence suggests that effectiveness of PBM treatment varies on both the energy and power density used (Chung et al., 2012). This is termed as 'biphasic dose response', whereby levels above or below the optimum exposure conditions result in diminished biological effects and PBM seems to follow Arndt-Schulz law: if insufficient energy is applied, no effect will be observed, and if too much energy is supplied, 'bio-inhibition' occurs (Hamblin and Demidova, 2006, Huang et al., 2009).

4.5.2 Double light treatment of Müller cells is more effective against oxidative stress compared to single treatments

When both the power and energy density (energy per unit mass) of NIR1068 nm treatment was increased (10 mW/sq cm^2 and 1.9 J/cm^2 respectively), there was modest improvement in cell viability of Müller cells when they were preconditioned with the treatment before oxidative insult or when they were treated after oxidative insult. Surprisingly though, when the cells were preconditioned with the NIR1068 nm treatment, they seemed to have a protective effect only against the higher dose of H_2O_2. This observation seems to contradict the observation made by Duggett et al. (2013), who showed that NIR1068 nm preconditioning is not required for a profound

level of neuroprotection against H_2O_2 induced oxidative stress. Thus, these results need further investigation with a greater number of repeats.

4.5.3 Double light treatment of Müller cells has no protective effect against inflammatory stress but seem to diminish cytokine release

Since double light treatment of human Müller cells was successful in fully protecting the cells against oxidative stress, we had expected to see similar results for glyoxal induced release of TNF-alpha from human Müller cells despite no effect upon gliotoxicity. Indeed, when the cell supernatant was analysed post-glyoxal treatment, the double NIR treatment seemed to be successful in eliciting complete inhibition of cytokine (TNF-alpha) release, a common example of cytokines that are the major causes of inflammatory damage in cells. Although the levels of TNF-alpha released by glyoxal appear not to be sufficient to elicit gliotoxity, they may elicit neurotoxicity. It will be interesting to repeat this and check for the expression of other cytokines such as IL-1beta, IL-6 and VEGF using real-time PCR along with ELISA and perform retinal neuroglial co-cultures. The reduction in cytokine release seems to be consistent with the study performed in rats where the rats were treated with PBM following focal brain damage and the treatment reduced TNF-alpha and other cytokine levels in the brain, but interestingly the TNF-alpha level increased significantly in the blood (Moreira et al., 2009).

4.6 Chapter Supplement and Support

This chapter provided optimal PBM treatment conditions to protect human Müller glial cells against oxidative insults and inflammatory responses such as those seen in diabetic retinopathy, but the treatment against inflammatory stress needs further repetition as the cell viability remained consistent throughout all the treatments, which could indicate a non-cytotoxic effect of glyoxal on the cells in that particular experiment. The chapter also provided further evidence and support to existing theory that therapeutic effect of PBM is dependent on the energy density and treatment regime.

Cell proliferation following PBM treatment has been reported in several cell types; such as osteoblasts (Kreisler et al., 2003, Pires Oliveira et al., 2008, Ueda and Shimizu, 2003), fibroblasts (Hawkins and Abrahamse, 2006, Taniguchi et al., 2009, Vinck et al., 2003), muscle cells (Sommer et al., 2001, epithelial cells (Whelan et al., 2001) and neuronal cells (Grillo et al., 2013), and it will be interesting to further investigate whether the increased cell viability with PBM treatment was due to increased proliferation of the Müller cells. This is unlikely as previously shown not to be the case for neurons (Duggett & Chazot, 2014).

Since it's already reported that there are increased ATP levels and mitochondrial electron transport activity following PBM (see introduction 1.6.3), further follow up experiments could measure ATP concentrations in sham and NIR1068 nm treated cell cultures to determine whether this was a factor in the increased cell viability.

Chapter 5

Overall Discussion and Future Work

There were two main aims of this project: The first aim was to characterize the histamine H_4 receptor in human Müller cells both anatomically and pharmacologically. The second aim was to determine where PBM at NIR wavelength 1068 nm has any therapeutic potential against diabetic retinopathy. Overall, through these two main objectives, we wanted to understand whether targeting the H_4 receptor in Müller cells or PBM of the retina could either act as a potential therapeutic treatment on its own or combining the two treatment methods could illicit better results.

Through a series of immunological and pharmacological studies, the following hypotheses have been addressed:

1. **Histamine H_4 receptor is expressed in Human Müller glial cells**

Prior immunological study performed by our group on fixed mouse retinal slices showed positive evidence for H_4 receptor expression in Müller cells in the plexiform layers. Thus, we decided to characterize the expression of H_4 receptor in Müller cells *in-vitro* because of their importance in diabetic retinopathy. Immunological studies using a selective H_4 receptor antibody confirmed the presence of the receptor in the projections of the Müller cells (consistent to the murine topology) when these cells were grown in both low and high glucose medium. Müller cells grown in high glucose medium seemed to show higher expression of the receptor. This does need further confirmation and quantification through western blot and PCR analysis. It wouldn't be surprising if H_4 receptor expression was indeed upregulated in high glucose environment as there is existing strong evidence for the upregulation of histamine and histamine H_4 receptor during glycemia in other organs (Gill et al., 1989; Fogel et al., 1990; Pini et al., 2018; Rosa et al., 2013). Since some of the histamine receptors

like H_3 have been shown to have both auto- and hetero-receptor activity, we further investigated whether H_4 receptor has autocrine activity as well in Müller cells. For this we carried out IF again using HDC-specific antibody, and we found evidence of the enzyme expression in the processes of the Müller cells. An interesting follow up of these results would be to carry out immunohistochemical studies of Müller cells to check for direct evidence for the presence of histamine in these cells, and whether histamine release is controlled by the histamine H_4 receptor, in order to better understand the potential cellular targets of histamine in the retina.

On functional characterization of the H_4 receptor through calcium imaging using agonists VUF8430 and ST-1006, both induced Ca^{2+} release from the intracellular calcium store in the cells but surprisingly ST-1006 induced a higher response even though VUF8430 is a full agonist for H_4 receptor (Lim et al., 2009). Overall, when carrying out dose response assays in Müller cells with different concentrations of H_2O_2, these glial cells seem to have a high tolerance for oxidative stress compared to when the same experiment was carried out with neuronal CAD cells. This result further emphasized the important protective and supportive role of Müller cells in the retina. To further characterize the presence of H_4 receptor in Müller cells, it's important to use other methods to evaluate the receptor at the gene (RT-PCR) and protein (western/blot) levels. Further pharmacological analysis of the receptor should also be done to measure the levels of messenger molecules such as IP3 and cAMP on H_4 receptor activation. In this study, only calcium imaging assay was deployed to characterize functional properties of the H_4 receptor, but other tools such as electrophysiological techniques could be used to further confirm functional properties.

2. **H_4 receptor ligands protect Müller cells against oxidative stress-induced toxicity and inflammatory stress**

The H_4 receptor is a potential therapeutic target against diabetic retinopathy because of its role in diabetes (Pini et al., 2015, Rosa et al., 2013) and vascular permeability (Karlstedt et al., 2013). When both H_4 receptor agonist VUF8430 and antagonist JNJ7777120 was checked for any protective effects against oxidative and inflammatory stress, there was no significant change in cell viability against oxidative

stress, but the ligands seem to make inflammatory stress induced toxicity worse as the cell viability decreases compared to control. On further analysis for the expression of the cytokine TNF-alpha in the supernatant from these cells, it was observed that VUF8430 in this treatment method increased the cytokine production compared to when cells were only stressed with glyoxal. This observation explains the decreased cell viability on treatment with the ligands. Further work needs to be done in terms of analyzing the production of other cytokines such as IL-1beta, IL-6 and VEGF using real-time PCR along with ELISA.

3. **PBM at NIR1068 nm protects Müller cells against oxidative stress-induced toxicity**

PBM treatment of Müller cells against oxidative and inflammatory stress was carried out using two different power and energy densities. When lower powered PBM treatment was used, not surprisingly, there was no beneficial effect in cell viability compared to sham treatments, but when higher powered PBM treatment was used, there was modest protective effect in the cells. Next, we decided to perform double treatments of the Müller cells with high powered PBM, and this seemed to have a complete protective effective against both oxidative stress and an inflammatory response; the treatment seemed to completely block expression of the cytokine. Again, it will be interesting to also check for the expression of other cytokines such as IL-1beta, IL-6 and VEGF, and protective effect upon neurons in a co-culture.

With PBM treatment, it is also important to understand the underlying mechanisms of light stimulation in the cells. There are already reports in the literature where PBM treatment causes increases in both cAMP (Karu, 1987) and ATP (Gao and Xing, 2009) levels. Bradford et al. (2005) also showed an increase in iNOS (inducible Nitric Oxide synthase) expression after NIR1072 treatment in human lymphocytes, and nitric oxide is known to act as a potent inhibitor of apoptosis. Therefore, it will be interesting to monitor the changes in protein expression levels during PBM treatment using RT-PCR. Also, it will be important to measure the basal levels of NO, ATP, Ca^{2+} and ROS before any PBM treatment as these components have been repeatedly reported to be increased following PBM treatment of cells; therefore,

depending on the basal levels of these components within a model, may determine the therapeutic output of the treatment.

5.1 Future Work

In this project, only two cellular stressors were used: glyoxal and H_2O_2. To have a broader understanding of the potential of the treatments used in this project as a potential therapeutic technique against diabetic retinopathy, it is important to test these protocols against other cellular stressors, including ER stressors.

Currently, for PBM treatments, there is no standard protocol in the literature for treatments. Wavelengths, dose and treatment time vary depending on the scientist's personal preference or experience; thus, it will be beneficial to further explore different protocols for treatment of Müller cells against various cellular stressors and develop a rigid and standard protocol that elicits the greatest benefit.

Since out of the two treatment methods for diabetic retinopathy proposed in this study, PBM showed the greatest promise, it is important to further investigate the effects of IR1068 nm in an *in-vivo* system to check for any discrepancies and provide further insights into the mechanism of action through which NIR1068 nm treatment of Müller cells has been shown to be protective.

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