ELECTROPHYSIOLOGICAL STUDIES OF RETINAL FUNCTION AT LOW LIGHT LEVELS IN TYPE 2 DIABETES MELLITUS

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SUMMARY

Diabetic retinopathy (DR) is the major cause of registerable blindness in the working population in Western countries. It has been proposed that the retina is subject to sub-clinical levels of tissue hypoxia prior to the development of DR, and that a rod–driven hypoxia during darkness may be a significant causal factor in its development.

The aim of this study was firstly to use the scotopic electroretinogram (ERG) in order to gain an objective measure of retinal function in subjects with diabetes mellitus (DM). Should there be a level of inner retinal hypoxia present this may be indirectly demonstrated by reduced oscillatory potential (OP) amplitudes, thought to arise predominantly from amacrine cells and known to be sensitive to vascular changes within the retina, and reduced b-wave amplitudes thought to arise predominantly from the bipolar cells of the inner retina. If hypoxia were present and reversable in the short term an increase in amplitude would be expected with oxygen (O_2) inhalation.

ERGs were recorded from subjects with Type 2 DM both with and without retinopathy before, during and following O_2 inhalation and compared to age-matched control subjects. No significant difference in amplitude was observed between subjects with DM and control subjects before O_2 inhalation, however both b-wave and summed OP amplitudes were significantly increased following O_2 inhalation in diabetic subjects with retinopathy, and OP3 significantly increased in subjects without retinopathy, yet remained unchanged in the control group.

The retinal O_2 demand has been reported to halve in light conditions compared to darkness and it has therefore been proposed that patients with DM may benefit from sleeping with night-time illumination in order to reduce the level of rod activity, and thus metabolic demand on the retinal tissue, to reduce inner retinal hypoxia. The amount of light required to significantly reduce rod activity was investigated by means of a simultaneous cone-rod ERG in DM subjects with no retinopathy and control subjects. It was found that a background illumination level as little as 3.4lux was sufficient to significantly reduce rod activity in all subjects.

To Tom,

and to my family,

Margaret, Hugh and Joanna

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Diabetic retinopathy is the major cause of registerable blindness in the working population in Western countries [Evans et al 1996]. This chapter will introduce the cells that make up the retinal tissue. Chapter 2 will then outline the physiology of diabetes mellitus (DM) and in particular its effects upon the retina. Chapter 3 introduces the electroretinogram (ERG), an objective method of assessing retinal function, with particular attention to the components of the ERG most affected by DM. Chapter 4 reviews retinal oxygenation in DM. Diabetic retinae are often thought to suffer from a level of sub-clinical hypoxia and Chapter 4 will outline current knowledge on the effects of oxygen inhalation on the retinal vasculature and visual function in subjects with DM.

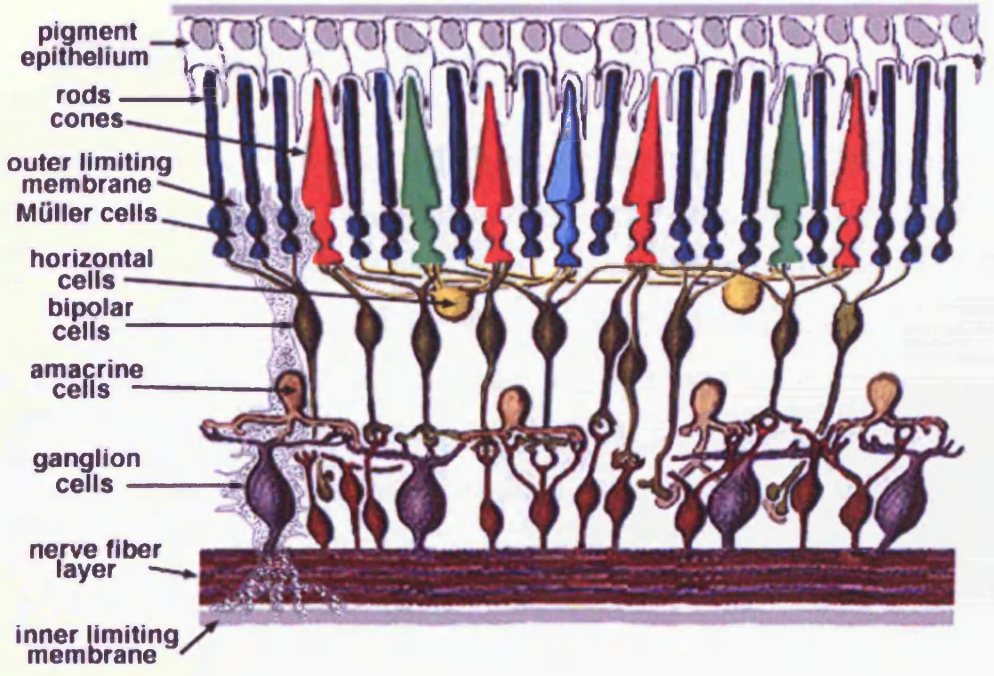

Figure 1.1 Simple organisation of the retina [After Kolb et al 2004].

1.1 Anatomy of the Retina

The retina is the innermost layer of the eyeball and is approximately 0.5 mm thick. It is continuous with the optic nerve posteriorly and extends forward to the ora serrata where it becomes the epithelium of the ciliary body and the iris. The inner surface of the retina is in contact with the vitreous body and the outer surface with Bruchs membrane of the choroid.

The retina can be divided into several distinct layers according to the cell bodies and synapses contained within them. The cells that make up the retina are shown in Figure 1.1. The vertebrate retina contains three layers of nerve cell bodies, the outer nuclear layer, the inner nuclear layer and the ganglion cell layer, and two layers of synapses, the outer and inner plexiform layers. It is within the retina that light energy is transformed into a neural signal before being passed to the visual cortex of the brain.

1.2 The Retinal Pigment Epithelium

The retinal pigment epithelium (RPE) is formed from a single layer of pigmented cells extending from the margin of the optic nerve head (ONH) to the ora serrata. The basal ends of these cells rest on a basement membrane forming part of Bruchs membrane of the choroid. The apical ends have multiple microvilli which project between the outer segments of the rods and cones, thus providing some mechanical stability to the photoreceptors. Numerous melanin granules are contained within these cells and may extend into the microvilli. Adjacent cells are bound together by tight junctions though gap junctions are also present between cells.

The RPE performs several important functions [Nilsson 1985] which include the:

- Formation of the outer blood-retinal barrier between the choriocapillaris and the neural retina. The tight junctions between cells prevent the

passage of large molecules or toxins from the systemic circulation to the photoreceptor layer.

- Phagocytosis of rod and cone outer segment discs.
- Metabolism of vitamin A (retinol), a molecule involved in phototransduction.
- Absorption of scattered light.

1.3 The Photoreceptor Layer and Outer Nuclear Layer

1.3.1 Densities of the Photoreceptors

The photoreceptor layer of the retina is comprised of the outer segments of the two types of photoreceptor cell, the rods and the cones. There are thought to be approximately 115 million rods in the retina and 6.5 million cones with the density of each type varying regionally across the retina, see Figure 1.2. Rods are absent at the fovea and their density increases towards the periphery reaching a maximum at 5mm (18°) from the centre of the fovea [Oesterberg 1935, Curcio et al 1990]. Their density then slowly decreases towards the extreme periphery. Cone density is maximal at the fovea, decreasing towards a reasonably even density across the periphery [Curcio et al 1990]. No photoreceptors are present at the optic nerve head thus leading to a blind spot in the visual field of each eye.

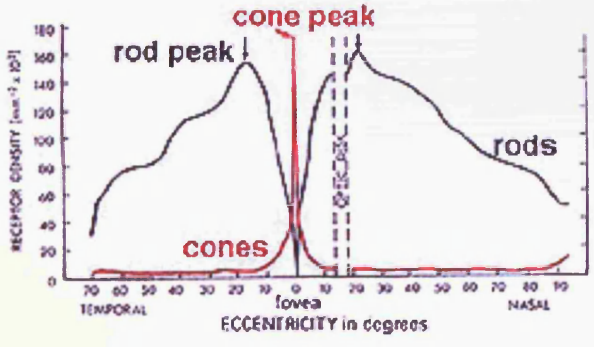

Figure 1.2 Densities of the rods and cones across the retina [After Oesterberg 1935].

1.3.2 Structure of the Photoreceptors

Each type of receptor consists of several defined regions, see Figure 1.3. The outer segments, situated in the photoreceptor layer, contain the visual pigment. They are connected to the inner segment, containing the metabolic machinery, by a modified cilium. An outer fibre then connects this area to the cell body or perikaryon region situated in the outer nuclear layer, containing the nucleus of the cell, which is in turn connected to the synaptic terminal by means of an inner fibre [Pipe and Rapley 1997].

1.3.2.1 Outer Segments

The outer segments of the photoreceptors contain a series of double-folded membranous discs. These discs are freely stacked within the rod outer segment membrane, but in the cones are continuous with the cell membrane and formed by infoldings of the cell membrane of the outer segment. The visual photopigments are embedded within the membranes of these discs with each disc containing many thousands of visual pigment molecules [Pipe and Rapley 1997]. Rods contain the visual pigment rhodopsin and are maximally sensitive to blue-green light with peak wavelength sensitivity of 496-500 nm. The cones also contain visual photopigments, similar in composition to rhodopsin, and are known as iodopsins. The cones can be divided into three groups based on the maximal wavelength sensitivity of the visual photopigment they contain. Those with a maximal sensitivity to long wavelength light, (peak sensitivity 564 nm), are known as L-cones or red cones. Those with a maximal sensitivity to medium wavelength light, (peak sensitivity 533 nm), are known as M-cones or green cones and those with a maximal sensitivity to short wavelength light, (peak sensitivity 437 nm) are known as S-cones or blue cones [Gouras 1984].

The membranous discs are formed at the base of the outer segments and move upwards towards the tip of the outer segment as new discs are formed below. The tips of the outer segments are pinched off and phagocytosed by the cells of the RPE in a diurnal cycle with the rod outer segment discs

maximally shed in the morning, at light onset, and the cone outer segment discs often at the end of the day at light offset [Besharse 1982].

1.3.2.2 Inner Segments

The inner segments of the rods are thinner than those of the cones, each having a diameter of 2 μm and 5 μm respectively [Pipe and Rapley 1997]. The outer segments of each are approximately 1.5 μm in diameter. However, at the fovea the cones have an inner segment diameter thinner than that of the rods at 1.5 μm, allowing dense packing of the cones in this area of the retina and thus greater visual discrimination.

The inner segments of the photoreceptors are comprised of two parts, the ellipsoid, closest to the outer segment, and the myoid, closest to the cell body of the photoreceptor. Opsin molecules are assembled in the inner segments of these cells before being passed to the discs of the outer segment where they form part of the visual photopigments. The other part of the rhodopsin molecule, retinal, is provided by the RPE via retinal binding proteins.

1.3.2.3 Synaptic Endings

The rod cells terminate in a round synapse known as a spherule, each containing many pre-synaptic vesicles. Each spherule contains a single round invagination formed by an infolding of the cell membrane [Pipe and Rapley 1997]. Here the rods may synapse with one to four rod bipolars, or horizontal cells, and up to seven processes fit within each spherule [Remington 1998].

The cones terminate in broad flattened hemispheres with up to twenty-five invaginations. Three processes may enter these forming a 'triad', the central element typically consisting of the dendritic terminal of an invaginating midget bipolar. The two lateral processes are dendritic terminals of horizontal cells. Cone pedicles have small projections which may contact neighbouring cone

pedicles and rod spherules allowing for the transfer of information between the photoreceptors [Pipe and Rapley 1997].

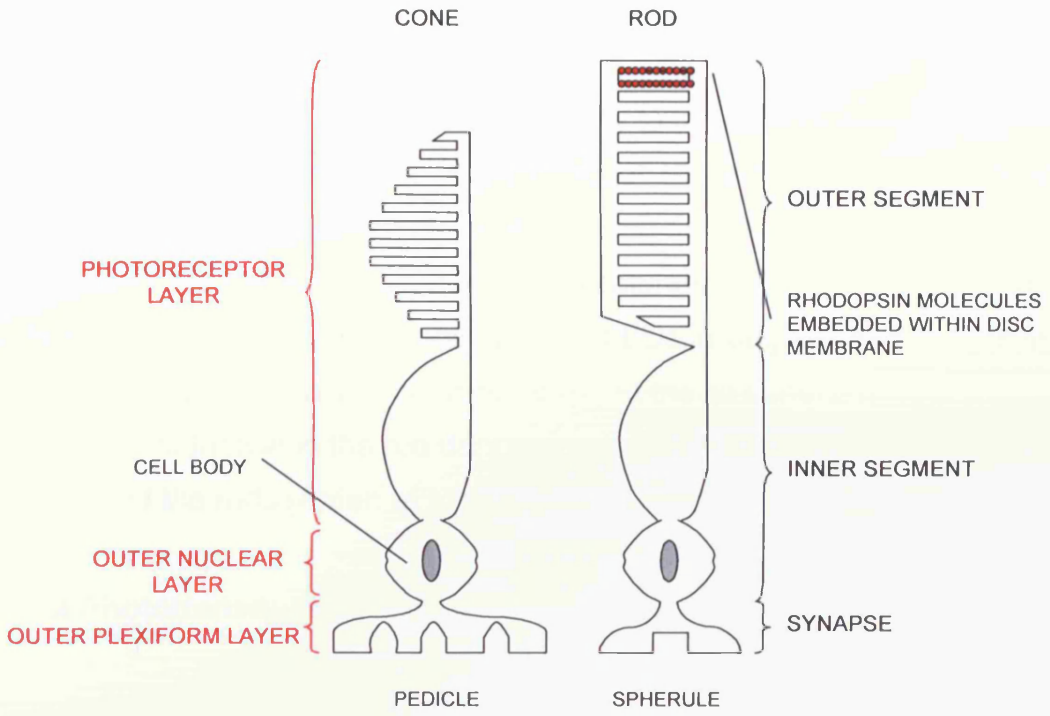

Figure 1.3 Schematic diagram of the photoreceptor cells.

1.3.3 Light Sensitivity of the Photoreceptors.

Rods provide the ability to see in scotopic conditions, (<0.003 $cd.m^{-2}$) [Kalloniatis and Luu 2005]. Their sensitivity is so great that they are able to detect a single quantum of light, however they are much slower to respond to light stimulation than cones, maybe by as much as 1/10 second [MacLeod 1972]. Cones provide the ability to see in photopic conditions, (>3cdm^{-2}) and at mesopic light levels, (0.003-3 $cd.m^{-2}$) both rods and cones function [Kalloniatis and Luu 2005].

As conditions change from scotopic to photopic light levels there is a shift in the spectral sensitivity of the eye, known as the Purkinje shift. The relative luminosity curve of the rods peaks earlier than that of the cones, the peaks

arising at 510 and 560 nm for the rods and cones respectively. Scotopic spectral sensitivity is higher than photopic spectral sensitivity at all but the longest wavelengths. At high levels of illumination the rod responses saturate and no longer increase as illumination levels continue to increase [Kalloniatis and Luu 2005].

It has been found that rod system sensitivity to increments begins to fall off rapidly with retinal illuminance levels of about 100 scotopic trolands. At about 2,000-5,000 scotopic Trolands, corresponding to photopic luminances of 120-300 $cd.m^{-2}$, the rod mechanism becomes saturated [Aguilar and Stiles 1954]. A later experiment found that using green LEDs through closed eyelids in humans a stimulus luminance of 10 $cd.m^{-2}$ at the lid surface is sufficient to produce a reduction in the rod dark current, and thus lower the retinal oxygen demand of the rods [Arden et al 1999].

1.3.4 Phototransduction

In the dark, a steady current flows between the inner and outer segments of the photoreceptors known as the 'dark current'. Sodium (Na^+) ions flow freely down their electrochemical gradient into the outer segments of the photoreceptors through open cation channels. This partially depolarises the cell and maintains a high release of the neurotransmitter glutamate from the synaptic terminals of the receptor. This release of neurotransmitter is high in the dark and reduced by light, proportionally to the number of photons absorbed [Yau 1994]. This partial depolarisation leads to leakage of potassium (K^+) ions from the inner segments and synaptic endings completing the loop of the circulating dark current. Internal Na^+ and K^+ concentrations are maintained by an ATP dependent Na^+/K^+ exchange pump [Baylor 1987]. As well as the influx of Na^+ through the cation channels, calcium (Ca^{2+}) ions also enter the outer segments and are released from the inner segments by means of a Na^+/Ca^{2+} exchanger, forming a smaller proportion of the dark current [Yau 1994].

Absorption of light causes hyperpolarisation of the cell membrane by indirectly closing the cation channels of the outer segments which are normally kept open by cyclic guanosine 3'-5'-monophosphate (cGMP). Photoisomerisation of rhodopsin molecules, embedded in the membranous discs of the outer segments, precipitates a series of reactions which result in a reduction of cGMP, closure of the cation channels and the dark current stops [Tovée 1996].

When a photon of light is absorbed by a rhodopsin molecule the retinal component of the visual pigment is isomerised from an 11-*cis* from to an all-*trans* form, freeing it from the opsin molecule. The protein then goes through a series of intermediate forms, one of which is the enzymatically active metarhodopsin II [Saari 2000]. Metarhodopsin II then binds to a disc membrane protein transducin. In its inactive state transducin is bound to a molecule of guanosine diphospahte (GDP). The association of metarhodopsin II leads to the exchange of the bound GDP for guanosine triphoshate (GTP), resulting in the activation of the transducin molecule [Fung et al 1990]. Activated transducin interacts with phosphodiesterase (PDE) and splits off its inhibitory subunits, and this complex then catalyses the hydrolysis of cGMP [Tovée 1996]. As the level of cGMP falls the cation channels of the outer segment close and the receptor hyperpolarises.

Metarhodopsin II is inactivated by phosphorylation which allows a protein called arrestin to compete with transducin for the metarhodopsin II which then inhibits further catalytic activity [Tovée 1996].

The transduction process leads to a fall in cGMP and cation channels close. This leads to a fall in intracellular Ca^{2+} since these ions can no longer enter the cell but are still being removed. Changing levels of intracellular Ca^{2+} are believed to act as a feedback mechanism speeding up the cells recovery from light stimulation and mediating light adaptation [Koutalos and Yau 1993]. The mechanism by which this occurs is still uncertain [Hurley et al 1993, Kawamura 1993].

1.4 The Inner Nuclear Layer

1.4.1 Bipolar Cells

The retinal bipolar cells synapse with the photoreceptors within the outer plexiform layer. Their cell bodies lie within the inner nuclear layer and their single axon is directed inwards towards the inner plexiform layer where they synapse with ganglion and amacrine cells. The bipolar cells can be classified according to their synaptic connections [Saude 1993]:

- Rod bipolar cells – connect 15-30 rod cells with only 1-4 ganglion cells via amacrine cells.
- Flat or diffuse bipolar cells – connect many cone cells with many ganglion cells.
- Midget bipolar cells – connect a single cone with a single midget ganglion cell.

More rods converge onto a single rod bipolar than cones onto a single cone bipolar, thus the rod system trades acuity for sensitivity [Masland 2001a]. The rod bipolar does not synapse directly with ganglion cells but instead information is passed via specific amacrine cells.

The bipolar cells can also be classified as either excitatory or inhibitory with ON or OFF centre receptive field types. Excitatory bipolars become activated in the dark and are deactivated by light. Inhibitory bipolars are opposite to this being suppressed by dark conditions and activated by light [Nelson and Kolb 1983].

1.4.2 Horizontal Cells

The horizontal cells, as their name suggests, transfer information in a horizontal manner across the retina. They are thought to perhaps be bi-directional with one long process, sometimes termed the axon, and several

long processes, sometimes termed the dendrites. Horizontal cells synapse with the photoreceptors, bipolar cells and with each other. When activated by a photoreceptor it is thought that they contact bipolar cells some distance away [Remington 1998] releasing an inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), which is thought to sharpen contrast and increase spatial resolution [Snell and Lemp 1998].

1.5 The Inner Plexiform Layer

The inner plexiform layer (IPL) can be divided into two sublaminas. In sublamina *'a'* the axons of OFF-centre bipolar cells end, and OFF-centre ganglion cells and OFF-centre amacrine cells branch. In sublamina *'b'* the axons of ON-centre bipolar cells end, and ON-centre ganglion and amacrine cells branch [Kolb et al 2004].

1.5.1 Amacrine Cells

The transfer of information from the bipolar cells to the ganglion cells is predominately via the amacrine cells. The amacrine cells synapse upon bipolar cells, other amacrines or ganglion cells. They are also thought to make reciprocal synapses to the bipolar cell axons from which they receive ribbon synapses [Kolb et al 2004]. This feed-back synapse to the bipolar cell within the IPL may provide a surround mechanism to the bipolar cell response.

There are thought to be at least 25 different morphological types of amacrine cell within the human retina [Kolb et al 1992]. They outnumber the horizontal cells by amounts ranging from 4:1 to 10:1 depending on the species [Masland 2001a]. The classification of amacrine cell type are based upon their dendritic tree size (found to increase with eccentricity from the fovea), their branching characteristics and on the stratification of their dendrites within the IPL.

Some amacrine cells perform particularly specific functions. All amacrine cells act as a bridge between the rod pathway and the ganglion cells [Smith

and Vardi 1995]. They are the most common type of amacrine cell in the mammalian retina and use the inhibitory amino acid glycine as a neurotransmitter. Dopaminergic amacrine cells are thought to control the sensitivity of many retinal neurons during light and dark adaptation [Masland 2001b]. Starburst amacrine cells make excitatory cholinergic synapses on specific retinal ganglion cells, particularly those sensitive to moving stimuli. These neurons release both acetylcholine (Ach) and GABA and by feed-forward excitation and/or inhibition are important for direction selectivity [Masland 2001a].

1.5.2 Interplexiform Cells

Interplexiform cells link the two plexiform layers of the retina, receiving synaptic input from the IPL and having synaptic output at the OPL. They receive input from amacrine cells and make synapses with rod and cone bipolar cell bodies and their dendrites [Remington 1998].

1.6 The Ganglion Cell Layer

1.6.1 Ganglion Cells

The ganglion cells are the final output neurons of the retina. They form a single layer across the majority of the retina though the number of layers increases from the periphery to the macula and decreases again towards the fovea where they are absent. They are classified according to cell size, dendritic field size and spread, which is known to increase with retinal eccentricity, and type of branching.

The most common type of ganglion cell is the midget ganglion cell, or P cell. This is a relatively small cell and projects to the parvocellular layer of the lateral geniculate body (LGN). They have the smallest dendritic trees at the fovea, though beyond 3mm eccentricity their dendritic tree size may be as large as 100μm [Dacey 1993]. At the fovea the P cells are connected to only

one midget bipolar cell, which in turn may be connected to a single cone photoreceptor. Beyond the fovea they may receive input from more than one midget bipolar by virtue of their increased dendritic tree size thus providing some convergence of the visual signal [Gouras and Zrenner 1981]. The parvocellular system is thought to provide high levels of visual acuity and colour vision [Kolb et al 2004].

The M cells are ganglion cells which project to the magnocellular system, which provides information regarding luminance and movement, and are thought to be the parasol cells described by Polyak [1941]. These cells have a larger spread of dendrites and thus larger receptive fields.

The remaining ganglion cells are designated G3 to G23. These cells vary by cell body size, dendritic tree size, branching characteristics and the location of dendrite termination [Remington 1998].

The ganglion cell axons are non-myelinated and run across the inner surface of the retina, forming the nerve fibre layer, and come together to leave the eye as the optic nerve. The optic nerve fibres pass through the sclera at the lamina cribosa at which point they become myelinated.

1.7 The Nerve Fibre Layer

This layer consists of the axons of the ganglion cells, neurogliei and astrocytes. In the nasal half of the retina the nerve fibres run in a radial pattern. Fibres originating in the central macula area form a thick pappillo-macular bundle which runs directly from the fovea to the temporal part of the disc. Fibres originating in the temporal periphery of the retina take a more arcuate course to the optic disc. Those fibres originating in the upper and lower quadrants form a well defined horizontal raphe running from the fovea to the temporal periphery.

1.7.1 Glial Cells

1.7.1.1. Müller Cells

The Müller cells are long, narrow cells with long processes that extend across almost the entire neural retina from the outer limiting membrane to the inner limiting membrane. They fill in most of the space within the neural retina not occupied by neurons and subsidiary branches extend horizontally providing mechanical support to the neurons that they surround. They are also rich in glycogen and have an important nutritive role as well as supportive. Müller cells protect the retinal neurons from exposure to excess neurotransmitters such as glutamate and mop up neural waste such as carbon dioxide (CO_2) and ammonia. They also control homeostasis within the retina by taking up extracellular K+ and redistributing it [Newman and Reichenbach 1996]. Müller cells are also believed to contribute to the generation of the electroretinogram (ERG) b-wave [Miller and Dowling 1970, Newman and Odette 1984], the slow P3 component of the ERG [Karwoski and Proenza 1977], and the scotopic threshold response (STR) [Frishman and Steinberg 1989], by their regulation of K+ distribution within the retina.

1.7.1.2 Astrocytes

Astrocytes consist of a flattened cell body with a series of fibrous radiating processes. They are found on the surface of bundles of ganglion cell axons, and sometimes on blood vessels running between the ganglion cell bundles, and are thought to form part of a blood-brain barrier [Zhang and Stone 1997]. They too are rich in glycogen and may play a nutritive role.

1.7.1.3 Microglial Cells

Microglial cells may be found in every layer of the retina. They are believed to provide a macrophagic function following trauma to the retina where they phagocytose degenerating retinal neurons.

1.8 The Rod Pathway

The rod photoreceptors are designed to mediate our vision under scotopic conditions and are so sensitive that they are able to reliably transduce information following the absorption of even a single photon. The classic rod pathway contains both specialised bipolar and amacrine cells, however the circuitry involved in the rod pathway is in part superimposed on existing cone circuitry [Kolb and Famiglietti 1974].

Both rods and cones are depolarised under dark conditions. Following an increase in light intensity both the rods and cones hyperpolarise and reduce their release of the neurotransmitter, glutamate. In mammals the rods are thought to synapse with a single type of bipolar cell, the rod ON-bipolar. The rod bipolar receives input from between 15 and 30 rod spherules. The rod bipolar depolarises and synapses with AII and A17 amacrine cells. This passage of signal via the amacrine cells allows for both convergence and divergence of signals from many rods and rod bipolars [Kolb et al 2004].

The AII amacrine cell is a small field bi-stratified cell, and A17 a wide-field cell. AII amacrine cells respond to light with a depolarising, ON-centre response. These cells then excite the ON (depolarising) cone bipolar cells and inhibit OFF (hyperpolarising) cone bipolar cells. The signal is then passed from the ON bipolars to excite the ON ganglion cells and from the OFF bipolars to excite the OFF ganglion cells.

A17 amacrine cells also respond to light with a depolarising, ON-centre response. They are thought to receive input from approximately 1,000 rod bipolars and are not known to synapse with either ganglion cells or other amacrines. Instead they interconnect rod bipolars via reciprocal synapses and thus provide convergence of the rod signal over a wide retinal area, increasing our sensitivity in scotopic conditions [Kolb et al 2004].

Dopaminergic amacrine cells may also influence the rod pathway via chemical synapses with AII and A17 in the inner plexiform layer. They may uncouple

AIIs across the retina and uncouple AII from the cone bipolars in order to increase the receptive field size of ganglion cells [Kolb et al 2004].

A second rod pathway is also present in which rods are able to contact the cone ON and OFF bipolars at an early stage. This is possible due to electrical synapses along gap junctions between the rod and cone photoreceptor cells. These gap junctions allow the rods access to the cone ON and OFF bipolars and thence to ON and OFF ganglion cells [Nelson and Kolb 1983, Smith et al 1986]. This duality of the rod system has been demonstrated with self-cancellation of flicker signals both perceptually and in the human electroretinogram (ERG) with stimuli at high rod intensity levels near 15 Hz in frequency [Stockman et al 1991]. This suggests that two rod signals are processed within the retina with different speeds of transmission. The phase delay between the two signals at 15 Hz is approximately half a cycle and leads to the presence of two rod signals of opposite phase which cancel each other out via destructive interference. This second rod pathway is thought to be active at low mesopic intensity levels, and is reflected in the faster rod signal in the human ERG. The slower signal is believed to reflect transmission of the rod response via the rod bipolars and AII amacrines, which is active at scotopic levels and saturates at approximately 1 scotopic troland (scot. td) [Stockman et al 1995].

A third pathway for rod signals through the retina has also been suggested [Soucy et al 1998, Hack et al 1999]. OFF-ganglion cell responses have been recorded in the coneless retina of a transgenic mouse whose primary rod pathway had been incapacitated pharmacologically. Since the second rod pathway relies on gap junctions between the rods and cones this is not occurring via this pathway due to the lack of cones. It is suggested that the survival of the OFF-response is due to a third rod pathway, perhaps connecting rods directly with OFF-bipolar cells [Soucy et al 1998]. This is supported by a later study which discovered a non-classical synaptic contact in the rodent retina where rod photoreceptors made synaptic contact with putative OFF-cone bipolar cells [Hack et al 1999]. It is, however, suggested that direct connections between rods and OFF-cone bipolars may be specific

to smaller eyes of rodents but absent in larger eyes, e.g. those of cats, monkeys and humans, and depend on rodent eyes receiving on average more quanta than rods in larger eyes [Sharpe and Stockman 1999].

In summary it is possible that three pathways exist for transmitting rod signals in the retina. The first pathway involves passage of the signal from rods to rod bipolar cells, to AII amacrine cells, to cone bipolar cells and finally to ganglion cells. The second involves direct transmission of the signal from rods to cones via gap junctions and then on to the ganglion cells via the cone bipolars. The third pathway may bypass the ON bipolar cells completely and rod photoreceptors may directly excite the OFF cone bipolars.

2. DIABETES MELLITUS

Diabetes Mellitus (DM) is a group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action or both [The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003]. Several processes are involved in the development of DM and can range from the auto-immune destruction of the pancreatic β-cells of the islets of Langerhans with consequent insulin deficiency, to abnormalities that result in resistance to insulin action. The chronic hyperglycaemia apparent in DM is responsible for the three main pathologies noted in this disease: peripheral neuropathy, nephropathy and vascular disease.

2.1 Physiology of Diabetes Mellitus

The pancreas is comprised of exocrine and endocrine cells. The exocrine portion synthesises and secretes digestive enzymes into the duodenum, the first portion of the small intestine, in order to aid digestion of food. The endocrine cells also secrete hormones to aid food digestion along with a number of hormones involved in regulation of metabolism. The endocrine cells of the pancreas are clustered together in small groups and this appearance led to the name 'pancreatic islets of Langerhans', named by Laguesse in 1889 after their original describer [Patel 2000]. These islets are surrounded by small blood vessels into which their hormones are secreted. Blood glucose levels are largely regulated by two hormones, glucagon and insulin, which are respectively secreted by the α (A) and β (B) cells of the islets of Langerhans. There are approximately one million islets of Langerhans in the normal adult. These are made up of four main cell types, α (A), β (B), δ (D) and pancreatic peptide (PP) cells.

2.1.1 A cells

The A cells are the second most abundant cell type of the islets and produce glucagon. Glucagon promotes an increase in the sugar content of the blood by increasing the rate of glycogenolysis in the liver, i.e. the breakdown of glycogen, the principle form in which carbohydrate is stored in animal tissue. Insulin inhibits the release of glucagon, while glucagon stimulates insulin secretion. Glucagon secretion is stimulated by low levels of glucose in the blood and inhibited by high levels [Tortora and Grabowski 1992].

2.1.2 B cells

The B cells are the most abundant of the islet cells and make up the bulky core of each islet. They produce insulin, which regulates metabolism via control of circulating plasma glucose levels. Insulin is a small protein consisting of a chain of 21 amino acids linked by two disulfide (S-S) bridges to a chain of 30 amino acids. When glucose is required insulin facilitates its utilisation. When glucose levels become excessive insulin stimulates skeletal muscle fibres and liver cells to take up glucose from the blood and convert it into glycogen. It also inhibits the production of enzymes involved in glycogenolysis, e.g. glucagon, to prevent the breaking back down of glycogen. Insulin also acts on fat/adipose cells to stimulate the uptake of glucose and the synthesis of fat. The amount of insulin released by the normal B cell immediately increases as blood glucose rises [Tortora and Grabowski 1992]. In Type 2 DM there is a progressive deficit of insulin secretion and this may be due in part to a deficit in B-cell mass [Butler et al 2003, Ritzel et al 2006], which may be caused by increased B-cell apoptosis [Ritzel et al 2006].

2.1.3 D cells

The D cells produce somatostatin, which suppresses the secretion of both insulin and glucagons [Tortora and Grabowski 1992].

2.1.4 PP cells

The PP cells synthesise pancreatic polypeptide whose function is as yet unknown [Tortora and Grabowski 1992].

2.2 The Role of Insulin

In DM the absence of, or resistance to, insulin results in a lack of uptake of glucose from the blood by skeletal muscle fibres and uncontrolled glucose output from the liver. This overall increase of glucose molecules in the bloodstream is termed hyperglycaemia. The actions and properties of insulin are summarised in Table 2.1.

Role of Insulin	
Tissue	**Insulin Action**
Skeletal Muscle	Stimulation of glucose uptake from blood and conversion to glycogen (gluconeogenesis)
	Stimulation of amino acid uptake from the blood and conversion to protein
Liver	Stimulation of glucose uptake from blood and conversion to glycogen
	Inhibition of breakdown of glycogen (glycogenolysis)
Fat	Stimulation of glucose uptake from blood and synthesis of fat
Properties of Insulin	
Secretion stimulated by	High blood sugar
	Glucagon
Secretion inhibited by	Low blood sugar
	Somatostatin

Table 2.1 Summary of role and properties of insulin.

2.3 Systemic Complications of Diabetes Mellitus

2.3.1 Diabetic Neuropathy

Diabetic neuropathy may affect any part of the nervous system. Complications may result from metabolic changes resulting in demyelination of nerve axons. Chronic peripheral neuropathy may result in a loss of touch, pain and temperature sensation. Subjects with diabetic peripheral neuropathy and vascular abnormalities are at high risk of diabetic foot ulceration, see Figure 2.1, with loss of sensation leading to periods of prolonged injury to the foot. Non-healing foot ulceration may lead to amputation and almost 50% of amputations not due to trauma are performed in diabetic patients [Boulton et al 1994]. Regular visits to the podiatrist are advised in order to avoid this. Peripheral neuropathy may also cause muscle weakness and loss of reflexes, particularly at the ankle which may result in a change in gait.

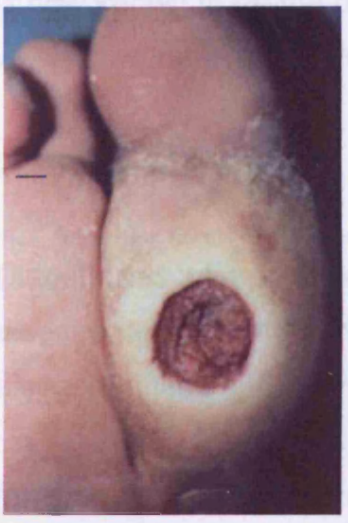

Figure 2.1 Diabetic foot ulcer [Armstrong and Lavery 1998].

Diffuse motor neuropathy is suggested by widespread, painless muscle wasting and weakness. Focal neuropathies, often due to vascular damage, may result in isolated palsies of either cranial or peripheral nerves. Palsies of the 3rd or 6th cranial nerves are characteristic of diabetic focal neuropathy.

Subnormal corneal sensitivity has also been reported with DM [Schwarz 1974, Macrae et al 1982, Ruben 1994]. A recent study found no significant relationship between the reduction in corneal sensitivity and the disease

duration, but did find a gradual reduction in sensitivity with age in both diabetic and non-diabetic subjects [Murphy et al 2004].

2.3.2 Diabetic Nephropathy

DM can also cause damage to the kidney, termed diabetic nephropathy. This generally involves sclerosis of internal kidney structures, particularly the glomerulus (the kidney membrane). The glomeruli provide the site of blood filtration and urine formation. As they are progressively destroyed filtration levels slow and protein may leak into the urine [Levine 2006]. Protein may be present in the urine for 5-10 years before other symptoms develop. The most serious long-term effect of diabetic nephropathy is kidney failure leading to end-stage renal disease. The subject will then require dialysis or a kidney transplant. Diabetic nephropathy is generally accompanied by other diabetic complications including hypertension, retinopathy and vascular damage, and is now known to be the principal cause of end stage renal disease in the western world [Russell 2006].

2.3.3 Vascular Disease

Diabetic vascular disease can be sub-divided into two groups, macro-vascular disease, affecting the larger blood vessels of the body, and micro-vascular disease, affecting the smaller blood vessels of the body.

Macro-vascular disease results in cardiac problems and increased blood pressure in diabetic subjects. Subjects with diabetes have a substantially increased risk of myocardial infarction, peripheral vascular disease, cerebro-vascular disease and retinopathy. Poor glycaemic control, obesity, smoking, physical inactivity and insulin resistance are all risk factors for development of vascular disease.

Microvascular dysfunction is paramount in the development and progression of diabetic retinopathy, the major cause of blindness in the working population

in the western world [Evans et al 1996]. This shall be discussed in more detail in section 2.7.

2.4 Diagnosis of Diabetes Mellitus

Three ways to diagnose diabetes are possible, and each must be confirmed on a subsequent day by any one of the three methods given [Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003], which are outlined in Table 2.2.

1. Symptoms of diabetes plus casual plasma glucose concentration ≥ 200mg/dl (11.1 mmol/l). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss.
or
2. FPG≥ 126mg/dl (7.0mmol/l). Fasting is defined as no caloric intake for at least 8h.
or
3. 2-h PG≥200mg/dl (11.1mmol/l) during an OGTT. The test should be performed as described by WHO [World Health Organisation 1985], using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water.
In the absence of unequivocal hyperglycaemia with acute metabolic decompensation, these criteria should be confirmed by repeat testing on a different day. The third measure (OGTT) is not recommended for routine clinical use.
FPG = Fasting Plasma Glucose
PG = Plasma Glucose
OGTT = Oral Glucose Tolerance Test
WHO = World Health Organisation

Table 2.2 Criteria for the diagnosis of diabetes mellitus [Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003].

2.5 Incidence of Diabetes Mellitus

Approximately 1.8 million people in the UK, approximately 3% of the population, are known to have diabetes today and it is thought that a further 1 million are currently undiagnosed [Diabetes UK 2006].

2.6 Classification of Diabetes Mellitus

DM can be divided into two main groups, Type 1 DM and Type 2 DM, though there are other specific types. The current classification is based on recommendations by an expert committee of the American Diabetes Association (ADA) and a World Health Organisation (WHO) consultation group [American Diabetes Association 2006].

2.6.1 Type 1 Diabetes Mellitus

2.6.1.1 Aetiology

This form of diabetes results from a cellular-mediated auto-immune destruction of the pancreatic B cells, usually leading to absolute insulin deficiency. Type 1 DM is thought to be triggered by a variety of environmental factors in those with a genetic susceptibility to this auto-immune process, though in some individuals there is no known aetiology and it is then termed idiopathic Type 1 DM [American Diabetes Association 2006]. Environmental factors appear to be more important than genetic factors which can explain only 30-40% of total susceptibility [Williams 2004]. Environmental triggers include viruses, including mumps, cytomegalovirus and rubella, bovine serum albumin from cows' milk and various toxins.

2.6.1.2 Prevalence

Type 1 DM accounts for between 7 and 10% of all diabetes with a population prevalence of 0.2 and 0.3% [Olefsky 1992], and shows no gender bias [Williams 2004].

2.6.1.3 Presentation

The symptoms of Type 1 DM have a relatively sudden onset although they generally become apparent when 80-85% of the B cells have been lost [Campbell and Lebovitz 1996]. These are outlined in Table 2.3. It appears

predominantly in childhood hence the previous description of 'juvenile-onset DM', however it can develop at any age. Most individuals with Type 1 DM become dependent on insulin for survival hence another previous description of 'insulin-dependent DM'.

Polyuria
Polydipsia
Polyphagia
Weight Loss
Muscular Weakness
Blurred Vision
Recurrent Infections
Diabetic Ketoacidosis

Table 2.3 Symptoms of Type 1 DM.

2.6.1.4 Treatment

Type 1 DM is generally controlled via subcutaneous injections of insulin at specific times each day. A low-fat diet, along with regulated intake of carbohydrates distributed throughout the day, is also recommended in order to help control blood glucose levels. Insulin may be given intravenously or intramuscularly in an emergency.

Bovine and porcine insulin are used to treat Type 1 DM. Bovine insulin differs chemically from human insulin by three amino acid residues and porcine by only one. However, these are now being superseded by insulin with the same structure as human insulin, manufactured in vitro via recombinant DNA techniques [Waller et al 2001].

Insulin half-life in plasma is very short, generally 8-16 minutes, and in order to avoid the need for frequent injections it is formulated either in a soluble

preparation or complexed with a different substance, protamine and/or zinc, to delay absorption from the injection site [Waller et al 2001].

Insulin analogues, e.g. Lispro, are chemical modifications of naturally occurring insulin. These act more rapidly than natural insulin but for a shorter time. In view of this they would generally be injected soon before the start of a meal [Rang et al 1999].

2.6.1.5 Prognosis

Before the introduction of insulin during the 1920s Type 1 DM was invariably fatal within a matter of months. With insulin treatment the risk of dying within 10 years is approximately fourfold higher for subjects with Type 1 DM throughout adult life in comparison to their non-diabetic peers [Williams 2004].

The greatest difficulties facing the Type 1 DM subject are now chronic tissue and vascular damage which may lead to renal failure, myocardial infarction and stroke.

2.6.2 Type 2 Diabetes Mellitus

2.6.2.1 Aetiology

Type 2 DM occurs as a result of insulin resistance and some B cell failure, the latter causing a relative rather than absolute insulin deficiency but resulting in an inability to overcome the insulin resistance. The specific aetiologies of this form are not known but auto-immune destruction of B cells does not occur. Genetic factors appear to play a major role in the pathogenesis of Type 2 DM and studies of identical twins have shown concordance rates of almost 100%. Having a first-order relative with Type 2 DM increases an individual's chance of developing it fivefold [Williams 2004, American Diabetes Association 2006]. Environmental factors which may play a role in the development of Type 2 DM include obesity, thought to increase insulin resistance, high carbohydrate diets and certain drugs [Campbell and Lebovitz 1996]. Obesity and physical

inactivity are also important risk factors in the development of Type 2 DM [Williams 2004].

2.6.2.2 Prevalence

Type 2 DM is by far the most prevalent form of diabetes accounting for approximately 90-95% of all patients with DM [Gutteridge 1999, American Diabetes Association 2006]. It is thought to affect 2% of the Caucasian populations in most westernised countries, the prevalence increasing with age to 10% of those over 70 and there is a 3:2 male preponderance among subjects with Type 2 DM [Williams 2004].

2.6.2.3 Presentation

Type 2 DM is generally diagnosed in those over 40 years of age, hence its previous description of 'mature-onset DM'. It is often of slow, insidious onset and the subject may not be aware of gradually increasing symptoms of polyuria, polydipsia and tiredness. Subjects are often obese with a family history of Type 2 DM. Insulin is not always required hence the previous classification of 'non-insulin dependent DM', though often insulin will be required to maintain stable blood glucose levels and thus the present classification was developed in order to avoid confusion [Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003]. Table 2.4 lists the complications found in a group of 2,337 subjects newly diagnosed with Type 2 DM [UK Prospective Diabetes Study 1990].

Complication	% Subjects
Retinopathy	21
Abnormal ECG	18
Myocardial Infarct	2
Angina	3
Absent Foot Pulses	13
Hypertension	35
Albinuria	3

Table 2.4 Complications present in a group of newly diagnosed subjects with Type 2 DM [UK Prospective Diabetes Study 1990].

2.6.2.4 Treatment

Type 2 DM is generally managed by a combination of lifestyle changes and oral hypoglycaemic agents. Weight loss and controlled physical exercise is particularly important for the obese DM subject, and a controlled and balanced diet is encouraged in all cases. Intakes of fat, salt and refined sugar are generally kept to a minimum and alcohol intake should not exceed three units per day in men and two units per day in women. Smoking is discouraged in all cases since it greatly increases the risk of vascular disease.

Oral hypoglycaemic drugs include sulfonylureas, biguanides, thiazolidinediones and glucosidase inhibitors.

Sulfonylureas, e.g. gliclazide, act by stimulating insulin release from the pancreatic B cells, and thus require functioning islet cells. Biguanides, e.g. metformin, increase glucose uptake in skeletal muscle and increase hepatic gluconeogenesis. Metformin also suppresses appetite and can therefore be useful in the obese DM subject. Biguanides do not require functioning B cells. Thiazolidinediones, e.g. rosiglitazone, act by stimulating glucose utilisation in peripheral tissues, particularly fat cells, and suppress hepatic gluconeogenesis. Glucosidase inhibitors, e.g. acarbose, act by delaying

carbohydrate absorption and thus reduce the postprandial increase in blood glucose levels. Like metformin this can be particularly helpful in the obese DM subject.

2.6.2.5 Prognosis

Life expectancy is shortened by up to a quarter in subjects with Type 2 DM presenting in their forties, with vascular disease being the main cause of death [Williams 2004].

2.6.3 Other Specific Types of Diabetes Mellitus

There are several other, less common, specific categories of diabetes and they are listed below [American Diabetes Association 2006].

- Genetic defects of the B cell/maturity-onset diabetes of the young (MODY)
- Genetic defects in insulin action
- Diseases of the exocrine pancreas
- Endocrinopathies
- Drug or chemical-induced diabetes
- Infections
- Uncommon forms of immune-mediated diabetes
- Other genetic syndromes associated with diabetes
- Gestational diabetes mellitus (GDM)

2.7 Diabetic Retinopathy

Diabetic retinopathy is a microvascular disorder affecting retinal capillary function. It is the major cause of registerable blindness in the working population in Western countries. This chapter will now review the histopathology and pathogenesis of diabetic retinopathy and some of its characteristic features.

2.7.1 Histopathology of Diabetic Retinopathy

2.7.1.1 Structural Changes In Retinal Microvessels.

Two groups of structural changes occur to the capillary network in diabetic retinopathy, occlusion of the capillaries and leakage from the capillaries.

2.7.1.1.1 Occlusion of the Retinal Capillaries

Occlusion of the retinal capillaries occurs due to a combination of the following factors:

1. Endothelial cell damage [Lawrenson 2000],
2. Thickening of the basement membrane of the vessel [Ashton 1974, Benson 1988, Lawrenson 2000],
3. Increase in blood platelet stickiness and aggregation of these cells. [Benson 1988],
4. Changes to red blood cells. [Benson 1988].

2.7.1.1.2 Capillary Leakage

The walls of the retinal vessels are formed from endothelial cells and pericytes. Pericytes are wrapped around the vessel wall, outside the endothelium, and are thought to give some structural integrity to the vessel wall. Pericytes may also play a role in regulating endothelial cell growth [D'Amore 1989]. It is thought that diabetic eyes may have a reduced number of pericytes in comparison to non-diabetic eyes and that this can result in areas of structural weakness. It is through these areas of weakness that blood and blood products can leak from the retinal vessels. The loss of pericytes is characteristically coupled with endothelial cell proliferations when exposed to elevated glucose levels [Ciulla et al 2002].

Capillary occlusion and leakage lead to many of the characteristic features of diabetic retinopathy that will be outlined later in this chapter.

2.7.1.2 Biochemical Changes

2.7.1.2.1 The Polyol Pathway

Hexose sugars, e.g. glucose and galactose, are converted into sugar alcohols via the polyol pathway. This metabolic pathway converts glucose to sorbitol and this conversion is catalysed by the enzyme aldose reductase. In normoglycaemia glucose is primarily metabolised by hexokinase, but in hyperglycaemia this pathway becomes saturated by the high levels of intracellular glucose and thus a higher proportion is metabolised via the polyol pathway [Lawrenson 2000]. In retinal capillaries the highest levels of aldose reductase have been observed within retinal pericytes and this increase in aldose reductase activity and sorbitol formation is thought to trigger pericyte degeneration [Robinson et al 1995]. Basement membrane thickening and endothelial proliferation may also be linked to this pathway [Robison et al 1995, Lawrenson 1997].

2.7.1.2.2 Non Enzymatic Glycation

Non-enzymatic glycation involves the covalent attachment of glucose or other sugar molecules to proteins. In hyperglycaemia diabetics show higher levels of glycated haemoglobin (HbA1c), i.e. haemoglobin containing a bound sugar molecule, and this is monitored clinically as a measure of glycaemic control. Early glycation products can combine with each other to form cross-linked proteins called advanced glycation end products (AGE). AGEs accumulate with hyperglycaemia and AGE-modified collagen may be responsible for basement membrane thickening of the retinal capillaries [Benson et al 1988]. AGEs may also contribute to impaired blood flow and alterations in permeability of the retinal vasculature [Lawrenson 2000].

2.7.1.3 Growth Factors and Neovascularisation

It is thought that retinal ischaemia and hypoxia initiate the release of an angiogenic signal within the retina that stimulates new vessel growth in order to form an alternative blood supply, and thus oxygen supply, to the existing compromised diabetic retinal vasculature [Ashton 1963]. The identity of this signal is still uncertain though recent studies investigating the vascular endothelial growth factor, VEGF, suggest that this may be a major factor in the pathogenesis of neovascularisation [Aiello 1997, Mathews et al 1997, Boulton et al 1998, Ishida et al 2000]. Levels of VEGF are increased in the diabetic retina and elevated VEGF has been associated with increased vascular permeability [Aiello 1997, Mathews et al 1997]. Several other angiogenic growth factors may also play a role in the proliferative stages of diabetic retinopathy including basic fibroblast factor, bFGF, and insulin-like growth factor, IGF, and it has been suggested that VEGF may work synergistically with these in initiating neovascularisation [Boulton et al 1998].

2.7.2 Features of Diabetic Retinopathy

2.7.2.1 Microaneurysms

Microaneurysms are often the earliest observable sign of diabetic retinopathy. They appear as small red dots approximately 10-100 μm in diameter and represent an area of weakness in the blood vessel wall where a small round cellular outgrowth of the wall occurs. Their aetiology is not completely understood, although pericyte loss may be a precipitating factor, either by creating a structural weakness at the point of loss, or via removal of their inhibitory control of endothelial proliferation [Lawrenson 1997], leading to a limited and localised proliferative response of endothelial cells at this point [Forrester et al 1993].

2.7.2.2 Intraretinal Haemorrhages

Intraretinal haemorrhages occur as blood leaks through damaged areas of the retinal blood vessel wall. There are three types of haemorrhage, classified according to their appearance as dot, blot or flame. Dot haemorrhages are small and round and are often difficult to distinguish from microaneurysms. They are located in the outer plexiform and inner nuclear layers of the retina. Blot haemorrhages appear slightly larger with less distinct margins. They originate from the deeper capillary network and are located in the inner and outer plexiform layers. Flame haemorrhages occur in the nerve fibre layer giving them an almost striated appearance as the blood follows the pattern of the nerve fibre arrangement in this more superficial layer.

2.7.2.3 Hard Exudates

Hard exudates form primarily in the outer plexiform layer from leakage of plasma proteins from the retinal vessels, particularly lipoprotein. They appear as yellow-white waxy lesions of variable size. They are often seen in individual clusters or streaks, or may be found associated with retinal microaneurysms in circinate patterns around the focal point of leakage. These may eventually reabsorb via phagocytosis but some long-term exudates can form a disciform-type scar.

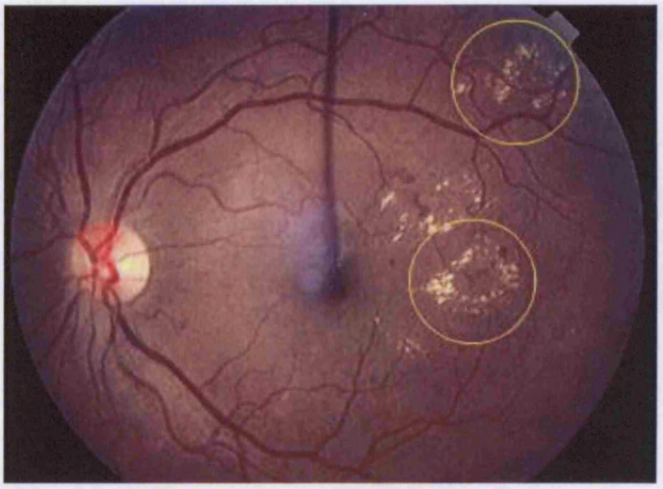

Figure 2.2 Circinate patterns of hard exudates [www.medweb2.bham.ac.uk 2003].

2.7.2.4 Cotton Wool Spots

Cotton wool spots are so called due to their poorly defined white appearance and they occur in association with areas of non-perfused retina. They consist of swollen ganglion cell axons containing an accumulation of degenerated axoplasmic organelles. These areas of swelling are thought to be caused by obstruction of axoplasmic transport along ganglion cell axons [McLeod et al 1977]. Large numbers of cotton wool spots indicate widespread retinal ischaemia.

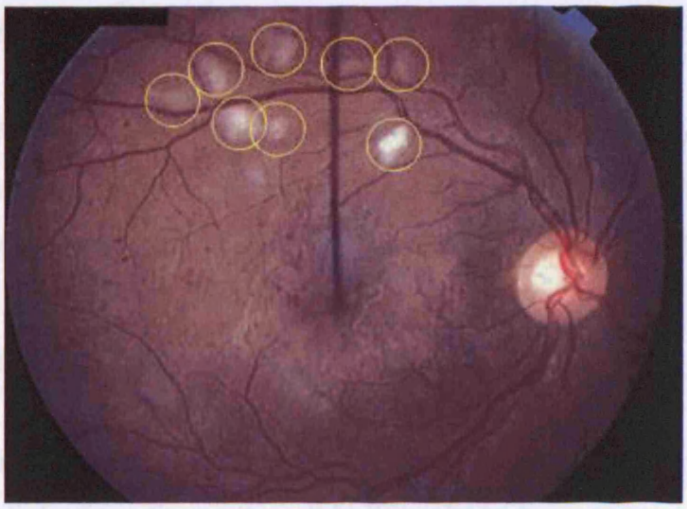

Figure 2.3 Cotton wool spots in pre-proliferative diabetic retinopathy [www.medweb2.bham.ac.uk 2003].

2.7.2.5 Retinal Oedema

The structural damage to the retinal vessels allows leakage of blood and blood products which then accumulate within the extracellular space. This may be localised i.e. focal leakage from a microaneurysm, or diffuse following extensive capillary dilatation and leakage. Should this leakage occur at the macula a significant reduction in vision will be apparent.

2.7.2.6 Intraretinal Microvascular Abnormalities / IRMA

IRMA consist of existing retinal capillaries that dilate and run between the retinal veins and arteries to bypass adjacent areas of capillary closure [Whitefield 1997]. They appear as areas of irregular vessel branching and may look like neovascularisation but lie in the deeper retinal layers, are less tortuous and are much less likely to haemorrhage. Since they lie within the retina they do not pose an immediate threat to vision but their presence indicates significant retinal ischaemia that warrants close monitoring.

2.7.2.7 Venous Changes

In the pre-proliferative stage of diabetic retinopathy the retinal veins can show changes in their appearance. They can become dilated, tortuous and may form venous loops or beading which gives a sausage-string like appearance.

2.7.2.8 Neovascularisation

Neovascularisation occurs in the advanced stages of retinopathy as a feature of proliferative diabetic retinopathy. It is thought to be a response to tissue hypoxia [Ashton 1957]. New vessels begin to grow at the optic disc, NVD, or elsewhere on the retina, NVE. They tend to arise as endothelial proliferations, most often from the retinal veins. These new vessels are structurally weaker than existing retinal vessels and are prone to leakage and rupture. After crossing the inner limiting membrane these vessels grow between the retina and vitreous in the retro-hyaloid space and can even penetrate the vitreous gel [Lawrenson 1997]. Fibrous and glial tissue grows with the new vessels and is laid down between the retina and the vitreous. As this tissue contracts it may lead to retinal tears or detachments. Haemorrhage of the new vessels can also lead to pre-retinal haemorrhages within the retro-hyaloid space, or intra-gel haemorrhages within the vitreous gel, both of which will significantly impede both vision and examination of the fundus.

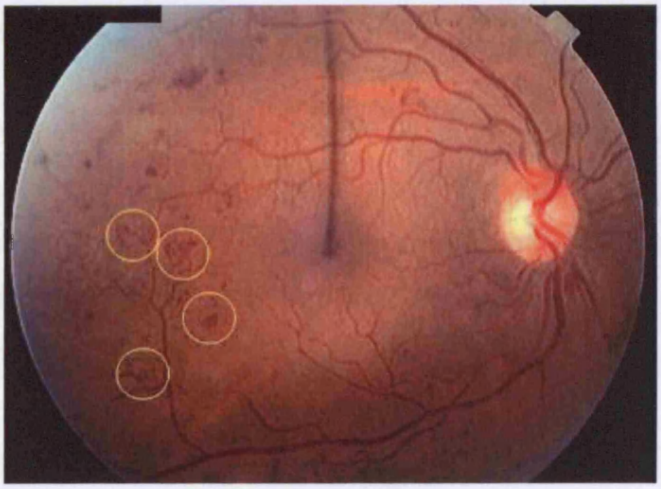

Figure 2.4 New vessel growth in proliferative diabetic retinopathy [www.medweb2.bham.ac.uk 2003].

2.7.3 Classification of Diabetic Retinopathy

2.7.3.1 Background Retinopathy

In background retinopathy the retina exhibits signs of microvascular leakage, away from the macula. Features include haemorrhages, microaneurysms, hard exudates and retinal oedema.

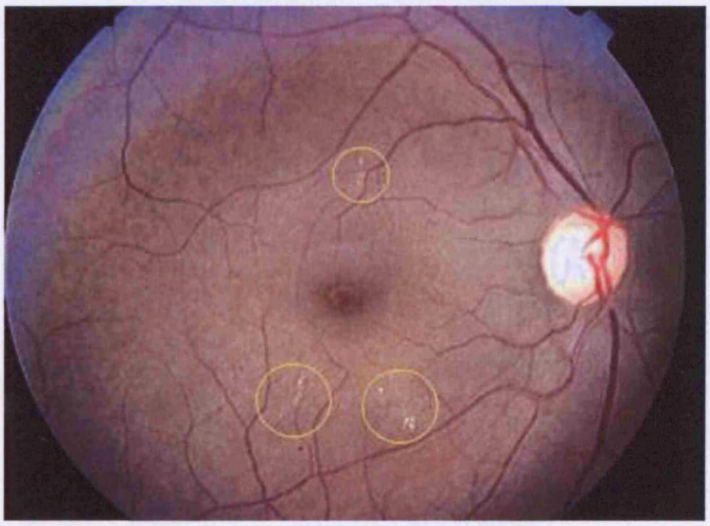

Figure 2.5 Background retinopathy [www.medweb2.bham.ac.uk 2003].

2.7.3.2 Pre-Proliferative Retinopathy

In the pre-proliferative stage of retinopathy there are signs of vascular occlusion, evidenced by cotton wool spots, and the retinal veins become irregular with the formation of venous loops and beading. There will also be increased retinal haemorrhages and cotton wool spots. IRMA may also be present at this stage.

2.7.3.3 Proliferative Retinopathy

In the proliferative stage of retinopathy retinal neovascularisation occurs in response to retinal hypoxia. This can occur at the disc, NVD, or elsewhere on the retina, NVE. Fibrous tissue is laid down between the retina and the vitreous as these new vessels progress which may lead to retinal tears or even detachment as described previously. Pre-retinal and intra-gel haemorrhages are also features of proliferative retinopathy.
New vessels may also begin to form on the iris, rubeosis iridis, and first become visible at the pupil margin. These may progress to form an extensive vascular network with scar tissue that can effectively block the anterior chamber angle. This will lead to a significant increase in the intraocular pressure, termed neovascular glaucoma, which can cause loss of vision as the central retinal artery and choroidal arteries may become occluded [Frank 1995].

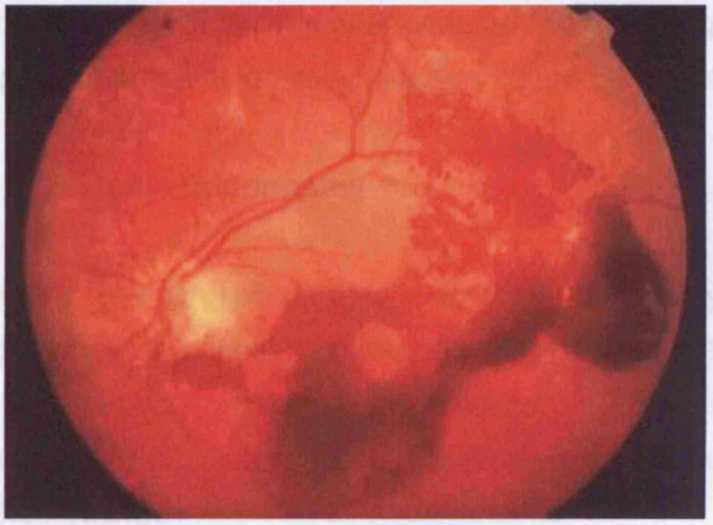

Figure 2.6 Intra-gel haemorrhages from new vessels in proliferative retinopathy [www.medweb2.bham.ac.uk 2003].

2.7.3.4 Diabetic Maculopathy

Macular oedema is caused by leakage of blood and blood constituents following a breakdown of the blood-retinal barrier in this area. This oedema may be localised e.g. focal leakage from a microaneurysm, and the limit of oedema is often characterised by a ring of exudates forming a boundary around the swollen area. It may also be diffuse occupying the vast majority of the macula region and may take on a cystoid appearance. Any reduction in vision should arouse suspicion of macular oedema and thus warrant stereoscopic examination of this region.

There are 3 main types of maculopathy, classified according to the appearance on fundoscopy and fluorescein angiography [Steele 2003].

1. Exudative: focal or diffuse leakage, with or without exudates. Haemorrhages, microaneurysms and retinal thickening seen.
2. Ischaemic: Capillary closure resulting in ischaemia.
3. Mixed: Combination of exudative and ischaemic together.

Treatment for clinically significant macular oedema is generally given with an argon laser applied to areas of vascular leakage and retinal thickening.

2.7.4 Grading of Diabetic Retinopathy

Diabetic retinopathy may be graded into distinct levels of severity. There are various grading systems available. In this study our findings will be graded according to a modified version of the Early Treatment of Diabetic Retinopathy Study, ETDRS, final retinopathy severity scale as outlined in Table 2.5 [ETDRS 1991b].

This grading system is an extension of the modified Airlie House Classification [ETDRS 1991a]. Seven standard photographic fields are assessed for the presence of the features of diabetic retinopathy and an overall grade assigned to each eye individually. Field 1 is centred on the optic disc, field 2 on the macula, field 3 is temporal to the macula and 4 to 7 are tangential to horizontal lines passing through the upper and lower poles of the optic disc and to a vertical line passing through its centre.

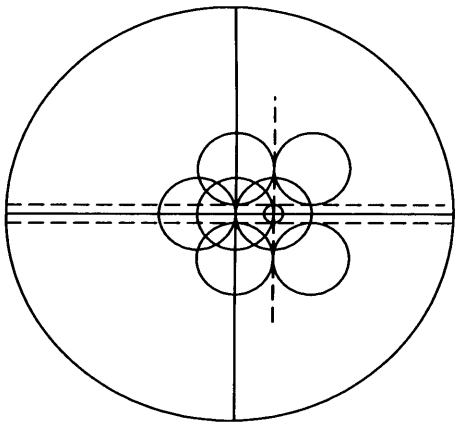

Fig 2.7 Seven standard fields of the Modified Airlie House Classification, shown for a right eye [ETDRS 1991a].

The ETDRS research group examined the power of each diabetic lesion, and combinations of lesions, to predict the progression to proliferative retinopathy in a five year clinical trial involving 3711 diabetic patients. Severity of IRMA,

haemorrhages and/or microaneurysms, and venous beading were determined to be the most important factors, and on the basis of their findings a final retinopathy scale was developed. This scale ranges from absence of retinopathy to severe vitreous haemorrhage over 15 levels [Table 2.5, ETDRS 1991b].

In this study fundus photography was performed by the author along with fundoscopy in the test eye of all subjects. Three 45° photographs were taken. One centrally including the optic disc and macula area, and one each of the nasal and temporal retina along the horizontal axis.

2.7.5 Treatment of Proliferative Diabetic Retinopathy

In the case of proliferative DR laser photocoagulation may be used in order to prevent further neovascularisation. Areas of retinal non-perfusion or profuse leakage are first assessed by fluorescein angiography in order to determine the areas to be treated. An Argon laser is used to destroy areas of ischaemic retina in order to reduce the retinal oxygen demand and to remove the stimulus for new vessel growth. Focal/grid photocoagulation is used in focal areas of ischaemia. Pan-retinal photocoagulation is used in the case of diffuse retinal ischaemia. In this case approximately 2,000 burns are placed over the entire peripheral retina.

2.8 Summary

Diabetes mellitus is characterised by hyperglycaemia and is thought to affect 2% of the Caucasian populations in most westernised countries. Diabetic retinopathy is the major cause of registerable blindness in the working population in western countries [Evans 1996]. Occlusion of the retinal capillaries and capillary leakage can result in microaneurysms, haemorrhages, exudates and new vessel growth which may lead to retinal detachments in some cases.

Level	Severity	Definition
10	DR absent	Microaneurysms and other characteristics absent.
14*	DR questionable	HE, SE or IRMA definite: microaneurysms absent.
15*	DR questionable	Haemorrhage(s) definite: microaneurysms absent.
20	Microaneurysms only	Microaneurysms definite, other characteristics absent.
35**	Mild NPDR	One or more of the following: Venous loops ≥ D/1; SE, IRMA or VB=Q; Retinal haemorrhages present; HE ≥ D/1; SE ≥ D/1
43	Moderate NPDR	H/MA = M/4-5 to S/1; *Or* IRMA = D/1-3 (not both)
47	Moderately severe NPDR	Both L43 characteristics and or one (only) of the following: Irma = D4-5; H/Ma = S/2-3; VB ≥= D/1
53	Severe NPDR	One or more of the following: ≥ 2 of the 3 L47 characteristics; H/Ma ≥ S/4-5; Irma ≥ M/1; VB ≥ D/2-3
61	Mild PDR	FPD or FPE present with NVD and NVE absent ; Or NVE = D
65	Moderate PDR	Either of the following: (1) NVE ≥M/1 or NVD = D; and VH and PRH = A or Q (2) VH or PRH = D and NVE < M/1 and NVD absent
71	High risk PDR	Any of the following: (1) VH or PRH ≥ M/1; (2) NVE ≥ M/1 and VH or PRH ≥ D/1; (3) NVD = 2 and VH or PRH ≥ D/1; (4) NVD ≥ M
75	High risk PDR	NVD ≥ M and VH or PRH ≥ D/1
81	Advanced PDR: Fundus partially obscured, centre of macula attached.	NVD = cannot grade, or NVD < D and NVE = cannot grade in ≥ 1 field and absent in all others; And retinal detachment at centre of macula < D.
85	Advanced PDR: Posterior fundus obscured, or centre of macula detached.	VH = VS in fields 1 and 2; Or retinal detachment at centre of macula = D
90	Cannot grade, even sufficiently for level 81 or 85.	

DR = diabetic retinopathy, HE = hard exudates, SE = soft exudates, IRMA = intraretinal microvascular abnormalities, NPDR = non proliferative DR, VB = venous beading, H/Ma = haemorrhages/microaneurysms, PDR = proliferative DR, NVE = new vessels elsewhere (>1DD from disc), NVD = new vessels disc (within 1DD of disc margin), FPD = fibrous proliferations disc, FPE = fibrous proliferations elsewhere, VH = vitreous haemorrhage, PRH = pre-retinal haemorrhage

*Levels 14 and 15 are not considered separate steps in the scale but are pooled with level 10 or 20

** NPDR levels 35 and above all require presence of Mas

Severity categories for characteristics graded in multiple fields are of the form (maximum severity/extent), where maximum severity can be absent (A), questionable (Q), definitely present (D), moderate (M), severe (S), or very severe (VS), and extent is the number of fields at that severity level.

Table 2.5 ETDRS final retinopathy severity scale [ETDRS 1991b].

3. THE ELECTRORETINOGRAM

The electroretinogram, ERG, is a graphical record of the summation of electrical responses of the retinal cells to light. These responses occur as ion currents, principally potassium and sodium, flow within the retina and changes in these currents occur with the onset and offset of light stimuli. As more is known regarding the origins of the components of the ERG the more useful this technique has become in the investigation of disorders affecting the retina, since it can provide a uniquely laminar distribution of retinal activity.

3.1 Granits Analysis

In 1933, Ragnar Granit carried out extensive investigations of the ERG in dark-adapted cats. He studied changes to the ERG under levels of deepening ether anaesthesia, and was able to isolate three components of the ERG, PI, PII and PIII, which when summed together give the total ERG waveform [Weisinger et al 1996]. These components were labelled in order of their disappearance with deepening anaesthesia, PI being the first to disappear and PIII the last. These processes, described by Granit, are now conventionally termed the c-, b- and a-waves of the ERG.

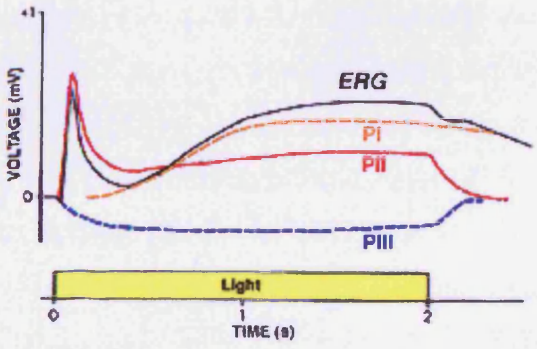

Figure 3.1 Components of the ERG identified by Granit, recorded from a cat in response to a two second light stimulus [Kolb et al 2003].

The PIII process was subsequently found to consist of two components, the initial phase termed the fast PIII or receptor potential and the second more slowly developing phase termed the slow PIII. The leading edge of the fast PIII is the first to develop and forms the corneal negative a-wave. Its short latency indicated that it arose early in the chain of events and thus probably reflects the activity of the photoreceptor cells [De Rouck 1991].

The next to develop is the corneal positive PII that, along with the slow PIII, forms the much larger b-wave. Granit believed the origin of the PII lay in the neural pathway between receptors and ganglion cells and was correlated with optic nerve activity [De Rouck 1991].

The PI is the last process to develop and thus forms the c-wave.

3.2 The Early Receptor Potential

The early receptor potential is a rapid waveform, which occurs almost immediately following a bright light flash. It reflects the bleaching of photopigments in the outer segments of the photoreceptors. It consists of a small positive portion followed by a larger negative portion and is essentially complete by the time the a-wave begins.

3.3 The a-wave

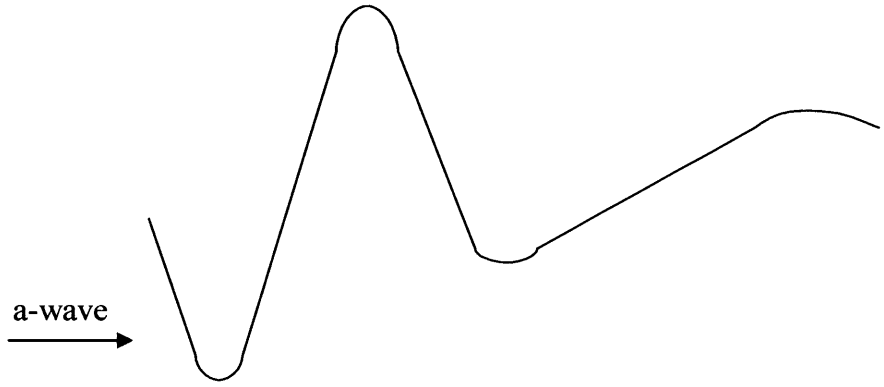

Figure 3.2 The a-wave of the photopic ERG.

The a-wave also provides information about the photoreceptor region of the retina. The leading edge of the a-wave is a corneal negative response believed to reflect light-induced hyperpolarisation of the photoreceptors [Fishman 2001], and the rest of the a-wave may reflect a glial cell response to decreased potassium concentration in the photoreceptor region of the outer retina [Wachtmeister 1998]. Experiments on the monkey retina found that part of the photopic a-wave was abolished following injection of the glutamate analogue *cis*-piperidine-2,3-dicarboxylic acid (PDA), known to block light responses of horizontal and hyperpolarising bipolar cells. This suggests that the a-wave may derive, in part, from activity post-synaptic to cone photoreceptors [Bush and Sieving 1994]. The a-wave occurs at approximately 15 ms after stimulus onset and peaks within 5ms [Weisinger et al 1996]. The a-wave latency has been shown to be determined solely by photoreceptor activity and any delay in a-wave latency would indicate a large area of photoreceptor damage [Qiu et al 2002]. The scotopic ERG a-wave reflects essentially rod photoreceptor cell activity [Fishman 2001].

3.4 The b-wave

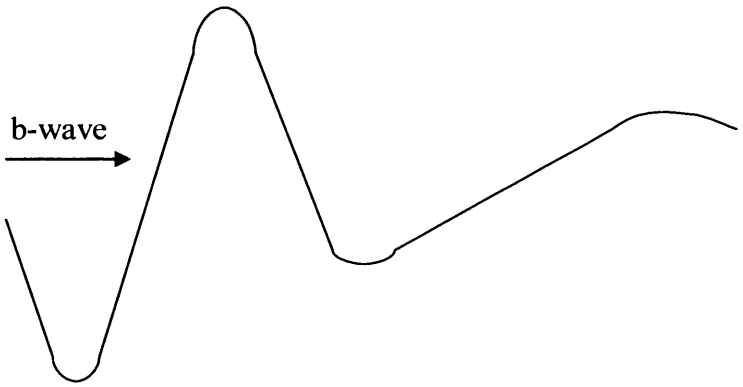

Figure 3.3 The b-wave of the photopic ERG.

Following the a-wave a corneal-positive waveform is observed, the b-wave, which is thought to be generated by the depolarisation of ON-bipolar cells [Tian and Slaughter 1994, Xu and Karwoski 1994a,b, Hood and Birch 1996, Green et al 1999, Shiells and Falk 1999]. The b-wave was thought to reflect the activity of depolarising Müller cells in response to increased potassium concentration in the inner retina, and indirectly represent the activity of ON bipolar cells [Dick and Miller 1978, Wachtmeister 1998, Fishmann 2001], though other experimental work has suggested that a portion of the b-wave may arise from Müller cells but that a stronger direct contribution arises from the depolarising bipolars [Xu and Karwoski 1994a,b].

3.4.1 Characteristics of the b-wave

The b-wave recorded under standard photopic conditions has an implicit time of 31-38 ms and an amplitude of 132-320 μV [Fishmann 2001]. Under scotopic conditions, where a maximal rod response is recorded, both implicit time and amplitude increase to 33-54 ms and 489-908 μV respectively [Fishmann 2001].

3.4.2 Origin of the b-wave

3.4.2.1 Current Source Density Analysis

Current source density (CSD) analysis of retinal field potentials was performed by Faber [1969], who identified a major current sink at the level of the OPL and a current source extending from the sink to the vitreal surface of the retina. He reasoned that this source sink pattern corresponded with current flow from Müller cells since they are the only retinal element to extend from the distal portion of the neural retina to the inner limiting membrane. More recent work by Xu and Karwoski [1994a] also looked at CSD analysis of retinal field potentials and identified a current sink for the b-wave at the OPL and a source at the IPL, corresponding to the anatomy of the bipolar cells.

3.4.2.2 Intracellular Recordings

Miller and Dowling [1970] recorded the intracellular responses of Müller cells in the mudpuppy retina. They found that light-induced Müller cell responses were always positive going and that these responses matched the ERG b-wave response at comparable flash intensities. The Müller cell response and b-wave were also found to be nearly identical in latency at all intensities. The similarity of these waveforms suggested that Müller cells may generate the b-wave of the ERG and they propose that this may be due to a potassium ion (K^+) regulated mechanism. Dick and Miller [1978] also looked at potassium activity within the mudpuppy retina. They found two sources of light evoked K^+ increases. One was located proximally and pharmacological properties suggested a post-bipolar origin. Another was located in the distal retina with pharmacological properties indicating a post-receptor origin, probably a reflection of depolarising bipolar cells. They proposed that this bipolar cell activity caused a rise in K^+ ions. Müller cells responded to this local increase in potassium and produced the extracellular currents that generate the b-wave.

3.4.2.3 Pharmacological Analysis

Analysis of the b-wave by Xu and Karwoski [1994b] suggested that a portion of the b-wave may originate from Müller cells, but that a stronger direct contribution from depolarising bipolar cells was likely. They found that in the presence of barium, Müller cell currents are blocked but the b-wave persists, suggesting that the b-wave may be produced directly by extracellular current flow from ON-bipolar cells. Tian and Slaughter [1995] looked at the effect of 2-amino-4-phosphono butyrate (APB), known to selectively suppress the activity of the ON-bipolars, on the b-wave in the vertebrate retina. Using slow drug application of APB they compared the progressive effects on the response amplitudes of the ON-bipolar cells and the b-wave. A strong positive correlation was found between the two waveforms, thus lending further support for the ON-bipolars as the direct generators of the b-wave. Later studies have confirmed that the b-wave is eliminated in the presence of APB [Shiells and Falk1999, Green et al 1995] and actually increased in the presence of barium [Green et al 1999].

3.5 The Scotopic ERG

3.5.1 Features of the Scotopic ERG

The scotopic ERG is recorded under conditions of dark adaptation but contains both rod and cone components. The dominant component is however a rod response which leads to an increase in both b-wave amplitude and implicit time when compared to the photopic ERG. This can be explained by both the increased sensitivity of the rods in comparison to the cones, and by the greater number of rods than cones within the photoreceptor population [Fishman 2001]. The recommended recording conditions for the scotopic ERG shall be discussed in more detail in section 3.5.2.

The main rod pathway runs from rods to ganglion cells via specific rod bipolars and AII amacrine cells. Müller cells also contribute to the dark adapted ERG as they respond to extracellular changes in K^+ concentration.

The relative contributions of both rods and cones to the scotopic ERG, and thus the form of the recorded ERG, can be influenced by the intensity, wavelength and frequency of the flash stimulus, along with th
the patient [Robson and Frishman 1999, Fishman 2001].

In the fully dark-adapted eye with a very dim flash stimulus a corneal negative deflection is recorded which peaks more than 100 ms after the flash. This response is known as the scotopic threshold response, STR, and is thought to arise in the proximal retina reflecting the activation of amacrine and ganglion cells [Aylward 1989, Robson and Frishman 1999]. The amplitude of the STR grows with increasing stimulus energy but saturates at fairly low levels, less than 100μV in cats [Robson and Frishman 1999].

As stimulus energy increases further an earlier positive wave appears, the b-wave, which represents primarily activation of the on-bipolar cells although other inner retinal cells may provide smaller positive contributions [Hood and Birch 1996, Robson and Frishman 1999]. It is the b-wave that dominates the ERG over most of its dynamic range. A further increase in stimulus energy reveals an earlier negative a-wave, which can grow to an amplitude several times greater than that of the saturated STR, and is thought to represent activity of the rods themselves [Robson and Frishman 1999].

3.5.2 Recording the Scotopic ERG

A scotopic response may be recorded following a period of dark adaptation. The International Society for Clinical Electrophysiology of Vision, ISCEV, recommends twenty minutes of dark adaptation, although this period may need to be extended if directly following photopic stimulation [Marmor et al 2004]. The extent to which this period should be lengthened is dependent on the luminance and duration of the flash used. If recording a rod response it is advisable to perform this measurement before others since it is the most sensitive to light adaptation.

When recording a rod response ISCEV standards recommend use of a dim white flash stimulus of strength 2.5 log units below that of the standard white flash, 1.5-3.0 cd.s.m^{-2}, with a minimum inter-flash interval of 2 s. A blue stimulus is thought to be equally appropriate providing it is equated to the white standard [Marmor et al 2004].

3.5.3 The Scotopic ERG in Diabetes Mellitus

Several studies have looked at possible changes in the scotopic ERG as a result of diabetes mellitus, and the findings are summarised in Table 3.1.

In a group of Type 1 diabetic subjects with a minimum disease duration of ten years, significant correlations were found between the grade of retinopathy and the amplitude and latency of the STR. No significant correlation was observed between the grade of retinopathy and any parameters of the scotopic b-wave. The b-wave was recorded following 30 minutes of dark adaptation to a blue flash stimulus, frequency 1Hz. Recordings were made to a range of flash luminance 1-2 to 4.2 log units above the psychophysical threshold, increasing in 0.3 log unit steps and the maximum rod amplitude chosen by inspection of the traces. Significant correlations were also found between all of the individual amplitudes of the OPs, the summed amplitude of the OPs and the implicit times of OP1 and OP2. The STR, although correlated with the grade of retinopathy, did not correlate as strongly as the OPs [Aylward 1989].

A later study also found no significant difference in scotopic b-wave amplitude between a group of diabetic subjects (four Type 1 and ten Type 2), and a group of age-matched controls. Retinopathy levels ranged from no retinopathy to microaneurysms with one or more other non-proliferative lesions of mild to moderate degree. The scotopic b-wave was recorded following 40 minutes of dark adaptation to a low luminance blue flash. The diabetic group did show significantly delayed b-wave implicit times in comparison to the controls and it is thought that this delay may indicate the presence of early retinal changes [Holopigian et al 1992].

Both delayed scotopic b-wave implicit time and a reduction in scotopic b-wave amplitude were observed in two groups of Type 1 juvenile diabetics when compared to age matched controls [Juen and Kieselbach 1990]. The groups consisted of patients with no retinopathy and background retinopathy, classified according to the ETDRS criteria. The reduction in b-wave amplitude and increase in implicit time were recorded to the highest flash energy, 0.25 J, in both groups. However, the scotopic ERG in this case was recorded after only fifteen minutes of dark adaptation, a shorter period than that recommended by ISCEV [Marmor et al 2004], directly following recording of a photopic ERG, and therefore does not represent a maximum rod response. As these changes are present with no retinopathy this may suggest a role for the scotopic b-wave as an indicator of early functional changes in diabetes before retinopathy becomes apparent.

A more recent study of Type 1 juvenile diabetics confirmed this reduction in scotopic b-wave amplitude though no significant difference was found in b-wave implicit time between the diabetic group and a group of age matched controls. This study looked at patients with no retinopathy or mild background retinopathy and recorded scotopic ERGs at two minute intervals during a 28.5 minute dark adaptation period. The b-wave was recorded to blue flashes at 1Hz delivered with an SLE stroboscope, lamp intensity 4, positioned 25cm in front of the patient. The b-wave amplitude was consistently smaller in the diabetic groups than that of the controls at equivalent times during the dark adaptation period, the smallest amplitudes being found in those with retinopathy [Papakostopoulos et al 1996].

It is possible that the difference in findings between the studies mentioned may, in part, be due to the different stimuli and recording conditions employed.

To summarise, these noted changes to the scotopic ERG with diabetes, and its relation to the level of retinopathy, suggests its use as an indicator of early retinal changes in diabetes, even before any visible retinopathy becomes apparent.

Finding	Study
B-wave amplitude significantly reduced in DM with no retinopathy and with mild BDR	Juen and Kieselbach 1990 Papakostopoulos et al 1996
No significant change in b-wave amplitude in DM with no retinopathy and mild BDR	Holopigian et al 1992
B-wave implicit time significantly delayed in DM with no retinopathy and with mild BDR	Juen and Kieselbach 1990 Holopigian et al 1992
No significant change in b-wave implicit time in DM with no retinopathy and mild BDR	Papakostopoulos et al 1996
Significant correlation found between level of retinopathy and both amplitude and latency of STR	Aylward 1989
No significant correlation between level of retinopathy and amplitude or latency of b-wave	Aylward 1989

Table 3.1 Summary of scotopic ERG findings in diabetes mellitus.

3.6 The c-wave

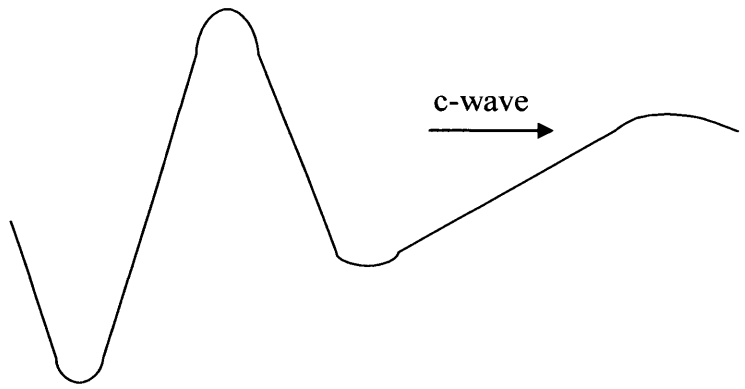

Figure 3.4 The c-wave of the photopic ERG.

The c-wave is a monophasic corneal positive response. It is thought to originate from transient hyperpolarisation at the apical surface of RPE cells following a light flash and hyperpolarisation of the distal end of Müller cell processes [Wachtmeister 1998, Fishman 2001].

3.7 The OFF Response

The OFF response, or d-wave, is a small positive deflection which can be seen with longer duration stimuli in the photopic ERG. It signals light offset and may be generated by hyperpolarising bipolars and photoreceptor cells [Wachtmeister 1998].

3.8 The Oscillatory Potentials

The oscillatory potentials, OPs, were first reported as 4-6 small waves superimposed on the ascending limb of the b-wave [Cobb and Morton 1954]. The OPs are not always clearly distinguishable in the unfiltered ERG waveform and thus filtering techniques are commonly applied to enhance their appearance and amplitude. Optimal recording conditions for observation of the OPs shall be discussed shortly. Each OP is named in order of its appearance, i.e. the first in the series of wavelets is named OP1.

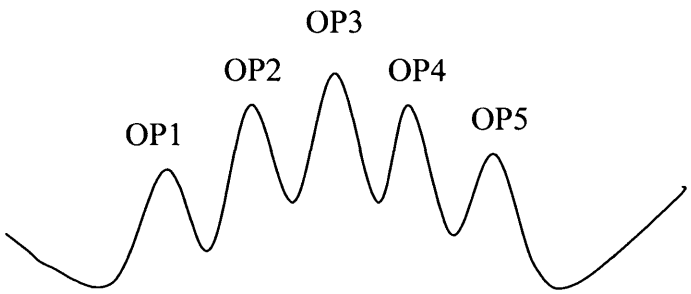

Figure 3.5 Diagram of the retinal oscillatory potentials.

3.8.1 Characteristics of the OPs

3.8.1.1 Basic Characteristics of the OPs

The OPs are known to be of much higher frequency than the other ERG components. In humans they are considered to have a dominant frequency of between 100-160 Hz, which is considerably higher than that of the a and b-waves of approximately 25 Hz [Wachtmeister 1991]. OPs recorded at

threshold have a high frequency of 160 Hz, but this frequency decreases to approximately 120 Hz with stronger stimuli on adaptation to increasing background illumination where cone sensitivity is higher than that of the rods [Wachtmeister 1973a]. They appear as rapid rhythmic sub-waves with a low and fairly uniform amplitude of approximately 30 μV [Cobb and Morton 1954].

The threshold of the OPs is approximately 2.5-3 log units higher than that of the b-wave and approximately the same as that of the a-wave [Algvere et al 1972].

3.8.1.2 Temporal Characteristics of the OPs

Temporal summation of the OPs has been investigated using stimuli of increasing duration under conditions of both weak and strong light adaptation. OP threshold was found to be the same for both short and long duration stimuli under conditions of strong light adaptation, however OP threshold was lower with longer duration stimuli under conditions of weak light adaptation and they were found to integrate temporally up to 400 ms. The b-wave showed temporal summation up to 10 ms under strong light adaptation and both a and b-waves showed temporal summation up to 40 ms under weak light adaptation. This difference in behaviour between the OPs and the other ERG components suggests a difference in their origin [Wachtmeister 1974b].

3.8.1.3 Spectral Characteristics of the OPs

Investigation of the OPs under conditions of light and dark adaptation with monochromatic stimuli has been performed to obtain relative spectral sensitivity values for the OPs. In light adapted eyes these values have been shown to approximate the CIE photopic luminosity function, and in dark adapted eyes to approximate the CIE scotopic luminosity function [Stodtmeister 1973]. A later experiment however found the spectral sensitivity of the OPs produced a composite curve with two peaks at 472 and 551 nm, and did not agree with the standard photopic or scotopic luminosity function

[Wachtmeister 1974a]. No shift of relative sensitivity of the OPs to long wavelength stimulus on light adaptation, and thus no Purkinje shift, is evident [Wachtmeister 1974b]. A comparative higher sensitivity to the short wavelengths of the spectrum suggest a close relation to scotopic rod activity and the composite curve suggests that the OPs may be generated by a mechanism of rod and cone interactions [Wachtmeister 1974a]. The existence of separate rod and cone OPs has also been suggested [King-Smith et al 1986] with bright white flashes to a dark-adapted eye eliciting both a rod and a cone system contribution [Peachey et al 1987].

3.8.1.4 Adaptational Characteristics of the OPs

OPs have been found to be optimally recorded in the mesopic range of the dark adaptation curve, at the level at which a change from photopic to scotopic vision occurs [Wachtmeister 1973b]. It is at this adaptation level that OPs of low frequency and maximum energy are recorded [Algvere and Westbeck 1972].

Mesopic conditions can be induced by the use of conditioning flashes, by light adaptation to a steady background illumination or during a period of recovery in the dark following bright illumination [Wachtmeister 1998]. Under scotopic conditions no prominent OPs can be recorded in response to a single flash [Algvere and Westbeck 1972, Wachtmeister 1973b]. Under scotopic conditions a series of three flashes is generally used and the response recorded to the 3rd flash [Wachtmeister 1991]. The amplitudes and energy of the OPs are found to be maximal with an interstimulus interval of 30 s [Wachtmeister 1973b, Gjotterberg 1974]. When the interstimulus interval is longer than this the energy of the oscillations is low, and retinal sensitivity is below the level that corresponds to the rod-cone break, about 6 x 10^{-3} lux [Wachtmeister 1991]. This energy increases with a decrease in interstimulus interval, peaking at 30 s. At intervals of less than 30 s sensitivity lies above the rod-cone break and is determined solely by the cones. The interstimulus interval necessary to elicit a maximal response will however depend on the

luminance of the flash. For bright 15 ms stimuli, about 5 x 10^4 photopic cd.m^2, an interval of 30 s or 15 s is advised [Wachtmeister 1991].

After a period of dark adaptation the amplitude of the photopic ERG is known to increase when the subject is exposed to an adapting field. This effect is known as the light adaptation effect, LAE. A study investigating the effects of different lengths of dark adaptation on the LAE found that the LAE appears to have a differential impact on the individual OPs. An early rapid process appears to affect OP4 whose amplitude diminished significantly with a period of dark adaptation as short as two minutes. A second slower process, activated later in the dark adaptation process, appeared to affect OP2 and OP3 whose amplitudes decreased after a ten minute dark adaptation period [Benoit and Lachapelle 1995].

The sensitivity of the OPs decreases on adaptation to bright background illumination when the sensitivity of the cones is higher than that of the rods [Wachtmeister 1973a].

The peak latency vs flash luminance functions, LI, for OPs 1-4 show similar slopes at low levels of light adaptation. However, as adaptation increases the LI slopes of OP3 and OP4 flatten which may suggest a saturation of rod activity. No saturation effect is observed for OP1 and OP2 and this may suggest a reflection of cone activity [Coupland 1987a].

3.8.1.5 Pharmacological Characteristics of the OPs

Gamma amino butyric acid, GABA, and glycine are inhibitory synaptic transmitters thought to act within the inner plexiform layer of the retina. OPs of the mudpuppy retina are selectively depressed by these substances with the earlier OPs abolished at low concentrations and OP3-5 still present, with no appreciable change to the b-wave [Wachtmeister and Dowling 1978]. OPs are also differentially sensitive to GABA antagonists, bicuculline and picrotoxin, with OP1-3 abolished at lower concentrations than OP4-5, and again no appreciable change to the suprathreshold b-wave. Disruption of

GABA mediated pathways may occur via saturation of GABA receptor sites or by blocking of these sites with bicuculline and picrotoxin [Wachtmeister 1980].

Glycine is known to selectively depress the OPs and the application of strychnine, a glycine antagonist, also abolishes the OPs. OP1 shows less sensitivity to strychnine at low concentrations but at high concentrations all OPs are abolished and the amplitude of the suprathreshold b-wave decreased. Disruption of glycine sensitive pathways may also occur via saturation or blocking of glycine receptors with glycine or strychnine respectively [Wachtmeister 1980]. The sensitivity of the OPs to these substances would suggest that the neural pathways involved in their generation are both GABA and glycine sensitive.

Cytopathological changes occurring within amacrine cells, in parallel to temporary loss of the OPs following intravitreal injection of glycine in the rabbit, may implicate these cells in the generation of the OPs [Korol et al 1975].

The oscillatory potentials of the mudpuppy retina are usually selectively depressed by dopamine [Wachtmeister and Dowling 1978]. Haloperidol, a dopamine antagonist, also selectively depresses the OPs in low concentrations, the amplitude of the earlier OPs decreasing without any significant alteration of the later OPs. High concentrations of haloperidol significantly reduce the amplitudes of all the OPs along with the suprathreshold b-wave. This would suggest that OPs are abolished either by saturation of dopaminergic receptors or by blocking the effect of dopamine with haloperidol. This sensitivity to dopamine and its antagonist would suggest that dopaminergic neurons may be involved indirectly or directly in the generation of the OPs [Wachtmeister 1981a].

Acetylcholine and carbocholine are thought to act as excitatory neurotransmitters within the inner plexiform layer of the retina. Their application to the mudpuppy retina does not affect the OPs or the b-wave [Wachtmeister and Dowling 1978], suggesting that the OPs may primarily

reflect inhibitory neuronal pathways and not excitatory pathways. In agreement with this view, blocking agents of the amino acids glutamate and aspartate, excitatory neurotransmitters thought to act within the outer plexiform layer between photoreceptors and second order neurons, decrease or abolish the OPs simultaneously with the b-wave. This lack of selective sensitivity of the OPs would suggest that glutamate or aspartate sensitive pathways are not directly involved in their generation. The OPs do however show differential sensitivity to glutamate which would suggest that this amino acid may be indirectly linked to their generation [Wachtmeister 1981a].

The excitatory amino acids kainic acid (KA), and N-methyl D-aspartate (NMDA), both cause a decrease in the amplitude of the OPs. KA is known to cause lesions of ganglion, amacrine and bipolar cell dendrites. NMDA was not shown to cause any specific structural changes and in the cat eye the decrease of the OPs with NMDA is reversible [Vaegan and Millar 1994].

β-alanine, an inhibitory amino acid, selectively and differentially suppresses the OPs with the earlier OPs, OP1-2, being more sensitive to this drug than the later ones, OP3-5, and with no change to the a and b-waves. This drug has been known to induce swelling in some amacrines of the rabbit retina. Valine, another inhibitory amino acid, does not affect the OPs or the amacrines. It would appear from these results that the activity of the amacrines may be linked to the neural activity generating the OPs [Wachtmeister 1981b].

Ethanol also has a differential and selective effect on the OPs, the later OPs, OP3-5, being more sensitive than the earlier ones [Wachtmeister 1981b]. Ethanol is also known to selectively abolish the off-component, d-wave, of the mudpuppy ERG. GABA is thought to be associated with 'on'-pathways in the retina and the depolarising bipolars, while glycine is linked to 'off'-pathways and the hyperpolarising bipolars. With the earlier OPs being more sensitive to disruption of GABA mediated pathways and later OPs being more sensitive to disruption of the glycine network and Ethanol it is thought that the earlier OPs

may be related to the on-response and the later OPs to the off-response system [Wachtmeister 1981b].

To summarise, the OPs are selectively depressed by the inhibitory amino acids GABA, glycine and β-alanine, but are unaffected by the excitatory neurotransmitters acetylcholine and carbocholine, suggesting that the OPs primarily reflect inhibitory neuronal pathways and not excitatory pathways. β-alanine and glycine are also known to cause destructive changes in some amacrine cells whilst valine, another inhibitory neurotransmitter, does not. Valine does not depress the OPs suggesting that the amacrine cells may be involved in the generation of the OPs. Dopamine also selectively depresses the OPs implicating dopaminergic neurons in their generation. Ethanol and glycine are thought to be linked to off-response pathways in the retina and the later OPs are more sensitive to these substances. GABA is thought to be linked to on-response pathways and the earlier OPs are more sensitive to disruption of GABA mediated pathways, suggesting that the earlier OPs may be related to the on-response and the later OPs to the off-response system. These results also suggest a difference in origin of the OPs from the a and b-waves, and also of the individual oscillatory peaks.

3.8.2 Origin of the OPs

Depth profile experiments have been undertaken in order to locate the origin of the oscillatory potentials, the theory being that each component of the ERG should have maximum amplitude as an electrode passes the cells that generate it. In the cynomolgus monkey the OPs increase in amplitude until approximately 16% retinal depth. The photoreceptors or cells of the INL are thus unlikely generators [Brown 1968]. Clamping of the retinal circulation abolishes the OPs also making the photoreceptors and the cellular structures of the deeper retinal layers, e.g. horizontal cells, unlikely generators [Brown 1968]. In the Rhesus monkey maximum amplitude was recorded at 25% retinal depth, centred upon the IPL [Ogden 1973]. The OPs have also been found to reverse their polarity with retinal depth in the mudpuppy retina, and

thus are thought to represent radial flows of current within the retina. The individual OP peaks reverse at different retinal levels, in sequence, suggesting that a difference in neuronal origin may underlie each peak [Wachtmeister and Dowling 1978]. This view is supported by various pharmacological studies as outlined previously. The polarity of OP1 reverses at the border between the inner plexiform and inner nuclear layers, at the level of the amacrine cells [Wachtmeister and Dowling 1978]. Amacrine cells however are thought to represent tangential current flow rather than radial and as such questions their direct role in the generation of the OPs [Heynen et al 1985, Wachtmeister and Dowling 1978]. The OPs are thought to be generated more proximally than the b-wave [Brown 1968, Ogden 1973, Heynen et al 1985, Wachtmeister and Dowling 1978] and a difference in origin between the OPs and the b-wave is again supported by pharmacological studies as outlined. It is thought from these depth profile studies that the bipolars, are the most likely generators of the OPs. This view would be supported since the bipolars receive inputs from rods and cones and this interaction of rod and cone mechanisms seems necessary to elicit the OPs [Wachtmeister 1974a, Peachy et al 1987], however the interplexiform cell should not be ruled out [Heynen et al 1985, Wachtmeister 1998].

The role of ganglion cells in the generation of the OPs is as yet undetermined. Unilateral intracranial optic nerve sectioning of the Rhesus Monkey resulted in marked reductions of the oscillatory potentials in one animal and virtual absence in another two years following the procedure, suggesting that an intact optic nerve is required for full expression of these potentials [Ogden 1973]. An earlier experiment however found no change in the OPs of the rabbit seven months after optic nerve sectioning [Winkler 1972]. Kainic acid (KA), as mentioned previously is known to cause lesions of ganglion, amacrine and bipolar cell dendrites. It is thought that this neural toxicity from KA in cats, as with mammals, starts with the ganglion cells and spreads to the more distal retinal cells with increasing dose. In the cat KA has been found to reduce all ERGs with the order of susceptibility being OPs, then pattern ERGs and focal ERGs, then STRs and b-waves. The OPs were significantly reduced at low doses of KA, below the 25 nmol dose at which ganglion cell

dendrites are selectively damaged, providing support for a role of ganglion cells in their generation [Vaegan and Millar 1994].

3.8.3 Recording the OPs

OPs of maximal energy are recorded during mesopic conditions, i.e. as the sensitivity of the eye changes from scotopic to photopic vision. In the dark adapted eye no prominent OPs can be recorded to a single flash [Algvere and Westbeck 1972], and the mesopic conditions required may be produced in the dark adapted eye by a series of flashes with the response recorded to the last flash [Wachtmeister 1991]. The first conditioning flashes operate by adapting the rod system contributions to the OPs so that those recorded to subsequent flashes result primarily from the cone system [Peachey et al 1987]. They may also be recorded by the use of a steady background illumination or during recovery in the dark following exposure to bright illumination [Wachtmeister 1991]. When recording OPs in the dark adapted eye the International Society for Clinical Electrophysiology of Vision, ISCEV, recommend twenty minutes of dark adaptation as well as using other standard ERGs as 'conditioning flashes' [Marmor et al 2004].

As mentioned previously the luminosity functions of the OPs contains both photopic and scotopic maxima [Stodtmeister 1973, Wachtmeister 1974a]. In view of this a white stimulus is preferred to monochromatic stimuli. ISCEV also state that where white stimuli are produced by a combination of narrow band sources, e.g. red, green and blue LEDs, it must be ensured that their stimulus output is of equivalent luminance to the standard white flash for all conditions [Marmor and Zrenner 1999]. The threshold of the OPs is approximately 2-3 log units higher than that of the b-wave, and the energy and amplitude of the OPs increase linearly over a range of about 3 log units, thus a very strongstimulating light should be used, about 5×10^4 photopic $cd.m^{-2}$ [Wachtmeister 1991].

With the use of a bright 15ms flash, i.e. about $75cd.s.m^{-2}$, an interstimulus interval of 30 s or 15 s is advised [Wachtmeister 1991]. This is both stronger

and three times longer than the ISCEV standard flash. In order to standardise the response, ISCEV recommend that an interstimulus interval of 15 seconds, and a flash luminance of 1.5-3 cd.s.m^{-2}, should be used [Marmor et al 2004].

Fourier analysis shows that the OPs have a dominant frequency of about 100-160 Hz. In order to enhance the amplitudes of the OPs and reduce the contribution of the a and b-waves a band pass filter is required, e.g. 80 to 500 Hz [Wachtmeister 1991].

3.8.4 The OPs in Diabetes Mellitus

Many studies have looked at changes to the OPs in DM. The findings are summarised in Table 3.2.

The oscillatory potentials have been found to reflect disturbances in the retinal circulation [Speros and Price 1981, Holopigian et al 1992], and are often affected in the very early stages of diabetic retinopathy, and as such are useful in the objective evaluation of retinal function in diabetes mellitus. Many studies have reported a decrease in the amplitudes of the OPs and often a delay in their implicit times.

A reduction in oscillatory potential amplitudes have been observed in diabetic subjects, often before the presence of any visible retinopathy [Moschos et al 1987, Van Der Torren and Van Lith 1989, Juen and Kieselbach 1990, Holopigian et al 1992], and with no change to the a and b-wave of the ERG [Simonsen 1980]. This reduction in amplitude increases progressively with increasing severity of retinopathy [Bresnick and Palta 1987b, Shirao et al 1991] and summed OP amplitude has been found to be a reliable predictor of the development of PDR [Simonsen 1980, Bresnick and Palta 1987a, Moscos et al 1987].

Simonsen [1980], studied a group of 137 diabetic subjects both with and without DR, aged 17-50 years, who had all been diagnosed with Type 1 diabetes mellitus before the age of 40. OPs were recorded following 20

minutes of dark adaptation to a 15 Hz flash of 100 ms duration and energy 0.6 J. This flash duration is significantly longer than the standard flash recommended by ISCEV [Marmor et al 2004]. After the initial recording of the OPs, the subjects were re-examined after 6-8 years and 13-15 years. At the 6-8 year examination, 54% of those who had initially presented with subnormal OP amplitudes, when compared to a group of healthy controls, had developed PDR. All of these subjects had disease duration of more than five years at the initial examination. No subjects with disease duration of less than five years at initial examination had developed PDR. Only 2% of those who initially presented with normal OP amplitudes had developed proliferative retinopathy, excluding those subjects whose PDR developed during pregnancy. After 13 –15 years, 62% of those with initial subnormal OP amplitudes had developed PDR, compared to only 26% of those with normal OP amplitudes at the beginning. It was concluded that the summed OP amplitude is valuable in selecting those at risk of developing PDR in Type 1 diabetes with disease duration of more than five years [Simonsen 1980].

Bresnick and Palta studied a group of 85 diabetic subjects aged between 20 and 67 over a period of four years. Thirty-six patients had been diagnosed before the age of 30 and the remaining 49 patients beyond this age. Disease duration ranged between one to thirty years and 78 of these subjects were insulin dependent. OPs were recorded following 30 minutes of dark adaptation. The light stimulus used was a Honeywell Strobonar flash and neutral density filters were used to provide a series of stimuli at the following minus log relative intensity levels: 2.1, 1.6, 1.1 and 0.5. The summed amplitudes of the oscillatory potentials were found to be independent predictors of progression to severe PDR and were found to be as predictive as fluorescein angiography [Bresnick and Palta 1987a].

Moschos et al [1987] studied a group of 240 diabetic subjects also over a four year period. The mean age was 50 years and the mean disease duration exceeded 20 years. The subjects were divided into three groups at initial examination consisting of either no visible retinopathy, early background retinopathy or PDR with the existence of NVD or NVE. Here subjects were

not dark adapted and a high intensity photic stimulus was used (2400 lux). The oscillograph sweep velocity and gain used were 10 ms div. $^{-1}$and 50 μV div. $^{-1}$. They found that OP amplitudes were reduced even in eyes with no visible retinopathy and that this finding was more pronounced in eyes which subsequently developed neovascularisation. They also found that disturbances of the OPs were more marked, and occurred earlier, in subjects with juvenile diabetes.

More recently Vadala et al [2002] also conducted a long-term follow up, over ten years, in a group of 80 subjects with Type 1 diabetes mellitus. Photopic OPs were recorded following 5 minutes of light adaptation, shorter than that recommended by ISCEV, to a stimulus flash of 2.6cd.s.m^{-2} at 3 Hz. All subjects presented with no visible retinopathy and normal OP amplitudes at first examination. It was found that eyes with reduced OP amplitudes at follow up visits had a greater probability of developing diabetic retinopathy, but that subnormal OP amplitudes are not proof of concomitant visible vascular damage since some subjects who went on to develop background retinopathy maintained normal OP amplitudes. Wanger and Persson [1985] compared the OPs of Type 1 diabetic subjects with no retinopathy, some with background retinopathy and a group of healthy controls. Here OPs were recorded following 15 minutes of dark adaptation, a shorter period than that recommended by ISCEV, to stimulation with a Grass MS2 photostimulator at intensity setting 16 with a 30 s interstimulus interval. They found no significant differences in the amplitudes or implicit times of the OPs between the three groups and felt that OPs do not reveal retinal dysfunction before retinopathy can be detected by means of retinal biomicroscopy.

Other studies have reported an increase in the implicit times of the OPs in diabetic subjects. Van Der Torren and Mulder [1993] compared the OPs of healthy control subjects with those of diabetic subjects with no visible retinopathy and those with early diabetic retinopathy. The OPs were recorded to a 10 J single white flash. The implicit times of OP2 and OP3 were found to be significantly delayed in both groups of diabetic subjects, though no significant difference was found between the amplitudes of OP2 and OP3

among the three groups. It was therefore concluded that the implicit times of OP2 and OP3 were better indicators of diabetic retinopathy than amplitude reductions.

A significant increase in the implicit time of OP1 has also been found in diabetic subjects with no visible retinopathy, along with a significant increase in the inter-peak interval between OP1 and OP2, when compared to healthy controls [Yoshida et al 1991]. OPs were recorded following 15 minutes of dark adaptation to a single 1 ms duration flash from a 25J xenon flashlamp. No significant decrease in the summed amplitudes of the OPs was apparent. This selective increase in the implicit time of OP1 was also present before any changes in the blood-retinal permeability barrier, measured by vitreous fluorophotometry, became apparent. Shirao et al [1991] also observed a significant increase in the implicit time of OP1 in eyes with no visible retinopathy and this finding occurred more often than a significant decrease in the summed amplitudes of the OPs or significant increase in the inter-peak interval. However, it was found that the reduction in the summed amplitudes of the OPs was closely correlated with the severity of diabetic retinopathy. It was concluded that the implicit time of OP1 may be suitable for the early detection of diabetic retinopathy and that the summed amplitudes may be more useful for quantitative evaluation of the severity of retinal functional disorder.

A recent study looked at the OPs in a group of 132 subjects with DM with no, mild, moderate and severe retinopathy [Kizawa et al 2006]. The type of DM was not stated. The implicit times of the OPs were found to be slightly delayed in subjects without retinopathy when compared to the controls, though OP amplitudes were not reduced. With advancing retinopathy the amplitudes of the OPs were progressively reduced along with prolongation of the implicit times.

These findings contrast with a later study also looking at diabetic subjects with no retinopathy and early retinopathy. No significant delay was found in the implicit times of the OPs when compared to those of a group of healthy

controls, and a uniform amplitude reduction of all the OPs was present in the diabetic group [Holopigian et al 1992]. This is also supported by another study in which no significant delay was found in the implicit times of diabetic subjects with no retinopathy [Coupland 1987b]. A reduction in amplitude was found in OP2 and OP3 of these subjects but only with stimuli of duration 20 ms and luminance ≥ 22 cd.m^{-2}. In those with background retinopathy a reduction in amplitude of all the OPs was present with flash luminances both above and below this level.

Kim et al [1998] found that a reduction in amplitude of the OPs occurred more frequently than delays in implicit times in diabetic subjects. They also found that OP2 may be of particular value in indicating retinal dysfunction prior to visible retinopathy on ophthalmoscopy. OPs were recorded following 20 minutes of dark adaptation to a white ISCEV standard flash, luminance 1.86 cd.s.m^{-2} with an interstimulus interval of 15 s. In diabetic subjects with no retinopathy the amplitude of OP2 was significantly reduced and the implicit time of OP2 significantly increased. In subjects with mild background retinopathy OP3 also became affected and both OP2 and OP3 were significantly impaired in both amplitude and timing. This contrasts with the findings of an earlier study by Li et al [1992] in which no significant difference in amplitude of OP2 was found between a group of healthy controls and a group of diabetic subjects with no retinopathy, however a significant reduction in amplitude of OP4 was apparent in this diabetic group. The implicit time of OP2 was significantly increased in a group of diabetic subjects with background retinopathy when compared to those with no retinopathy and the controls. Here the OPs were recorded to a flash brighter than that recommended by ISCEV of 3.7cd.s.m^{-2} with an interstimulus interval of 30s. This study also found that the total power of the OPs, calculated by integrating the power spectrum of each isolated OP, in light adaptation was significantly reduced even in those with no visible retinopathy.

In summary, many studies have looked at the OPs in subjects with DM and the results are sometimes contrasting. This may be due to many factors

including the different population groups studied, the varying disease durations and amount of DR present and also to the different stimuli and recording conditions employed by each study. Of the papers reviewed, in most cases insufficient details of stimulus conditions were given to allow direct comparisons to be made. However, the OPs appear to be sensitive indicators of functional changes in the retina in diabetes mellitus even before retinopathy becomes visible. Disturbance of the OPs may indicate an increased risk for progression to proliferative retinopathy and the extent of disturbance may provide an objective quantitative measure of retinal functional disorder.

Finding	Study
Summed OP amplitudes significantly reduced with no retinopathy	Simonsen 1980 Moschos et al 1987 Van Der Torren and Van Lith 1989 Juen and Kieselbach 1990 Holopigian et al 1992
Total Power of OPs significantly reduced with no retinopathy	Li et al 1992
Eyes with no retinopathy which have significant reduction in summed OP amplitudes have greater probability of developing retinopathy.	Vadala et al 2002
Reduction in OP amplitude increases with increasing retinopathy	Bresnick and Palta 1987b Shirao et al 1991 Kizawa et al 2006
OP amplitudes are a reliable predictor of progression to proliferative retinopathy	Simonsen 1980 Bresnick and Palta 1987a Moschos et al 1987
OP4 amplitude significantly reduced in diabetic subjects with no retinopathy	Li et al 1992
No significant difference in OP amplitudes in diabetic subjects with no retinopathy and control subjects	Wanger and Persson 1985 Van Der Torren and Mulder 1993
Implicit times of OP2 and OP3 significantly delayed with no retinopathy	Van Der Torren and Mulder 1993
Implicit time of OP2 significantly delayed and amplitude of OP2 significantly reduced with no retinopathy	Kim et al 1998
Implicit times of OP2 significantly delayed and amplitude of OP2 significantly reduced with background retinopathy	Kim et al 1998
Implicit time of OP2 significantly delayed with background retinopathy, not with no retinopathy	Li et al 1992
Implicit time of OP1 significantly delayed with no retinopathy	Shirao et al 1991 Yoshida et al 1991
No significant delay in OP implicit times with no retinopathy	Coupland 1985b Wanger and Persson 1985 Holopigian et al 1992
Significant increase in interpeak interval between OP1 and OP2 with no retinopathy	Yoshida et al 1991

Table 3.2 Summary of oscillatory potential findings in diabetes mellitus.

3.9 Summary

The electroretinogram can be used as an objective method of assessing retinal function in humans. Both the b-wave and the OPs of the ERG appear to be sensitive indicators of the effects of diabetes mellitus on the retina.

4. RETINAL OXYGENATION IN DIABETES MELLITUS

Blood flow characteristics of the retinal vasculature are known to be affected by increased oxygen (O_2) supply. Alterations to blood flow can produce changes in visual function, particularly when existing hypoxia is reduced, for example in diabetic retinae. This chapter outlines current knowledge of the effects oxygen inhalation on the retinal vasculature and visual function in man. This topic is of particular clinical interest at the moment as a recent study has shown that supplemental oxygen may decrease macular thickness in subjects with diabetic macular oedema, and improve visual acuity in some cases [Nguyen et al 2004]. Other studies have also shown improvements in colour vision [Dean et al 1997], contrast sensitivity [Harris et al 1996], and summed scotopic oscillatory potential amplitudes [Drasdo et al 2002], in subjects with diabetes with O_2 inhalation. This chapter will also outline current knowledge on the effects of illumination level on the retinal O_2 consumption and blood flow.

4.1 The Retinal Blood Supply

All arteries of the eyeball originate from the ophthalmic artery (OA), which in turn devolves from the internal carotid artery. Likewise all major veins of the eye usually discharge into the ophthalmic vein. As the arteries extend they develop into arterioles which then supply blood to capillaries, the smallest of the blood vessels, which connect arterioles and venules allowing exchange of nutrients and waste. Both arteries and veins divide into two separate vascular systems before entering the eye, the retinal system and the ciliary system, which then remain separate within the globe.

The inner retina is supplied by the central retinal artery (CRA), arising from the ophthalmic artery, which enters the eye via the optic nerve head. The CRA divides into two equal superior and inferior branches which then divide again, into the superior and inferior temporal and nasal branches near the surface of the optic nerve head. Each branch then supplies a quadrant of the retina and

there is no anastomosis between branches within each quadrant. The temporal branches of the CRA follow an arched course above and below the fovea centralis before passing to the ora serrata. The nasal branches follow a fairly straight course out to the periphery.

The branches of the CRA run within the nerve fibre layer (NFL), just below the internal limiting membrane. Since this membrane is transparent the vessels can be viewed directly on fundoscopy. The vessels form two networks of capillaries. The superficial layer lies in the NFL or ganglion cell layer, whilst the deeper network lies in the inner nuclear layer (INL), close to the outer plexiform layer (OPL) [Duke Elder and Wybar 1961, Remington 1998]. Arterial disease, such as vascular hypertension, is generally thought to affect the superficial layer of capillaries whilst venous disease such as DM is more likely to involve the inner nuclear layer capillaries [Newell and Ernest 1974]. The outer retinal layers beyond the OPL, i.e. the retinal pigment epithelium (RPE), and the photoreceptors, are avascular and are instead primarily nourished by the choroidal circulation.

The vascular bed of the choriocapillaris is made up from the short posterior ciliary arteries, again derived from the OA. These arteries penetrate the sclera and form a dense vascular layer adjacent to Bruchs membrane and the RPE. This layer is arranged in a lobular fashion at the posterior pole with alternating arterioles and venules, whilst at the periphery the arrangement becomes more spindle- or ladder shaped [Alm 1992]. Some of the short posterior arteries form a partial or complete vascular ring around the optic nerve known as the Circle of Zinn-Haller. The long posterior ciliary arteries typically form two branches, each supplying a sector of the nasal or temporal peripheral choroidal supply [Alm 1992]. Typically four or five vortex veins drain the choroid and pierce the sclera to join the ophthalmic vein.

A capillary free zone directly surrounds the retinal vessels and at the fovea there lies an area, approximately 0.5 mm in diameter, which is completely

devoid of any retinal blood vessels. This allows transmission of light to the foveal photoreceptors without obstruction from a blood vessel.

4.1.1 Autoregulation

Autoregulation describes the ability of blood vessels to control the level of blood flow in accordance with the metabolic demands of the retinal tissue. The retinal vessels receive no autonomic innervation and thus are reliant on local factors to regulate retinal blood flow. The retinal blood vessel wall is comprised from a single layer of endothelial cells surrounded by intramural pericytes and a basement membrane. Pericytes are contractile cells and are known to respond to certain vasoactive agents, neurotransmitters and hormones as well as to local metabolic needs. When blood flow is inadequate the resulting hypercapnia, where levels of carbon dioxide (CO_2) are increased above normal levels, and hypoxia, where O_2 levels are subnormal, stimulate relaxation of the pericytes and thus the retinal vessels will dilate [Matsugi et al 1997]. Retinal pericytes are known to contract in response to hyperoxia, angiotensin II and high concentrations of endothelin-1 (ET-1) [Kohner 1993, Cai and Boulton 2002].

4.1.2 Autoregulation in Diabetes Mellitus.

Autoregulation is reduced in the early stages of DM [Alm 1992]. In response to increased oxygen inhalation the retinal vessels would normally respond by vasoconstriction in order to control retinal blood flow. Hyperglycaemia has been shown to both increase retinal blood flow and interfere with retinal autoregulation [Kohner 1993, Kohner et al 1995].

In subjects with DM, there is a reduction in vasoconstriction with hyperoxia which may be due to the high glucose levels found in diabetes. Retinal pericytes are known to contract in response to hyperoxia, ET-1, a potent vasoconstrictor, and angiotensin II. The production of ET-1 by endothelial cells

is decreased in the presence of high glucose levels [Kohner 1993]. Hyperglycaemia is linked to pericyte damage and loss, and damage to these cells will significantly disrupt retinal haemodynamics [Ciulla et al 2002]. An increasing loss of retinal pericytes will lead to capillary dilatation and consequently increased retinal blood flow. This hyperperfusion leads to increased sheer stress on the vessel wall, which can damage the vessel wall leading to capillary non-perfusion and thus retinal ischaemia. This may initiate the onset of proliferative retinopathy [Kohner 1993, Kohner et al 1995].

4.2 Retinal Vascular Effects of Hyperoxia

4.2.1 Retinal Vascular Effects of Hyperoxia in Normals

Inhalation of pure oxygen, is known to reduce retinal blood flow in the normal eye [Riva et al 1983, Grunwald et al 1984, Pakola and Grunwald 1993, Grunwald et al 1996, Rassam et al 1996, Langhans et al 1997, Kiss et al 2002, Luksch et al 2002], and it is thought that this may be secondary to constriction of the retinal vessels [Langhans et al 1997, Kiss et al 2002]. A significant reduction in retinal arterial diameter [Deutsch et al 1983, Fallon et al 1985, Kiss et al 2002, Luksch et al 2002], and retinal venous diameter [Deutsch et al 1983, Grunwald et al 1984, Fallon et al 1985, Hague et al 1988, Grunwald et al 1996, Kiss et al 2002, Luksch et al 2002], has been demonstrated. Most of this vasoconstrictive response has been shown to occur within the first five minutes of 100% O_2 inhalation, thereafter vessel diameters remain stable [Kiss et al 2002]. This vasoconstriction has also been shown to vary regionally with temporal vessels constricting more than those of the nasal retina [Rassam et al 1996, Kiss et al 2002], though this difference has shown statistical significance only in the latter study. Likewise retinal blood flow showed a greater reduction the temporal circulation with hyperoxia [Rassam et al 1996], though this difference was not significant. However a more recent study has found a significant reduction in retinal blood flow in the inferior temporal retina when

compared to that of the superior temporal retina with hyperoxia [Chung et al 1999].

The control of retinal oxygen levels via changes in retinal vessel diameter may be attributed to autoregulation [Funk 1997], which operates to maintain total oxygen delivery to the retinal tissue at a constant level. Autoregulation is believed to occur within 1.5 minutes of pure oxygen breathing [Riva et al 1983]. It has also been demonstrated by a significant reduction in red blood cell velocity with hyperoxia, [Riva et al 1983, Pakola and Grunwald 1993, Rassam et al 1996, Grunwald et al 1996, Luksch et al 2002], slowed arteriovenous passage time of fluorescein dye [Harris et al 1996], and a reduction in leukocyte velocity in the perimacular capillary bed [Fallon et al 1985, Sponsel et al 1992,].

Hyperoxia has been reported to produce no change in the global response of choroidal blood flow [Kergoat and Faucher 1999], and no change in choroidal red blood cell velocity, flow or volume in the foveal region [Geiser et al 2000]. Conversely, fundus pulsation amplitudes at the macula, which have been shown to estimate local pulsatile ocular blood flow (POBF) [Schmetterer et al 1995], and are determined solely by the choroidal circulation are known to be significantly reduced with hyperoxia. They are further reduced at the optic disc where they are determined by both the choroidal and retinal circulation in an unknown ratio [Schmetterer et al 1996].

Ninety minutes of hyperbaric oxygen (HO) treatment has also been shown to constrict the retinal vessels. Inhalation of 100% O_2 at 2.5 atmospheres absolute pressure (ATA) has been found to constrict the retinal arterioles by 9.6% and venules by 20% of their size in air at ambient pressure. Ten minutes following the treatment much of this effect had reversed with the arteries dilating to 94.5% of their original diameter and the venules to 89% of their primary size [Vucetic et al 2004].

Isocapnic hyperoxia, where levels of carbon dioxide (CO_2) are maintained at a constant level, has been shown to significantly reduce end diastolic velocity (EDV), and significantly increase resistance index (RI), in the CRA of normal subjects [Evans et al 1997]. In this study, measurements were initiated after end tidal oxygen expiration levels had reached or exceeded 80%. This finding however is contradicted by a later study where five minutes of 100% O_2 inhalation had no effect on the peak systolic velocity (PSV) or EDV of either the CRA or short posterior ciliary arteries, whereas PSV, and EDV were significantly reduced in the OA indicative of reduced volumetric flow [Hosking et al 2004]. The absence of changes in the CRA and short posterior ciliary arteries was attributed to a concurrent reduction in intra-ocular pressure (IOP). The smaller calibre vessels are thought to be more sensitive to changes in IOP than larger vessels such as the ophthalmic artery, with a decrease in IOP leading to an increase in ocular perfusion pressure (OPP) and a resultant increase in blood flow [Findl et al 1997].

4.2.2 Retinal Vascular Effects of Hyperoxia in Diabetics

The significant decrease in retinal blood flow in normal subjects with hyperoxia has been found to be reduced in the diabetic eye where retinopathy is present [Grunwald et al 1984, Grunwald et al 1992]. Autoregulation has also been shown to be impaired in Type 1 diabetic subjects with no visible retinopathy, (mean disease duration 7 years) suggesting that it may be affected early in the disease process [Grunwald et al 1984]. This is supported by the significant delay in arteriovenous passage time of fluorescein dye with hyperoxia in a group of Type 1 diabetic subjects with minimal or no retinopathy when compared to control subjects [Harris et al 1996]. However, a later study showed no abnormalities in autoregulatory function in a group of Type 1 diabetic subjects with no visible retinopathy and disease duration less than four years. No significant difference in retinal blood flow, vasoconstriction of retinal veins or decrease in red blood cell velocity with hyperoxia was found between these subjects with DM and a control group, implying that impairment of

autoregulation may be linked to the duration of disease [Grunwald et al 1996]. A significant correlation has been found between the degree of autoregulation and the severity of retinopathy, with autoregulation becoming further impaired as retinopathy progresses [Grunwald et al 1992].

Instigation of strict diabetic control does not appear to affect autoregulation in previously poorly controlled Type 1 diabetic subjects with retinopathy [Davies et al 1990, Grunwald et al 1994], and autoregulation has been shown to be the same under conditions of normoglycaemia and hyperglycaemia [Davies et al 1990].

As mentioned previously, hyperoxia has been shown to affect retrobulbar blood flow in control subjects. In a group of Type 1 diabetic subjects, with no or minimal retinopathy, significant differences have been found between their response to hyperoxia and that of the control group. No change was apparent in PSV, EDV or RI in the CRA of the diabetic group whereas the control subjects experience reduced blood flow in retrobulbar vessels during hyperoxia. This finding highlights the irregularities in vascular reactivity in the major blood vessels feeding the eye in diabetic subjects [Evans et al 1997].

The retinal vascular effects of hyperoxia are summarised in Table 4.1.

Normals	Diabetic Subjects
Retinal Blood Flow	
Significantly reduced [Kiss et al 2002, Luksch et al 2002, Langhans et al 1997, Grunwald et al 1996, Rassam et al 1996, Pakola and Grunwald 1993, Grunwald et al 1984, Riva et al 1983]	Significantly **less reduction** in subjects with Type 1 DM with retinopathy than in normals [Grunwald et al 1992, Grunwald et al 1984]
	Not significantly different to normals with Type 1 DM and no retinopathy, disease duration < 4 years [Grunwald et al 1996]
	Reduction significantly correlated to degree of retinopathy in subjects with Type 1DM [Grunwald et al 1992]
	No significant difference in reduction in subjects with Type 1 DM with retinopathy under conditions of normoglycaemia and hyperglycaemia [Grunwald et al 1994, Davies et al 1990]
Vasoconstriction	
Significant reduction in arterial [Kiss et al 2002, Luksch et al 2002, Langhans et al 1997, Fallon et al 1985, Deutsch et al 1983], and venous diameter [Kiss et al 2002, Luksch et al 2002, Grunwald et al 1996, Hague et al 1988, Fallon et al 1985, Grunwald et al 1984, Deutsch et al 1983].	Significantly **smaller reduction** in venous diameter in subjects with Type 1 DM with retinopathy than in normals [Grunwald et al 1984].
	Not significantly different to normals in subjects with Type 1 DM with no retinopathy, disease duration < 4 years [Grunwald et al 1996].
	Arterial calibre not significantly different in subjects with Type 1 DM under conditions of normoglycaemia and hyperglycaemia [Davies et al 1990].
Red Blood Cell Velocity	
Significantly reduced [Pakola and Grunwald 1993, Riva et al 1983].	No significant difference in subjects with Type 1 DM with no retinopathy and disease duration < 4 years, when compared to normals [Grunwald et al 1996].
Arteriovenous Passage Time of Fluorescein Dye	
Significantly slowed [Harris et al 1996].	Not significantly slowed in subjects with Type 1 DM [Harris et al 1996].
Retrobulbar Blood Flow	
Reduced blood flow measured at the ophthalmic and central retinal artery [Hosking et al 2004, Evans et al 1997].	Not significantly altered in subjects with Type 1 DM measured at the ophthalmic and central retinal artery [Evans et al 1997].

Table 4.1 Summary of retinal vascular effects of hyperoxia in normals and in subjects with diabetes mellitus.

4.3 Functional Effects of Hyperoxia

4.3.1 Functional Effects of Hyperoxia in Normals

Both contrast sensitivity [Harris et al 1996], and colour vision [Dean et al 1997], are known to be unaffected in normals under conditions of hyperoxia. No change in contrast sensitivity was observed in a group of twelve normal subjects following fifteen minutes of isocapnic hyperoxia [Harris et al 1996]. Likewise no change in colour thresholds was found in a group of twenty-seven normal subjects after five minutes of 100% oxygen inhalation through a 60% Ventimask [Dean et al 1997].

More recent studies have looked at changes to the scotopic electroretinogram (ERG) of normals in hyperoxia [Drasdo et al 2002, Faucher and Kergoat 2002]. In a group of eight subjects, b-wave amplitude and summed oscillatory potential (OP) amplitudes were recorded both before O_2 inhalation and following two minutes of 100% O_2 inhalation through a 60% Ventimask. Both were found to be unaltered during O_2 inhalation [Drasdo et al 2002].

Another study looked at changes to the a-wave, b-wave and oscillatory potential amplitudes of the scotopic ERG in seventeen controls under conditions of hyperoxia. After a five minute period of 100% O_2 inhalation the amplitudes of the a- and b-waves and all OPs, with the exception of the third OP (OP3), again remained unaltered [Faucher and Kergoat 2002]. The a-wave is known to reflect the activity of photoreceptors [Fishman et al 2001, Qiu et al 2002], and thus may be resistant to hyperoxia since choroidal blood flow has been shown to be unaffected by pure O_2 breathing [Kergoat and Faucher 1999, Geiser et al 2000], and therefore photoreceptor activity should remain stable. The b-wave is believed to be generated by depolarisation of bipolar cells [Xu and Karwoski 1994a, Xu and Karwoski 1994b, Tian and Slaughter 1995, Hood and Birch 1996, Green and Kapousta-Bruneau 1999, Shiells and Falk 1999], and although the exact origin of the OPs is as yet unknown they are believed to be generated

more proximally than the b-wave [Brown 1968, Ogden 1973, Wachtmeister and Dowling 1978, Heynen et al 1985, Kergoat and Tinjust 2004], and are known to be reliant on the retinal circulation [Brown 1968]. Maintenance of the b-wave amplitude and the majority of the OPs is therefore thought to arise via autoregulation of the inner retinal arterial network. The amplitude of OP3 showed a significant increase during O_2 inhalation of 33.5%. This difference in behaviour from the other OPs is suggested to arise from its different site of origin within the retina, and that it may represent an interaction between rod and cone system inputs [Faucher and Kergoat 2002].

It was also found that the latencies of all the OPs were unaffected by O_2 inhalation. The latency of the a- and b-waves also remained unaltered during the period of O_2 breathing, but were found to be delayed ten minutes later. The reason for this is not clear although a sudden reduction in haemoglobin oxygen saturation (SaO_2), and possibly also local retinal oxygen tension (PO_2) along with a transient swelling of the sub-retinal space following hyperoxia are each suggested as a possible cause [Faucher and Kergoat 2002].

The photopic ERG has been shown to be slightly more resistant to hyperoxia than components of the scotopic ERG [Kergoat and Tinjust 2004]. The delay observed in the latencies of the scotopic a- and b-waves ten minutes after a five minute period of O_2 inhalation was not apparent following O_2 inhalation in the photopic ERG. The amplitudes of the photopic a- and b-waves and all OPs were found to be unaltered both during and after five minutes of 100% O_2 inhalation in a group of eighteen normals. The latencies of the a- and b-waves, along with OP1-3 also remained stable, however the latency of OP4 was delayed ten minutes after O_2 inhalation was stopped. This sensitivity of OP4 in the photopic ERG, and OP3 of the scotopic ERG may suggest component specific elements within the OP complex with varying reactivity under altered vascular conditions.

4.3.2 Functional Effects of Hyperoxia in Diabetes

Several functional effects of hyperoxia in diabetic subjects have previously been investigated and are summarised in Table 4.2 (see page 83).

Contrast sensitivity is known to be reduced in diabetic subjects when compared to age-matched controls. In a group of Type 1 diabetic subjects, with no or minimal retinopathy, hyperoxia has been shown to reverse this defect [Harris et al 1996]. In room air the diabetic group showed reduced contrast sensitivity at 12 cycles per degree (cpd), and at 18 cpd when compared to the control group. These contrast sensitivity thresholds are shown in Figure 4.1. As previously described the diabetic group also show slowed arteriovenous passage time of fluorescein dye in comparison to the controls. 100% oxygen was added to a mixing chamber with small amounts of CO_2 in order to maintain arterial CO_2 at a constant level. This was then inhaled for fifteen minutes. The control group showed no alterations in their contrast sensitivity thresholds with hyperoxia, and their arteriovenous passage time slowed substantially thus keeping total oxygen delivery to the retina at a reasonably constant level. The diabetic group showed improved contrast sensitivity at 12 cpd with hyperoxia, eliminating the difference in sensitivity between the two groups. Hyperoxia did not significantly alter arteriovenous passage time suggesting a reduced autoregulatory capacity in this group. The improvement shown in contrast sensitivity in the diabetic subjects with hyperoxia suggests that some visual deficits may be reversible, and imply that retinal hypoxia may play a significant role in their development.

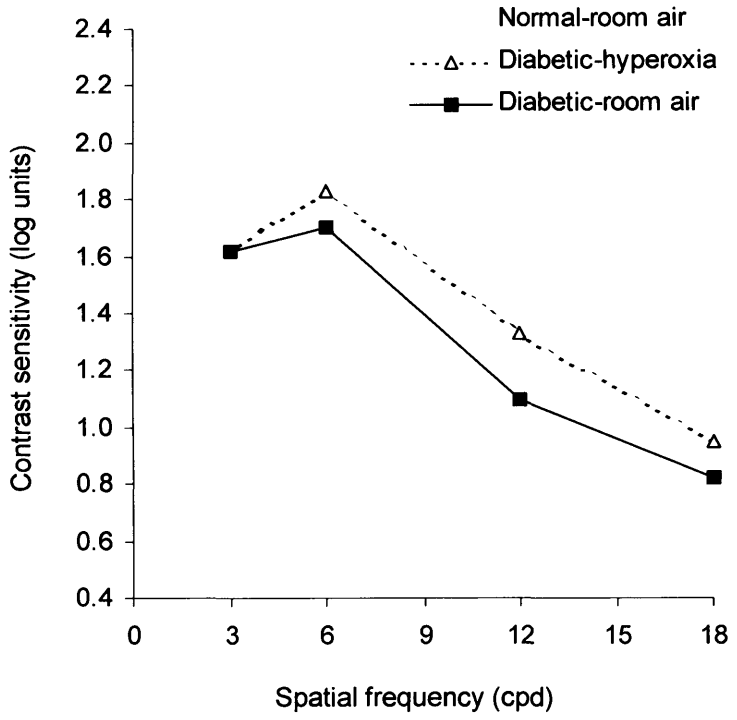

Figure 4.1 Contrast sensitivity thresholds in controls and subjects with Type 1 DM during hyperoxia [*After* Harris et al 1996].

Protan and tritan colour thresholds are known to be significantly higher in diabetic subjects when compared to controls. It has also been shown that these thresholds increase both with the level of diabetic retinopathy and the duration of disease. In a group of Type 1 diabetic subjects, with no or minimal retinopathy, it was found that these raised thresholds could be lowered during conditions of hyperoxia by inhalation of 100% O_2 through a 60% Ventimask for a period of five minutes, see Figure 4.2. As mentioned previously no change in either the protan or tritan thresholds were apparent in the control group under identical conditions. Again this suggests that this visual deficit may be reversible in the early stages of diabetic eye disease and that retinal hypoxia may be a causative factor of colour vision defects in diabetes [Dean et al 1997].

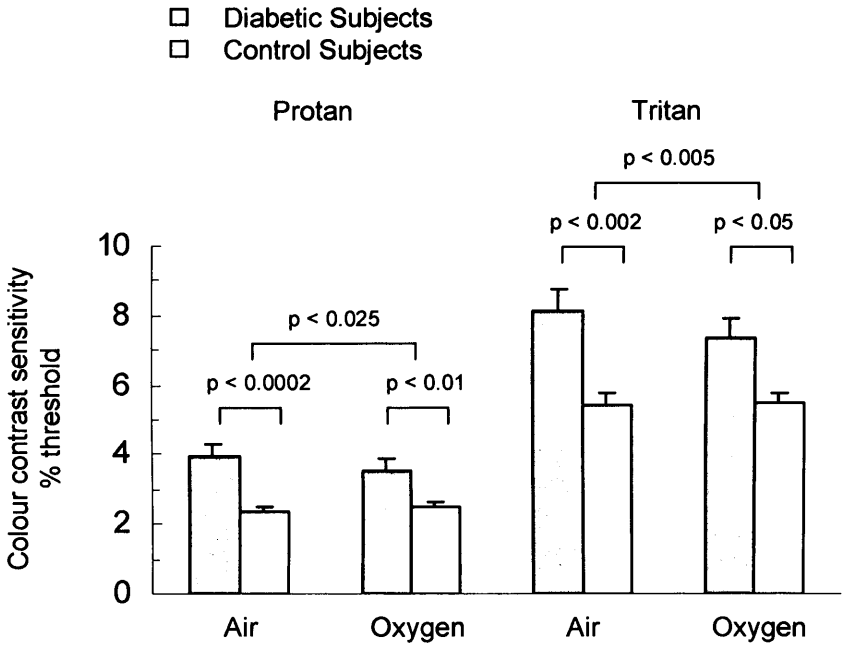

Figure 4.2 Colour vision thresholds in controls and subjects with Type 1 DM during hyperoxia [*After* Dean et al 1997].

A recent study looked at changes to the scotopic ERG with hyperoxia in a group of Type 2 diabetic subjects with no visible retinopathy [Drasdo et al 2002]. B-wave amplitudes and summed OP amplitudes were recorded simultaneously before O_2 inhalation and repeated after two minutes of 100% O_2 inhalation through a 60% Ventimask.

The protocol used for the Drasdo et al experiment [2002] was primarily designed to record a dark adaptation curve followed by the OPs. Subjects were pre-adapted at 950 $cd.m^{-2}$ for 5 minutes and subsequently dark-adapted for 20 minutes. Two white conditioning flashes (4.4×10^4 ph.td) of 6 ms and 12 ms duration were presented to and signals then recorded to four flashes of 12 ms duration presented 15 s apart and averaged offline.

Before O_2 inhalation commenced the diabetic group showed significantly smaller summed OP amplitudes in relation to the control group. These amplitudes increased by 30% during O_2 inhalation to a level not significantly

different to that of the controls before oxygen, see Figure 4.3. The summed OP amplitude remained stable during O_2 inhalation in the control group, suggesting that autoregulation is adequate in healthy individuals during dark adaptation but impaired in diabetic subjects with no visible retinopathy.

A small increase in b-wave amplitude of 4.8% was found in subjects with DM following the O_2 inhalation which was not found to be significant. Control b-wave amplitudes also remained unaltered.

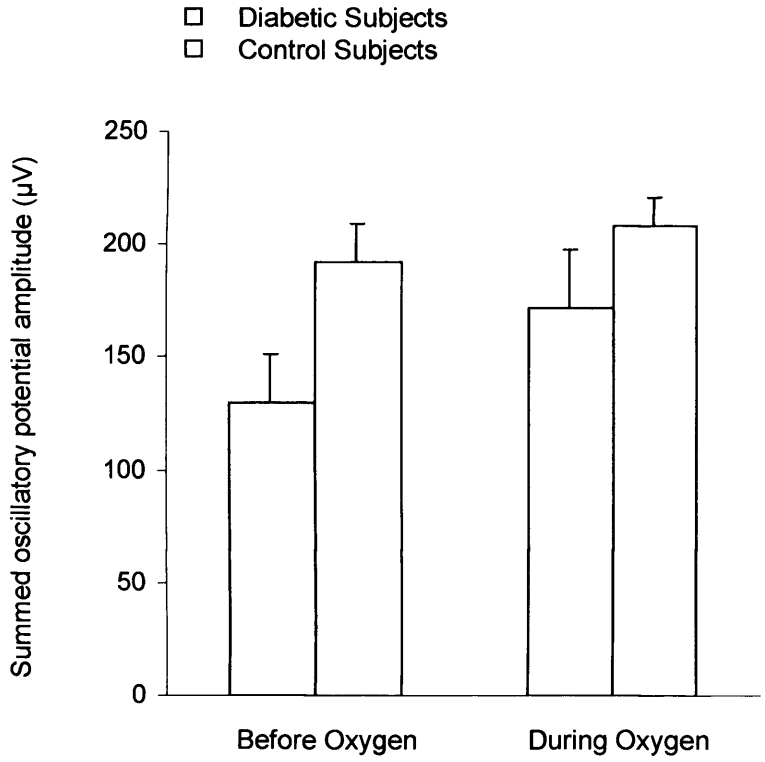

Figure 4.3 Summed OP amplitudes in controls and subjects with Type 2 DM during hyperoxia [*After* Drasdo et al 2002].

A recent study also looked at the effects of hyperoxia upon dark adaptation thresholds in a group of twelve subjects with Type 1 DM and no, or minimal retinopathy [Kurtenbach et al 2006]. Rod thresholds measured following four minutes of room air inhalation were found to be significantly higher in these subjects when compared to a group of age-matched controls. However

following four minutes of 100% O_2 inhalation these thresholds lowered and rod sensitivity improved to a level not significantly different to that of the control group.

Normals	Diabetic Subjects
Contrast Sensitivity	
No alteration in contrast sensitivity thresholds at 3, 6, 12 or 18cpd [Harris et al 1996].	**Significant improvement in contrast sensitivity** at 12cpd in subjects with Type 1 DM and no, or minimal, retinopathy [Harris et al 1996].
Colour Vision	
Protan and tritan colour thresholds unaltered [Dean et al 1997].	**Significant reduction in protan and tritan colour thresholds** in subjects with Type 1 DM and no, or minimal, retinopathy [Dean et al 1997].
Rod Sensitivity	
Rod sensitivity unaltered [Kurtenbach et al 2006]	**Rod sensitivity improved** in subjects with Type 1 DM and no, or minimal retinopathy [Kurtenbach et al 2006]
Scotopic Electroretinogram	
a- [Faucher and Kergoat 2002] and b-wave [Drasdo et al 2002, Faucher and Kergoat 2002], amplitudes unaffected a- and b-wave latencies delayed 10mins after hyperoxia [Faucher and Kergoat 2002]. OP1, OP2, OP4 amplitudes unaffected [Faucher and Kergoat 2002]. OP3 amplitude increased [Faucher and Kergoat 2002]. OP1-4 latencies unaffected [Faucher and Kergoat 2002]. Summed OP amplitude unaffected [Drasdo et al 2002].	b-wave amplitude unaffected in subjects with Type 2 DM and no retinopathy [Drasdo et al 2002]. **Summed OP amplitude increased** to level of normals before oxygen in subjects with Type 2 DM and no retinopathy [Drasdo et al 2002].
Photopic Electroretinogram	
a- and b-wave amplitudes unaffected [Kergoat and Tinjust 2004]. a- and b-wave latencies unaffected [Kergoat and Tinjust 2004]. OP amplitudes unaffected [Kergoat and Tinjust 2004]. OP1-3 latencies unaffected [Kergoat and Tinjust 2004]. OP4 latency delayed 10 minutes after hyperoxia [Kergoat and Tinjust 2004].	

Table 4.2 Summary of the functional effects of hyperoxia in normals and in subjects with diabetes mellitus.

4.4 Effect of Illumination on Retinal O_2 Consumption

In the early stages of diabetic retinopathy, changes to the retinal capillaries occur. There are endothelial cell changes along with a loss of pericytes from the vessel walls [Ciulla et al 2002]. The changes to these vessels, along with changes to the red blood cells and platelets, result in microaneurysms, capillary dropout and leakage and a level of local hypoxia within the tissue. It is this hypoxia within the retina that is believed to trigger the neovascularistaion observed in proliferative retinopathy [Ashton 1963]. In capillaries within the brain, which otherwise resemble retinal capillaries, only thickening of the basal membrane occurs and it has been suggested that local factors within the retina must be responsible for the changes observed in DR [Kern and Engerman 1996]. The main difference between retinal and brain tissue is the photoreceptors.

In subjects with DM, a lack of insulin and increased levels of blood glucose can cause abnormal glycation of some proteins, and increased metabolism via the polyol pathway. The high metabolic load on the retinal cells as a result of this is thought to trigger the development of DR [Lawrenson 1997, Lawrenson 2000]. Strict glycaemic control may delay the onset and slow the progression of diabetic retinopathy [Hansen et al 1990, DCCT 1993, UKPDS 1998]. However for severe background or pre-proliferative retinopathy, laser photocoagulation currently remains the only effective treatment.

It has recently been hypothesized that a rod-driven hypoxia within the retina is a significant contributory causal factor in the development of DR [Arden et al 1998, Arden et al 2006]. The retina contains approximately 115 million rods [Curcio et al 1990]. The rods have a very high metabolic demand which allows them to signal the absorption of single light quanta. In darkness an ionic current, the 'dark current', flows between the inner and outer segments. The rods also shed and resynthesise the membranous discs of their outer segments each day [Besharse 1982]. This high level of metabolic activity requires a high

blood supply. The photoreceptor layer is avascular and its O_2 requirements are met via diffusion from the choroid. The inner layers of the retina are supplied by the retinal circulation. Experiments have found that in normal eyes retinal anoxia is present in dark adaptation [Linsenmeier 1986].

Micro-electrode studies have found significantly increased O_2 consumption of the retina in the dark in monkeys [Stefansson et al 1983], cats [Linsenmeier 1986, Linsenmeier and Braun 1992, Braun et al 1995], toads [Haugh-Scheidt et al 1995a, b] and rats [Cringle et al 2002, Yu and Cringle 2002]. Studies in the cat retina have found that during dark adaptation a minimum in O_2 tension is found at retinal depths of 65-85%, corresponding to the level of the outer plexiform level, where it falls to almost 0mmHg [Linsenmeier 1986]. It has also been shown that light reduced the O_2 consumption of the cat and rat retina by approximately 50% of its dark adapted value [Linsenmeier 1986, Yu and Cringle 2002]. However, in the macaque monkey a slightly higher minimum O_2 tension of 9mmHg was found at 70% retinal depth in the dark, and light reduced the O_2 consumption of the retina by 16-36%. It was also found that during dark adaptation 90% of the O_2 requirement of the photoreceptors was met by the choroid and 10% by the retinal circulation [Ahmed et al 1993]. Although in the cat retina dark adaptation rendered some regions of the outer retina anoxic [Linsenmeier 1986], this was not found to be the case in the rat retina. Under dark-adapted conditions increased O_2 extraction occurred from both the choroidal capillaries, and also from the deep retinal capillary layer. Although the retinal capillaries do not penetrate the outer retinal layers they were able to create a sufficient O_2 gradient to support the outer retina via O_2 diffusion. Whilst this did not render the inner retina anoxic, which is supported vascularly by the deep retinal capillary layer, it may still leave it vulnerable to ischaemic insult where there is vascular disease [Yu and Cringle 2002].

A recent study in mice lends further support to the hypothesis that the high oxygen consumption of the rods leads to inner retinal hypoxia during dark adaptation [Gooyer et al 2006]. Retinae from rhodopsin knockout mice ($Rho^{-/-}$)

were evaluated along with those of wild-type (WT) control mice. Hypoxia was observed in the inner nuclear and ganglion cell layers of the WT retina and was significantly reduced in $Rho^{-/-}$ mice. This hypoxia significantly increased in the WT mice during dark adaptation however it remained unaltered in the $Rho^{-/-}$ mice. This would suggest that photoreceptor loss reduces retinal oxygen usage and thus retinal hypoxia.

There is also evidence to support the hypothesis that rod driven anoxia triggers the development of DR. DR does not occur in patients with DM and retinitis pigmentosa, where rod outer segments are reduced [Arden 2001]. DR is also often successfully treated with pan-retinal photocoagulation which may simply destroy enough rods to lower the O_2 demand [Yu and Cringle 2001]. As previously outlined, three months of supplemental O_2 inhalation improved visual acuity and reduced macular thickness in subjects with diabetic macular oedema [Nguyen et al 2004]. This treatment provided less than 10% more oxygen to the retina and it is proposed that light adaptation during sleep could reduce the oxygen requirement of the retina by 50% and should therefore be at least as effective [Arden and Schlingemann 2005].

It has been proposed that if people with diabetes and no retinopathy were to sleep in light levels of 1-10 $cd.m^{-2}$ that sufficient light would pass through the eyelids to protect against the development of DR by significantly lowering the level of O_2 consumption of the rod photoreceptors [Arden 2001, Arden et al 2005].

Although we do not know the effect of illumination level on O_2 consumption in subjects with diabetes, it has been proposed that retinal O_2 consumption significantly increases during hyperglycaemia [Tiedeman et al 1998].

4.5 Effect of Illumination Level on Ocular Blood Flow

Retinal blood flow has been found to be initially reduced in subjects with DM before retinopathy develops [Kawagashi et al 1995, Bursell et al 1996]. As retinopathy develops a significant increase in blood flow has been observed [Patel et al 1992], which is sufficient to damage the vascular endothelial cells [Kohner 1993, Grunwald et al 1996]. However as the vascular endothelium is dysfunctional the retina may remain hypoxic even with increased blood flow [Porta 1996]. Pulsatile ocular blood flow has also been shown to be significantly reduced in subjects with DM and no retinopathy, and is found to reduce further as retinopathy progresses [Langham et al 1991].

4.5.1 Effect of Darkness on Human Retinal Blood Flow

Riva et al [1983] recorded a significant increase in retinal blood flow of approximately 67% in a group of three control subjects after only five minutes of dark adaptation using laser Doppler velocimetry (LDV). Following twenty minutes of dark adaptation retinal venous diameter had increased by 5% and red blood cell velocity by 65%. This increase in red blood cell velocity occurred quickly in darkness, plateaued within five minutes of dark adaptation, and persisted for as long as eighty minutes which was the longest period tested. However, it was found to be eliminated by eight minutes of 100% O_2 inhalation. They concluded that the observed increase in retinal blood flow during darkness was primarily due to an increase in blood velocity rather than a change in vessel diameter. This is supported by a later study which also found no significant change in retinal vessel diameters under photopic and scotopic conditions [Barcsay et al 2003]. It was also concluded that the retina consumes more O_2 in darkness than light since this effect was eliminated by O_2 inhalation, and that this increase in O_2 consumption was presumably due to maintenance of the retinal dark current.

This finding is supported by another study [Feke et al 1983] where a 40-70% increase in retinal blood flow was observed using LDV in a group of three control subjects following thirty minutes of dark adaptation. Again it was felt that this increase in blood flow is linked to the increase in metabolic demand of the rod photoreceptors under scotopic conditions.

A later study looked at blood flow in the CRA and OA of control subjects in light and dark conditions using colour Doppler imaging. No significant changes were observed in systolic or diastolic flow velocities in the OA on changing from photopic to scotopic conditions, nor on returning to photopic conditions. In the CRA the systolic and diastolic flow velocities were markedly increased in darkness. After re-exposure to light, the systolic flow velocity decreased. It was concluded that darkness is associated with increased blood flow velocity in the CRA, presumably reflecting the increased retinal metabolic demands of the photoreceptors [Havelius et al 1999].

4.5.2 Effect of Darkness on Human Choroidal Blood Flow

Somewhat unexpectedly choroidal blood flow has been found to be reduced under conditions of darkness. This seems unusual given the large increase in O_2 demand of the photoreceptors. Choroidal blood flow has been found to be significantly reduced by approximately 15% following twenty minutes of dark adaptation using laser Doppler flowmetry. This reduction was found to be reversible following just six minutes of re-light adaptation [Longo et al 2000]. This is supported by a later study which also found a significant 12-14% reduction in choroidal blood flow and ocular fundus pulsation amplitude following twenty minutes of dark adaptation, measured with laser Doppler flowmetry and laser interferometry respectively [Fuchsjäger-Maryl et al 2001]. In this study a similar, but less pronounced, reduction of 8-10% of both parameters was also shown in the contralateral eye which was continuously exposed to light throughout. This suggests that choroidal perfusion rate is adapted to retinal illumination conditions by a neural control mechanism. A

further pharmacological study has found that this effect is not controlled by muscarinic or β-receptor pathways in the choroid and the exact mechanism involved in this is as yet unknown [Fuchsjäger-Maryl et al 2003].

4.6 Summary

Hyperoxia is believed to reduce retinal blood flow and constrict retinal vessels by means of autoregulation in normal subjects. In subjects with diabetes impairment of autoregulation reduces the haemodynamic effects of hyperoxia, and this level of impairment is believed to correlate with the severity of existing diabetic retinopathy. Diabetic subjects also do not appear to experience the reduced retrobulbar blood flow apparent in normal subjects with hyperoxia [Harris et al 1996, Evans et al 1997], again highlighting the irregularities in their vascular reactivity. These irregularities may be useful in order to increase perfusion of the inner retina with hyperoxia, which is often hypoxic in diabetic subjects.

Three months of oxygen inhalation has already been shown to reduce macular thickness in the case of diabetic macular oedema and in some cases improve visual acuity. This finding not only highlights the role of hypoxia in the development of macular oedema but suggests that inhalation therapy warrants further investigation regarding the treatment of this condition [Nguyen et al 2004]. Oxygen inhalation has also been shown to improve levels of contrast sensitivity [Harris et al 1996], colour vision [Dean et al 1997], and scotopic oscillatory potential amplitudes [Drasdo et al 2002], with diabetes, again highlighting the detrimental effects of hypoxia on visual function in diabetic subjects.

It has been hypothesised a rod-driven hypoxia, due to the extremely high metabolic activity of the rods, may be a causal factor in the development of DR [Arden et al 1998, Arden et al 2006]. Studies of the dark-adapted cat retina have found a minimum O_2 tension at the level of the outer plexiform level of

almost 0 mmHg [Linsenmeier 1986], and in monkeys it has been shown that in darkness the oxygen requirement of the photoreceptors is not met by the choroidal circulation alone, but 10% is drawn from the retinal circulation [Ahmed et al 1993]. Light has been shown to approximately halve the O_2 consumption of the cat and rat retina [Linsenmeier 1986, Yu and Cringle 2002], and it has therefore been suggested that people with diabetes may benefit from sleeping under low levels of illumination [Arden 2001, Arden et al 2005].

Since O_2 consumption increases so markedly under dark conditions an increase in ocular blood flow in the dark may also be expected. This has been shown to be true within the retinal circulation of control subjects [Feke et al 1983, Riva et al 1983, Havelius et al 1999], but somewhat unexpectedly not within the choroidal circulation where it has been shown to reduce [Longo et al 2000, Fuchsjäger-Maryl et al 2001]. As yet the effect of illumination on retinal blood flow in subjects with DM is unknown.

5. HYPOTHESES AND AIMS OF THE STUDY

Although there is extensive literature on diabetic retinopathy its evolution is still not fully understood. Capillary function is known to be affected throughout the body in DM but the development of uncontrolled vasculopathy confined to the retina needs to be explained [Wiedemann 1992, Aiello 1997].

It has been proposed that the retina is subject to sub-clinical levels of tissue hypoxia prior to the development of DR [Arend et al 1991, Linsenmeier et al 1998]. It has also been hypothesized that it is a rod-driven hypoxia within the retina that may be a significant contributory causal factor in the development of DR [Arden et al 1998, Arden et al 2006]. The rods, which function at low light levels, have a very high metabolic demand and thus a markedly high level of O_2 consumption. Autoregulation of blood flow is little more than adequate to cope with the O_2 demand in the normal retina during dark adaptation [Arden et al 1998], and given the changes in the blood and capillary function in subjects with DM a sub-clinical tissue hypoxia seems inevitable. The retinal O_2 demand is halved in light conditions [Linsenmeier 1986], and thus it has been suggested that patients with DM may benefit from sleeping with night time illumination in order to slow the progression, or even prevent the development of DR [Arden et al 2006].

An objection to this hypothesis might be that the O_2 supply for the rods is predominantly via diffusion from the choroidal circulation, and not from the retinal vascular system where retinopathy develops. Animal studies have found that during dark adaptation a minimum in O_2 tension is found at retinal depths of 65-85%, corresponding to the level of the outer plexiform layer, which represents the watershed between the retinal and choroidal blood supply where it falls to almost 0mmHg [Linsenmeier 1986]. It has also been found that during dark adaptation only 90% of the O_2 requirement of the photoreceptors was met by the choroid and 10% was met by the retinal circulation [Ahmed et al 1993]. Induced hypoxia has also been shown to

reduce the b-wave of the ERG in DM [Rimmer at al 1996], which is believed to reflect the activity of the bipolar cells within the inner retina.

The most direct demonstration of the role of hypoxia in the development of DR, could be provided if there was clear evidence of significant hypoxia in the tissues between the superficial and deep capillary networks of the central retinal circulation after dark adaptation in subjects with DM. A pilot study by our group supported the hypothesis that inner retinal hypoxia is present prior to the development of visible DR [Drasdo et al 2002]. OPs, which are highly sensitive indicators of activity in the proximal layers of the retina and reflect the functional efficiency of the inner retinal circulation [Holopigian et al 1992], were found to be reduced in summed amplitude in subjects with Type 2 DM without retinopathy, and these were increased during O_2 inhalation to an amplitude that was not significantly different to that of the controls [Drasdo et al 2002].

5.1 Aims of the Study

This study will aim to investigate the following:

1. The effect of O_2 inhalation upon retinal function by measuring scotopic b-waves and OPs in control subjects and DM subjects both with and without DR. The aim was to expand the number of patients from the pilot study. (Since the b-wave is believed to arise from bipolar cells it was felt that this may provide further information about inner retinal function along with the scotopic OPs. These investigations are presented in Chapters 7 and 8).
2. The minimum light level required to saturate rod activity, and thus reduce O_2 consumption, within the retina by means of a simultaneous cone-rod ERG. (This investigation is described fully in Chapter 9).
3. The ocular blood flow during dark adaptation in subjects with DM by means of the OPs and pulsatile ocular blood flow amplitudes. (This is presented in Chapter 10).

5.2 Hypotheses

1a) Subjects with DM are believed to have retinal hypoxia causing OPs and scotopic b-waves with smaller amplitudes and prolonged implicit times.

1b) BDR will be associated with a further reduction in scotopic OP and b-wave amplitudes.

1c) Hypoxic retinae lack autoregulation and thus OP and b-wave amplitudes will increase with O_2 inhalation.

2) It is expected that rod activity will become saturated with increasing levels of background illumination and thus the rod-b-wave of the simultaneous cone-rod ERG will be extinguished.

3) An increase in retinal blood flow is known to occur during dark adaptation in control subjects due to the increased metabolic demands of the retina. The effect of dark adaptation on blood flow in subjects with DM is as yet unknown though retinal blood flow may be expected to be lower than in control subjects if retinal autoregulation is impaired.

6. EXPERIMENTAL TECHNIQUES

6.1 Electrophysiology

As mentioned previously the retina will produce an electrical current in response to stimulation by a light source. In order to record this response special equipment is required and is outlined below.

6.1.1 Electrodes

6.1.1.1 Ground Electrode

Two skin electrodes are required for ERG recording, the ground electrode and the reference electrode. Nine mm silver chloride coated cup electrodes were used. The ground electrode was placed at an indifferent point and connected to the ground of the amplifier. The ground electrode was positioned on the forehead at the centre of the hairline. Its purpose is to reduce mains interference which can add large 50-60 Hz signals to the biological signals and hamper the recording of the desired response signal.

6.1.1.2 Reference Electrode

The reference electrode was placed near the orbital rim alongside the outer canthus. This completes the physiological circuit and provides a comparison for normal biological noise so that this is not confused as part of the desired response.

6.1.1.3 Active Electrode

The active electrode used was a Dawson Trick and Litzkow electrode [Dawson et al 1979], or DTL fibre. Although corneal contact lens electrodes are thought to be optimal for recording full-field flash ERGs they have several disadvantages in that they require corneal anaesthesia and may cause discomfort in prolonged recording sessions. Corneal abrasions are also

possible. The DTL fibre consists of seven low-mass, fine nylon fibres, 50 μm in diameter, impregnated with silver which are intertwined in a helix to form a fine thread. The DTL fibre may be worn for long periods and causes minimal discomfort for subjects. It does not require corneal anaesthesia and is less likely to cause a corneal abrasion, which is especially important in diabetic subjects where healing rate may be slowed. The DTL fibre is disposable since it cannot be sterilised, so a new fibre is required for each patient. It has been shown to permit the recording of highly reproducible retinal potentials [Hebert et al 1999], and to be adequate to record the high frequency oscillatory potentials [Lachapelle et al 1993].

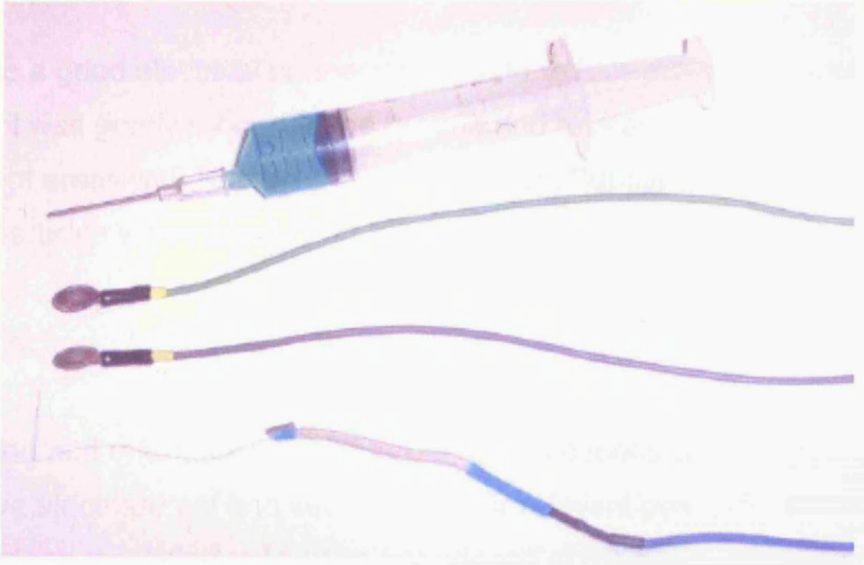

Figure 6.1 Electrodes and electrode gel used in recording the ERG.

6.1.2 Subject Preparation

6.1.2.1 IOP Measurement and Anterior Chamber Angle Assessment

To minimise the risk of closed angle glaucoma with pupillary dilatation the suitability of subjects was assessed by measurement of the intra-ocular pressure with a non-contact tonometer, and the anterior chamber angle depth using Van Herick's technique [Doshi and Harvey 2003]. Patients with shallow

angles, Grade 0 and 1, or an intra ocular pressure >21 mmHg were excluded from the study.

6.1.2.2 Dilatation

The pupil of the test eye, assigned at random, was dilatated with 1 drop of 0.5 or 1% Tropicamide to a minimum of 6 mm, unless otherwise stated. 1% Tropicamide was used in more heavily pigmented eyes in order to obtain maximal dilatation.

6.1.2.3 Skin Preparation

To ensure a good electrical connection, and to reduce electrical resistance of the skin, it was gently rubbed at the ground and reference electrode attachment areas with Nuprep (Medelec Medical Supplies), a suspension of silicone particles within a gel.

6.1.2.4 Electrode Attachment

The ground and reference silver chloride cup electrodes were filled with conductive electrode gel and secured to their relevant positions on the face with Blenderm insulating tape. The impedence of the electrodes was maintained below 5 Ω in accordance with ISCEV standards [Marmor et al 2004]. The DTL fibre was wrapped around a special holder and attached to a wire connected to the amplifier. The holder was secured to the side of the face with Blenderm insulating tape. With the patient looking upwards the remaining fibre was then draped in the lower fornix where it lay in contact with the bulbar conjunctiva. The end of the fibre was then secured to the side of the nose between two pieces of insulating tape so that it was not in direct contact with the skin.

6.1.3 Averager

The averager used was a Medelec Sapphire[II] 4E system (Oxford Instruments, Old Woking, UK). This was used to acquire and process the responses obtained.

6.1.4 Stimulator

The stimulator used was a commercially available Mini-Ganzfeld LED stimulator (CH Electronics, Bromley, Kent, UK), see Figure 6.2. This was used to provide the necessary flash stimuli to the subject. The stimulator contains 5 LED channels whose wavelengths are shown below in Table 6.1. These LEDs can be manipulated in the domains of time and luminance.

Channel	LED Colour	Peak Wavelength (nm)
Channel 1	Red	655
Channel 2	Green	515
Channel 3	Blue	425
Channel 4	Amber	627
Channel 5	White	552 and 463

Table 6.1 LED wavelength values for LED stimulator.

The stimulator is designed to deliver square pulses of light rather than complex waveforms. Additional intensity control is provided by means of pulse width control, which is precisely variable over a range of microseconds to tens of seconds. The frequency of the waveform is controlled by means of a 3-position switch. High or low frequency ranges may be selected and, in the third position which selects the higher frequency range, the pulse width control is bypassed to provide a 50% duty cycle drive waveform.

The groups of LEDS are mounted on a small planar circuit board with current limiting resistors. The circuit board is mounted at one end of an internally partially mirrored plastic tube (160 mm long x 50 mm diameter), with a diffusing screen approximately mid way along the tube and a hemispheric translucent diffuser mounted at the far end of the tube. An 'eye cup' is mounted at the end of the tube to assure patient comfort.

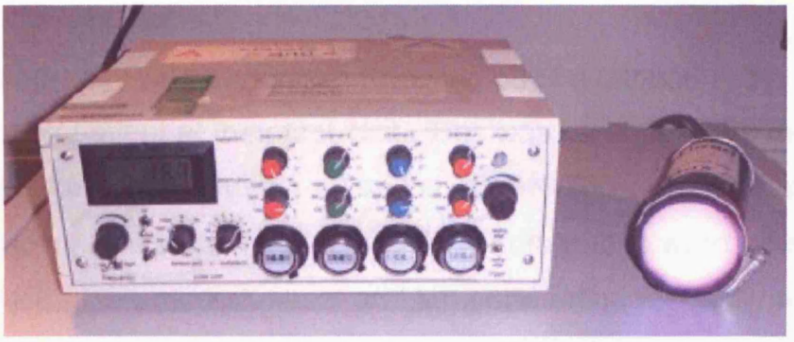

Figure 6.2 LED stimulator used in recording the ERG.

6.1.5 Patient Instruction

Following twenty minutes of dark adaptation, as recommended by ISCEV [Marmor et al 2004] for recording of the scotopic electroretinogram, the subject was instructed to hold the ganzfeld tube close to their eye in order to provide full-field stimulation. The non-test eye was patched. The subject was then advised to look into the tube during response recording, to try to keep their face muscles relaxed and to avoid blinking when requested. Signals larger than 2.5 x the input sensitivity were automatically rejected by the averager during data acquisition.

6.1.6 Photometer and Illuminance meter.

A Minolta LS-110 photometer (Minolta Camera Co., Japan) was used for all luminance measurements of the LED stimulator. Illuminance was measured using the Minolta Illuminance Meter T-1 (Minolta Camera Co., Japan).

6.2 Visual Acuity

Distance Snellen visual acuity was recorded from the test eye of each subject prior to pupillary dilatation. All subjects had VA of 6/12 or better in the test eye.

6.3 Fundus Photography and Fundoscopy

Fundus photography was performed following pupil dilatation in the selected test eye of each patient at their first visit. This was carried out by the author at the end of the examination using a Topcon TRC-NW6S non-mydriatic digital camera (Topcon, Berkshire, UK). Three 45° photographs were taken in each case, one each of the central, nasal and temporal retina respectively. Indirect or direct ophthalmoscopy was also performed on the test eye of each subject at the end of the examination. If subsequent appointments occurred more than two months from the first then fundoscopy and/or fundus photography was repeated.

6.4 Arterial Oxygen Saturation

When equipment was available, arterial oxygen saturation was measured using a Biopac MP100 pulsoximeter (Biopac Systems, California, USA). The pulsoximeter measures beat by beat blood oxygen saturation level. Two wavelengths of light, 660 and 940 nm, are transmitted via LEDS through a pulsating vascular bed, in this case the finger, to a receiving photodiode. Oxygen saturated blood absorbs different fractions of light at different wavelengths compared to unsaturated blood. Accordingly the ratio of light absorbed can be used to calculate the ratio of oxygenated haemoglobin to total haemoglobin, which is then expressed as a percentage. The transmitter and receiver were taped either side of the subjects finger with Blenderm insulating tape and this finger was then wrapped in a thick black cloth to prevent light from escaping.

6.5 Experimental Protocol

Subjects attended for two appointments usually within one month of each other. All investigations were carried out in the School of Optometry and Vision Sciences at Cardiff University. Ethical Committee approval for this study was provided by Bro Taf Local Research Ethics Committee, Cardiff, and the Human Science Ethical Committee of the School of Optometry and Vision Sciences, Cardiff University.

The experimental protocol for each visit is outlined in Table 6.2

Visit One	**Visit Two**
History and symptoms	
Visual acuity measurement	
IOP measurement and anterior chamber angle assessment	IOP measurement
Pupillary dilatation	Pupillary dilatation
Dark adaptation	Dark adaptation
Recording of scotopic ERG and arterial oxygen saturation	Recording of scotopic OPs
Plasma glucose measurement	Plasma glucose measurement
Fundus photography and fundoscopy	
IOP measurement	IOP measurement

Table 6.2 Experimental protocol for first and second visit.

6.6 Subject Selection

6.6.1 Subjects with Diabetes Mellitus

These subjects were recruited from the University Hospital of Wales and Llandough Hospital. The age, duration of diabetes, HbA1c, type of management and blood pressure was recorded from records of the most

recent hospital visit. Records were only available for thirty-two of the thirty-nine diabetic subjects seen. Of these 78% had been seen at the hospital within six months of this study and only one subject had not been seen for over twelve months. Plasma glucose level was recorded at each visit using an Accu-Check Advantage blood glucose meter (Roche Diagnostics, Sussex UK). Exclusion criteria included patients with a previous history of any ocular condition other than diabetic retinopathy, family history of open angle glaucoma, amblyopia, epilepsy, cardiovascular or respiratory disorders or migraine.

Subjects were divided into two groups. Group one consisted of those subjects with no visible diabetic retinopathy (NDR) as assessed by fundus photography and fundoscopy and graded as level 10 ETDRS retinopathy scale (Table 2.5). Group two consisted of those subjects with mild to moderate background retinopathy (BDR) ranging from level 14-43 ETDRS retinopathy scale [ETDRS 1991a].

6.6.2 Control Subjects

A control group of healthy age-matched volunteers was recruited from spouses and students, staff and friends at University Hospital of Wales, Llandough Hospital and Cardiff University. Subjects with a family history of open angle glaucoma, ocular disorders, epilepsy, cardiovascular or respiratory disorders or migraine were excluded.

Three control subjects had a family history of Type 2 DM. All were regular control group participants in studies with the Diabetic Research Group Unit at Llandough Hospital and as such had all been regularly and recently checked for DM.

6.7 Statistical Analysis

The analysis of data was carried using the SPSS 12.0 program according to the following requirements:

- Prior to carrying out statistical analysis of the results, the spread of data were investigated by interpretation of histograms, box-plots and Q-Q plots and using the Shapiro-Wilks test, to test for normality. In cases where data was found not to be normally distributed it was then log-transformed to improve the spread of normality and, if found to be normal, analysis was performed on these log-transformed data.
- To compare two different conditions on the same group of patients, the paired t-test was used to assess the significance when the data were normally distributed (or parametric). In cases in which the data were non-parametric the Wilcoxon signed ranks test was applied.
- When more than two conditions were being compared in the same group of subjects, the repeated measures analysis of variance (RM ANOVA) was used to look at differences across the groups of data, with suitable post-hoc tests to identify individual group differences.
- Comparisons of data involving the same conditions but different groups of subjects were carried out using the independent (or unpaired) t-test or Mann-Whitney test for parametric and non-parametric data respectively.
- To assess the degree of association between two variables in a single group, the correlation coefficient was measured. By plotting the two numerical variables against each other their linear correlation could be assessed. For parametric data the Pearson correlation coefficient was used and for non-parametric data the Spearmans rank correlation coefficient was used.
- On all charts error bars represent ±1 SE

7. INVESTIGATION OF THE EFFECTS OF OXYGEN INHALATION ON THE SCOTOPIC OPS

7.1 Background to the Study

The OPs are believed to arise from the amacrine cells, located between the superficial and deep capillary networks of the retinal circulation. As outlined in Chapter 5 the aim of this study was to record OPs in order to investigate possible signs of hypoxia in the inner retinal layers, and, if found, to what extent its effects on retinal function may be reversed by O_2 inhalation.

This study investigated the effect of five minutes of O_2 inhalation on the scotopic OPs in subjects with DM. If inner retinal hypoxia were present in these subjects OP amplitudes may be expected to be initially depressed, and to increase in amplitude as hypoxia is reduced.

7.2 Protocol Development for the Scotopic OPs with O_2 Inhalation

7.2.1 Preliminary Trials

The ISCEV standard for the flash luminance is 1.5-3 cd.s.m^{-2} [Marmor et al 2004]. The luminance of the white LED was 1100 cd.m^{-2} and therefore to produce a flash luminance of 3 cd.s.m^{-2} a stimulus duration of 3 ms was required.

There was some concern that the output of the white LED may decrease over the length of the study. Should this occur it was not possible to increase the luminance of the white LED in order to ensure the output remained constant. In view of this it was decided that OPs would also be recorded using a combination of the red, green and blue LEDs to produce a white flash, whose luminance could be altered if required. The luminance of these three LEDs at the second attenuation setting, attenuation 3, was measured with the photometer and found to be 490 cd.m^{-2}. In order to produce a flash

luminance of 3 cd.s.m^{-2} a stimulus duration of 6 ms was therefore required. The flashes were presented 15 seconds apart to the dilatated, dark-adapted eye in accordance with ISCEV standards [Marmor et al 2004].

The settings for recording the OPs are outlined in Table 7.1.

Parameter	**Setting**	
Time window (ms)	200	
Low frequency filter (Hz)	100	
High frequency filter (Hz)	1000	
Sensitivity (μV)	50	
Number of Sweeps	1	
Field Size	Ganzfeld	
Peak Wavelength of Stimulation (nm)	White	Red, Green, Blue
	552 and 463	655, 515, 425
Frequency of Stimulation (Hz)	1/15	
Stimulus duration (ms)	White	Red, Green, Blue
	3	6
Stimulus Intensity (cd.s.m^{-2})	3	

Table 7.1 Settings for recording the oscillatory potentials.

7.2.2 Stability Measurements of the OPs

Using the settings outlined above a series of eight sets of OPs were recorded from a 29 year old female subject, following 20 minutes dark adaptation, using both stimuli in order to investigate the number of conditioning flashes required for the response to be stable. A period of 5 minutes of dark adaptation was given between each series. Each flash was presented 15 seconds apart. The outcomes of these experiments are shown in Figures 7.1 and 7.2.

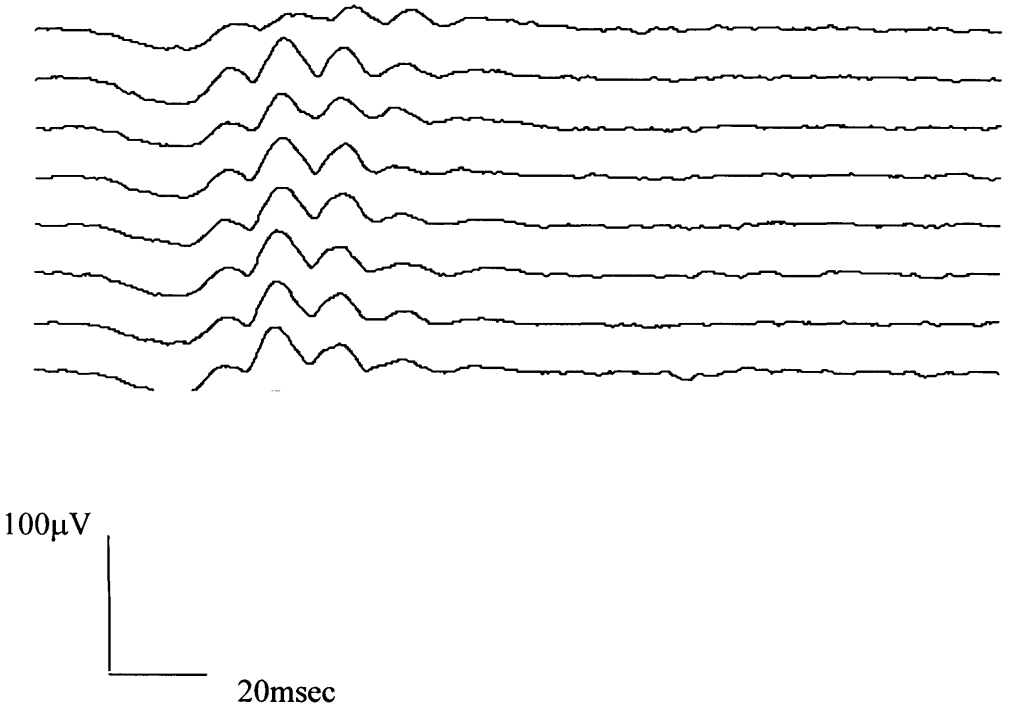

Figure 7.1 Series of eight flashes presented in order to record OPs from a 29-year-old female subject using the white LED. OPs recorded to each single flash shown.

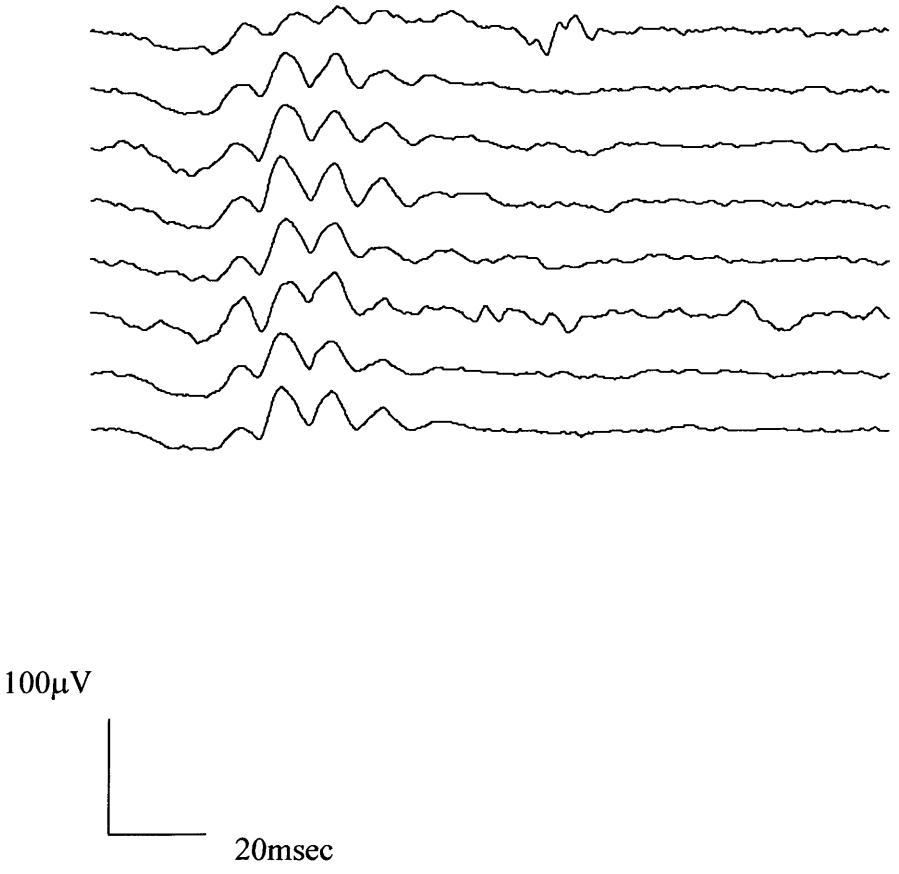

Figure 7.2 Series of eight flashes presented in order to record OPs from a 29-year-old female subject using the red, green and blue LEDs. OPs recorded to each single flash shown.

As shown in Figures 7.1 and 7.2, the OPs appear to stabilise following the first two flashes. In view of this, two conditioning flashes were presented to the subject, 15 seconds apart, before the first response was recorded.

7.2.3 The Effect of Repeated Measurements upon the Scotopic OP Recordings

In order to determine the effect of O_2 inhalation on the OPs, they were initially recorded at five minute intervals on five occasions over a period of twenty minutes. On each occasion, a series of four sets of OPs was required, following two conditioning flashes, and in order to do this, allowing for possible stimulus rejection, it was felt that a three minute period to record the response would be sufficient. To assess if any significant retinal adaptation effects occur in subjects who receive a greater number of flashes due to increased stimulus rejection, the following protocol was developed.

- 20 minutes dark adaptation
- Time 0 min: OPs recorded, Set 1
- Time 5 min: OPs recorded, Set 2.
- Time 10 min: OPs recorded, Set 3, flash stimulus running for a 3 minute period in total.
- Time 15 min: OPs recorded, Set 4, flash stimulus running for a 3 minute period in total.
- Time 20 min: OPs recorded, Set 5.

This procedure is outlined in Figure 7.3

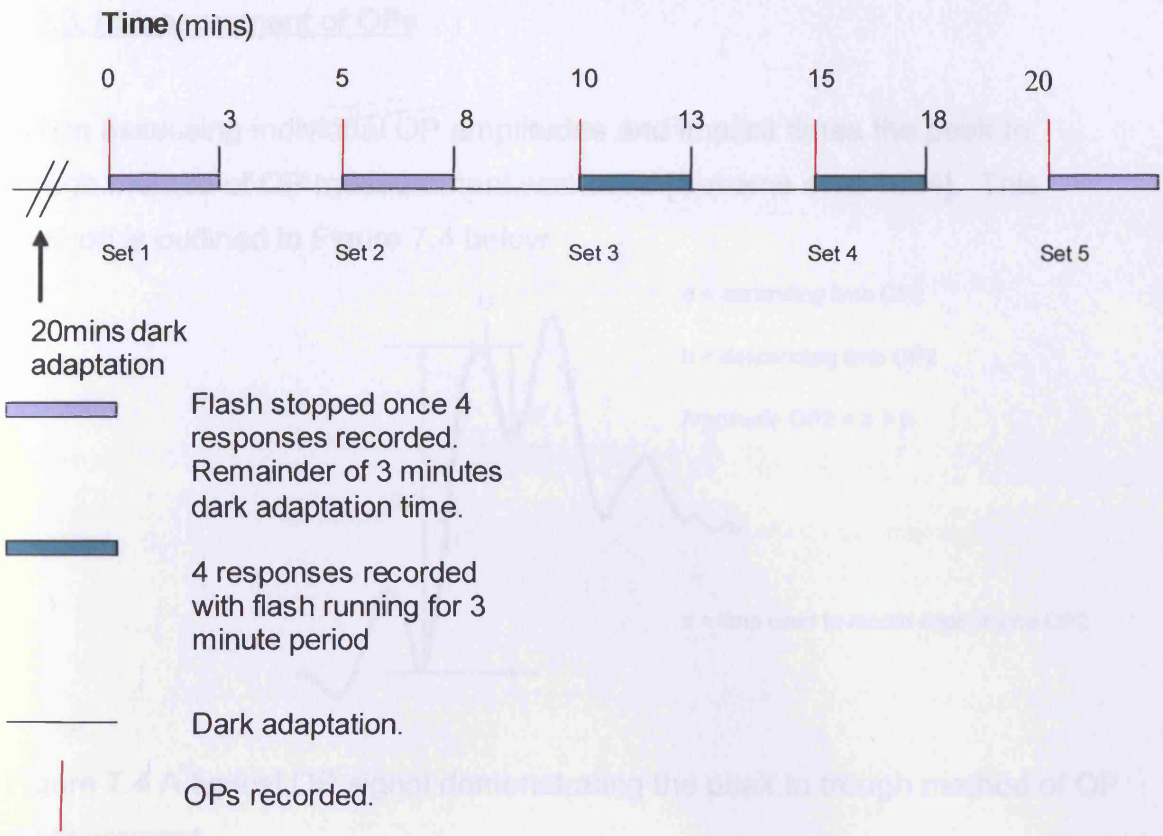

Figure 7.3 Protocol for repeated measurements of scotopic OPs.

7.2.3.1 Measurement of OPs

When assessing individual OP amplitudes and implicit times the peak to trough method of OP measurement was used [Severns et al 1994]. This method is outlined in Figure 7.4 below

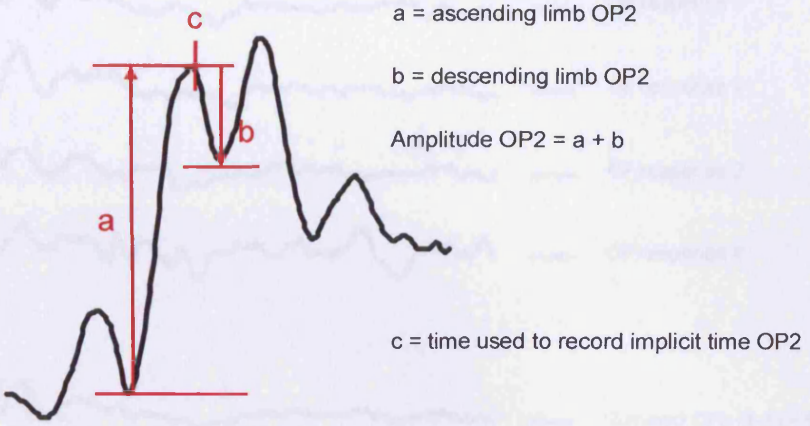

Figure 7.4 A typical OP signal demonstrating the peak to trough method of OP measurement.

In this investigation, summed OP amplitudes were calculated by adding the amplitudes of each ascending and descending limb of the first four individual OPs. The response to the two conditioning flashes were not recorded, and the following four OP responses recorded and averaged. This gave the summed OP amplitude for each of the five sets recorded in this experiment. Figure 7.5 shows the measurements taken to calculate the summed OP amplitude of each OP response. The amplitude of each red arrow was added together to give the final result.

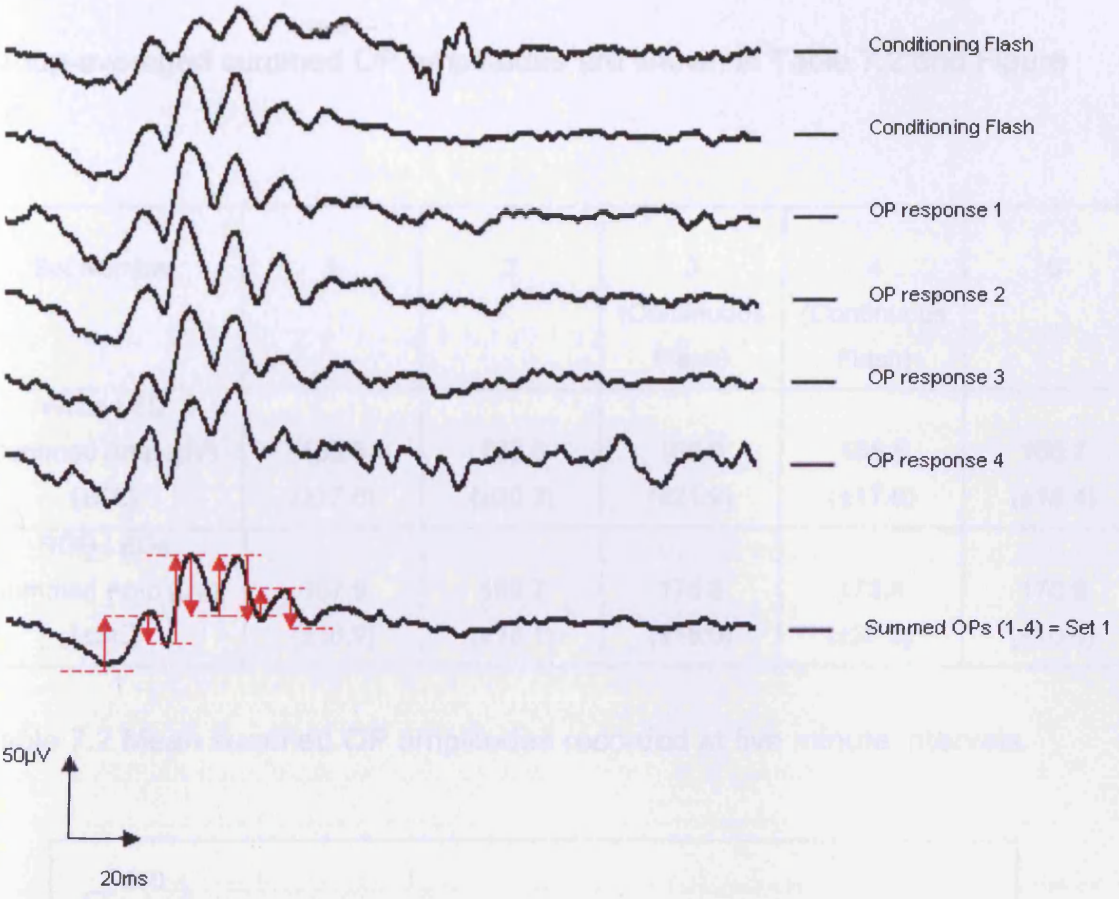

Figure 7.5 Diagram outlining the measurement of the summed OP amplitude.

7.2.3.2 Subject Group

The subject group consisted of 7 healthy controls, 2 male and 5 female with no history of ocular disease. Ages ranged from 24-31 years, mean age 26 years (SD ±2.6). The test eye was assigned at random.

7.2.3.3 Results

Group-averaged summed OP amplitudes are shown in Table 7.2 and Figure 7.6.

Set Number	1	2	3 (Continuous Flash)	4 (Continuous Flash)	5
White LED Summed Amp (μV) (±SE)	156.5 (±17.8)	163.5 (±20.3)	166.9 (±21.9)	165.8 (±17.6)	166.7 (±18.4)
RGB LEDs Summed Amp (μV) (±SE)	167.9 (±16.9)	169.7 (±18.1)	175.8 (±18.0)	173.4 (±20.0)	170.8 (±10.6)

Table 7.2 Mean summed OP amplitudes recorded at five minute intervals.

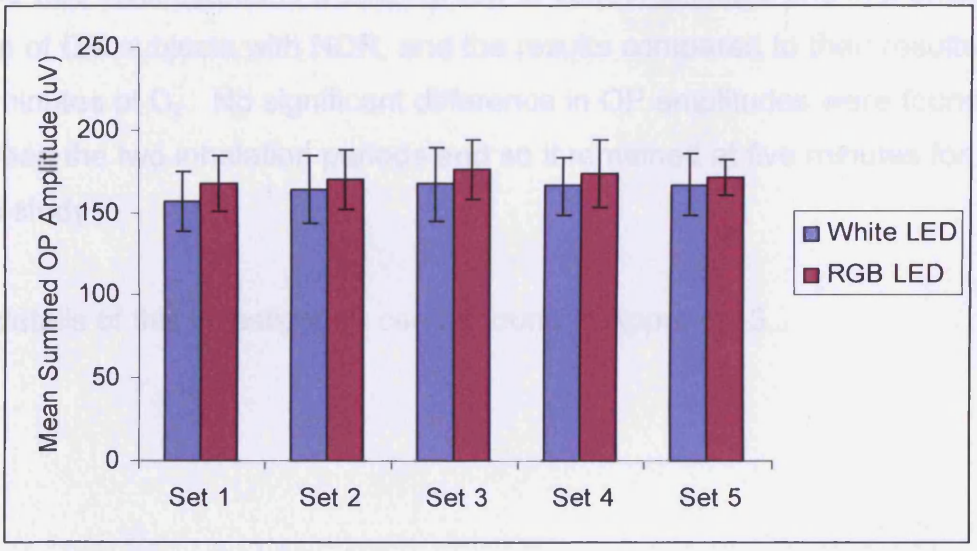

Figure 7.6 Mean summed OP amplitudes of repeatability measurements of scotopic OPs.

With the white LED RM ANOVA found no significant change in summed OP amplitude across time, p=0.724. With the red, green and blue LEDs RM

ANOVA again found no significant change in summed OP amplitude across time, p=0.894.

7.2.3.4 Conclusion

Since no significant difference was found between the five sets of OPs it was decided that the flash could be stopped once four OP responses had been obtained and did not need to be left running for the whole three minute period.

7.2.4 Effect of Length of Inhalation Period

At the beginning of this study a five minute O_2 inhalation period was chosen since this had been used in previous work with positive results in subjects with DM [Dean et al 1997, Drasdo et al 2002]. In the early stages of this investigation, only a small change in OP amplitudes was apparent following the five minute inhalation period. In view of this, a fifteen minute inhalation period was investigated in a small group of control subjects and in a small group of DM subjects with NDR, and the results compared to their results with five minutes of O_2. No significant difference in OP amplitudes were found between the two inhalation periods and so it remained at five minutes for the main study.

Full details of this investigation can be found in Appendix 3.

7.3 Final Protocol for the Investigation of the Effects of O_2 Inhalation on the Scotopic OPs

The final protocol for the recording of the scotopic OPs with O_2 inhalation is outlined below and shown in Figure 7.7. 100% O_2 was provided through a 60% Ventimask for a period of five minutes.

- 20 minutes dark adaptation
- Time 0 min: Record 4 responses (Baseline Response).
- Time 3 min: Begin O_2 breathing
- Time 5 min: Record 4 responses (During O_2).
- Time 8 min: Finish O_2 breathing, remove mask.
- Time 10 min: Record 4 responses (2 min after mask removal).
- Time 15 min: Record 4 responses (7 min after mask removal).
- Time 20 min: Record 4 responses (12 min after mask removal).

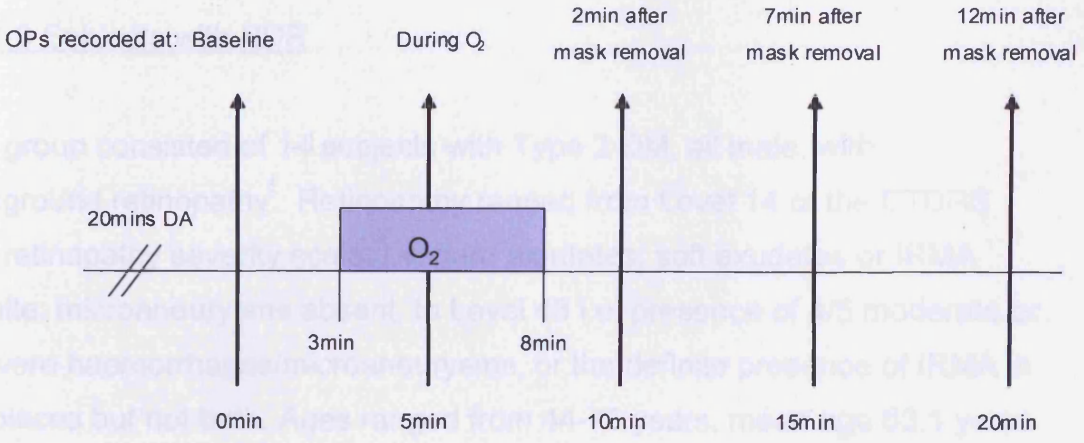

Figure 7.7 Final protocol for scotopic OPs.

Stimulus settings for recording the OPs can be found in section 7.2.1 and are given in Table 7.1. At this stage of the study, advice was finally received from the manufacturer of the LED stimulator that the output of the white LED would not change throughout the duration of this investigation, therefore the white stimulus was used on all occasions.

7.3.1 Subject Groups

Subject characteristics are summarised in Table 7.3

7.3.1.1 Control Subjects

This group consisted of 21 control subjects, 9 female and 12 male, with no history of ocular disease. Ages ranged from 41-80 years, mean age 60.0 years (SD ±10.8). The test eye was assigned at random.

7.3.1.2 Subjects with NDR

This group consisted of 23 subjects with Type 2 DM, 6 female and 17 male, with no visible retinopathy. Ages ranged from 55-72 years, mean age 62.3 years (SD ±5.6). Disease duration ranged from 1.5-18 years, mean duration 7.3 years (SD ±4.8). The test eye was assigned at random.

7.3.1.3 Subjects with BDR

This group consisted of 14 subjects with Type 2 DM, all male, with background retinopathy. Retinopathy ranged from Level 14 of the ETDRS final retinopathy severity scale, i.e. hard exudates, soft exudates or IRMA definite: microaneurysms absent, to Level 43 i.e. presence of 4/5 moderate or 1 severe haemorrhages/microaneurysms, or the definite presence of IRMA in 1-3 places but not both. Ages ranged from 44-75 years, mean age 63.1 years (SD ±8.3). Disease duration ranged from 4-22 years, mean duration 11.7 years (SD ±6.3). The test eye was assigned at random.

Group	Gender	Age (yrs)	Disease Duration (yrs)	Plasma Glucose Level (mmol/L)	Management Type	Retinopathy Level (ETDRS Scale)
Control	9 ♀ 12 ♂	Mean 60 (SD ±10.8) Range 41-80	-	-	-	-
NDR	6 ♀ 17 ♂	Mean 62.3 (SD ±5.6) Range 55-72	Mean 7.3 (SD ±4.8) Range 1.5-18	Mean 9.1 (SD ±3.1) Range 6.3-16.2	8=diet control 8=oral hypoglycaemics 3=insulin 4=insulin + oral hypoglycaemics	23=L10
BDR	0 ♀ 14 ♂	Mean 63.1 (SD ±8.3) Range 44-75	Mean 11.7 (SD ±6.3) Range 4-22	Mean 9.5 (SD ±3.6) Range 6.1-13.4	2=diet control 5=oral hypoglycaemics 4=insulin 3=insulin + oral hypoglycaemics	4=L14 1=L35 9=L43

Table 7.3 Subject characteristics.

7.3.2 Results

7.3.2.1 Analysis of Results

Some difficulties arose as to the choice of the most suitable statistical test for the analysis of these results across time with O_2 inhalation. It was felt that a repeated measures ANOVA was the most appropriate test. Post hoc tests were done for the four comparisons of interest, i.e. the difference between baseline values and each of the following:

1. During O_2 inhalation
2. Two minutes after mask removal
3. Seven minutes after mask removal
4. Twelve minutes after mask removal

Firstly, a RM ANOVA was performed then the paired samples t-tests were performed between baseline and each of the four time-points above and the p-values stated have been Bonferroni adjusted for four comparisons.

7.3.2.2 Summed OP Amplitudes at Baseline

The group averaged summed OP amplitudes at baseline are shown in Table 7.4 and Figures 7.8 and 7.9. Four well-defined OPs are clearly shown for each group, as shown in Figure 7.8. Individual summed OP amplitudes are shown in Figure 7.10. The percentage of NDR and BDR subjects who fell below the control group mean amplitude at baseline are given in Table 7.5.

It was hypothesised that if hypoxia were present within the inner retinal layers in subjects with DM this may be reflected by reduced summed OP amplitudes. No significant difference was observed between the groups at baseline, $p>0.05$ (Bonferroni pairwise comparisons). The NDR group show the greatest summed OP amplitudes, followed by the control group with the BDR group showing the smallest amplitudes.

In the control group the summed OP amplitude at baseline was not found to be significantly correlated with age, $p>0.05$.

In subjects with DM, the summed OP amplitude was not found to be significantly correlated with age, disease duration or plasma glucose level at the time of recording, $p>0.05$. Summed OP amplitude at baseline was also not found to be correlated with HbA1c level, systolic blood pressure or diastolic blood pressure recorded at their most recent hospital visit, $p>0.05$. One way ANOVA also did not find a significant difference between summed OP amplitude and management when subjects were grouped according to their treatment type, i.e. diet alone, oral hypoglycaemics, insulin or insulin plus oral hypoglycaemics, $p>0.05$.

Multiple linear regression analysis was performed with the following explanatory variables:

- Subject age
- Disease duration
- Plasma glucose level

- Systolic blood pressure
- Diastolic blood pressure
- Management type, grouped as a) diet control or oral hypoglycaemics or b) insulin or insulin plus oral hypoglycaemics
- Retinopathy grouped as a) no retinopathy or b) any retinopathy

None of the explanatory variables were found to be related to summed OP amplitude at baseline, p=0.619, and the adjusted R^2 value of -0.071 indicates that only 7% of the variability in OPs could be explained by the regression model.

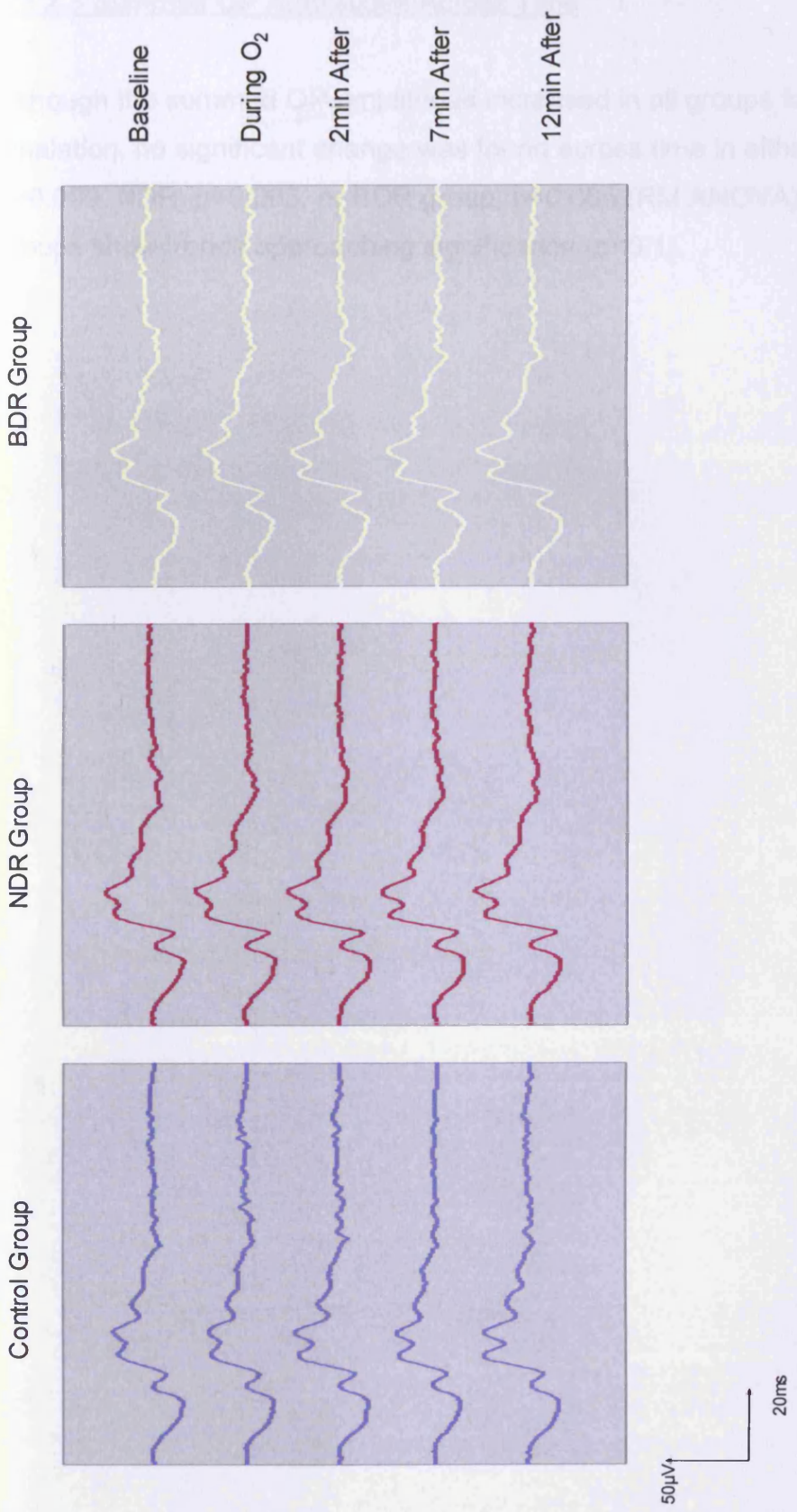

Figure 7.8 Group-averaged summed OP amplitudes.

7.3.2.3 Summed OP Amplitudes Across Time

Although the summed OP amplitudes increased in all groups following O_2 inhalation, no significant change was found across time in either the control, $p=0.099$, NDR, $p=0.065$, or BDR group, $p=0.065$ (RM ANOVA). All three groups show trends approaching significance ($p<0.1$).

Group		Summed OP Amplitudes (μV)				
		Baseline	During O_2	2min After	7min After	12min After
Control	Mean	132.4	129.2	140.7	142.8	145.7
	SD	*49.6*	*69.7*	*63.7*	*68.6*	*65.1*
	SE	*10.8*	*15.2*	*13.9*	*15.0*	*14.2*
NDR	Mean	146.0	148.9	158.3	164.1	165.9
	SD	*50.0*	*43.2*	*60.2*	*60.0*	*72.2*
	SE	*10.4*	*9.0*	*12.6*	*12.5*	*15.0*
BDR	Mean	115.4	123.4	137.7	133.2	148.0
	SD	*48.8*	*51.0*	*51.6*	*57.4*	*56.0*
	SE	*13.0*	*13.6*	*13.8*	*15.3*	*15.0*

Table 7.4 Group-averaged summed OP amplitudes across time with O_2 inhalation.

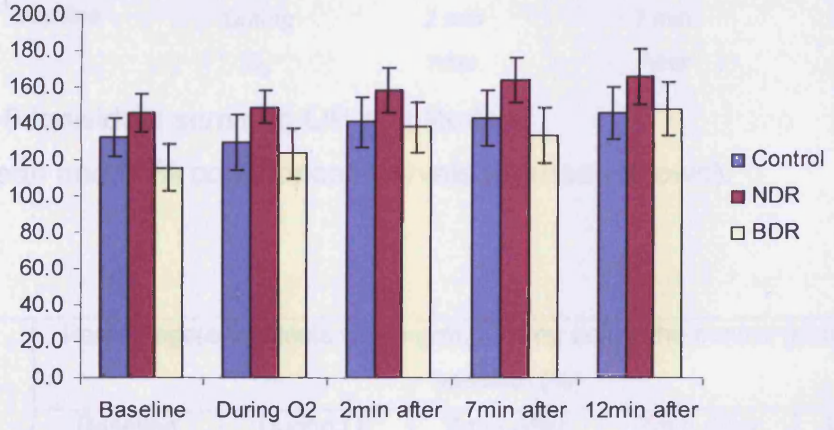

Figure 7.9 Group-averaged summed OP amplitudes across time with O_2 inhalation.

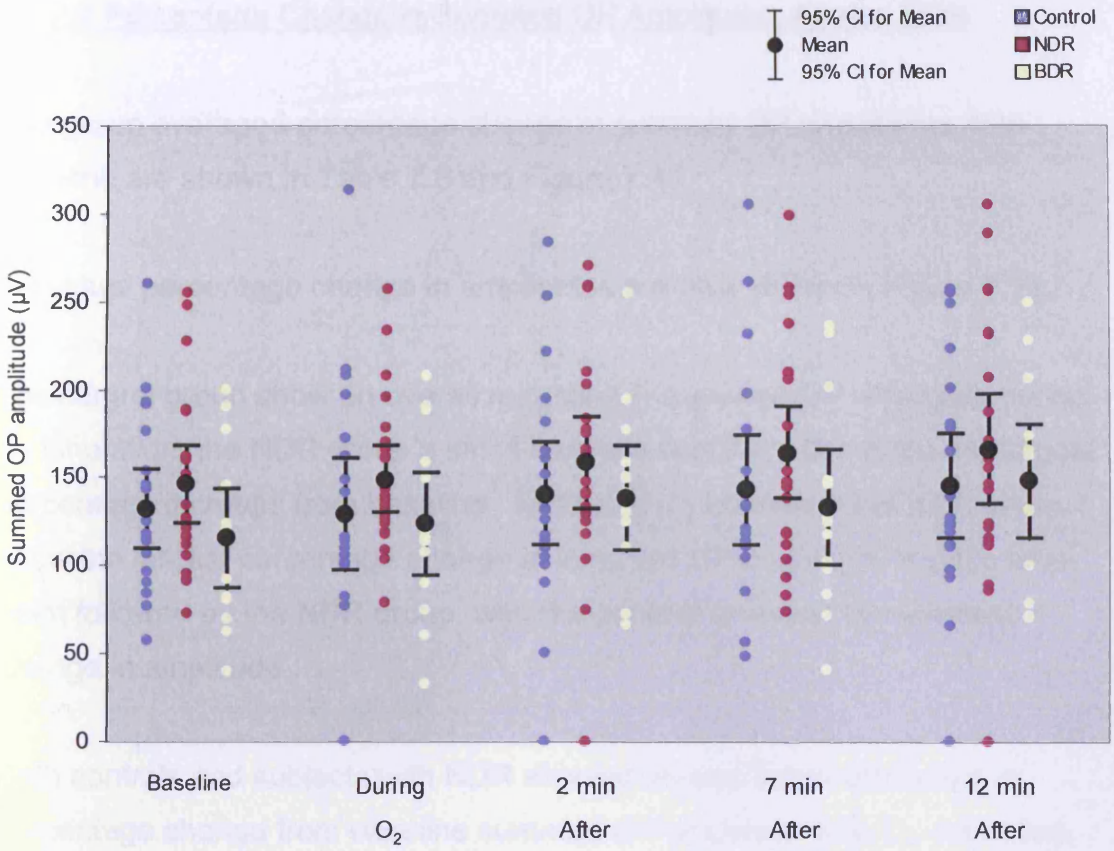

Figure 7.10 Individual summed OP amplitudes.
(Group mean and 95% confidence intervals for mean shown).

	Percentage of subjects within group falling below the control group mean at baseline (%)				
	Baseline	During O_2	2min After	7min After	12min After
NDR	53	43	30	30	35
BDR	271	79	50	71	36

Table 7.5 Percentage of subjects within each group falling below the control group mean summed OP amplitude at baseline.

7.3.2.4 Percentage Change in Summed OP Amplitudes Across Time

The group averaged percentage change in summed OP amplitudes from baseline are shown in Table 7.6 and Figure 7.11.

Individual percentage change in amplitudes are also shown in Figure 7.12.

The control group show an overall reduction in summed OP amplitude during O_2 inhalation, the NDR group a small increase and the BDR group the largest percentage increase from baseline. Following O_2 inhalation the BDR group show the largest percentage change in summed OP amplitude at each time-point followed by the NDR group, with the controls showing the smallest change in amplitude.

Both controls and subjects with NDR showed no significant difference in percentage change from baseline summed OP amplitude with O_2 inhalation, $p=0.087$ and $p=0.359$ respectively (RM ANOVA) and no significant change from baseline was apparent with paired samples t-tests, $p>0.05$.

The BDR group did show a significant change in summed OP amplitude with O_2 inhalation, $p=0.006$ (RM ANOVA), and paired samples t-tests found a statistically significant increase in amplitude of 36.7% twelve minutes after mask removal, $p=0.040$, and a significant increase in amplitude of 25.1% two minutes after mask removal, $p=0.024$.

Group		% Change in Summed OP Amplitude From Baseline			
		During O_2	2min After	7min After	12min After
Control	Mean	-7.4	+4.8	+7.0	+9.2
	SD	*34.9*	*31.2*	*27.4*	*33.1*
	SE	*7.6*	*6.8*	*6.0*	*7.2*
NDR	Mean	+1.3	+8.1	+10.0	+9.8
	SD	*29.3*	*43.6*	*37.7*	*41.5*
	SE	*6.1*	*9.1*	*7.9*	*8.6*
BDR	Mean	+11.3	**+25.1***	+20.0	**+36.7***
	SD	*35.9*	*28.6*	*33.5*	*40.3*
	SE	*9.6*	*7.6*	*9.0*	*10.8*

Table 7.6 Percentage change in summed OP amplitudes across time with O_2 inhalation.
(Statistical significance, compared to baseline value within each group indicated by: * = $p<0.05$, paired samples t-test).

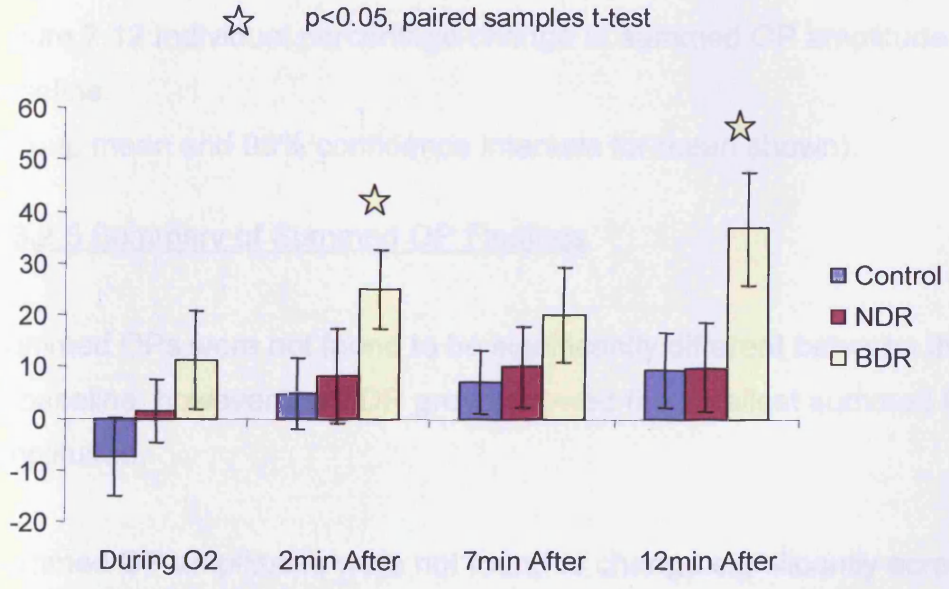

Figure 7.11 Percentage change in summed OP amplitude across time with O_2 inhalation.

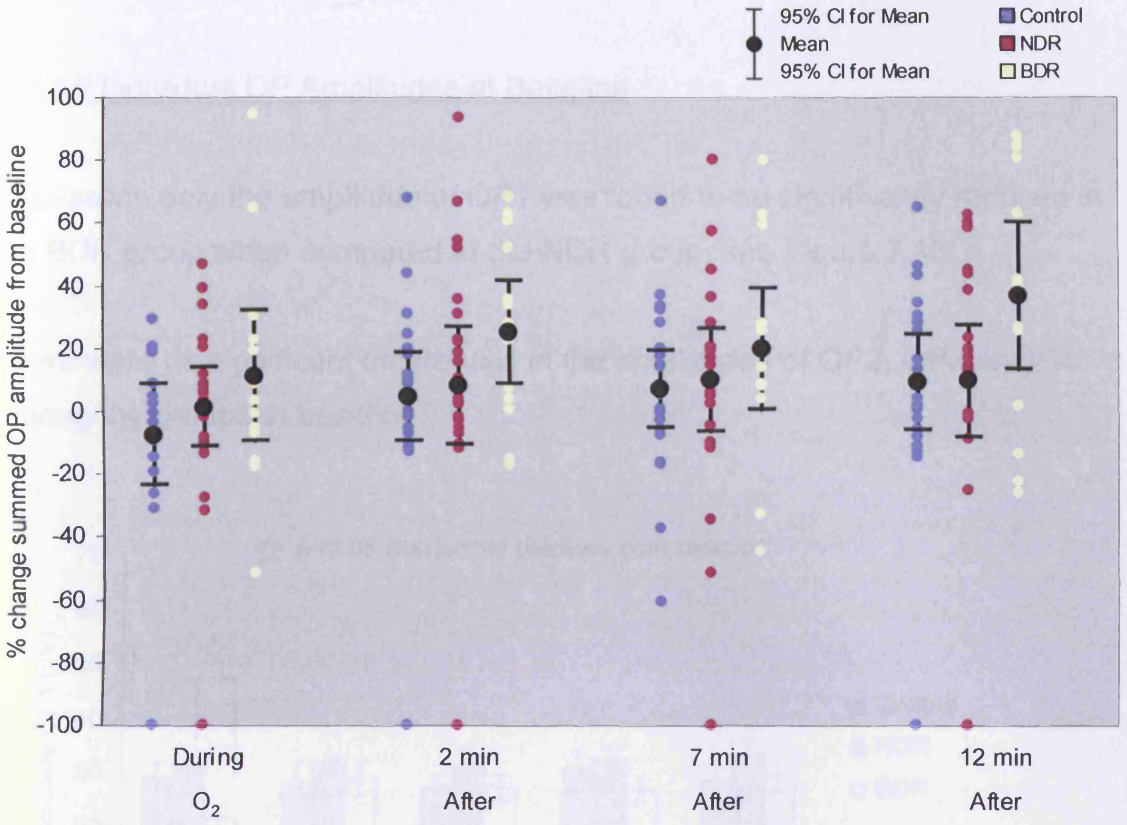

Figure 7.12 Individual percentage change in summed OP amplitude from baseline.
(Group mean and 95% confidence intervals for mean shown).

7.3.2.5 Summary of Summed OP Findings

Summed OPs were not found to be significantly different between the groups at baseline, however the BDR group showed the smallest summed OP amplitudes.

Summed OP amplitudes were not found to change significantly across time within any group.

However, when looking at percentage change in summed OP amplitude from baseline. a significant increase across time was found within the BDR group, p=0.006 (RM ANOVA) paired samples t-tests found a significant 36.7%

increase twelve minutes after mask removal, p=0.020 and a significant 25.1% increase two minutes after mask removal, p=0.024.

7.3.2.6 Individual OP Amplitudes at Baseline

At baseline only the amplitude of OP1 was found to be significantly reduced in the BDR group when compared to the NDR group, see Figure 7.13.

There were no significant differences in the amplitudes of OP2, OP3 or OP4 among the groups at baseline.

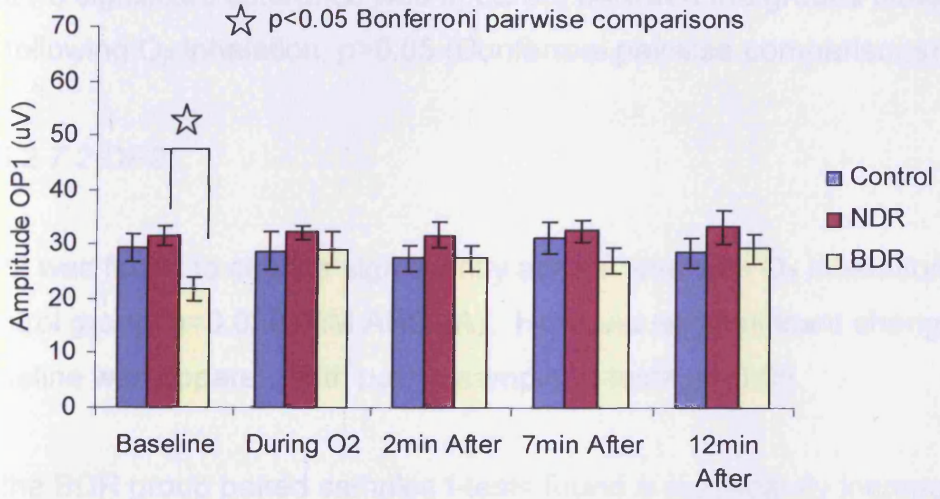

Figure 7.13 Group-averaged OP1 amplitudes.

7.3.2.7 Individual OP Amplitudes Across Time

Group averaged individual OP amplitudes are given in Table 7.7 and Figure 7.14.

7.3.2.7.1 OP1

In some cases the situation arose where RM ANOVA found a significant change in amplitude with O_2 inhalation status, however paired samples t-tests did not find significant differences between amplitudes. OP1 amplitude was found to increase significantly across time with O_2 inhalation in the BDR group only, p=0.041 (RM ANOVA), but no significant increase from baseline was apparent with paired samples t-tests, p>0.05.

The increase with O_2 inhalation reduced the difference in amplitude of OP1 between the BDR group and the NDR group which was apparent at baseline, and no significant difference was apparent between the groups either during or following O_2 inhalation, p>0.05 (Bonferroni pairwise comparisons).

7.3.2.7.2 OP2

OP2 was found to change significantly across time with O_2 inhalation in the control group, p=0.020 (RM ANOVA). However no significant change from baseline was apparent with paired samples t-tests, p>0.05.

In the BDR group paired samples t-tests found a significantly increased OP2 amplitude from baseline two minutes after mask removal, p=0.016.

7.3.2.7.3 OP3

OP3 amplitude was found to increase significantly across time with O_2 inhalation in the NDR group only, p=0.005 (RM ANOVA). Paired samples t-tests found significantly increased amplitude from baseline seven minutes after mask removal in this group, p=0.020.

7.3.2.7.4 OP4

OP4 amplitude did not change across time with O_2 inhalation in any group, p>0.05 (RM ANOVA and paired samples t-tests).

Group		Baseline	During O_2	2min After	7min After	12min After
		Amplitude OP1 (μV)				
Control	Mean	29.4	28.8	27.4	31.2	28.5
	SD	*11.1*	*15.8*	*11.5*	*13.2*	*11.8*
	SE	*2.4*	*3.4*	*2.5*	*2.9*	*2.6*
NDR	Mean	31.4	32.1	31.7	32.5	33.3
	SD	*8.4*	*6.8*	*11.0*	*9.5*	*14.8*
	SE	*1.8*	*1.4*	*2.3*	*2.0*	*3.1*
BDR	Mean	21.8	28.9	27.4	27.0	29.2
	SD	*8.6*	*12.3*	*8.2*	*8.2*	*10.2*
	SE	*2.3*	*3.3*	*2.2*	*2.2*	*2.7*
		Amplitude OP2 (μV)				
Control	Mean	46.9	46.5	52.6	51.4	57.2
	SD	*19.3*	*28.4*	*28.3*	*29.8*	*30.8*
	SE	*4.2*	*6.2*	*6.2*	*6.5*	*6.7*
NDR	Mean	54.2	49.8	52.9	56.8	58.6
	SD	*21.8*	*20.5*	*23.9*	*26.2*	*30.7*
	SE	*4.5*	*4.3*	*5.0*	*5.5*	*6.4*
BDR	Mean	40.3	41.6	**50.3***	49.1	53.2
	SD	*19.2*	*19.0*	*20.3*	*21.0*	*25.7*
	SE	*5.1*	*5.1*	*5.4*	*5.6*	*6.9*
		Amplitude OP3 (μV)				
Control	Mean	36.4	33.2	38.8	39.1	38.0
	SD	*17.5*	*20.9*	*21.4*	*21.6*	*22.4*
	SE	*3.8*	*4.6*	*4.7*	*4.7*	*4.9*
NDR	Mean	40.7	41.2	45.8	**51.0***	47.4
	SD	*20.6*	*16.6*	*19.5*	*21.9*	*23.0*
	SE	*4.3*	*3.5*	*4.1*	*4.6*	*4.8*
BDR	Mean	34.3	33.3	39.0	37.0	41.5
	SD	*16.0*	*15.5*	*19.1*	*21.1*	*20.9*
	SE	*4.3*	*4.1*	*5.1*	*5.6*	*5.6*
		Amplitude OP4 (μV)				
Control	Mean	20.4	23.2	20.6	22.0	21.9
	SD	*9.6*	*14.8*	*10.5*	*11.5*	*10.5*
	SE	*2.1*	*3.2*	*2.3*	*2.5*	*2.3*
NDR	Mean	23.5	23.5	25.2	25.6	25.0
	SD	*11.7*	*7.4*	*11.2*	*12.9*	*11.3*
	SE	*2.4*	*1.5*	*2.3*	*2.7*	*2.4*
BDR	Mean	19.0	19.6	21.1	20.1	24.2
	SD	*9.4*	*9.8*	*10.5*	*10.9*	*7.6*
	SE	*2.5*	*2.6*	*2.8*	*2.9*	*2.0*

Table 7.7 Group-averaged individual OP amplitudes across time with O_2 inhalation. (Statistical significance, compared to baseline value within each group indicated by: * = $p<0.05$, paired samples t-test).

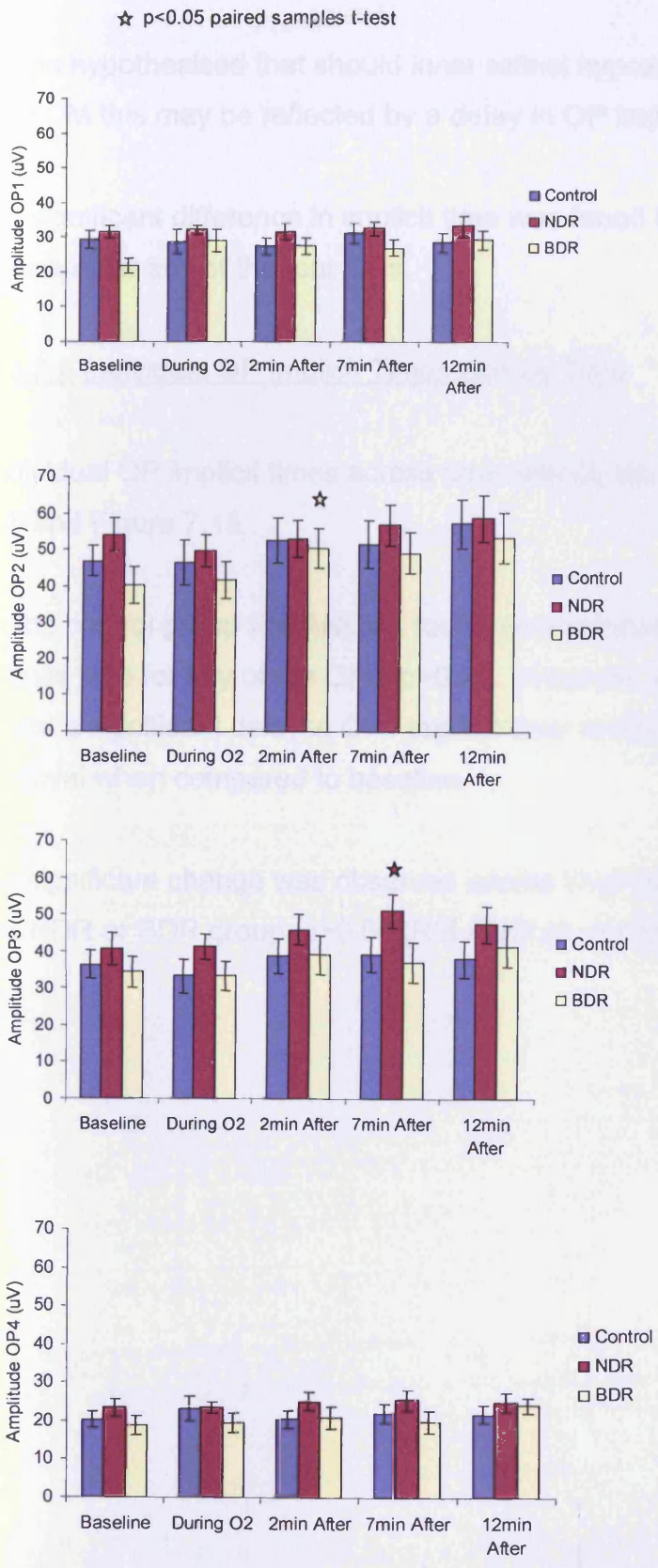

Figure 7.14 Group-averaged individual OP amplitudes (OP1-4) across time.

7.3.2.8 Individual OP Implicit Times at Baseline

It was hypothesised that should inner retinal hypoxia be present in subjects with DM this may be reflected by a delay in OP implicit times.

No significant difference in implicit time was found between the groups at baseline for any of the four OPs.

7.3.2.9 Individual OP Implicit Times Across Time

Individual OP implicit times across time with O_2 inhalation are shown in Table 7.8 and Figure 7.15.

In the control group RM ANOVA found no significant change in implicit time across time for any of the OPs, $p>0.05$. However, a paired samples t-test found a significant delay in OP1 implicit time seven minutes after mask removal when compared to baseline.

No significant change was observed across time for any of the OPs in either the NDR or BDR group, $p>0.05$ (RM ANOVA, paired samples t-tests).

Group		Baseline	During O_2	2min After	7min After	12min After
		Implicit Time OP1 (ms)				
Control	Mean	19.5	17.5	18.0	**20.0***	18.7
	SD	*1.7*	*6.0*	*4.6*	*1.8*	*4.6*
	SE	*0.4*	*1.3*	*1.0*	*0.4*	*1.0*
NDR	Mean	19.8	19.7	19.1	19.6	19.1
	SD	*0.7*	*0.6*	*4.2*	*1.1*	*4.2*
	SE	*0.1*	*0.1*	*0.9*	*0.2*	*0.9*
BDR	Mean	20.9	20.7	20.8	21.1	21.4
	SD	*2.6*	*2.7*	*2.0*	*2.3*	*2.3*
	SE	*0.7*	*0.7*	*0.5*	*0.6*	*0.6*
		Implicit Time OP2 (ms)				
Control	Mean	27.1	24.6	25.8	27.1	26.0
	SD	*3.7*	*8.7*	*6.8*	*2.8*	*6.6*
	SE	*0.8*	*1.9*	*1.5*	*0.6*	*1.4*
NDR	Mean	28.3	28.0	26.7	27.3	26.7
	SD	*1.8*	*1.5*	*5.9*	*2.1*	*6.0*
	SE	*0.4*	*0.3*	*1.2*	*0.4*	*1.3*
BDR	Mean	28.2	28.2	28.5	28.7	28.7
	SD	*2.0*	*2.0*	*2.1*	*2.6*	*2.1*
	SE	*0.5*	*0.5*	*0.6*	*0.7*	*0.6*
		Implicit Time OP3 (ms)				
Control	Mean	33.1	29.6	31.6	33.8	31.7
	SD	*3.4*	*10.3*	*7.8*	*3.9*	*7.7*
	SE	*0.7*	*2.2*	*1.7*	*0.9*	*1.7*
NDR	Mean	33.9	33.5	31.9	33.3	32.0
	SD	*2.2*	*1.3*	*7.0*	*1.5*	*7.1*
	SE	*0.5*	*0.3*	*1.5*	*0.3*	*1.5*
BDR	Mean	34.0	33.6	33.8	34.3	34.0
	SD	*1.5*	*1.5*	*1.5*	*1.9*	*1.5*
	SE	*0.4*	*0.4*	*0.4*	*0.5*	*0.4*
		Implicit Time OP4 (ms)				
Control	Mean	40.5	36.2	38.1	40.9	38.3
	SD	*4.2*	*12.5*	*9.4*	*4.0*	*9.2*
	SE	*0.9*	*2.7*	*2.1*	*0.9*	*2.0*
NDR	Mean	38.6	40.4	38.5	38.3	38.8
	SD	*8.6*	*1.6*	*8.5*	*8.5*	*8.6*
	SE	*1.8*	*0.3*	*1.8*	*1.8*	*1.8*
BDR	Mean	40.9	40.6	40.9	41.1	41.0
	SD	*1.6*	*1.5*	*1.5*	*1.8*	*1.6*
	SE	*0.4*	*0.4*	*0.4*	*0.5*	*0.4*

Table 7.8 Group-averaged individual OP implicit times across time with O_2 inhalation. (Statistical significance, compared to baseline value within each group indicated by: * = $p<0.05$, paired samples t-test).

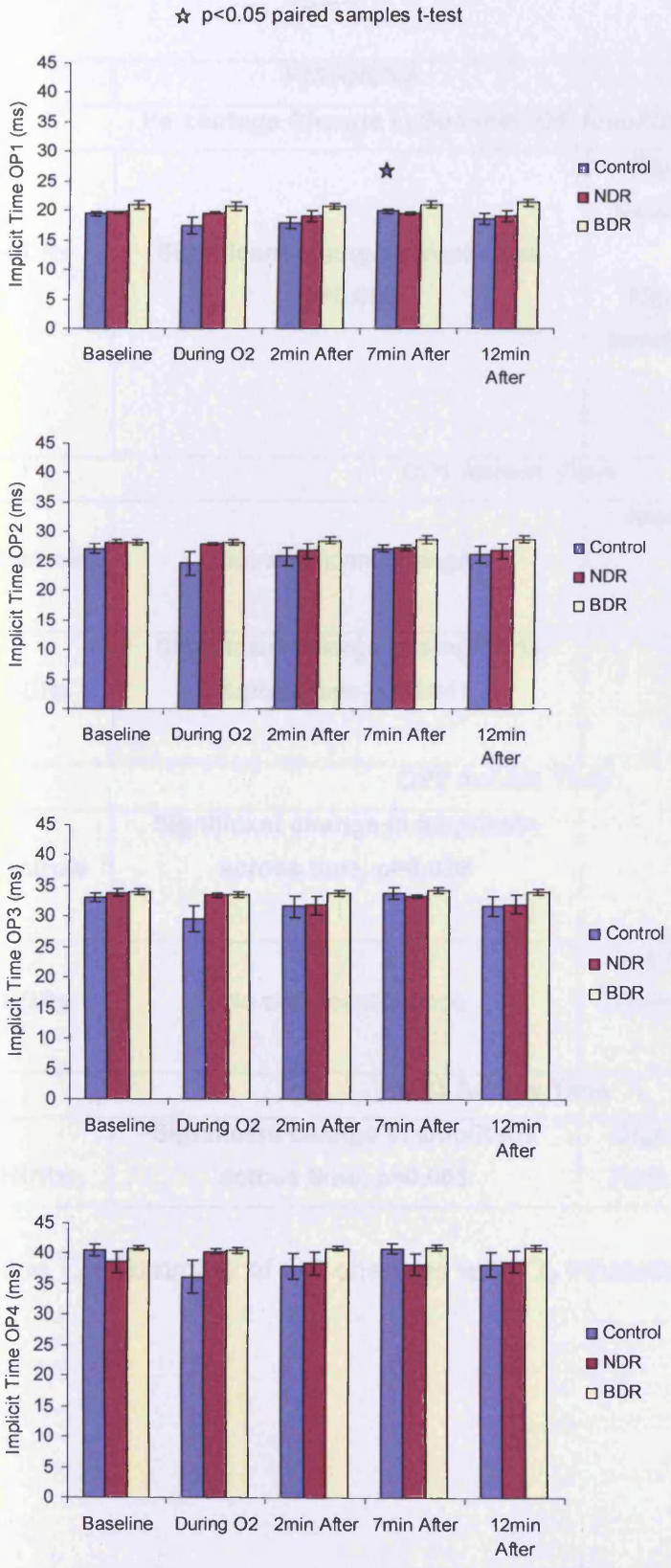

Figure 7.15 Group-averaged individual OP implicit times across time.

7.3.3 Summary of Significant Findings

	RM ANOVA	Paired samples t-tests
Percentage Change in Summed OP Amplitudes Across Time		
BDRs	**Significant change across time, p=0.006**	**Significant 25.1% increase from baseline 2 min after mask removal (p=0.024). Significant 36.7% increase from baseline 12min after mask removal, p=.020**
OP1 Across Time		
Controls	No significant change	**Implicit time delayed 7min after mask removal, p=0.048**
BDRs	**Significant change in amplitude across time, p=0.041**	No significant change
OP2 Across Time		
Controls	**Significant change in amplitude across time, p=0.020**	No significant change
BDRs	No significant change	**Significant increase in amplitude 2min after mask removal, p=0.016**
OP3 Across Time		
NDRs	**Significant change in amplitude across time, p=0.005**	**Significant increase in amplitude 7min after mask removal, p=0.020**

Table 7.9 Summary of OP changes with O_2 inhalation.

7.3.4 Arterial Oxygen Saturation

Unfortunately, due to equipment failure, measurement of arterial O_2 saturation (SaO_2) was only possible on a small sample of subjects. Subject characteristics are given in Table 7.10.

Group	Gender	Age (yrs)	Disease Duration (yrs)	Plasma Glucose Level (mmol/L)	Management Type	Retinopathy Level (ETDRS Scale)
Control	1 ♀ 2 ♂	Mean 58 (SD ±11.5) Range 45-67	-	-	-	-
NDR	3 ♀ 3 ♂	Mean 61.2 (SD ±1.9) Range 59-64	Mean 9.2 (SD ±5.9) Range 3-18	Mean 9.9 (SD ±4.2) Range 6.3-15.6	1=diet control 1=oral hypoglycaemics 2=insulin 2=insulin + oral hypoglycaemics	6=L10
BDR	0 ♀ 5 ♂	Mean 58 (SD ±8.5) Range 44-65	Mean 10.2 (SD ±5.6) Range 4-18	Mean 6.3 (SD ±0.6) Range 5.7-6.9	1=diet control 2=oral hypoglycaemics 2=insulin 0=insulin + oral hypoglycaemics	2=L14 3=L43

Table 7.10 Subject characteristics.

Mean group-averaged arterial SaO_2 in the twenty-three minutes before, the five minutes during and the twelve minutes following O_2 inhalation are shown in Table 7.11 and Figure 7.16. It can be seen that SaO_2 begins to rise within approximately two minutes of inhalation, increases by 1-2% and peaks approximately two minutes following the end of the inhalation period, returning to baseline within about five minutes of the end of inhalation. It therefore does not correlate with OP amplitudes which continued to increase with O_2 inhalation up to twelve minutes after mask removal.

No significant difference in SaO_2 was found between the groups at any point, $p>0.05$ (Bonferroni pairwise comparisons).

Group		SaO_2 Before Inhalation (%)	SaO_2 During Inhalation (%)	SaO_2 After Inhalation (%)
Control	Mean	94.8	96.5	93.6
	SD	*1.4*	*0.4*	*4.7*
	SE	*0.8*	*0.2*	*2.7*
NDR	Mean	95.3	96.2	95.1
	SD	*0.6*	*1.1*	*1.6*
	SE	*0.3*	*0.4*	*0.7*
BDR	Mean	95.0	96.5	96.0
	SD	*1.3*	*0.6*	*0.9*
	SE	*0.6*	*0.3*	*0.4*

Table 7.11 Group-averaged SaO_2 before, during and following O_2 inhalation.

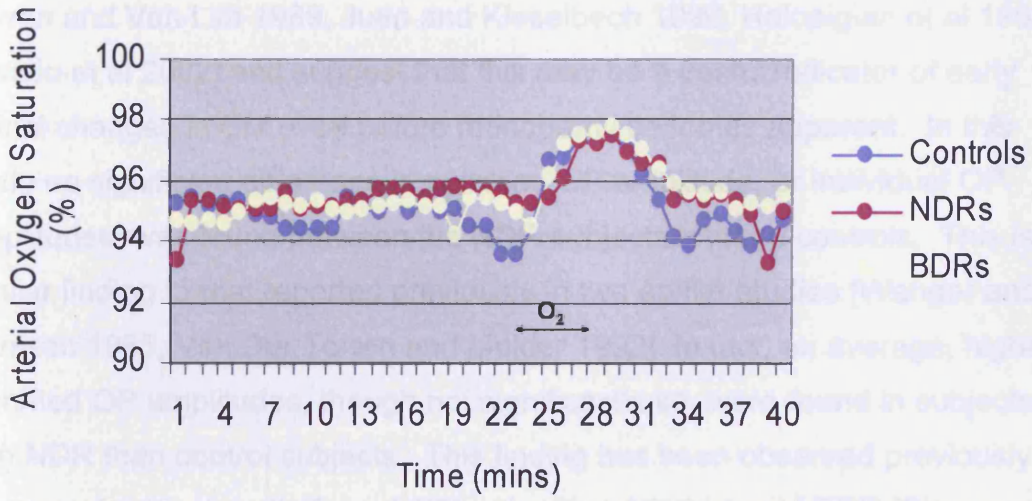

Figure 7.16 Group-averaged SaO_2 before, during and following O_2 inhalation.

7.3.5 Discussion

It has been proposed that the retina is subject to sub-clinical levels of tissue hypoxia prior to the development of DR [Arend et al 1991, Linsenmeier et al 1998]. Autoregulation of blood flow in the normal retina is little more than adequate to cope with the high O_2 demand of the retina during dark adaptation [Arden at al 1998]. In subjects with DM autoregulation is known to be impaired [Alm 1992], and changes in the blood and capillary function may then lead to hypoxia within the inner retinal tissue. If hypoxia were present in the tissues between the superficial and deep capillary networks of the central retinal circulation after dark adaptation in subjects with DM, this may be indicated by reduced OP amplitudes which are known to reflect the functional efficiency of the inner retinal circulation [Holopigian et al 1992].

Previous studies have found reduced summed OP amplitudes in subjects with DM and no visible retinopathy [Simonsen 1980, Moschos et al 1987, Van Der Torren and Van Lith 1989, Juen and Kieselbech 1990, Holopigian et al 1992, Drasdo et al 2002] and suggest that this may be a useful indicator of early retinal changes in DM even before retinopathy becomes apparent. In this study no significant difference in summed OP amplitude, or individual OP amplitudes, was found between the NDR subjects and the controls. This is a similar finding to that reported previously in two earlier studies [Wanger and Persson 1985, Van Der Torren and Mulder 1993]. In fact, on average, higher summed OP amplitudes, though not significantly so, were found in subjects with NDR than control subjects. This finding has been observed previously in a group of subjects with Type 1 DM with either NDR or mild BDR [Simonsen 1980], where the hypernormal oscillatory potential observed excluded the development of proliferative retinopathy within the next six to eight years. Previous studies have also shown that the reduction in summed OP amplitude in subjects with DM increases with increasing severity of retinopathy [Bresnick and Palta 1987b, Shirao et al 1991]. Our results lend support to these findings since subjects with BDR had the smallest summed OP amplitudes of the three groups at baseline, however this did not reach statistical significance.

Whilst the average summed OP amplitude was slightly higher in the NDR group than the controls, some individual subjects within the group did show initially depressed amplitudes. NDR subjects with initially depressed summed OP amplitudes were investigated to see if they showed the greatest increases with O_2 inhalation but this was not found to be the case. Likewise when examining those NDR subjects with initially depressed amplitudes no similarities were apparent between the subject characteristics i.e. disease duration, management type, plasma glucose etc.

No significant difference in OP implicit times was observed between control subjects and either of the diabetic subject groups. This has also been reported previously, although again these studies only examined diabetic subjects with no retinopathy [Coupland 1985b, Wanger and Persson 1985, Holopigian et al 1992].

Drasdo et al [2002] found the significantly reduced summed OP amplitude observed in NDR subjects increased to the level of the control group at baseline following two minutes of O_2 inhalation. In this study following O_2 inhalation the greatest increase in summed OP amplitude was apparent in the BDR group, followed by the NDR group with the least effect visible in the control group. O_2 was not expected to affect control group amplitudes since autoregulation should compensate for the increased O_2 by reducing retinal blood flow [Riva et al 1983, Grunwald et al 1984, Kiss et al 2002, Luksch et al 2002]. In subjects with DM, where autoregulation has been found to be impaired, an increase in summed OP amplitude may be expected as any inner retinal hypoxia is reduced. These results may indirectly reflect a level of hypoxia within the retinae of subjects with DM, since OPs are known to reflect inner retinal function and summed OP amplitude increased significantly following O_2 inhalation in subjects with BDR and the OP3 amplitude significantly increased following O_2 inhalation in subjects with NDR.

This study found differing results to that of Drasdo et al [2002] in subjects with NDR. This may be explained by the fact that 11 of the 23 subjects displayed summed OP amplitudes falling above the control group mean summed OP

amplitude at baseline. This may suggest that these subjects are at less risk of developing retinopathy than those subjects with depressed OP amplitudes [Simenson 1980], and as such it may be presumed that the retinae may therefore be less hypoxic. In the Drasdo et al study [2002] the subject group exhibited initially depressed OP amplitudes. This suggests that this group of subjects were more at risk of developing retinopathy and the retinae were in a more hypoxic state. If this were the case a greater effect with O_2 inhalation would be expected.

The amplitudes of OP1 and OP2 were found to increase significantly with O_2 inhalation in subjects with BDR. Interestingly the amplitude of these two OPs have been found to be significantly reduced following induced systemic hypoxia in healthy individuals [Janáky et al 2007]. In their study fourteen healthy male subjects were exposed for fifteen minutes to a simulated altitude of 5500m in order to induce systemic hypoxia. ERGs were recorded before, immediately following and twenty minutes after the hypoxic exposure. No significant changes were observed in either the a or b-waves of the ERG however OP1 and OP2 were found to be significantly reduced in amplitude immediately after the hypoxic exposure. These results again highlight the high sensitivity of the OPs towards circulatory and/or hypoxic challenges [Janáky et al 2007]. The increase in OP amplitudes observed in the current study may be explained by a reduction of inner retinal hypoxia with O_2 inhalation.

The retinal structures underlying the origin of the OPs are a subject of controversy. Since rod function has been found to be impaired in subjects with DM even before the development of significant retinopathy [Henson and North 1979, Greenstein et al 1993], and it is believed that the high metabolic demands of the rod photoreceptors may cause a level of hypoxia within the normal retina under dark adaptation, it may be expected that the rod-dominated OPs would be more affected by O_2 inhalation than the cone-dominated OPs. The OPs are believed to be generated by a mechanism of rod and cone interactions [King-Smith et al 1986, Peachey et al 1987] with the cone system influencing the earlier OPs (OP1-2) and the rod system

influencing the later OPs (OP3-4) [Lachapelle et al 1983, Coupland 1987a, Jánaky et al 1996]. It is therefore unclear why in this study the earlier OPs appear to be more affected by O_2 inhalation in subjects with BDR, however another study did suggest that the earlier OPs may be more rod-dominated than the later OPs [Wang et al 2001].

The values of arterial O_2 saturation found in this study are lower than that found in a previous study [Faucher and Kergoat 2002]. Normal arterial O_2 saturation is known to vary between 94-100% [www.webmd.com 2007]. Faucher and Kergoat [2002] found an average level of 98.1% before breathing 100% O_2 for five minutes, which rose to 99.4% during the inhalation period in a group of thirty-five control subjects. The differences between the two studies may be due to the fact that their subjects were considerably younger with an age range of 19-26 years and a mean age of 22.3yrs, and arterial O_2 saturation is known to reduce with age [Cerveri et al 1995].

The arterial O_2 saturation measurements were taken from the subjects' fingertip and therefore does not directly indicate the level of oxygenation within the retinal tissue. A review of the literature did not reveal how long the increase in inspired O_2 would take to reach the retinal tissue. From personal communication with a local anaesthetist [Dr Jim Stewart] it was felt that it would take some time for the increase in inspired O_2 to result in an increase of oxygenation to the tissue and, though dependent on many factors, subtle changes in previously hypoxic retinas may take ten to fifteen minutes from the initial increase in inspired O_2. This may explain the increases observed in OP amplitudes two, seven and twelve minutes after mask removal.

In summary the results of this study did reflect signs of inner retinal hypoxia in subjects with DM, with an increase in OP amplitudes observed following O_2 inhalation.

7.4 General Summary

This chapter aimed to investigate the possible presence of hypoxia in subjects with DM in the retinal tissue between the superficial and deep capillary networks of the central retinal circulation, lying in the nerve fibre and inner nuclear layers respectively, following dark adaptation. This may be indicated by reduced OP amplitudes in these subjects. OP amplitudes were found to be smallest in subjects with visible retinopathy and both summed OP amplitudes and the amplitudes of OP1 and OP2 were found to significantly increase following O_2 inhalation in thus group. OP3 was also found to significantly increase with O_2 inhalation in the NDR group. These increases may reflect a reduction of inner retinal hypoxia in these subjects with O_2 inhalation.

8. INVESTIGATION OF THE EFFECTS OF OXYGEN INHALATION ON THE SCOTOPIC B-WAVE

8.1 Aim of the Study

The aim of this study was to investigate signs of possible hypoxia in the inner retina of subjects with DM which may be indicated by reduced b-wave amplitudes, believed to reflect the activity of the bipolar cells.

This study investigated the effect of five minutes of O_2 inhalation on the scotopic b-wave in subjects with DM. If inner retinal hypoxia were present in these subjects, an increase in b-wave amplitudes may be expected as the hypoxia is reduced.

8.2 Preliminary Trials of the Scotopic ERG

The technique used to elicit the scotopic (dim flash) ERG is based on that previously used by our department in the investigation of visual deficits in dyslexia as reported by Greatrex and Drasdo [1998]. The stimulus used was a dim flash provided by green LEDs, 0.027 $cd.m^{-2}$, peak wavelength 565 nm at 1.3 Hz. The b-wave elicited by a bright flash used by Drasdo et al [2002] contained contributions from both the rod and cone systems, and since the rod system is known to be more vulnerable to hypoxia in subjects with DM a scotopic ERG may offer a better reflection of rod-driven hypoxia.

In order to be more compliant with ISCEV standards the stimulus duration was shortened from 400 ms to 5 ms [Marmor et al 2004]. The ISCEV standard stimulus is a dim white flash of strength 2.5 log units below the standard flash of 1.5-3 $cd.s.m^{-2}$ [Marmor et al 2004], and the stimulus used fell within this recommended luminance range. A minimum interval of two seconds between flashes is also advised [Marmor et al 2004], and thus the frequency of stimulation was changed from 1.3 to 0.5 Hz. The final settings are outlined in Table 8.1 and were used on all occasions unless otherwise stated.

Parameter	Setting
Time window (ms)	500
Low frequency filter (Hz)	1
High frequency filter (Hz)	100
Sensitivity (μV)	100
Number of Sweeps	8
Field Size	Ganzfeld
Peak Wavelength of Stimulation (nm)	515
Frequency of Stimulation (Hz)	0.5
Stimulus duration (ms)	5
Stimulus Intensity ($cd.s.m^{-2}$)	0.0012

Table 8.1 Final settings for the scotopic ERG.

8.3 Protocol Development for the Scotopic ERG with O_2 Inhalation

8.3.1 The Effect of Repeated Measurements of the Scotopic ERG

It was intended that ERGs should be recorded before, during and after five minutes of O_2 inhalation. A total of five sets of measurements would be recorded at five minute intervals. As some subjects may have a higher rate of stimulus rejection, due to unwanted blinks etc, enough time had to be allowed to obtain eight responses. It was felt that a period of three minutes would be long enough to record the required number of responses.

In the event of multiple stimulus rejection, the situation may arise where some subjects are actually receiving more flashes than others in order to obtain eight desired responses, and thus their retinal adaptation may be different. In order to keep the level of adaptation the same for each subject the flash could be left running for the whole of the three minute recording period. To investigate whether or not this was necessary and whether or not adaptational effects were likely to occur the following experiment was carried out (similar to that in Chapter 7):

- 20 minutes dark adaptation
- Time 0 min: 1st set of responses recorded, Trace 1.
- Time 5 min: 2nd set of responses recorded, Trace 2.
- Time 10 min: 3rd set of responses recorded, Trace 3, flash stimulus running for a 3 minute period in total.
- Time 15 min: 4th set of responses recorded, Trace 4, flash stimulus running for a 3 minute period in total.
- Time 20 min: 5th set of responses recorded, Trace 5.

This procedure is summarised in Figure 8.1

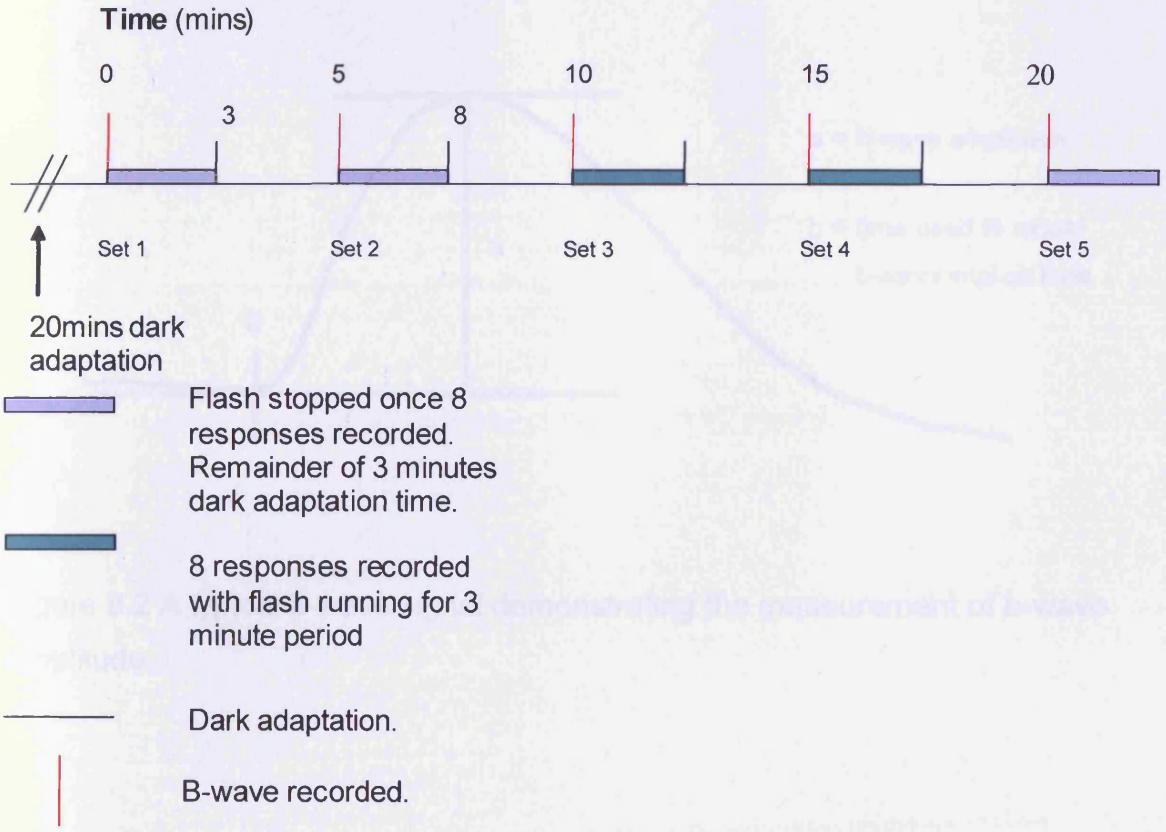

Figure 8.1 Protocol for repeated measurements of the scotopic ERG.

These data were then analysed to determine if any significant adaptational effects had occurred.

8.3.1.1 Subject Group

The subject group consisted of 8 healthy controls, 2 male and 6 female, with no history of ocular disease. Ages ranged from 24-31 years, mean age 26 years (SD ±3.6). The test eye was assigned at random and pupils dilatated.

8.3.1.2 Results

B-wave amplitudes were measured from trough to peak as indicated by the red arrow in Figure 8.2. Results for the mean b-wave amplitude are given in Table 8.2 and Figure 8.3.

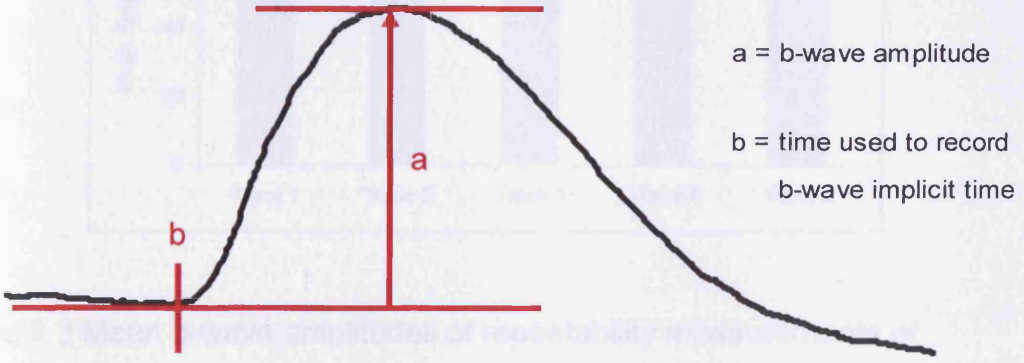

Figure 8.2 A typical b-wave signal demonstrating the measurement of b-wave amplitude.

Trace Number	1	2	3 (Continuous Flash)	4 (Continuous Flash)	5
Mean Amp (μV) (±SE)	78.8 (±6.9)	77.4 (±7.9)	84.7 (±8.0)	87.3 (±11.5)	91.5 (±6.9)

Table 8.2 Mean b-wave amplitudes of repeated measurements of scotopic ERG.

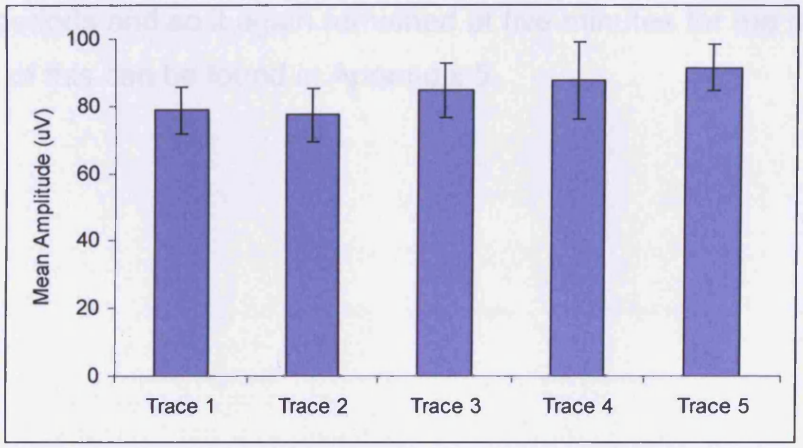

Figure 8.3 Mean b-wave amplitudes of repeatability measurements of scotopic ERG.

RM ANOVA found no significant change in b-wave amplitude across time, p=0.303.

8.3.1.3 Conclusion

Since no significant difference was found between any of the five traces it was decided that the flash could be stopped once eight responses had been obtained and did not need to be left running for the whole three minute period.

8.3.2 Effect of Length of Inhalation Period

As in Chapter 7, a five minute O_2 inhalation period was chosen since this had been used in previous work with positive results in subjects with DM [Dean et al 1997, Drasdo et al 2002]. Again in the early stages of this investigation only a small change in b-wave amplitudes were apparent following the five minute inhalation period. A fifteen minute inhalation period was investigated in a small group of control subjects and a small group of DM subjects with NDR, and the results compared to those with five minutes of O_2. No significant difference in b-wave amplitudes were found between the two inhalation periods and so it again remained at five minutes for the main study. Full details of this can be found in Appendix 5.

8.4 Final Protocol for the Investigation of the Effects of O_2 Inhalation on the Scotopic B-wave

The protocol for the recording of the scotopic ERG with O_2 inhalation, similar to that for recording the OPs in Chapter 7, is outlined below and shown in Figure 8.4. Subjects inhaled 100% O_2 through a 60% Ventimask for a period of 5 minutes.

- 20 minutes dark adaptation
- Time 0 min: Record response, stop flash. (Baseline Response).
- Time 3 min: Begin O_2 breathing
- Time 5 min: Record response, stop flash. (During O_2).
- Time 8 min: Finish O_2 breathing, mask removed.
- Time 10 min: Record response, stop flash. (2 min after mask removal).
- Time 15 min: Record response, stop flash. (7 min after mask removal).
- Time 20 min: Record response, stop flash. (12 min after mask removal).

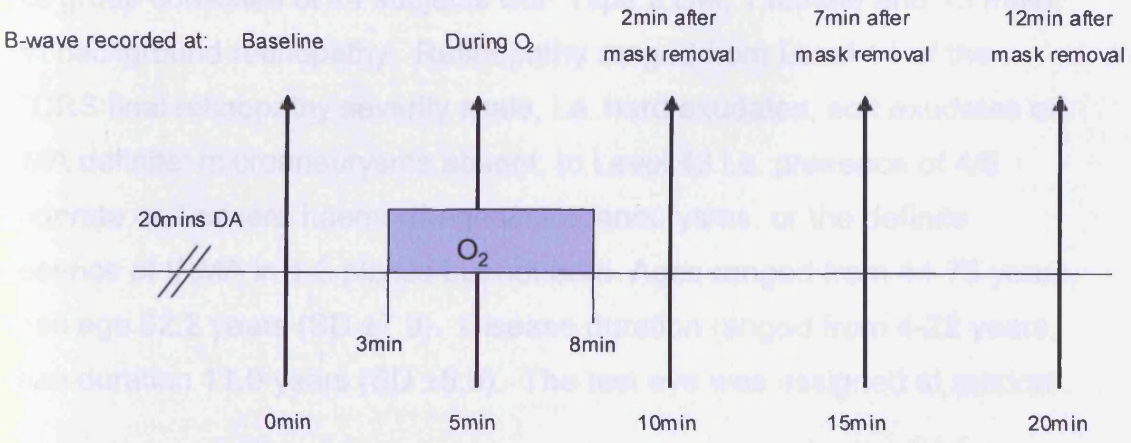

Figure 8.4 Final protocol for the scotopic ERG.

8.4.1 Subject Group

Subject group characteristics are summarised in Table 8.3

8.4.1.1 Control Subjects

This group consisted of 24 control subjects, 10 female and 14 male, with no history of ocular disease. Ages ranged from 41-80 years, mean age 60.0 years (SD ±10.6). The test eye was assigned at random.

8.4.1.2 Subjects with NDR

This group consisted of 25 subjects with Type 2 DM, 7 female and 18 male, with no visible retinopathy. Ages ranged from 53-72 years, mean age 62.3 years (SD ±6.0). Disease duration ranged from 1.5-18 years, mean duration 7.7 years (SD ±4.8). The test eye was assigned at random.

8.4.1.3 Subjects with BDR

This group consisted of 14 subjects with Type 2 DM, 1 female and 13 male, with background retinopathy. Retinopathy ranged from Level 14 of the ETDRS final retinopathy severity scale, i.e. hard exudates, soft exudates or IRMA definite: microaneurysms absent, to Level 43 i.e. presence of 4/5 moderate or 1 severe haemorrhages/microaneurysms, or the definite presence of IRMA in 1-3 places but not both. Ages ranged from 44-75 years, mean age 62.2 years (SD ±7.9). Disease duration ranged from 4-22 years, mean duration 11.0 years (SD ±5.9). The test eye was assigned at random.

Group	Gender	Age (yrs)	Disease Duration (yrs)	Plasma Glucose Level (mmol/L)	Management Type	Retinopathy Level (ETDRS Scale)
Control	10 ♀ 14 ♂	Mean 60 (SD ±10.6) Range 41-80	-	-	-	-
NDR	7 ♀ 18 ♂	Mean 62.3 (SD ±6.0) Range 53-72	Mean 7.7 (SD ±4.8) Range 1.5-18	Mean 8.8 (SD ±3.6) Range 5.2-17.8	7=diet control 9=oral hypoglycaemics 3=insulin 6=insulin + oral hypoglycaemics	25=L10
BDR	1 ♀ 13 ♂	Mean 62.2 (SD ±7.9) Range 44-75	Mean 11.0 (SD ±5.9) Range 4-22	Mean 10.3 (SD ±4.1) Range 4.8-18.0	2=diet control 5=oral hypoglycaemics 3=insulin 4=insulin + oral hypoglycaemics	4=L14 1=L35 9=L43

Table 8.3 Subject characteristics.

8.4.2 Results

8.4.2.1 Analysis of Results

In this study the results have again been analysed two ways, as in Chapter 7. Firstly, a RM ANOVA was performed. Secondly, paired samples t-tests were performed between baseline and each of the four time-points tested and the p-values stated have been Bonferroni adjusted for four comparisons.

8.4.2.2 B-wave Amplitudes at Baseline

The group averaged b-wave amplitudes can be seen in the traces of Figure 8.5. Well defined b-waves can be seen for each group at each of the time-points tested. Group-averaged b-wave amplitudes are shown in Table 8.4 and Figure 8.6. The percentage of NDR and BDR subjects falling below the control group mean amplitude at baseline are given in Table 8.5.

Individual amplitudes are also shown in Figure 8.7

At baseline the control group showed the largest b-wave amplitude, followed by subjects with NDR, and BDR subjects showed the smallest amplitude. However there was no significant difference in amplitude between the groups, $p>0.05$ (Bonferroni pairwise comparisons).

In the control group, the b-wave amplitude at baseline was not found to be significantly correlated with age, $p>0.05$. In subjects with DM the b-wave amplitude was also found not to be significantly correlated with age, disease duration or plasma glucose level at the time of testing, $p>0.05$. No significant correlation was found between b-wave amplitude and HbA1c level or diastolic blood pressure recorded at their most recent hospital visit, $p>0.05$.

One way ANOVA found no significant difference in b-wave amplitudes between subjects with DM when grouped according to their management type

$p>0.05$, i.e. diet-controlled, oral hypoglycaemics, insulin or insulin plus oral hypoglycaemics.

A significant correlation was found between baseline b-wave amplitude in subjects with DM and systolic blood pressure recorded at their most recent hospital visit, $r=-0.396$, $p=0.027$, with amplitudes decreasing with increasing blood pressure.

Multiple linear regression analysis was performed with the following explanatory variables:

- Subject age
- Disease duration
- Plasma glucose level
- Systolic blood pressure
- Diastolic blood pressure
- Management type, grouped as a) diet control or oral hypoglycaemics or b) insulin or insulin plus oral hypoglycaemics
- Retinopathy grouped as a) no retinopathy or b) any retinopathy

None of the explanatory variables were found to be related to b-wave amplitude at baseline, $p=0.290$.

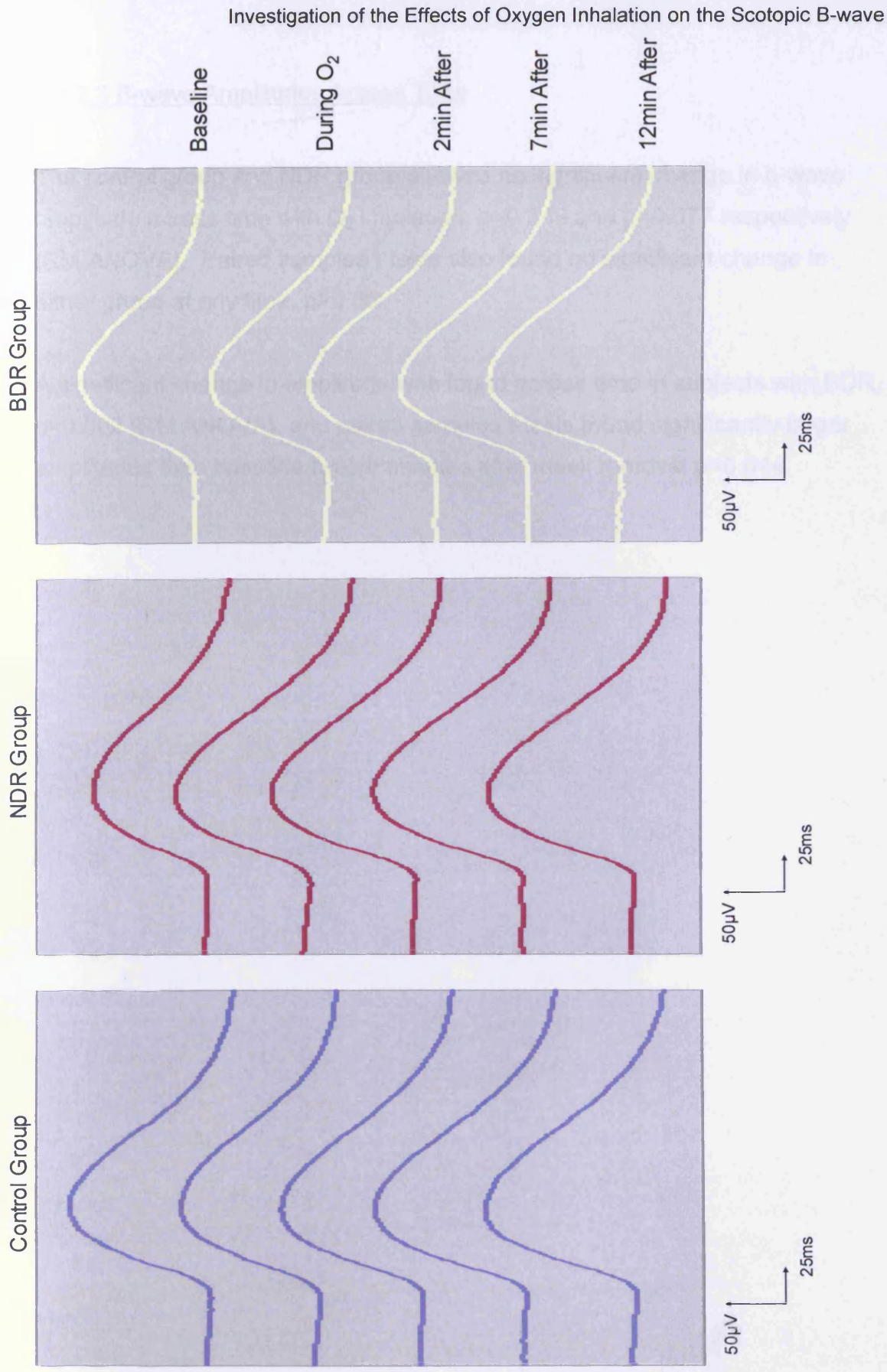

Figure 8.5 Group-averaged b-wave traces.

8.4.2.3 B-wave Amplitudes Across Time

The control group and NDR group showed no significant change in b-wave amplitude across time with O_2 inhalation, p=0.249 and p=0.077 respectively (RM ANOVA). Paired samples t-tests also found no significant change in either group at any time, p>0.05.

A significant change in amplitude was found across time in subjects with BDR, p=0.002 (RM ANOVA), and paired samples t-tests found significantly larger amplitudes than baseline twelve minutes after mask removal p=0.044.

Group		B-wave Amplitudes (μV)				
		Baseline	During O_2	2min After	7min After	12min After
Control	Mean	111.7	112.4	113.9	119.1	118.1
	SD	*34.2*	*52.5*	*41.0*	*46.0*	*51.8*
	SE	*7.0*	*10.7*	*8.4*	*9.4*	*10.6*
NDR	Mean	108.0	108.5	114.4	117.4	116.4
	SD	*35.8*	*46.3*	*42.4*	*41.5*	*45.6*
	SE	*7.2*	*9.3*	*8.5*	*8.3*	*9.1*
BDR	Mean	90.4	93.1	102.0	104.0	**105.4***
	SD	*30.6*	*30.6*	*34.4*	*34.0*	*29.3*
	SE	*8.2*	*8.2*	*9.2*	*9.1*	*7.8*

Table 8.4 B-wave amplitudes across time with O_2 inhalation.
(Statistical significance, compared to baseline value within each group indicated by: * = $p<0.05$, paired samples t-test).

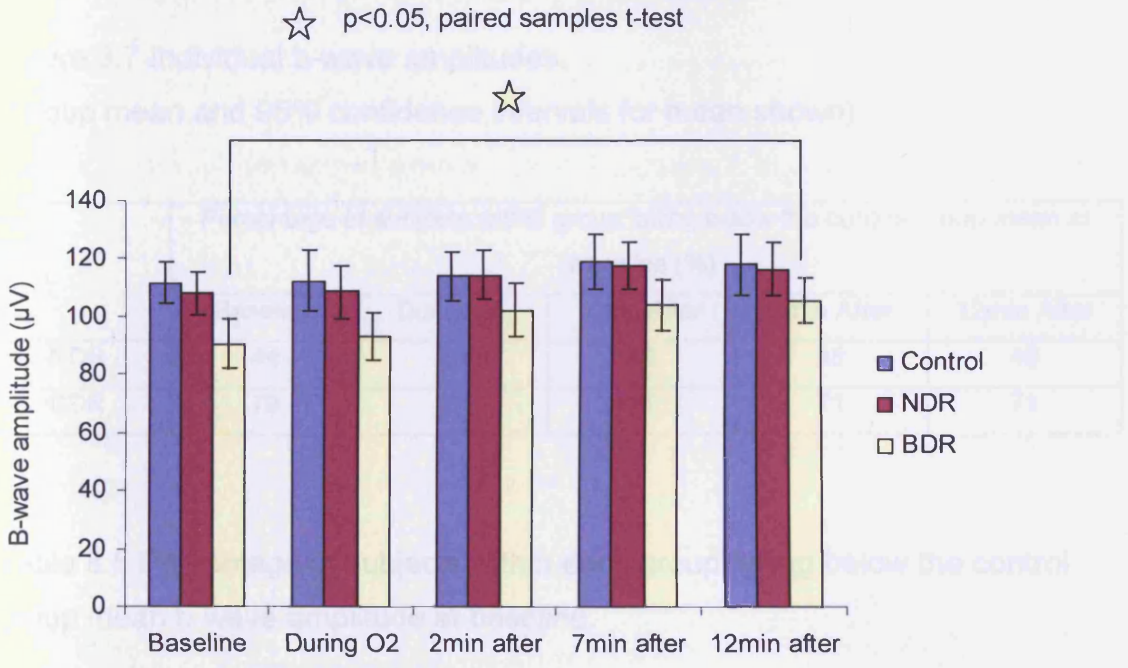

Figure 8.6 B-wave amplitudes across time with O_2 inhalation.

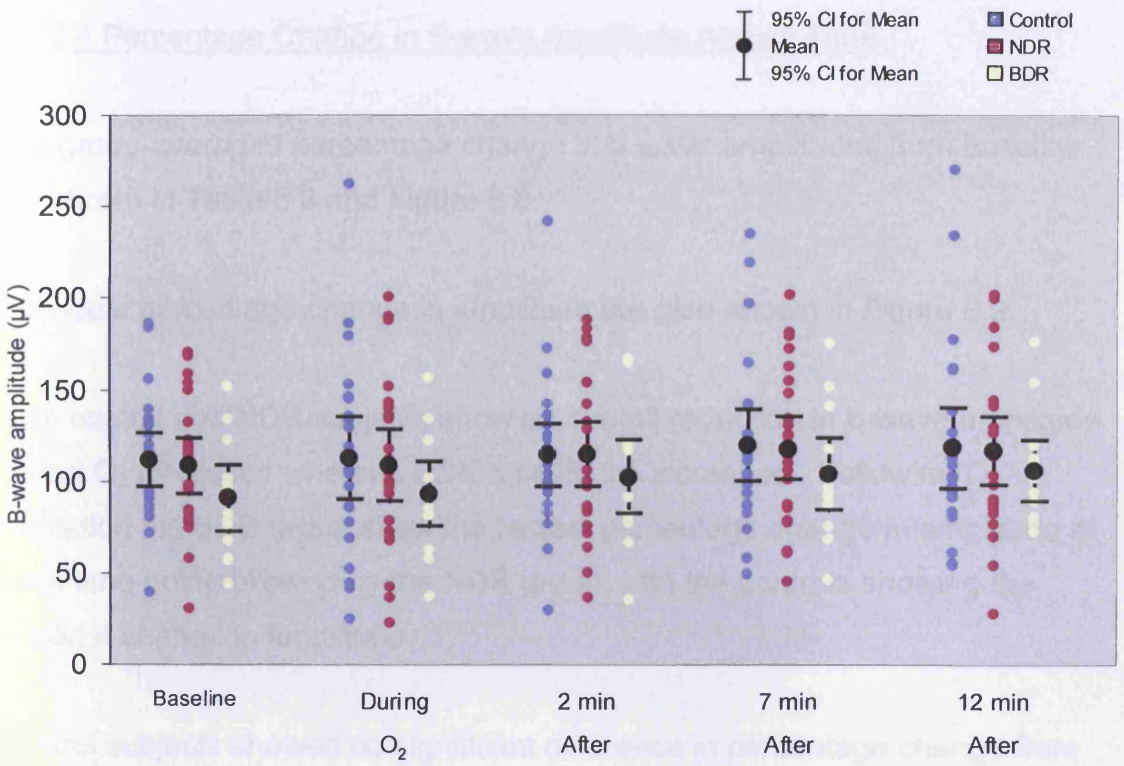

Figure 8.7 Individual b-wave amplitudes.
(Group mean and 95% confidence intervals for mean shown)

	Percentage of subjects within group falling below the control group mean at baseline (%)				
	Baseline	During O_2	2min After	7min After	12min After
NDR	44	60	48	48	48
BDR	79	79	71	71	71

Table 8.5 Percentage of subjects within each group falling below the control group mean b-wave amplitude at baseline.

8.4.2.4 Percentage Change in B-wave Amplitude Across Time

The group-averaged percentage change in b-wave amplitudes from baseline are shown in Table 8.6 and Figure 8.8.

Individual percentage change in amplitude are also shown in Figure 8.9.

Both control and NDR subjects show an overall reduction in b-wave amplitude during O_2 inhalation whereas BDR amplitudes increased. Following O_2 inhalation the BDR group show the largest percentage change in amplitude at each time-point followed by the NDR group, with the controls showing the smallest change in amplitude.

Control subjects showed no significant difference in percentage change from baseline b-wave amplitude during or following O_2 inhalation, $p=0>0.05$ (RM ANOVA, paired samples t-tests).

The NDR group also showed no significant change in amplitude from baseline with O_2 inhalation, $p>0.05$ (RM ANOVA, paired samples t-tests).

The BDR group did show a significant change in amplitude with O_2 inhalation, $p=0.001$ (RM ANOVA), and paired samples t-tests found a statistically significant increase in amplitude of 21.3% twelve minutes after mask removal, $p=0.016$.

Group		% Change in B-wave Amplitude From Baseline			
		During O_2	2min After	7min After	12min After
Control	Mean	-2.8	+1.3	+6.3	+5.3
	SD	*25.1*	*15.1*	*19.5*	*25.0*
	SE	*5.1*	*3.1*	*4.0*	*5.1*
NDR	Mean	-1.1	+5.9	+10.3	+8.2
	SD	*22.1*	*15.0*	*19.1*	*24.1*
	SE	*4.4*	*3.0*	*3.8*	*4.8*
BDR	Mean	+4.8	+14.8	+18.3	**+21.3***
	SD	*20.2*	*23.6*	*28.2*	*22.9*
	SE	*5.4*	*6.3*	*7.5*	*6.1*

Table 8.6 Percentage change in b-wave amplitudes across time with O_2 inhalation.
(Statistical significance, compared to baseline value within each group indicated by: *= $p<0.05$, paired samples t-test).

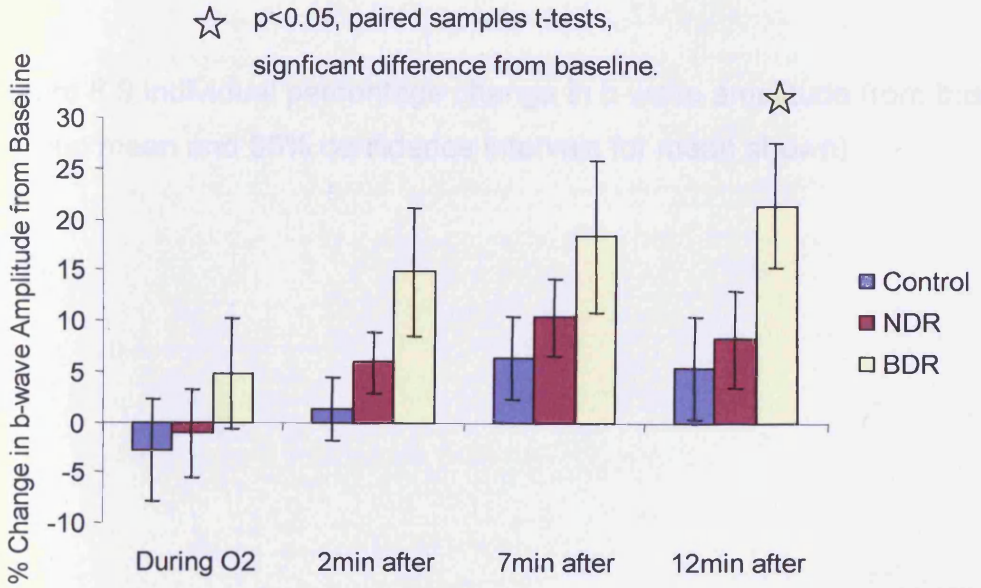

Figure 8.8 Percentage change in b-wave amplitudes across time with O_2 inhalation.

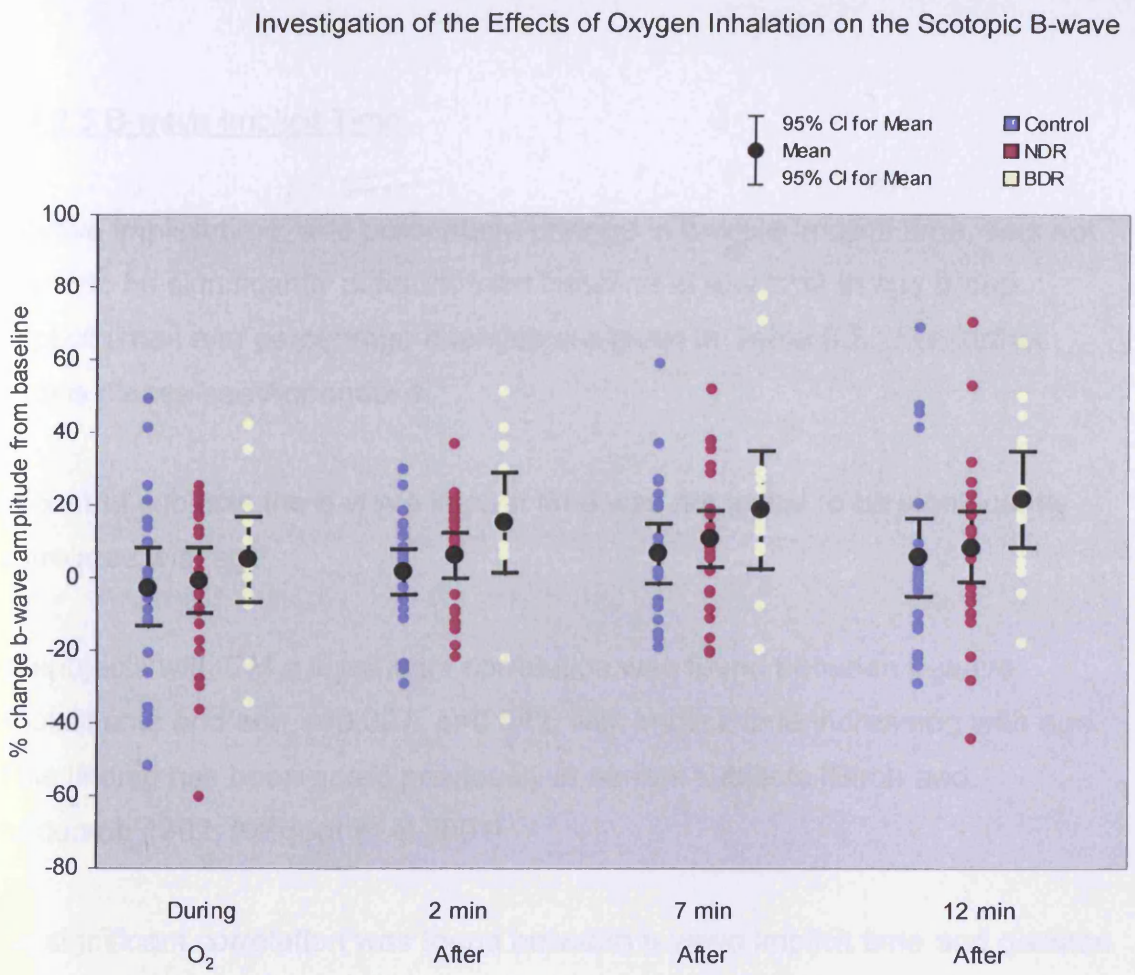

Figure 8.9 Individual percentage change in b-wave amplitude from baseline. (Group mean and 95% confidence intervals for mean shown)

8.4.2.5 B-wave Implicit Time

B-wave implicit time, and percentage change in b-wave implicit time, was not found to be significantly different from baseline at any time in any group. Implicit times and percentage changes are given in Table 8.7. For further details please see Appendix 4.

In control subjects the b-wave implicit time was not found to be significantly correlated with age.

In subjects with DM a significant correlation was found between b-wave implicit time and age, $r=0.327$, $p=0.042$, with implicit time increasing with age. This finding has been noted previously in normal subjects [Birch and Anderson 1992, Kergoat et al 2001].

No significant correlation was found between b-wave implicit time and disease duration or plasma glucose level at the time of recording, $p>0.05$. Also no significant correlation was found between b-wave implicit time and HbA1c level, systolic or diastolic blood pressure recorded at their most recent hospital visit $p>0.05$. One way ANOVA also found no significant difference in b-wave implicit time when grouped according to their management type, $p>0.05$

B-wave implicit times did not change significantly across time with O_2 inhalation in any group, $p>0.05$ (RM ANOVA, paired samples t-tests).

Group		B-wave Implicit time (ms)				
		Baseline	During O_2	2min After	7min After	12min After
Control	Mean	50.0	49.8	49.0	49.5	49.8
	SD	*4.7*	*4.5*	*5.9*	*4.3*	*4.8*
	SE	*1.0*	*0.9*	*1.2*	*0.9*	*1.0*
NDR	Mean	49.7	49.5	50.8	50.4	51.6
	SD	*5.5*	*5.5*	*4.8*	*5.3*	*4.0*
	SE	*1.1*	*1.1*	*1.0*	*1.1*	*0.8*
BDR	Mean	48.0	49.7	49.5	51.3	51.4
	SD	*6.7*	*6.2*	*5.8*	*6.9*	*4.1*
	SE	*1.8*	*1.7*	*1.5*	*1.8*	*1.1*
Group		% Change B-wave Implicit time From Baseline				
		Baseline	During O_2	2min After	7min After	12min After
Control	Mean		-0.1	-1.7	-0.7	-0.1
	SD	NA	*8.5*	*10.2*	*6.6*	*6.6*
	SE		*1.7*	*2.1*	*1.4*	*1.3*
NDR	Mean		+0.6	+3.2	+2.2	+5.2
	SD	NA	*13.0*	*13.0*	*11.4*	*16.1*
	SE		*2.6*	*2.6*	*2.3*	*3.2*
BDR	Mean		+4.9	+4.4	+8.3	+8.8
	SD	NA	*17.4*	*15.3*	*18.8*	*16.3*
	SE		*4.7*	*4.1*	*5.0*	*4.4*

Table 8.7 B-wave implicit times and percentage change in b-wave implicit times across time with O_2 inhalation.

8.4.3 Summary of Significant Findings

	RM ANOVA	Paired Samples T-Test
B-wave Amplitudes Across Time		
BDRs	**Significant change across time p=0.002**	**Significantly higher amplitude 12min (p=0.044) after mask removal.**
Percentage Change in B-Wave Amplitudes Across Time		
BDRs	**Significant change across time, p=0.001**	**Significant 21.3% 12min after mask removal, p=0.016**

Table 8.8 Summary of significant b-wave changes with O_2 inhalation.

8.4.4 Arterial Oxygen saturation

As mentioned in the previous chapter, measurement of arterial O_2 saturation (SaO_2) was only possible on a small sample of subjects. Subject characteristics are given in Table 8.9.

Group	Gender	Age (yrs)	Disease Duration (yrs)	Plasma Glucose Level (mmol/L)	Management Type	Retinopathy Level (ETDRS Scale)
Control	3 ♀ 3 ♂	Mean 56.8 (SD ±10.9) Range 41-67	-	-	-	-
NDR	3 ♀ 5 ♂	Mean 61 (SD ±3.4) Range 59-64	Mean 8.3 (SD ±5.4) Range 3-18	Mean 8.7 (SD ±4.4) Range 6.1-17.8	2=diet control 1=oral hypoglycaemics 2=insulin 3=insulin + oral hypoglycaemics	8=L10
BDR	0 ♀ 3 ♂	Mean 57 (SD ±11.4) Range 44-65	Mean 7.7 (SD ±4.7) Range 4-13	Mean 9.2 (SD ±7.6) Range 4.9-18	1=diet control 0=oral hypoglycaemics 2=insulin 0=insulin + oral hypoglycaemics	2=L14 1=L43

Table 8.9 Subject characteristics.

Mean group-averaged arterial SaO_2 in the twenty-three minutes before, the five minutes during and the twelve minutes following O_2 inhalation are shown in Table 8.10 and Figure 8.10. As in the previous chapter it can be seen that SaO_2 begins to rise within approximately two minutes of inhalation, increases by about 1% and peaks approximately two minutes following the end of the inhalation period, returning to baseline within about five minutes of the end of inhalation. It therefore does not correlate with b-wave amplitude which continued to increase with O_2 inhalation up to seven minutes after mask removal.

No significant difference in SaO_2 was found between the groups before, during or after O_2 inhalation, $p>0.05$ (Bonferroni pairwise comparisons).

Group		SaO_2 Before Inhalation (%)	SaO_2 During Inhalation (%)	SaO_2 After Inhalation (%)
Control	Mean	94.7	95.4	95.6
	SD	*1.3*	*1.0*	*1.6*
	SE	*0.5*	*0.4*	*0.7*
NDR	Mean	95.2	96.1	95.6
	SD	*1.0*	*0.8*	*0.8*
	SE	*0.3*	*0.3*	*0.3*
BDR	Mean	95.4	96.3	96.1
	SD	*0.9*	*0.6*	*1.2*
	SE	*0.5*	*0.3*	*0.7*

Table 8.10 Group-averaged SaO_2 before, during and following O_2 inhalation.

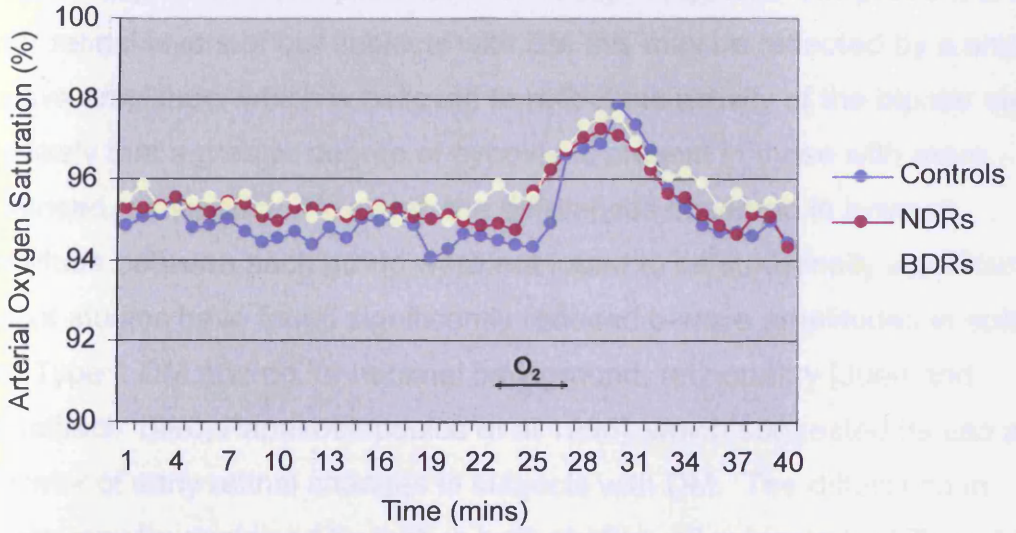

Figure 8.10 Group-averaged SaO_2 before, during and following O_2 inhalation

8.4.5 Discussion

It is known that the autoregulation of blood flow in the normal retina is little more than adequate to cope with the high O_2 demand of the retina during dark adaptation [Arden at al 1998]. In subjects with DM this situation becomes more worrying since autoregulation is known to be impaired and changes in the blood saturation and capillary function are likely to lead to a level of sub-clinical hypoxia within the inner retinal tissue.

As expected, the control group showed the highest b-wave amplitudes, followed by the NDR group with the BDR group showing the smallest amplitudes. Induced hypoxia has been shown to reduce the b-wave of the ERG in subjects with DM [Rimmer et al 1996]. If hypoxia was present in the inner retinal layers of our subjects with DM this may be reflected by a smaller b-wave amplitude, which is believed to reflect the activity of the bipolar cells. It is likely that a greater degree of hypoxia is present in those with more advanced retinopathy. However, the differences observed in b-wave amplitude between each group were not found to be statistically significant. Earlier studies have found significantly reduced b-wave amplitudes in subjects with Type 1 DM and no, or minimal background, retinopathy [Juen and Kieselbech 1990, Papakostopoulos et al 1996], which suggested its use as an indicator of early retinal changes in subjects with DM. The difference in results may be explained by that, in both studies, all subjects had Type 1 DM and all were receiving insulin, whereas in the present study all subjects had Type 2 DM and only nine subjects were receiving insulin. Disease duration was similar between the earlier studies and the present study however in the present study DM subjects were considerably older.

However, in another study of subjects with both Type 1 and Type 2 DM, with no or minimal BDR, no significant difference in b-wave amplitude was found when compared to a control group [Holopigian et al 1992]. Management type was not stated so the number of patients receiving insulin is unknown. In this study b-wave amplitudes in the BDR group were not different to the control group amplitudes at any point. The difference in findings between the studies

may be explained by the fact that those subjects with Type 1 DM are known to be more at risk of developing retinopathy [Klein et al 1984a,b] than those with Type 2 DM, and as such their retinae are likely to be in a more hypoxic state. This may be reflected by a reduction in b-wave amplitudes.

Earlier studies have also found a delay in b-wave implicit time in subjects with DM and no, or minimal, BDR when compared to controls [Juen and Kieselbech 1990, Holopigian et al 1992]. As with the study by Papakostopoulos et al [1996], this investigation found that b-wave implicit time was not significantly different among the groups.

Following O_2 inhalation only the BDR group showed an increase in b-wave amplitude. O_2 inhalation would not be expected to affect control group amplitudes since autoregulation should compensate for the increased O_2 by reducing retinal blood flow. In subjects with DM, where autoregulation has been found to be impaired, an increase in b-wave amplitude might indicate that inner retinal hypoxia was reduced. These results provide support for this hypothesis with the significant increase in b-wave amplitudes in those subjects with visible retinopathy. Earlier work by our research group also found a significant increase in b-wave amplitude in a small group of subjects with Type 2 DM and NDR (n=7) following five minutes of O_2 inhalation, two minutes after mask removal [Chiti 2001].

It may be expected that those NDR subjects with the smallest b-wave amplitudes at baseline may have a greater degree of inner retinal hypoxia, and therefore may show the greatest increase with O_2 inhalation. NDR subjects with initially low b-wave amplitudes were investigated and this was not found to be the case. Those DM subjects with initially low b-wave amplitudes were also investigated for similarities in subject characteristics i.e. disease duration, plasma glucose level, management type etc, however none were found.

As in Chapter 7 the arterial O_2 saturation measurements were taken from the subjects' fingertip and therefore do not directly indicate the level of

oxygenation within the retinal tissue. From personal communication with a local anaesthetist it was felt that it would take some time for the increase in inspired O_2 to result in an increase of oxygenation to the tissue and subtle changes in previously hypoxic retinas may take ten to fifteen minutes from the initial increase in inspired O_2. This may explain the increase observed in b-wave amplitude twelve minutes after mask removal, and may explain why they had not increased during inhalation as not enough time from breathing the O_2 had elapsed.

B-wave implicit time did not appear to be affected by O_2 inhalation in either group in this study. This was also found to be the case in previous work by our research group [Chiti 2001].

8.5 General Summary

This chapter aimed to investigate the effect of O_2 inhalation upon retinal function in subjects with DM. This study does provide support for the suggestion of inner retinal hypoxia during dark adaptation in subjects with DM and visible background retinopathy since a significant increase in b-wave amplitude was found in subjects with BDR following O_2 inhalation.

9. INVESTIGATION OF THE OPTIMUM LIGHT LEVEL REQUIRED TO SUPPRESS THE ROD RESPONSE

9.1 Aim of the Study

It has been proposed by Arden [2001] that a reduction in rod activity, and thus lower metabolic demands, may avoid the rod driven hypoxia which could trigger the development of DR, see Chapter 4 section 4.4. The aim of this study was therefore to use electrophysiological techniques to investigate the amount of light required to suppress the rod response whilst mimicking conditions of sleep.

9.2 Preliminary Trials

In order to find a suitable technique to investigate the optimum light level required to suppress the rod response two preliminary trials were conducted. Firstly, stimulus duration and luminance required in order to gain a good rod response were investigated. Secondly, the techniques for the introduction of background illumination whilst mimicking conditions of sleep were investigated.

9.2.1 Stimulus Duration and Luminance

The use of a dim red stimulus in the dark adapted eye has been shown to produce a simultaneous ERG recording from the rods and cones, with a phase difference since the rods respond more slowly than the cones [Carr and Siegel 1982]. The technique used in the current study was based on one previously employed by our group in the investigation of visual deficits in dyslexia [Greatrex and Drasdo 1998]. The original settings used for this are outlined in Table 9.1

Parameter	Setting
Time window (ms)	500
Low frequency filter (Hz)	1
High frequency filter (Hz)	100
Sensitivity (μV)	100
Number of Sweeps	8
Field Size	Ganzfeld
Wavelength of Stimulation (nm)	655
Frequency of Stimulation (Hz)	1.3
Stimulus duration (ms)	400
Stimulus Luminance ($cd.m^{-2}$)	1.3875, 2.41, 5.915

Table 9.1 Original settings for the cone-rod ERG.

In order to bring the settings outlined in Table 9.1 nearer to ISCEV recommendations for flash ERGs it was decided to investigate the use of a 5 ms flash. The luminance of the LEDS required to produce the above luminance levels for flash durations of both 5 ms and 400 ms were calculated and measured for the LED stimulator with a photometer. It was not possible to produce the highest of the three levels with the LED stimulator in Table 9.1 with the 5 ms flash duration.

Following twenty minutes of dark adaptation, a luminance-response series was produced with the two lowest stimulus intensities and 5 ms flash duration, and with three stimulus intensities and 400 ms flash duration. The LED stimulator was held directly over the eye. A four minute gap was given between the recordings at each luminance. It has been previously shown that the retinal sensitivity will return to baseline within this time and thus any adaptation effects avoided [Greatrex 1999]. An example of the traces is shown in Figure 9.1

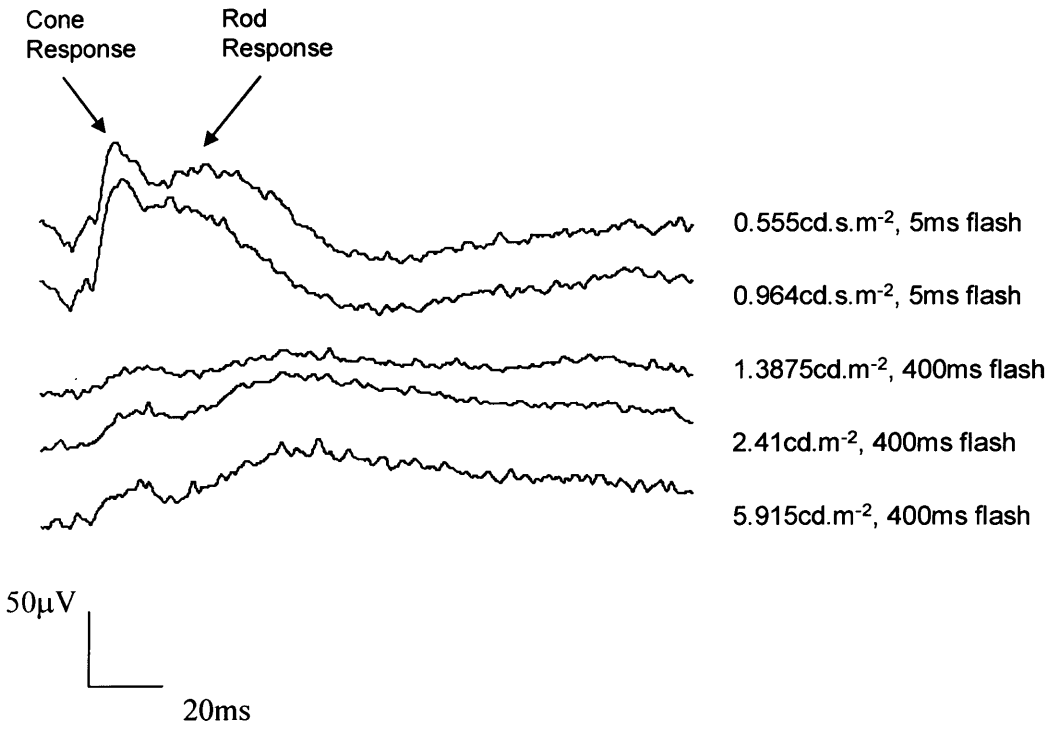

Figure 9.1 Luminance-response series of the cone-rod ERG from a 24 year-old male subject with dilatated pupils.

As shown above the 400 ms flash did not provide as good a separation of the cone response and rod response as the 5 ms flash, which showed particularly good separation at the lower luminance level. In view of this, further luminance-response series were constructed at lower luminance levels for both the 5 ms and 400 ms flash on a small group of subjects. The stimulus intensities used are given in Table 9.2.

9.2.1.1 Method

Pupils were dilatated to a minimum of 6 mm diameter with 0.5% Tropicamide. Subjects were then dark-adapted for twenty minutes before recording the first response. A series of cone-rod ERGs were then recorded with the 5ms duration flash beginning with the lowest luminance level. This was then repeated with the

400ms flash. Four minutes of dark adaptation were given between each ERG recording.

Luminance Level for 5msec Flash Duration ($cd.s.m^{-2}$)	Onset Luminance Setting for 400msec Flash Duration ($cd.m^{-2}$)
0.022	0.1625
0.095	0.26
0.135	0.3675
0.175	0.47
0.264	-

Table 9.2 Stimulus intensities used to record the cone-rod ERG.

9.2.1.2 Subject Group

The subject group consisted of 7 healthy controls, 2 male, 5 female, aged 20-29 years, mean age 24.1 years (SD ±3.1). The test eye was assigned at random.

9.2.1.3 Results

The group averaged traces for the 5 ms and 400 ms flash are shown in Figures 9.2 and 9.3 respectively.

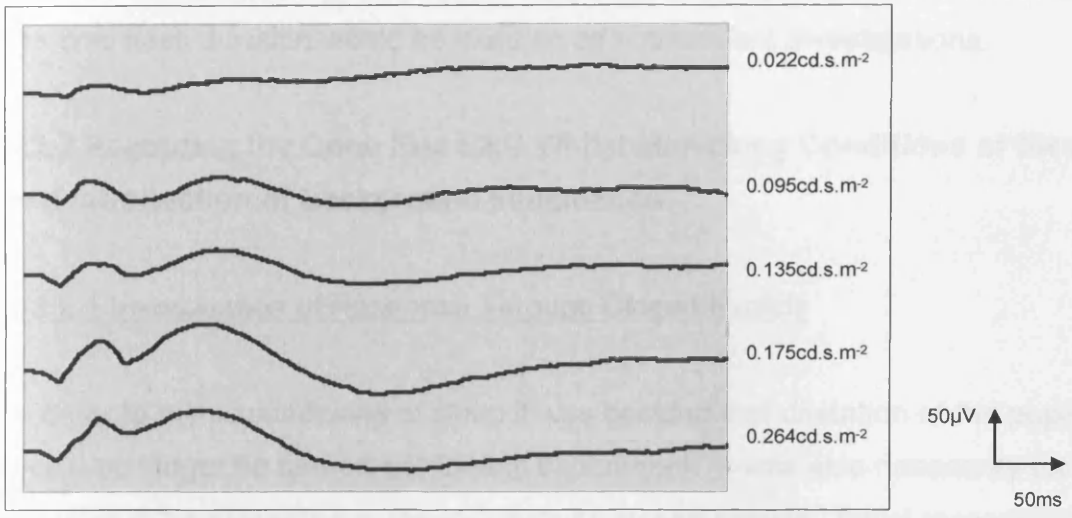

Figure 9.2 Group-averaged cone-rod ERGs, 5 ms flash duration.

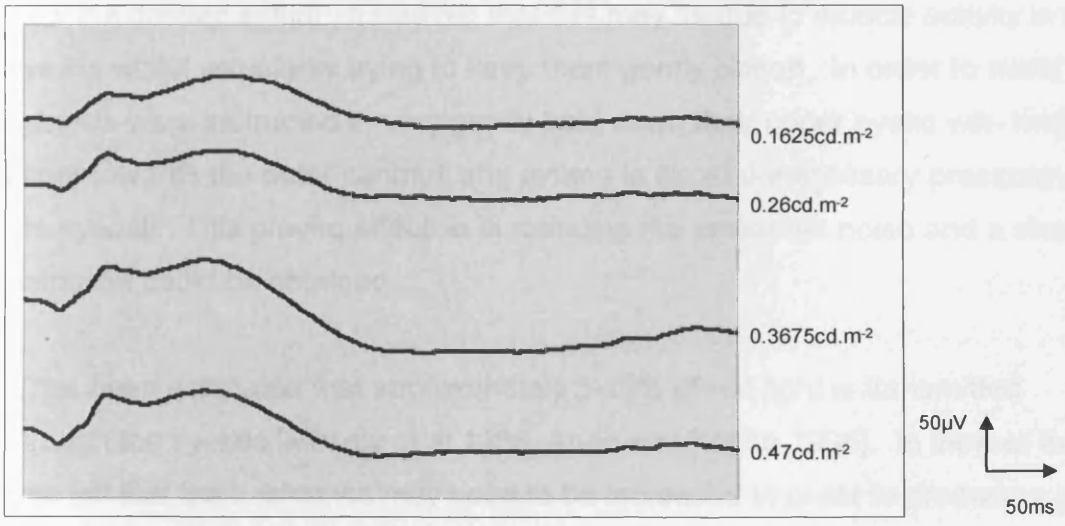

Figure 9.3 Group-averaged cone-rod ERGS, 400 ms flash duration.

9.2.1.4 Conclusion

The 5 ms flash duration proved to provide the best separation of the cone-rod response in all cases. It was also found to be subjectively much more comfortable for the subject than the 400 ms flash. It was therefore decided that the 5ms flash duration would be used on all subsequent investigations.

9.2.2 Recording the Cone Rod ERG Whilst Mimicking Conditions of Sleep and Introduction of Background Illumination

9.2.2.1 Investigation of Response Through Closed Eyelids

In order to mimic conditions of sleep it was decided that dilatation of the pupil would no longer be carried out for this experiment. It was also necessary for the stimulus to be presented to the eye through closed eyelids. Initial recordings through closed eyelids appeared to be very noisy making it almost impossible to view the desired signal. It was felt that this may be due to muscle activity in the eyelids whilst voluntarily trying to keep them gently closed. In order to avoid this, subjects were instructed to very gently hold down their upper eyelid with their finger towards the outer canthus and aiming to avoid unnecessary pressure on the eyeball. This proved effective in reducing the unwanted noise and a clear response could be obtained.

It has been estimated that approximately 5-10% of red light is transmitted through the eyelids [Mosely et al 1988, Ando and Kripke 1996]. In view of this it was felt that the luminance may need to be increased in order to produce a good response. This did not prove to be the case in initial trials on three subjects and good separation of the cone and rod components was apparent even at the lower luminance levels, as shown in Figure 9.4.

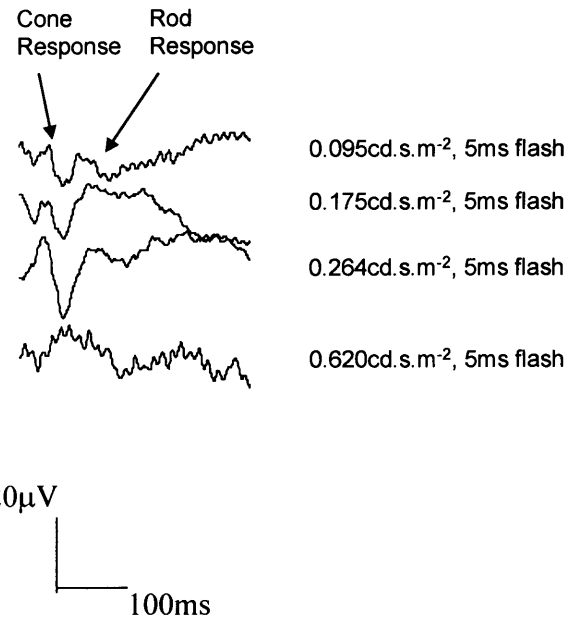

Figure 9.4 Luminance-response series for 5 ms flash from a 48 year-old female subject through closed eyelids and without pupil dilation.

9.2.2.2 Introduction of Background Illumination

9.2.2.2.1 Equipment Used

From the previous experiment it can be seen that a well-defined cone-rod response can be obtained with the 5 ms flash duration in closed eyes with natural pupils, and the best separation was apparent with a stimulus intensity of 0.264 $cd.s.m^{-2}$.

The aim of this experiment was to determine the lowest level of background illumination necessary to reduce or eliminate the rod response. In order to investigate the levels of diffuse background light required to saturate or significantly reduce rod activity, an angle poise lamp, fitted with a diffusing screen, was placed behind the LED stimulator, as shown in Figure 9.5. In order to prevent the LED stimulator blocking background light from the lamp it was

necessary for the LED stimulator to be held 5 cm in front of the subjects' eyelid rather than directly over the eyelid. The luminance of the flash was adjusted to provide equivalent stimulus luminance at 5 cm from the eye and this was found to be true at stimulus intensity 1.21 cd.s.m^{-2}.

The angle poise was then placed behind the LED stimulator at a distance of approximately 70 cm from the subjects' eye. The illuminance from the angle poise was then reduced by means of a combination of 0.3, 0.6 and 0.9 neutral density filters placed over the diffusing screen. This provided a range of illuminance between approximately 0.8 and 30 lux.

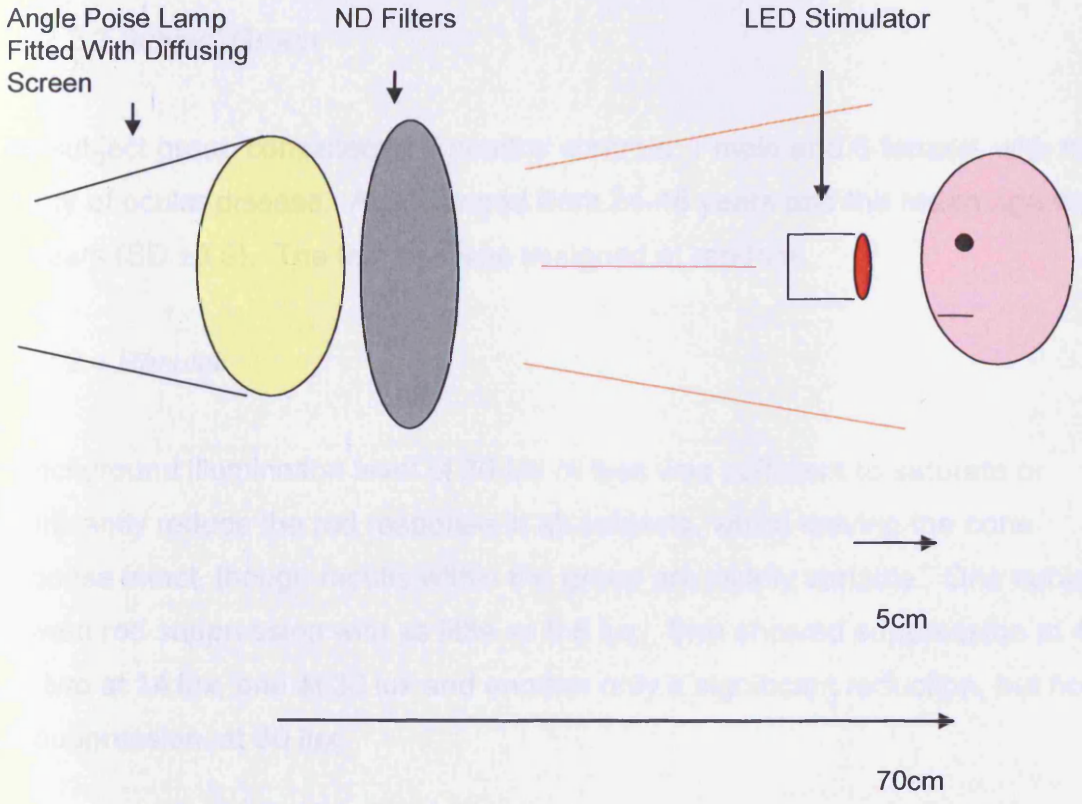

Figure 9.5 Equipment set up to investigate the effect of background illumination on the rod response.

9.2.2.2.2 Method of ERG Recording

Following twenty minutes dark adaptation a cone-rod response was obtained with the stimulator at a distance of 5 cm from the subjects' eye, stimulus luminance of 1.21 cd.s.m^{-2}, and no background illumination. The lowest level of background illumination was then introduced and the subject was allowed to adapt to this light level, with eyes still closed, for five minutes. A second response was then recorded. The background illumination was then increased and again five minutes allowed for light adaptation. This procedure was repeated with increasing levels of background illumination until the rod response had been eliminated or a level of 30 lux had been reached.

9.2.2.2.3 Subject Group

The subject group consisted of 7 healthy controls, 1 male and 6 female, with no history of ocular disease. Ages ranged from 24-48 years and the mean age was 29 years (SD ±8.9). The test eye was assigned at random.

9.2.2.2.4 Results

A background illumination level of 30 lux or less was sufficient to saturate or significantly reduce the rod response in all subjects, whilst leaving the cone response intact, though results within the group are widely variable. One subject showed rod suppression with as little as 0.8 lux. Two showed suppression at 4 lux, two at 14 lux, one at 30 lux and another only a significant reduction, but not full suppression, at 30 lux.

An example of the cone-rod responses is shown in Figure 9.6.

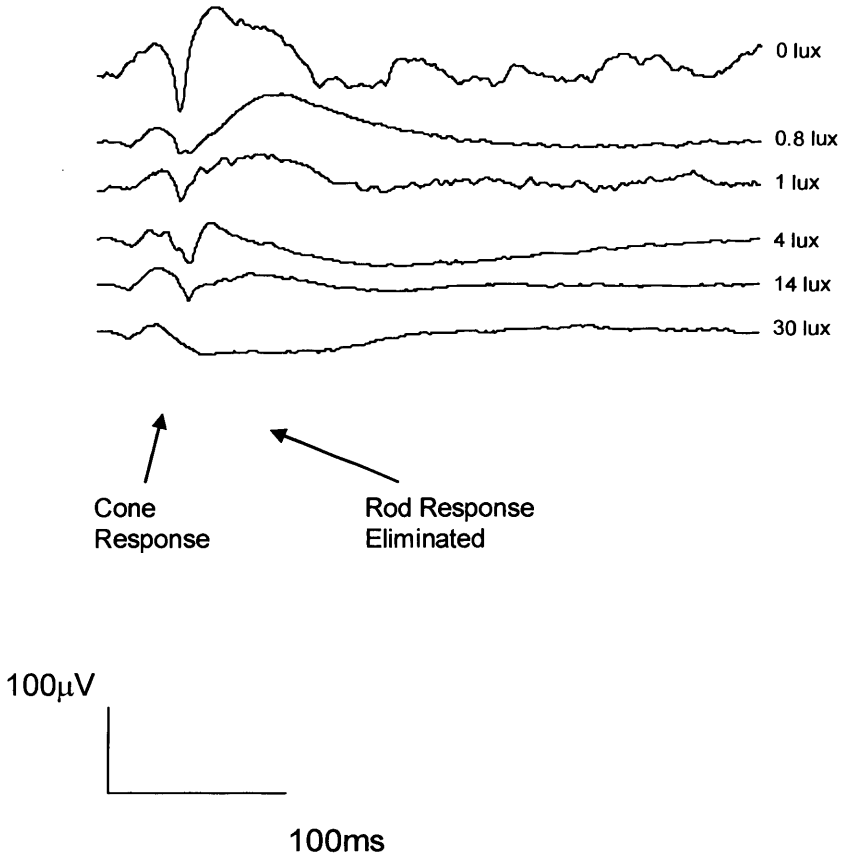

Figure 9.6 Cone-rod ERGs recorded from a 24 year-old female subject with increasing background illumination. Level of background illumination is given beside each trace.

9.2.2.2.4 Discussion

Good separation of the cone and rod responses was obtained using the technique described through closed eyelids and with natural pupils. A background illumination level of 30 lux or less was sufficient to saturate or significantly reduce the rod response, whilst leaving the cone response intact.

However, the results within the group were widely variable and it was felt that this may in part be due to the equipment used. Difficulties arose in keeping the

angle-poise lamp at an exact distance of 70 cm from the patient. Moreover, it was unlikely that the LED stimulator remained at exactly 5 cm from the subjects' eye for each recording. In view of this it was decided that employing the LED stimulator to produce the background illumination and the stimulus simultaneously would be preferable. This also provided an opportunity to alter the wavelength of the background illumination. It was decided that the green LED may provide the best background for these recordings since white light may attenuate both the rod and the cone response more than green light, and therefore not be able to show as clear a depression of the rod response as the background illumination increased. The settings on the LED stimulator allowed provision of a good range of illuminance at the eyelid, as well as a completely dark-adapted response.

Initial recordings on control subjects showed less variable responses with the green background and the highest stimulus intensity setting on inspection of the traces. The final settings for the cone-rod ERG are given in Table 9.3.

Parameter	Setting
Time window (ms)	500
Low frequency filter (Hz)	1
High frequency filter (Hz)	100
Sensitivity (μV)	100
Number of Sweeps	24
Field Size	Ganzfeld
Peak Wavelength of Stimulation (nm)	655
Frequency of Stimulation (Hz)	1.3
Stimulus duration (ms)	5
Stimulus Flash Luminance Setting ($cd.s.m^{-2}$)	1.21
Peak Wavelength of Background (nm)	515
Illuminance Levels at Eyelid (lux)	0, 0.7, 3.4, 15.1, 62.4

Table 9.3 Final settings for recording the cone-rod ERG.

9.3 Final Protocol

Subjects were dark-adapted for thirty minutes. Initially some subjects showed no definite cone-rod response after twenty minutes of dark adaptation and it was felt that a further ten minutes of dark adaptation may improve this.

Cone-rod ERGs were then recorded as outlined in Table 9.3. A total of thirty-two flashes were recorded as four groups of eight on separate channels. Signal to noise ratio often proved variable and therefore three sets were chosen and averaged off-line. Responses were recorded through closed eyelids, with natural pupils, with the subject gently holding shut the upper lid as previously described.

Subjects were then adapted to the lowest intensity background for five minutes before recording the next response. This was repeated for each of the subsequent backgrounds in ascending order of intensity.

9.3.1 Subject Group

9.3.1.1 Control Subjects

This group consisted of 11 healthy controls, 7 female and 4 male, with no history of ocular disease. Ages ranged from 39-72 years, mean age 54.8 years (SD ±10.0). The test eye was assigned at random.

9.3.1.2 Subjects with DM

This group consisted of 10 subjects with Type 2 DM, 4 female and 6 male with no visible retinopathy. Ages ranged from 57-72 years, mean age 65.3 years (SD ±5.2). Disease duration ranged from 2.5-12 years, mean duration 6.4 years (SD ±3.0). The test eye was assigned at random.

9.3.2 Results

The individual and group averaged rod b-wave amplitudes are given in Table 9.4 and can be seen in Figures 9.7 and 9.8.

Within the NDR group subjects showed a mean reduction in rod b-wave amplitude from their fully dark-adapted response of 34% (±10.8) at 0.7 lux, 57% (±10.2) at 3.4 lux, 72% (±10.6) at 15.1 lux and 97% (±3.0) at 62.4 lux. As can be seen in Figure 9.7 results within the group were again quite variable. Subject DM1 was the only subject with DM to show full rod suppression at just 3.4 lux. All subjects, except DM3 and DM4, showed a significant reduction in rod response at 3.4 lux. Subject DM2 also appeared to show full rod suppression at 3.4 lux

however a small rod response can be observed again at 15.1 lux. Three more subjects, DM6, DM8 and DM9 showed full suppression at 15.1 lux and all bar one subject, DM10, showed full suppression at 62.4 lux.

As can be seen in the group averaged trace it would appear that 3.4 lux is enough to significantly reduce the rod response in the subjects with DM, where it is less than half the amplitude of the fully dark-adapted response. This is supported by the results of a RM ANOVA where a significant change with background illumination was found, $p<0.001$. Bonferroni pairwise comparisons found that the reduction at 0.7 lux of background illumination was not statistically significant, $p=0.126$, however the further reduction in rod b-wave amplitude with 3.4 lux, 15.1 lux and 62.4 lux were all found to be statistically significant, $p=0.004$, $p=0.001$ and $p<0.001$ respectively.

Any correlation between the fully dark-adapted rod b-wave amplitude and the duration of DM was assessed using Pearson's correlation, and none was found, $r= -0.001$, $p=0.998$. Any correlation with age was also assessed and again none was found, $r=0.218$, $p=0.545$. Also the amount of light required to suppress the rod response was not found to correlate with either duration of DM, $r=0.506$, $p=0.165$, or with age, $r=0.302$, $p=0.429$.

In general, the rod response appeared to saturate with lower levels of background illumination in the control group than the NDR group. Within the control group subjects showed a mean reduction in rod b-wave amplitude from their fully dark-adapted response of 39% (±12.3) at 0.7 lux, 84% (±4.8) at 3.4 lux, 97% (±2.1) at 15.1 lux and 98% (±2.3%) at 62.4 lux. One subject, C6 showed full rod suppression with as little as 0.7 lux. A further 5 subjects, C2, C3, C4, C6 and C10 showed full suppression at 3.4 lux. Those remaining all showed a greater than 60% reduction in their rod b-wave amplitude at this level. At 15.1 lux only two subjects, C7 and C8, still had some rod response remaining though both

showed a greater than 80% reduction in amplitude and at 62.4 lux only C7 had a small rod response remaining.

As with the NDR group 3.4 lux appears to be sufficient in either suppressing or significantly reducing the rod response. Again this is supported by the results of a RM ANOVA where a significant reduction in rod b-wave amplitude with increasing background illumination was found, $p<0.001$. Significant reductions in rod b-wave amplitude were found with 3.4 lux, 15.1 lux and 62.4 lux, $p<0.001$ in all 3 cases.

Again no correlation was found between the subjects age and the amplitude of the fully dark-adapted b-wave using Pearson's correlation, $r= -0.379$, $p=0.263$. The amount of light required to suppress the rod response was also found not to correlate with age, $r=-0.497$, $p=0.143$.

There was no significant difference in amplitude between the groups at any light level, $p>0.05$ (Bonferroni pairwise comparisons).

In summary, a light level of 3.4 lux at the closed eyelid appears to be sufficient in significantly reducing the rod response in all subjects.

Subject	Age (yrs)	Disease Duration (yrs)	Rod b-wave amplitudes (μV)				
			0 lux	0.7 lux	3.4 lux	15.1 lux	62.4 lux
DM1	72	3	36.2	20.8	0	0	0
DM2	63	2.5	15.9	8.64	0	2.19	0
DM3	61	10	15.4	18.9	13.9	11.8	0
DM4	72	7	12.5	14.5	10.9	9.28	0
DM5	66	7	9.43	9.26	6.32	6.97	0
DM6	60	4	11.4	5.87	2.28	0	0
DM7	68	7	14.7	5.67	6.62	3.6	0
DM8	64	5	26.7	16.9	15.1	0	0
DM9	57	12	32.1	12.5	12	0	0
DM10	70	6	47	10.5	13.9	9.23	13.9
Group Mean (±SE)	65.3 (±1.6)	6.4 (±0.9)	22.1 (±4.0)	12.4 (±1.7)	8.1 (±1.9)	4.3 (±1.5)	1.4 (±1.4)
C1	55	NA	24.6	6.95	7.76	0	0
C2	41	NA	18.9	18.8	0	0	0
C3	63	NA	9.17	6.19	0	0	0
C4	62	NA	8.34	8.31	0	0	0
C5	72	NA	15.2	21.1	4.45	0	0
C6	64	NA	14.9	0	0	0	0
C7	55	NA	23.6	10	5.53	4.28	6.03
C8	39	NA	29.3	23.6	10.5	4.8	0
C9	51	NA	36.2	15.3	12.8	0	0
C10	51	NA	12.6	6.15	0	0	0
C11	50	NA	78.3	17.6	16.7	0	0
Group Mean (±SE)	54.8 (±3.0)	NA	24.6 (±6.0)	12.2 (±2.3)	5.2 (±1.8)	0.8 (±0.6)	0.5 (±0.5)

Table 9.4 Rod b-wave amplitudes with increasing levels of background illumination.

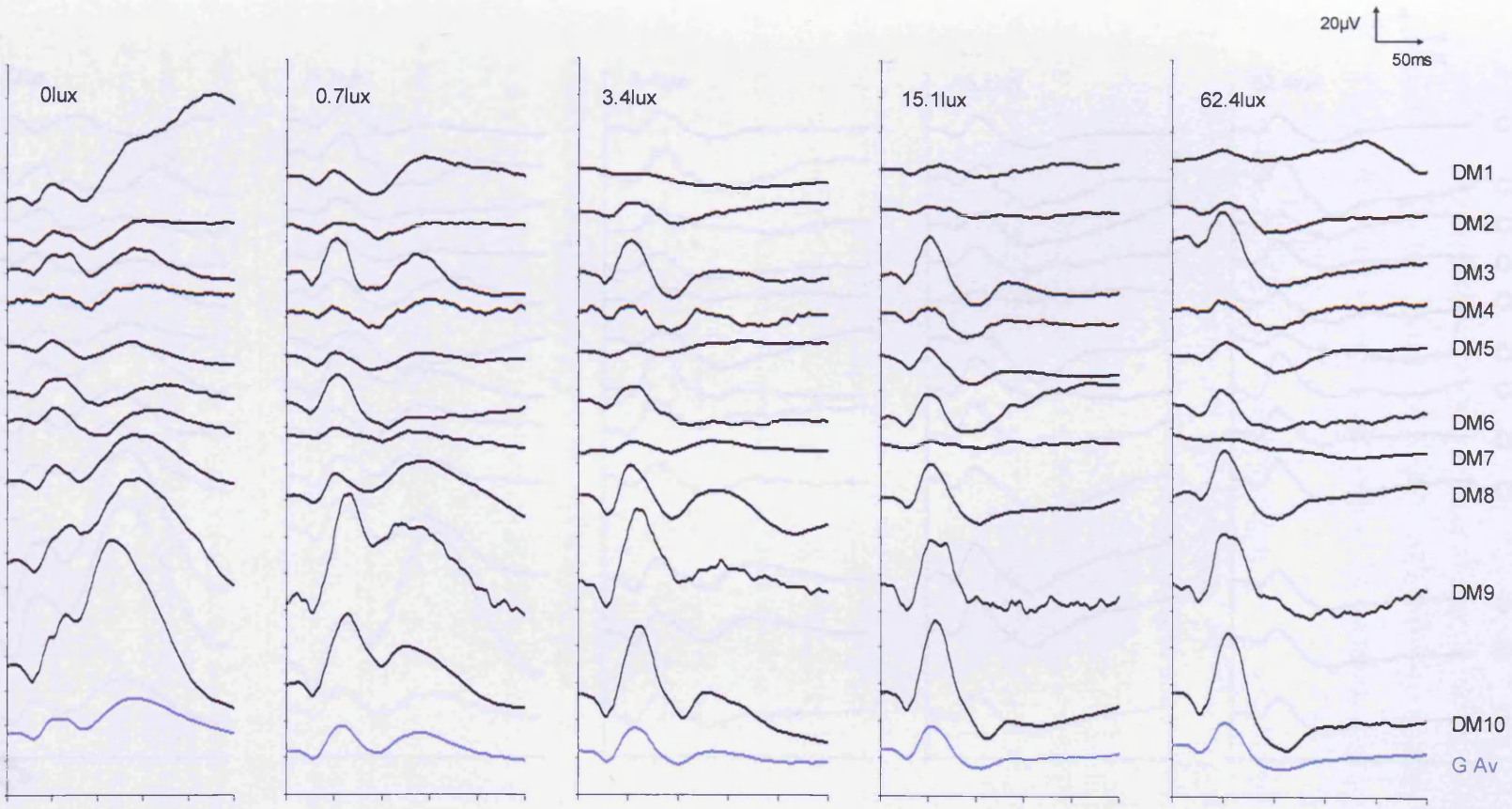

Figure 9.7 Individual and group-averaged cone-rod ERGs from subjects with NDR with increasing background illumination

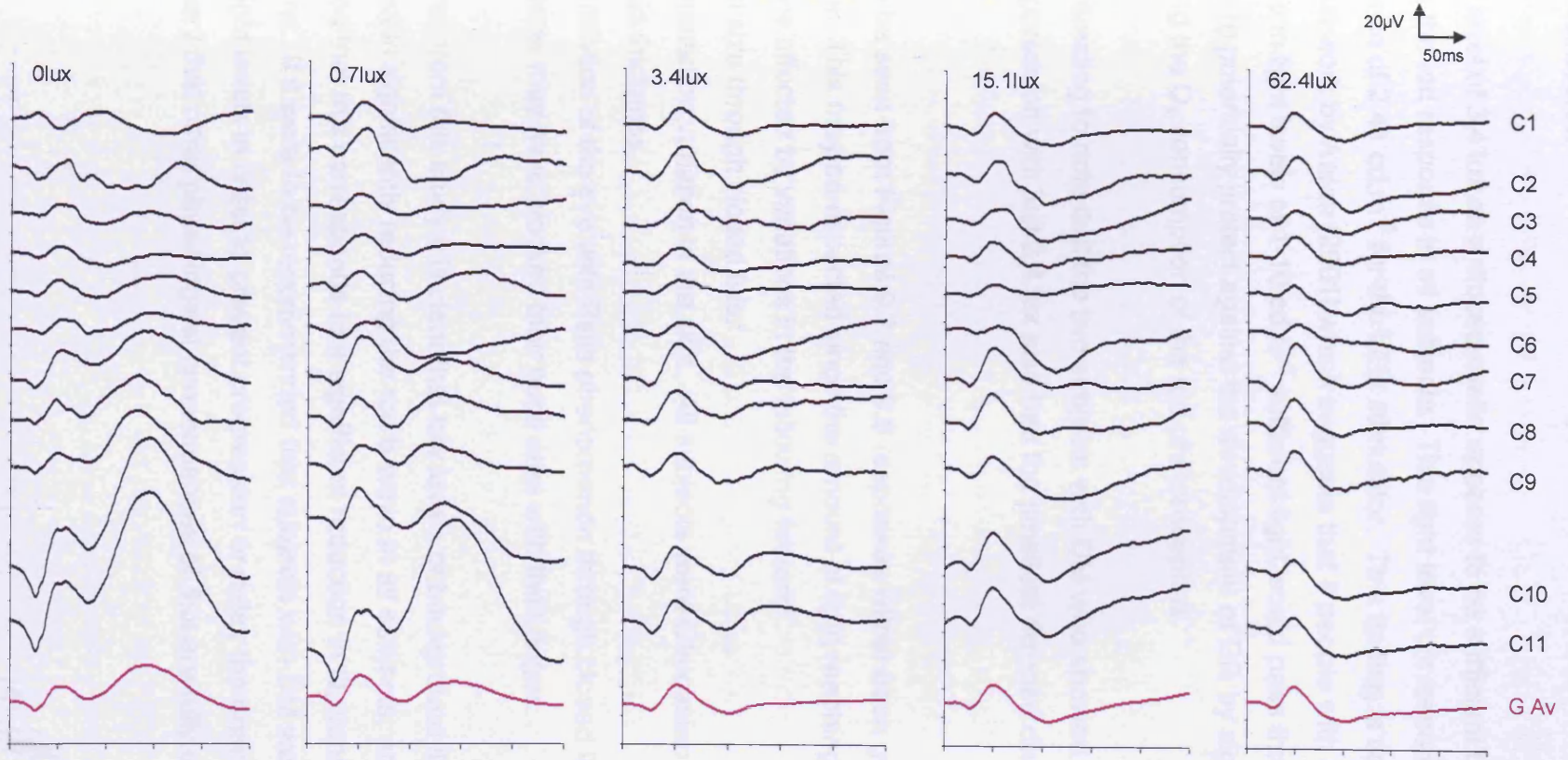

Figure 9.8 Individual and group-averaged cone-rod ERGs from control subjects with increasing background illumination

9.3.3 Discussion

A light level of 3.4 lux on a closed eyelid appears to be sufficient to significantly reduce the rod response in all subjects. This light level corresponds to a stimulus luminance of 2.41 $cd.m^{-2}$ for the LED stimulator. This finding is consistent with previous work by Arden [2001] which suggests that if people with diabetes were to sleep in light levels of 1-10 $cd.m^{-2}$ sufficient light would pass through the eyelids to potentially protect against the development of DR by significantly reducing the O_2 consumption of the rod photoreceptors.

It is interesting to note that the two subjects with DM who showed the earliest full rod suppression with just 3.4 lux also had the smallest disease duration within the group.

As can be seen from Figures 9.7 and 9.8 responses within each group were quite variable. This may be expected since the amount of light reaching the retina would be affected by variations in the following factors:

1. Pupil size through closed lids.
2. Pigmentation variation in the skin. All subjects were Caucasian.
3. Eyelid thickness.
4. Any rotation of the eye with Bells phenomenon through closed lids.
5. Subjects may have blocked their pupil area with their finger.

However, from this study it is clear that low levels of background illumination are sufficient in significantly reducing the rod b-wave in all subjects, and it is assumed that this corresponds to a significant reduction in O_2 consumption within the retina. If it were to be recommended that subjects with DM were to sleep in these light levels in order to prevent progression or delay the onset of DR, it is necessary that other physiological consequences of this are fully understood.

It has been suggested that light exposure at night within the first two years of life may be a causal factor in the development of myopia. Quinn et al [1999] reported a strong association between ambient light exposure during sleep at night, and that the relationship between refraction and night-time light was dose-dependent, in a group of 479 children attending an out-patients ophthalmology clinic. This study, however, did not control for parental myopia and many studies since have shown no link between light exposure at night and myopia development [Zadnik et al 2000, Gwiazda et al 2000, Saw et al 2001, Saw et al 2002, Guggenheim et al 2003].

A recent study in Japan found that alternating-shift work is a significant independent risk-factor the development of diabetes mellitus [Suwazono et al 2006]. Increased incidence of high blood pressure, sleep disorders and stress have also been reported in night-shift and alternating-shift workers when compared to day-shift workers [Boggild and Knutsson 1999, Knutsson 2003].

Melatonin is secreted by the pineal gland and its secretion is known to peak during the night. Exposure to light at night has been shown to suppress the production of melatonin [McIntyre et al 1989b, Reiter 1991]. Melatonin is known to have oncostatic actions and the most prominent mechanisms proposed to explain this are its antimiotic and antioxidant activity [Brzezinski 1997], as well as potential modulations of some cell cycle lengths [Mediavilla et al 1999].

Blask et al [1999] also described circadian stage-dependent interactions between melatonin and fatty acid metabolism which have been associated with inhibition of carcinogenic tumour growth in rats. It has been proposed that exposure to light at night may not only reduce the oncostatic actions of melatonin by its reduced production, but may increase the risk of breast cancer in women. The decrease in melatonin production led to a rise in levels of reproductive hormones such as oestrogens, thereby stimulating the growth of hormone-sensitive tumours in the breast [Davis et al 2001]. Observational studies using night-shift work as a

surrogate for light at night show fairly strong evidence for a relationship between this and breast cancer risk [Tynes et al 1996, Davis et al 2001, Schernhammer and Schulmeister 2004a,b]. A later study however found no association between night shift work and an increased risk of breast cancer amongst a group of women in Long Island, USA. Interestingly though they did find an increased risk in those women who reported rising frequently during the week and turning on lights multiple times during the night [O'Leary et al 2006].

Exposure to light at night may also increase the risk of other cancers within the body. A significantly elevated risk of colorectal cancer has also been reported in a group of female nurses who had worked for more than fifteen years on rotating night shifts than those who had never worked on a rotating shift [Schernhammer et al 2003].

This study has shown that a light level of 3.4 lux is sufficient to significantly reduce the rod response. This level of light would not be sufficient to suppress the release of melatonin which requires levels of 350 lux or greater [McIntyre et al 1989a, Mayeda et al 1998]. It has also been shown that short wavelength light has the greatest effect on suppression of melatonin [Thapan et al 2001, Wright and Lack 2001].

If sleeping under low levels of background illumination were to be recommended to subjects with DM, it is important that all the physiological and psychological consequences of this are fully understood. Possible intermittent illumination for short periods during the night, or illumination only on a few nights a week could be investigated as potential treatment, but it is clear that further research into the effects of this is required.

9.4 General Summary

A background illumination level of 3.4 lux was sufficient in significantly reducing the rod response in all subjects, which is believed to correspond to a significant reduction in O_2 consumption within the retina. As it is hypothesised that hypoxia triggers the development of DR, sleeping under low levels of background illumination may be beneficial for subjects with DM by delaying the development, or slowing the progression of DR. However, since light exposure at night may have other detrimental physiological and psychological consequences, further investigation is required before this could be recommended as a treatment for subjects with DM.

10. INVESTIGATION OF OCULAR BLOOD FLOW FOLLOWING LIGHT AND DARK ADAPTATION

10.1 Aim of the Study

In normal healthy subjects retinal blood flow is known to increase in scotopic conditions (see Chapter 4, section 4.5.1), but it is not known if this is also true in subjects with DM. The original aim of this study was to assess the effects of light and dark adaptation on retinal and ocular blood flow directly using the Heidelberg Retinal Flowmeter (HRF) and pulsatile ocular blood flow (POBF) respectively, and indirectly by recording photopic and scotopic OPs, in both control subjects and subjects with DM and no visible retinopathy. Unfortunately it was not possible to measure retinal blood flow by means of the HRF due to persistent equipment failure.

10.2 Preliminary Trial

POBF reflects a combination of retinal and choroidal blood flow, predominantly the choroidal blood flow which is responsible for 85% of the total blood flow [Langham et al 1989]. Pulsatile variation in IOP is due to the systolic bolus of blood entering the choroidal circulation. The OBF system (OBF Labs, UK) measures this pulsatile component of ocular blood flow. The IOP is monitored continuously and the pressure pulse wave form recorded by a modified pneumotonometer. This waveform is then converted to a volume pulse by a computerised system.

10.2.1 Repeatability of POBF Measurements

Since more than one measure of POBF would be recorded it was first necessary to determine whether or not it was repeatable over consecutive measurements.

10.2.1.1 Subject Group

The subject group consisted of 9 healthy controls, 3 female and 6 male, with no history of ocular disease. Ages ranged from 21-63 years, mean age 31 years (±13.5 SD). The test eye was assigned at random.

10.2.1.2 Method

POBF was measured using the Langham OBF Tonometer (OBF Labs, UK) subsequent to the cornea being anesthetised with 0.5% proxymetacaine. Measurements were carried out by a single trained optometrist and the integrity of the cornea checked following recordings with the instillation of 0.25% fluorescein sodium stain.

Subjects were instructed to sit for five minutes before measurements commenced to reduce the effects of any changes in blood pressure following exercise [Lanzi et al 1996]. POBF was then recorded in normal room light conditions at time 0 min, 2 min, 7 min and 17 min.

10.2.1.3 Results

Time (min)	0	2	7	17
Mean POBF (µl/min) (±SE)	818.8 (±104.6)	823.9 (±97.2)	764.4 (±92.9)	801.0 (±97.3)

Table 10.1 Repeatability measurements of POBF over time.

RM ANOVA found no significant change in POBF over time, p=0.132.

10.2.1.4 Conclusion

The measurements of POBF were found to be repeatable over time. This is in agreement with previous work where POBF measurement with the OBF tonometer has proven to be a repeatable, reliable technique [Spraul et al 1995, Massey et al 1996, Yang 1997].

10.3 Final Protocol

- Pupils dilatated to a minimum of 5.5 mm diameter with 1 drop of 1% Tropicamide.
- Subjects were light adapted using the LED stimulator to a white light, intensity 1090 $cd.m^{-2}$ for five minutes
- Photopic OPs recorded monocularly to four white flashes, intensity 3 $cd.s.m^{-2}$, 3 ms duration at 1.5 s intervals (ISCEV standard).
- POBF was recorded once in the same eye using the POBF tonometer (OBF Labs UK Ltd) and a hand-held adaptor.
- Subjects were then dark adapted for twenty minutes.
- POBF recorded again with the use of long wavelength dim illumination.
- Scotopic OPs were recorded monocularly to six white flashes, 3 $cd.s.m^{-2}$, 3 ms duration at 15 s intervals (ISCEV standard), with the first two flashes treated as conditioning flashes.
- Subjects were then light adapted again to a white background, intensity 1090 $cd.m^{-2}$, for five minutes.
- Photopic OPs and POBF measurements repeated.
- Plasma glucose levels were recorded at the end of the visit.

10.3.1 Subject Groups

10.3.1.1 Control Subjects

This group consisted of 7 healthy, age-matched control subjects, 4 female and 3 male, with no history of ocular disease. Ages ranged from 49-72 years, mean age 61 years (SD ±10.2). The test eye was assigned at random.

10.3.1.2 Subjects with DM

This group consisted of 9 subjects with Type 2 DM, 2 female and 7 male, with no visible retinopathy. Ages ranged from 56-74 years, mean age 65 years (SD ±5.2). Disease duration ranged from 3-18 years, mean duration 8.4 years (SD ±6.0).

There was no significant difference in age between the groups, p=0.381.

10.3.2 Results

10.3.2.1 POBF Results

The mean POBF was greater in the DM group than the controls, but not significantly so, p>0.05.

POBF showed an increase of 9.1% following dark adaptation in the control group, and a further increase of 2.1% following re-light adaptation. These changes were not significant, p=0.081 RM ANOVA, see Figure 10.1 and Table 10.2.

It was only possible to record POBF in six of the nine subjects with DM. In these subjects, POBF showed a similar increase of 10.9% following dark adaptation,

but also decreased by 10.4% following re-light adaptation. Again, these changes were not significant, p=0.448 RM ANOVA, see Figure 10.1 and Table 10.2.

There was no significant difference in POBF between the two groups at any of the three time points, $p>0.05$.

In subjects with DM no correlation was found between baseline POBF and age, $r=-0.070$, $p=0.895$, or between baseline POBF and plasma glucose level, $r=0.143$, $p=0.787$. However, a significant positive correlation was found between POBF and disease duration, $r=0.986$, $p<0.01$, see Figure 10.2.

Group	POBF Light (µl/min) (±SE)	POBF Dark (µl/min) (±SE)	POBF Light (µl/min) (±SE)
Control	777.9 (±95.0)	849.0 (±78.0)	866.4 (±91.6)
NDR	881.9 (±118.8)	978.0 (±109.5)	876.5 (±110.5)

Table 10.2 POBF results following light and dark adaptation.

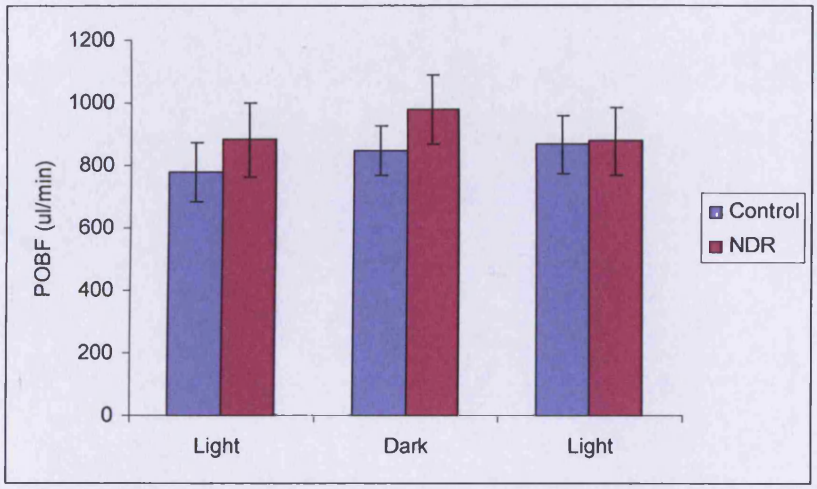

Figure 10.1 POBF results following light and dark adaptation.

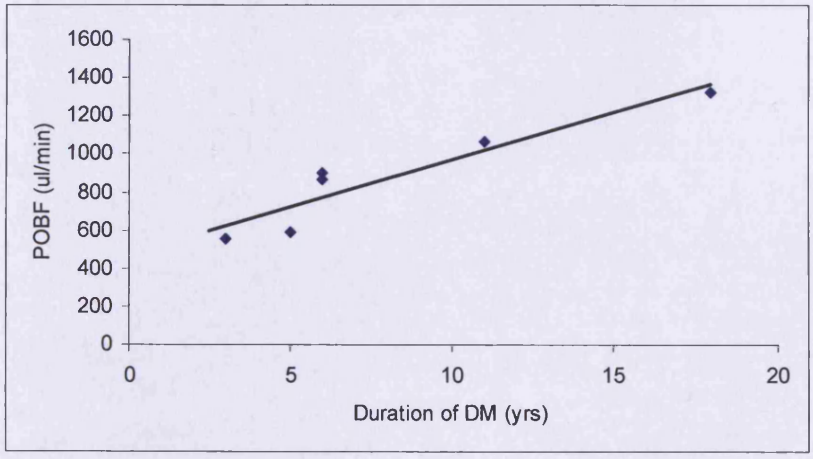

Figure 10.2 Relationship between POBF and duration of DM.

10.3.2.2 OP Results

OPs showed an increase in summed OP amplitude of 4% following dark adaptation in the control group. They then decreased by 9.3% following re-light adaptation, however these changes were not statistically significant, p=0.877 RM ANOVA, see Figures 10.3, 10.5 and Table 10.3.

In subjects with DM a greater increase in summed OP amplitude of 22.6% following dark adaptation was found. They too decreased following re-light adaptation by 31%, see Figures 10.4, 10.5 and Table 10.4. These changes were found to be significant, p=0.012 RM ANOVA. Bonferroni pairwise comparisons found no significant increase in summed OP amplitude from photopic to scotopic conditions p=0.314, though found the decrease in amplitude from scotopic back to photopic conditions statistically significant, p=0.036.

There was no significant difference in amplitude between the two groups at any of the three time points, p>0.05 (Bonferroni pairwise comparisons).

In subjects with DM no significant correlation was found between either the scotopic or photopic amplitudes with POBF, age, plasma glucose level or disease duration, p>0.05.

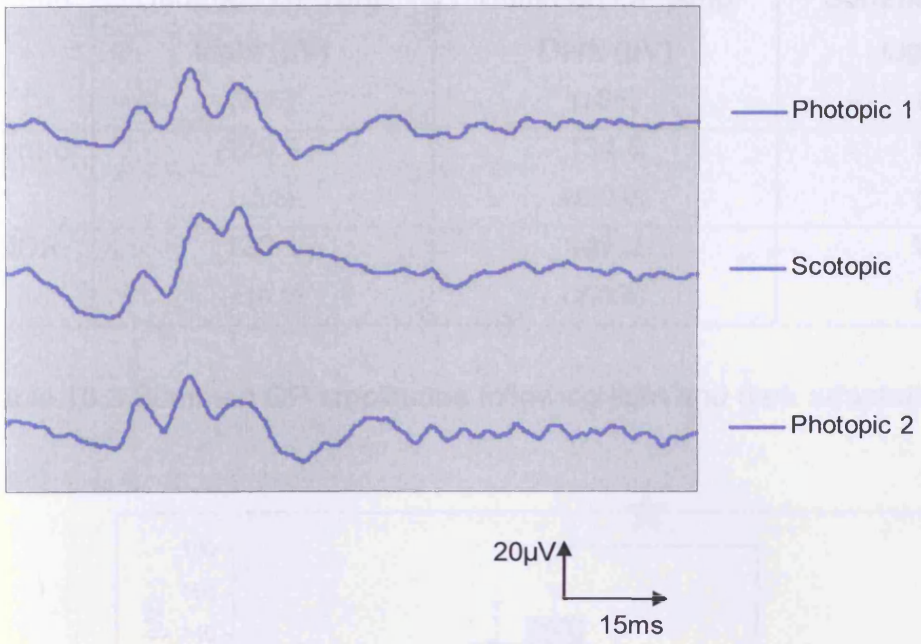

Figure 10.3 Group averaged summed OP amplitudes in the control group.

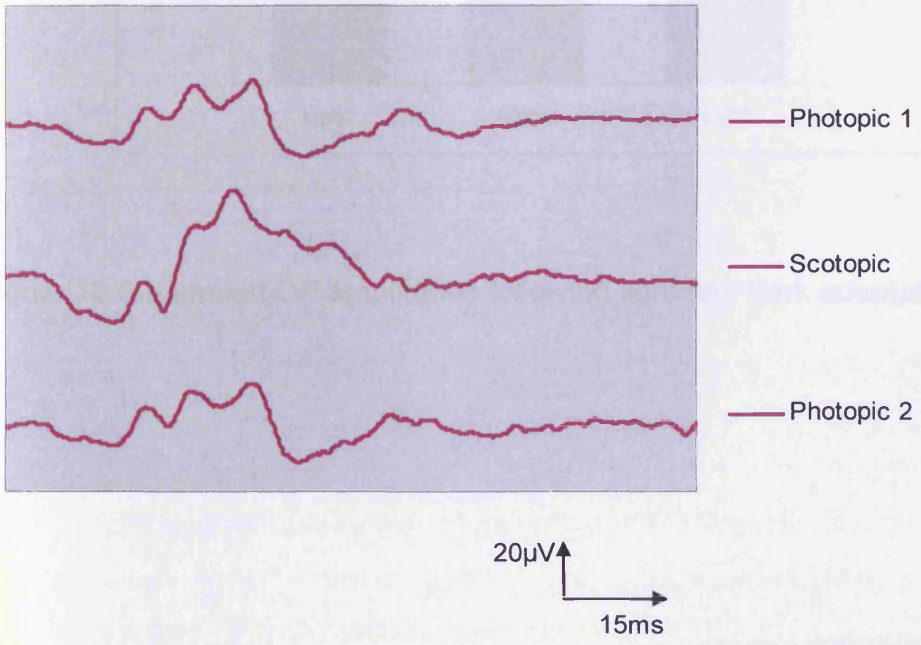

Figure 10.4 Group averaged summed OP amplitudes in subjects with DM.

Group	Summed OP Amp Light (μV) (±SE)	Summed OP Amp Dark (μV) (±SE)	Summed OP Amp Light (μV) (±SE)
Control	129.4 (±5.3)	134.5 (±20.6)	122.0 (±8.9)
NDR	120.1 (±10.0)	147.2 (±15.2)	101.6 (±9.7)

Table 10.3 Summed OP amplitudes following light and dark adaptation.

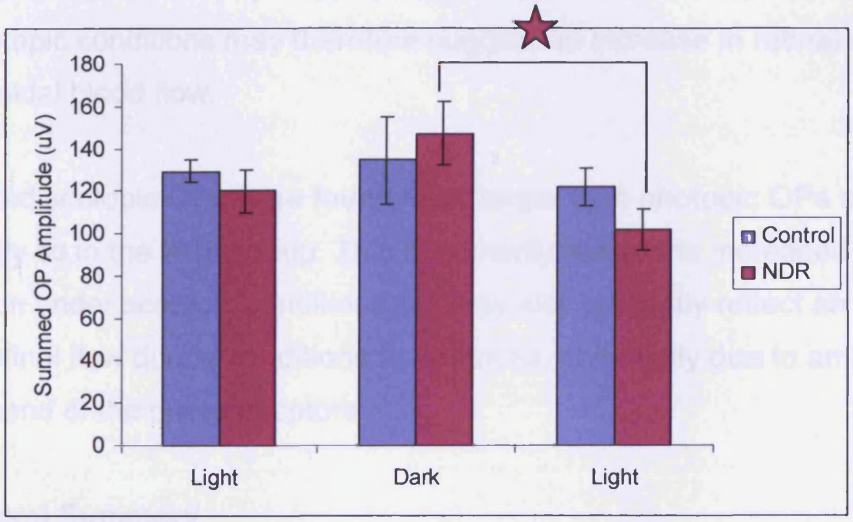

Figure 10.5 Summed OP amplitudes following light and dark adaptation.

10.3.3 Discussion

POBF showed no significant change in either group between conditions of light and dark. POBF was found to be larger in scotopic conditions and decrease following light adaptation in the NDR group, a pattern similar to that found with the OPs, though these changes were not significant. POBF is thought to reflect both the choroidal and retinal circulation. Previous studies have shown that choroidal flow decreases following dark adaptation in control subjects and returns to normal following six to ten minutes of light adaptation [Longo et al 2000, Fuchsjäger-Maryl et al 2001]. The non-significant increase observed in POBF under scotopic conditions may therefore suggest an increase in retinal rather than choroidal blood flow.

As expected scotopic OPs were found to be larger than photopic OPs and significantly so in the NDR group. This is primarily due to the increased rod contribution under scotopic conditions but may also indirectly reflect an increase in inner retinal flow during conditions of darkness, potentially due to an increase in O_2 demand of the photoreceptors.

10.4 General Summary

From this study it appears that ocular blood flow may increase in the dark in subjects with DM and no visible retinopathy, however the small number of subjects make it difficult to draw any definite conclusions. Further study of the retinal blood flow in subjects with DM is required, potentially using Laser Doppler Flowmetry to gain a more direct measure of retinal blood flow under both photopic and scotopic conditions. Subjects with BDR were difficult to recruit for this investigation but it would be useful to extend this investigation to include them to see if there is any difference in response.

11. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

11.1 General Discussion

The first aim of this study was to use the ERG in order to gain an objective measure of retinal function in subjects with DM. Diabetic retinopathy is the major cause of registerable blindness in the working population in Western countries [Evans 1996]. It has been proposed that in DM a sub-clinical level of tissue hypoxia is present within the retina prior to the development of DR [Arend et al 1991, Linsenmeier et al 1998] and it is believed that this hypoxia may be a causal factor in the development of some functional visual defects in subjects with DM. Contrast sensitivity [Harris et al 1996], colour vision [Dean et al 1997] and summed OPs [Drasdo et al 2002] have all found to be reduced in subjects with DM with no or minimal retinopathy, and have been shown to be reversible with O_2 inhalation.

There are thought to be approximately 115 million rods within the human retina which function at low light levels. They have a very high metabolic demand and, as such, have a markedly high level of O_2 consumption. Autoregulation of retinal blood flow is little more than adequate to cope with the O_2 demand of the normal retina during dark adaptation [Arden et al 1998] and, since subjects with DM are known to often have changes to their blood and capillary function, it seems likely that a sub-clinical tissue hypoxia may develop during darkness.

Chapters 7 and 8 investigated the effects of O_2 inhalation upon the ERG following dark adaptation. If inner retinal hypoxia were present in subjects with DM following dark adaptation, this may be reflected by reduced OP amplitudes, thought to arise mainly from the amacrine cells of the inner retina [Holpigian et al 1992, Wachtmeister 1998], and by reduced b-wave amplitudes or delayed b-wave implicit times, believed to reflect the activity of the bipolar cells [Green et al 1995, Shiells and Falk 1999].

In Chapter 7 no significant difference was found in either summed or individual OP amplitudes between subjects with DM, either with or without retinopathy, and the age-matched control group. Previous studies have shown reduced OP amplitudes in subjects with DM [Simonsen 1980, Moschos et al 1987, Van Der Torren and Van Lith 1989, Juen and Kieselbech 1990, Holopigian et al 1992, Drasdo et al 2002], however some other studies have shown no difference in OP amplitudes between subjects with DM and no visible retinopathy and age-matched controls [Wanger and Persson 1985, Van Der Torren and Mulder 1993].

In this investigation higher mean summed OP amplitudes, though not significantly so, were found in the NDR group compared with the control group mean. This finding has been observed previously in a group of subjects with Type 1 DM with either NDR or mild BDR [Simonsen 1980], where it was found that the hypernormal oscillatory potential observed excluded the development of proliferative retinopathy within the next six to eight years.

No significant difference in OP implicit times was observed between control subjects and either of the diabetic subject groups, which has also been reported previously [Coupland 1985b, Wanger and Persson 1985, Holopigian et al 1992].

Summed OP amplitude did not increase significantly in subjects with NDR following O_2 inhalation, which differs from the findings of Drasdo et al [2002]. This may be explained by the fact that 11 of the 23 subjects with NDR displayed summed OP amplitudes falling above the control group mean amplitude at baseline. This may suggest that these subjects were at less risk of developing retinopathy than those subjects with depressed OP amplitudes [Simenson 1980], and as such it may be presumed that the retinae may therefore be less hypoxic. In the Drasdo et al study [2002] the subject group exhibited initially depressed OP amplitudes which suggests that this group of subjects were more at risk of developing retinopathy and the retinae were in a more hypoxic state. If this were the case, a greater effect with O_2 inhalation would be expected.

O_2 inhalation was not expected to affect the control group OP amplitudes since retinal autoregulation should compensate for the extra O_2 by reducing retinal blood flow and this investigation found that they did not change significantly. In subjects with DM, where autoregulation has been found to be impaired, an increase in summed OP amplitude was expected as any inner retinal hypoxia is reduced. Subjects with BDR on average showed lower OP amplitudes compared with the control group but not significantly so. The results from this study may indirectly reflect a level of hypoxia within the retinae of subjects with DM, since OPs are known to reflect inner retinal function and summed OP amplitude increased significantly following O_2 inhalation in subjects with BDR. Also, OP3 amplitude, which is believed to have a more dominant rod contribution [Lachapelle et al 1983, Coupland 1987a], significantly increased following O_2 inhalation in subjects with NDR.

The amplitudes of OP1 and OP2 were found to increase significantly with O_2 inhalation in subjects with BDR. This may not have been expected since the earlier OPs are believed to be cone-dominated and the later OPs rod-dominated [Lachapelle et al 1983, Coupland et al 1987a, Janáky et al 1996], however this is subject to some controversy [Wang et al 2001].

In Chapter 8, no significant difference was observed in either b-wave amplitude or implicit time between subjects with DM, either with or without retinopathy, and the age-matched control group. Again results from previous studies are mixed. Some have shown reduced b-wave amplitudes [Juen and Kieselbach 1990, Papkostopoulos et al 1996] and delayed b-wave implicit times [Juen and Kieselbach 1990, Holopigian et al 1992] in subjects with DM and no or minimal BDR when compared to controls. Others have shown no difference in b-wave amplitude [Holopigian et al 1992] or implicit time [Papkostopoulos et al 1996] in subjects with DM and no or minimal BDR when compared to controls. In this investigation control subjects showed the highest b-wave amplitudes, followed by the NDR group with the BDR group showing the smallest amplitudes, however the difference in amplitudes of the diabetic groups were not significant.

Following O_2 inhalation, the greatest increase in b-wave amplitude was apparent in the BDR group, followed by the NDR group with the least effect visible in the control group. O_2 inhalation was not expected to affect control group amplitudes since autoregulation should compensate for the extra O_2 by reducing retinal blood flow. In subjects with DM, where autoregulation has been found to be impaired, the increase in b-wave amplitude may indicate that any inner retinal hypoxia was reduced. These results provide support for this hypothesis with the significant increase in b-wave amplitudes in those subjects with visible retinopathy.

Since it has been hypothesised that a rod-driven hypoxia may be a significant contributory causal factor in the development of DR [Arden at al 1998], and that subjects with DM may benefit from sleeping with night-time illumination [Arden et al 2006], the amount of background light required to saturate rod activity within the human retina was investigated. In Chapter 9, by means of a simultaneous cone-rod ERG, it was found that a light level of just 3.4 lux was sufficient in either saturating or significantly reducing the rod response in subjects with DM and no visible retinopathy. This level is consistent with previous work by Arden [2001] who suggests that if subjects with DM were to sleep in levels of 1-10 $cd.m^{-2}$ sufficient light would pass through the eyelids to protect against the development of DR. This level of 3.4 lux is not sufficient to suppress the release of melatonin [McIntyre et al 1989a, Mayeda et al 1998] and therefore should not be associated with an increased risk of cancers which have been reported amongst night-shift workers [Tynes et al 1996, Davis et al 2001, Schernhammer et al 2003, Schernhammer and Schulmeister 2004a,b,].

Retinal blood flow is known to increase in healthy subjects following dark adaptation which is thought to reflect the increased metabolic demands of the photoreceptors [Feke et al 1983, Riva et al 1983, Havelius et al 1999]. Surprisingly choroidal blood flow has been shown to decrease in darkness [Longo et al 2000, Fuchsjäger-Maryl et al 2001]. Chapter 10 aimed to investigate ocular blood flow following dark adaptation in subjects with DM. As expected, scotopic OPs were found to be significantly larger than photopic

OPs in subjects with DM and no visible retinopathy. POBF was also found to be larger in scotopic conditions than photopic but not significantly so and the increase observed was similar to that of the control subjects. Since POBF is thought to reflect both the choroidal and retinal circulation it is presumed that the increase observed in POBF is associated with an increase in retinal rather than choroidal blood flow.

11.2 Conclusions

Scotopic OPs and b-wave were not found to be significantly reduced in amplitude, or delayed in implicit time, in subjects with DM, both with and without retinopathy when compared to age-matched control subjects.

Both scotopic OP and b-wave amplitudes were found to increase significantly following O_2 inhalation in subjects with DM and BDR, and OP3 in subjects with NDR, which lends support to the suggestion of inner retinal hypoxia during dark adaptation in these subjects.

A background illumination level of 3.4 lux is sufficient in saturating or significantly reducing rod activity in subjects with DM and no visible retinopathy.

Ocular blood flow appears to increase in subjects with DM in darkness, presumably due to the increased metabolic demand of the photoreceptors, however these changes were not found to be significant

11.3 Future Work

It may be interesting to investigate the effects of carbogen inhalation on scotopic OPs and b-waves in subjects with DM. As described hyperoxia is believed to significantly reduce blood flow in the human retina and this is believed to be secondary to vasoconstriction of the retinal vessels. It is thought that the addition of CO_2 may reduce the vasoconstrictive effects of hyperoxia [Hickam and Frayser 1966], and as such a hyperoxic-hypercapnic

gas mixture such as carbogen, usually 5% CO_2 + 95% O_2, may be more effective in oxygenating the retinal circulation than inhalation of O_2 alone. A recent study found no change in a-wave or OP1-4 amplitudes with hypercapnia however b-wave and OP5 amplitudes have been shown to decrease ten minutes after a ten minute carbogen inhalation period in normal subjects [Faucher and Kergoat 2002]. As yet the effects of carbogen inhalation on visual function in subjects with DM are unknown. It may be that it will produce the same effects as inhalation of O_2 alone since subjects with DM have been shown to display a reduced vasoconstrictive response to hyperoxia when compared to control subjects [Grunwald et al 1984].

Previous work in monkeys has found that retinal oxygen consumption continues increasing with increasing dark adaptation for up to one hour [Stefánsson et al 1983]. A review of the literature did not reveal a longer testing period than this. In subjects with DM, as the retinal oxygen consumption continues to increase, the retina itself is likely to become increasingly hypoxic. It is unknown exactly how long this would continue for. In this study, OP and b-wave measurements were taken following twenty minutes of dark adaptation and the findings of the above study would suggest that the retina continued to get more hypoxic beyond this time. It would be of interest to investigate whether or not this is the case with prolonged dark-adaptation over several hours, and OP or b-wave amplitudes may reflect signs of any increase in hypoxia during this time.

Chapter 9 investigated the optimum light level required to saturate the rod-response of the simultaneous cone-rod ERG in control subjects and DM subjects with no visible retinopathy. It would be interesting to extend this investigation to subjects with DM who have already developed visible retinopathy, which was not possible here due to problems with recruitment. Since rod function is known to be affected in DM it may be that a different amount of light is required as retinopathy progresses.

In Chapter 9 ERGs were recorded following just thirty minutes of dark adaptation. Following sleep, the retina may well be more hypoxic after much

longer periods in the dark. It would also be of interest to investigate the amount of light required to suppress rod activity after a longer period of dark adaptation where presumably the retina is under a greater degree of hypoxic stress.

In Chapter 10 OP and POBF amplitudes were used to investigate retinal blood flow under photopic and scotopic conditions in subjects with DM. LDV investigations of retinal blood flow would be very useful in gaining a more direct measure of retinal blood flow under both photopic and scotopic conditions. Again, it would also be interesting to investigate whether this is affected by increased diabetic retinopathy.

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Appendix 1
Subject Characteristics

This appendix contains the characteristics of the control subjects and subjects with DM who participated in this study.

Abbreviations used are as follows:

- NDR: diabetic subject with no retinopathy
- BDR: diabetic subject with background retinopathy
- Mgmt: management
- BP: blood pressure
- Ret: retinopathy
- M: male
- F: female

Management type is divided into the following categories:

- 1 = diet control
- 2 = oral hypoglycaemics
- 3 = insulin
- 4 = insulin + oral hypoglycaemics

Plasma glucose was recorded at both the Study One visit and the Study Three visit. They are reported as Study One result / Study Three result.

1a: Control Subject Characteristics

Subject ID	Gender	Age (yrs)
JH	F	49
MG	F	79
GO	M	80
CG	M	71
DJ	M	70
JC	M	52
HC	M	53
MC	F	51
RW	M	64
BA	M	51
BV	F	71
PH	F	72
RB	F	53
SS	M	63
CS	F	61
RC	M	65
TH	M	54
MB	M	50
BH	M	62
PG	F	67
RM	M	45
PG2	F	62
DJ2	M	64
LH	F	53
PM	M	41
RE	M	63
RN	F	51
SH	F	49

Table A1.1 Control subject characteristics

1b: NDR Subject Characteristics

Subject ID	Gender	Age (yrs)	Disease Duration (yrs)	Mgmt Type	Systolic BP (mmHg)	Diastolic BP (mmHg)	Hba1c (%)	Plasma Glucose (mmol/L)
JG	M	65	5	2	-	-	-	10.6/10.8
HP	M	55	12	2	-	-	-	7.6/8.0
CA	M	70	7	1	130	76	7.1	6.8/7.1
AB	M	64	1.5	2	115	69	7.1	5.2/5.2
AD	M	61	1.5	1	142	82	5.9	5.6/-
TD	M	72	10	2	152	76	5.9	12.4/8.3
RF	M	55	18	3	157	77	6.5	10.8/12.2
CJ	M	55	2	1	134	87	4.3	12.1/-
PG3	F	69	5	4	148	90	6.6	7.1/-
PD	M	70	5	4	162	76	7.5	-/5.8
EC	F	69	9	2	163	70	6.9	16.2/9.2
AJ	M	56	6	2	163	101	6.2	6.9/6.9
SP	F	62	4	1	150	83	7.1	9.8/10.0
PM	M	56	11	2	154	94	8.5	9.2/15.5
GM	F	53	5	2	107	63	6.8	-/6.8
PM2	M	62	18	4	136	84	7.3	15.6/13.3
JC2	M	64	5	4	153	97	7.7	6.3/6.1
CP	M	55	8	1	119	79	5.9	6.5/5.9
HT	M	72	3	1	106	60	6.7	8.2/9.8
JW	F	59	3	1	130	53	6.2	-/5.8
AT	F	61	10	3	122	80	10.2	-/17.8
SW	F	59	14	3	144	85	6.4	10.3/6.5
WP	M	66	3	2	140	89	5.7	6.7/6.2
RY	M	64	6.5	4	-	-	-	-/-
RC	M	62	5	4	133	81	5.5	7.4/8.1

Table A1.2 NDR Subject Characteristics

1c: BDR Subject Characteristics

Subject ID	Gender	Age (yrs)	Disease Duration (yrs)	Mgmt Type	Systolic BP (mmHg)	Diastolic BP (mmHg)	Hba1c (%)	Ret Level	Plasma Glucose (mmol/L)
GL	M	67	9	1	180	102	6.6	35	6.1/6.9
DJ3	M	64	18	4	150	85	6	14	-/6.6
RG	M	65	22	3	152	84	7.3	14	13.4/9.6
DJ4	M	62	6	1	137	90	5.6	14	5.7/4.9
WM	M	72	16	4	141	65	8.1	43	16.3/12.9
RF2	M	55	10	4	142	87	8.5	43	11.2/11.4
JD	F	57	18	4	-	-	8.8	43	-/-
CD	M	63	18	2	166	92	9.4	43	-/17.0
RS	M	56	10	2	72	10	7.2	43	6.7/11.0
KB	M	75	4	2	-	-	-	43	11.3/9.6
TJ	M	58	8	2	-	-	-	43	-/9.8
GH	M	75	21	3	-	-	-	43	8.9/-
RJ	M	62	5	2	-	-	-	43	12.0/12.0
VB	M	65	13	3	178	91	7.8	43	5.8/18.0
KW	M	44	4	3	123	93	6.5	14	6.9/4.8

Table A1.3 BDR subject characteristics

Appendix 2
OP Measurements

2a: Control Summed OP Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
JH	106.2	105.7	132.0	111.9	123.6
MG	145.3	172.4	131.0	56.6	126.8
GO	113.7	109.6	125.4	121.2	97.1
DJ	89.7	81.5	89.8	84.5	93.2
JC	83.8	77.0	100.3	111.0	122.4
MC	176.5	129.6	194.4	177.2	178.0
RW	76.9	0	0	48.1	0
BA	192.8	196.9	252.4	231.2	248.2
BV	191.1	207.9	181.6	261.4	257.2
PH	57.6	0	50.2	56.0	65.3
CS	143.4	98.9	160.3	132.6	180.3
SS	132.3	113.2	117.1	110.7	121.6
TH	118.0	128.6	109.6	117.5	111.0
MB	114.8	148	164.9	153.2	164.3
BH	95.9	115.8	113.2	113.6	122.7
PG	201	210.6	220.6	230.9	223.1
RM	153.9	161.4	151	197.1	200.3
PG2	117.4	141.4	104.0	134.2	119.2
DJ2	124.9	100.5	131.7	104.0	111.3
LH	260.2	313.4	283.4	305.1	254.7
PM	84.2	101.0	141.6	140.5	138.7

Table A2.1 Control subject summed OP amplitudes with 5 minutes of O_2 inhalation.

2b: NDR Summed OP Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
JG	120.5	108.4	133.1	164.4	167.4
HP	92.3	123.5	140.4	111.4	114.0
CA	188.6	137.0	182.9	209	207.2
TD	125.9	127.5	129.0	146.9	143.1
AB	105.7	103.8	125.2	117.7	121.4
AD	122.4	132.2	166.3	145.9	146.8
RF	188	162.2	175.1	178.9	187.1
CJ	159.9	178.4	157.5	163.9	231.8
PG3	247.3	256.6	258.1	258.3	288.7
EC	112.0	118.5	129.9	118.6	111.6
AJ	186.8	166.3	177.2	165.3	170.6
SP	95.6	117.6	147.3	138.4	155.6
PM2	134.2	139.0	163.9	153.6	160.4
PM3	226.9	208.1	202.6	237.5	256.2
JC2	91.5	134.4	177.4	143.9	146.7
CP	115.2	160.4	141.5	103.6	86.2
HT	160.4	170.3	209.7	205.5	231.0
JW	151.7	103.7	0	73.2	0
AT	254.4	232.9	259.2	299.2	304.6
SW	116.8	143.3	103.4	210.3	115.3
WP	72.3	78.8	74.3	92.7	89.3
RY	126.8	126.4	118	83.3	123.8
RC	161.7	195	269.7	254	256.5

Table A2.2 NDR subject summed OP amplitudes with 5 minutes of O_2 inhalation.

2c: BDR Summed OP Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
GL	81.2	90.4	110.3	102.0	132.5
DJ3	176.6	149.2	149.5	98.5	131.5
RG	124.2	60.1	124.8	201.5	228.5
DJ4	115.4	107.6	141.6	125.3	100.2
WM	116.2	120.2	177.0	148.9	163.1
RF2	199.1	188.1	254.7	236.6	250
CD	39.5	32.5	66.6	41.2	71.6
RS	92.5	113.5	150.8	117.6	173.7
KB	120.8	120.1	129.6	130.5	165.9
TJ	145.4	189.9	194.6	232.2	206.5
GH	62.4	74.4	67	71.2	79.2
RJ	81.4	158.3	130.7	146.7	150.2
VB	69.8	115.3	72.6	83.8	71.0
KW	190.8	207.9	158.4	128.1	148.3

Table A2.3 BDR subject summed OP amplitudes with 5 minutes of O_2 inhalation.

2c: BDR Summed OP Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
GL	81.2	90.4	110.3	102.0	132.5
DJ3	176.6	149.2	149.5	98.5	131.5
RG	124.2	60.1	124.8	201.5	228.5
DJ4	115.4	107.6	141.6	125.3	100.2
WM	116.2	120.2	177.0	148.9	163.1
RF2	199.1	188.1	254.7	236.6	250
CD	39.5	32.5	66.6	41.2	71.6
RS	92.5	113.5	150.8	117.6	173.7
KB	120.8	120.1	129.6	130.5	165.9
TJ	145.4	189.9	194.6	232.2	206.5
GH	62.4	74.4	67	71.2	79.2
RJ	81.4	158.3	130.7	146.7	150.2
VB	69.8	115.3	72.6	83.8	71.0
KW	190.8	207.9	158.4	128.1	148.3

Table A2.3 BDR subject summed OP amplitudes with 5 minutes of O_2 inhalation.

2d: Control Individual OP Amplitudes and Implicit Times

OP1 = amplitude of OP1, OP2 = amplitude of OP2, IT1 = implicit time of OP1 etc.

Subject ID	Parameter	Baseline	During O_2	2min After	7min After	12min After
JH	OP1	35.2	36.1	29.2	35.9	27.1
	OP2	50.3	37.5	37.1	49.2	43.1
	OP3	24.9	22.8	19.1	36.0	29.0
	OP4	13.2	9.8	20.2	11.0	12.6
	IT1	20	18.8	20	19.8	20
	IT2	26.6	28.4	26	26.4	26.4
	IT3	29.6	30.6	31	31	31.2
	IT4	37.8	37	37.2	37.2	38.6
MG	OP1	28.2	35.7	18.6	16.0	22.6
	OP2	42.5	67.7	72	7.6	34.6
	OP3	44.4	28.6	28.8	27.1	40.8
	OP4	30.2	40.4	11.6	6.0	28.8
	IT1	19.6	20	20.4	20.8	19.6
	IT2	27.6	33.6	33.6	26.6	28.2
	IT3	33	40	39.8	34	33.6
	IT4	39.6	49	48.2	42	39.8
GO	OP1	36.5	25.8	16.5	37.2	19.6
	OP2	31.7	47.5	34.9	43.2	61.8
	OP3	30.4	28	51.5	26.2	6.6
	OP4	15.1	58.8	22.7	13.9	18.4
	IT1	18.8	20	10.8	19.8	21.2
	IT2	20	29.6	20.4	29.8	34.2
	IT3	34.6	34	30	35	38.8
	IT4	40	41	34.4	43.2	42
DJ	OP1	6.9	16.2	10.1	6.3	9.0
	OP2	26	20.0	25.7	23	25.8
	OP3	41.3	29.7	34.8	37.0	32.6
	OP4	15.5	15.5	19.2	18.2	25.8
	IT1	14.6	14	16.6	16	14.6
	IT2	19.6	19	21	20.2	19.6
	IT3	27.4	27.2	29.2	29	28.6

	IT4	34.8	31.8	32	32.6	32.6
JC	OP1	17.1	13.1	18.7	23.7	24.6
	OP2	36.2	32.3	47.9	55.2	58.2
	OP3	10.4	10.4	10.2	13	15.7
	OP4	20.1	21.3	23.5	19.1	26.9
	IT1	21.8	21.2	20.8	20.6	20.4
	IT2	34	33.2	32.8	32.6	32.6
	IT3	39.4	39.2	39.4	38.8	39.6
	IT4	45.2	45.6	46.4	43.8	46.4
MC	OP1	33.1	28.5	34.2	32.5	32
	OP2	65.5	43.5	64.8	66.8	65.7
	OP3	47	29.2	59.2	45.4	52.1
	OP4	30.9	28.4	36.2	32.5	28.2
	IT1	19	19.2	20	19.8	19.4
	IT2	25.8	26	26	26	25.8
	IT3	32	32.4	32	32.2	32.2
	IT4	40.2	39	39.4	39.2	39.2
RW	OP1	17.3	0	0	13.4	0
	OP2	34.1	0	0	10.0	0
	OP3	16.7	0	0	17.8	0
	OP4	8.8	0	0	7.0	0
	IT1	23	-	-	26	-
	IT2	35.8	-	-	34.4	-
	IT3	41.4	-	-	47.8	-
	IT4	55.2	-	-	54.2	-
BA	OP1	28.5	24.0	41.1	32.6	28.5
	OP2	74.9	82.3	98.6	85.3	103.6
	OP3	50.7	50.4	67.8	70.8	70.1
	OP4	38.7	39.9	44.9	42.5	46
	IT1	19.6	20.2	19.2	20	19.4
	IT2	28	27.6	27.6	26.8	26.8
	IT3	33.2	33.2	33	32.4	32.6
	IT4	40.6	39.8	39.8	40.6	40.6
BV	OP1	41.7	49	37.2	48.8	45.8
	OP2	65	69.6	68.2	96.8	109.7
	OP3	56.4	63.1	50.1	79.1	76.1
	OP4	28	26.2	26.1	36.7	25.6
	IT1	19.4	19.6	19	19.4	19.4
	IT2	28	27.8	27.6	27.4	27.2

	IT3	33	32.8	32.8	32.8	32.6
	IT4	38.8	38.4	39	38.8	39.2
PH	OP1	10.7	0	9.5	7.9	11.5
	OP2	14.8	0	13.5	13.1	22.4
	OP3	15.0	0	12.6	17.6	14.5
	OP4	17.2	0	14.5	17.5	16.9
	IT1	22.6	0	19	22.6	22.2
	IT2	31.4	0	35	29	30.6
	IT3	37.4	0	39.4	38	36.8
	IT4	45.2	0	44.8	44.2	46
CS	OP1	21.8	17.3	20.2	21.9	26.9
	OP2	51.6	31.6	60.7	48	64.1
	OP3	43	26.9	50.1	35.1	55.9
	OP4	27	23.1	29.3	27.6	33.4
	IT1	20	19.6	20.6	20	20
	IT2	26.6	26.6	26.2	26.2	26.2
	IT3	33.2	32.6	32.8	32.6	32.8
	IT4	40.4	41.2	40.4	39.8	41
SS	OP1	23.8	18.6	25.2	24.6	20.7
	OP2	45.4	45.8	44.2	42	48.5
	OP3	49.9	42.8	38.2	34.8	48.4
	OP4	13.2	6.0	9.5	93	4.0
	IT1	19.8	19.4	19.8	20.6	22.4
	IT2	27	27.4	26.6	27.2	27.8
	IT3	34.2	34.2	33.6	34.4	34.8
	IT4	42.4	39.2	40.6	42.8	38.4
TH	OP1	39.9	38.9	38.8	44.1	40.4
	OP2	35.4	33.0	30.5	31.2	29.6
	OP3	33.9	31.4	28.4	27.1	27.5
	OP4	8.9	25.3	11.9	15.1	13.5
	IT1	18.8	19.4	19.2	19.4	19.6
	IT2	24.4	26.2	26.4	26.6	26.4
	IT3	30.4	30.2	31	31.2	31.2
	IT4	38.4	34.6	37.2	37.4	38.4
MB	OP1	22.0	28.2	30.7	24.9	30.7
	OP2	43.1	47.4	57.4	47.5	54.3
	OP3	30.4	38	48.1	46.4	49.3
	OP4	19.3	34.4	28.7	34.4	30
	IT1	19	18.6	19.2	19.2	19.2

	IT2	29.4	28.4	28.4	28.2	28.4
	IT3	34.2	33	33.4	33.6	33.2
	IT4	41	40.4	40.8	40.8	39.8
BH	OP1	36.8	38.1	35.9	42	41.4
	OP2	28.9	34.5	36.6	35.8	43.9
	OP3	20.8	31.3	26.2	26.4	20.6
	OP4	9.4	12.0	14.5	9.5	16.8
	IT1	18.8	19.2	19.2	19.2	19
	IT2	25.8	25.2	25.4	25.6	26
	IT3	27.8	30.2	30.2	30.8	30.8
	IT4	35	37.4	38.6	38	38.8
PG	OP1	36.8	45	39.2	41.3	42.5
	OP2	76.1	75.4	87.8	92.8	96.4
	OP3	58.8	64	63.4	69.4	60.7
	OP4	29.3	26.2	30.2	27.4	23.5
	IT1	20	20	20	20	20.2
	IT2	27.2	27.4	27.4	27.6	27.4
	IT3	33.2	33.6	33.4	33.4	33.4
	IT4	40.6	40.8	40.6	41	41
RM	OP1	29.9	26.6	32.4	43.3	38
	OP2	52.1	63.4	56.8	73.1	86.2
	OP3	45.2	48.5	40.8	52.4	49
	OP4	26.7	22.9	21	28.3	27.1
	IT1	20.2	20.8	19.4	20	19.4
	IT2	26.8	27.2	27.4	26	26.2
	IT3	32.8	32.8	33.4	32.6	32.4
	IT4	40	39.8	40.4	39.8	40.2
PG2	OP1	46.8	53	34.3	42.6	34.4
	OP2	31.0	33.3	35.9	42.7	45.6
	OP3	22.7	24.4	20.9	27.3	22.5
	OP4	16.9	30.7	12.8	21.5	16.8
	IT1	18.4	19.2	18.6	19.4	18.4
	IT2	25.2	24.6	26.2	25	26.4
	IT3	28.6	28.8	32.4	32.2	32
	IT4	40	41.4	40.2	38.8	40
DJ2	OP1	33.5	24.0	29.2	28.2	31.3
	OP2	50.3	48.4	55	40.3	46.1
	OP3	31	23.4	34	23.6	23.7
	OP4	10.1	4.7	13.5	11.8	10.2

	IT1	19.8	20.2	19.4	19.4	19.8
	IT2	26.6	26.2	27	26.2	26.6
	IT3	33.6	32.6	32.6	32.4	32
	IT4	39.8	41.8	41	40.4	39
LH	OP1	48.2	61.2	38.2	53.7	31.6
	OP2	94.4	127.6	123.8	123.1	119.3
	OP3	78.8	86.8	90.1	89.2	77.5
	OP4	38.8	37.8	31.3	39.1	26.3
	IT1	18.4	19.2	18.6	18.8	18
	IT2	25.4	25.2	25.2	25	25.2
	IT3	32	31.8	32	32.2	32.4
	IT4	39	42	40.2	42.4	40.8
PM	OP1	23.2	25.7	35.9	34.3	40.2
	OP2	36.1	35.2	52.8	52.3	42.6
	OP3	13.1	17.2	40.9	19.6	26.1
	OP4	11.7	22.9	12.0	34.3	29.9
	IT1	18.8	19.4	18.8	19.4	20.2
	IT2	27.4	28	26.2	27	27.6
	IT3	33.2	33	32.2	32.4	34
	IT4	37.4	40	38.4	41.2	41.6

Table A2.4 Control subject individual OP amplitudes (μV) and implicit times (ms) with 5 minutes of O_2 inhalation.

2e: NDR Individual OP Amplitudes and Implicit Times

Subject ID	Parameter	Baseline	During O_2	2min After	7min After	12min After
JG	OP1	32.4	30.9	29.9	40.6	41.5
	OP2	47.6	35.2	60.6	53.4	54.1
	OP3	25.7	25.5	31.4	41.6	47.7
	OP4	14.9	16.8	11.2	28.8	24.1
	IT1	20	19.6	19.8	19.6	20
	IT2	27.6	28	28	28	31.6
	IT3	33.4	34	33.6	34	34.6
	IT4	38.4	40.6	40.2	40	41.2
HP	OP1	28.9	30	35.7	25.8	29.9
	OP2	44.2	41.4	37.8	31.4	33.5
	OP3	7.8	28.5	39	33.8	29
	OP4	11.4	23.5	27.9	20.5	21.6
	IT1	20.2	19.2	19.6	19.6	19.4
	IT2	28.8	27	28.2	26.4	26.4
	IT3	36.8	31	31.8	31.6	32.2
	IT4	38.4	37.6	37.8	37.2	38
CA	OP1	36.4	33.9	33.2	38.2	38.7
	OP2	39.8	17.1	19.1	32.4	27.0
	OP3	24.0	40.2	34.3	33.7	39
	OP4	21.2	14.5	17.2	20.9	13
	IT1	19.8	19.8	19.6	20.2	20.6
	IT2	30.6	26.2	28	28.4	28
	IT3	33.8	32.8	33.6	34.8	33.8
	IT4	41.2	42.6	40.2	40.8	42
TD	OP1	28.8	27.4	38.3	22.2	26.6
	OP2	39.6	43.4	45.1	53.8	46.3
	OP3	32.5	39.3	56.8	45.6	45
	OP4	21.5	22.1	26.1	24.3	28.9
	IT1	18.8	18.8	19.2	20	19.4
	IT2	26.4	26	26.6	26	26
	IT3	31.2	31	31.6	31	31.4
	IT4	37.8	37.2	38	37.4	38
AB	OP1	30.4	32.1	28.4	30.4	30.1
	OP2	37.4	38.8	46.5	53.8	53.3

	OP3	36.5	37.8	39.3	41.6	39.5
	OP4	21.6	18.8	14.8	21.1	20.2
	IT1	19.6	19.4	19.2	20.4	19.6
	IT2	27.8	29	28.2	28.2	27.8
	IT3	33.8	33.8	34.4	34.6	34
	IT4	40.8	41	40.8	41.4	41.6
AD	OP1	32.6	27.6	36.6	47.2	36.3
	OP2	73.7	40.3	61.2	67.9	67.5
	OP3	56	44	53.5	60.3	68.3
	OP4	26.3	25.1	31.6	33.6	35.1
	IT1	19.4	19.8	20.4	19.4	19.8
	IT2	28.2	29.2	28.6	27.8	27.8
	IT3	34.2	34.4	33.6	33.6	33.8
	IT4	41.6	41.4	40.6	40.8	41.4
RF	OP1	36.6	34.8	37	33.9	31.2
	OP2	62.3	54.3	57.9	58	65.4
	OP3	58.8	47.2	48	54.6	60.2
	OP4	30.3	25.9	32.2	32.4	30.3
	IT1	19	19.2	19.4	19.2	19.6
	IT2	26.2	26	26.2	26.2	26.2
	IT3	31.4	31.2	31.6	31.8	31.8
	IT4	37	37.6	37.2	37.6	38.2
CJ	OP1	39.6	39	35	32	56.3
	OP2	50.4	61.2	57.5	52.7	80.3
	OP3	47.9	60	42.8	51.7	67.4
	OP4	22	18.2	22.2	27.5	27.8
	IT1	20.6	20	20.4	21	20
	IT2	27.6	28.6	28.6	28.2	27.8
	IT3	35.2	34.6	35.2	34.4	35
	IT4	43	44.6	43.8	42.6	43.6
PG3	OP1	38.5	39.4	40.9	38.5	43.6
	OP2	98.7	96.6	103.7	111.2	123.1
	OP3	77.7	89.4	81.6	79.7	96.2
	OP4	32.4	31.2	31.9	28.9	25.8
	IT1	20.4	20.2	20.4	20.2	20.4
	IT2	27	26.6	26.8	26.6	27.2
	IT3	32.6	32.4	32.6	32.4	32.4
	IT4	39.6	39.4	40	39.8	39.8
EC	OP1	24.5	30.3	24.4	26.9	18.7

	OP2	38.0	35.3	49.4	42.3	40.8
	OP3	28.6	31.1	37.4	31.4	33.8
	OP4	20.6	21.8	18.8	18.0	18.4
	IT1	19.6	19.4	19.4	19.8	20
	IT2	28.4	28.2	28.4	28.2	28.4
	IT3	33.6	33.8	33.8	33.8	33.8
	IT4	39.6	40	40.8	41	40.4
AJ	OP1	27.8	42.6	40.6	33.5	40
	OP2	41.2	58.1	40.6	37.7	36.3
	OP3	62.3	40.4	59	55.5	54.3
	OP4	53.5	25.2	37	38.6	40
	IT1	20.4	19.6	20	19	19.6
	IT2	26.4	26.8	26.2	27.2	27.6
	IT3	32.4	33.6	32.8	32.8	32.4
	IT4	39.4	39.6	39.6	39.4	39.4
SP	OP1	22.1	21.9	27.5	23.7	27.6
	OP2	55.2	41.2	54.9	48.5	56.9
	OP3	18.3	34.3	35.8	41	48.2
	OP4	0	20.2	28.8	25.2	22.9
	IT1	19.4	21	20	20	19.8
	IT2	33.4	28	27.6	27.4	28.2
	IT3	41.6	33.6	33.8	33.8	34.2
	IT4	0	41.6	41.4	41.6	41.2
PM2	OP1	24.1	37.3	38.2	30.9	35.9
	OP2	54.5	35.7	58.0	62.7	62
	OP3	38	19.1	39.7	43	42.1
	OP4	17.6	30.2	28.0	17.0	20.4
	IT1	20.4	20	20.4	20	20.4
	IT2	28.4	30.4	29	29.2	27.6
	IT3	32.4	34.4	33.4	33.4	33.4
	IT4	38.2	39.8	39.6	39.2	38.4
PM3	OP1	34.2	32.7	37.1	39.1	24
	OP2	73.9	68	81.7	87.8	80
	OP3	62.5	64.8	72.7	81.4	73.7
	OP4	37.5	37.1	46	47.9	49.2
	IT1	202	20.2	20.2	19.4	20.2
	IT2	28	28.2	27.4	27	27.6
	IT3	33.2	33.6	33.4	33	33.2
	IT4	40	39.8	39.4	39.6	39.4

JC2	OP1	16.4	18.1	24.7	16.9	20.6
	OP2	20.9	31.8	44.4	36.8	44
	OP3	26.5	49.9	67.1	49.3	41.5
	OP4	27.8	35	41.2	40.9	40.6
	IT1	21	20.6	21	21.2	21.2
	IT2	29.6	30.4	29	29	28.6
	IT3	35.8	34.6	34.8	35.4	34.8
	IT4	41.4	41.4	42	42.6	42.4
CP	OP1	17.4	33.3	24.2	24.6	18.9
	OP2	61.1	72.6	51.9	41.2	32.3
	OP3	29.6	26.9	30.5	27	21.6
	OP4	7.0	27.6	3.9	10.7	13.5
	IT1	20	19.4	21.6	21	20.8
	IT2	30.2	29.8	30.6	30.4	31.6
	IT3	35.2	34.6	34.8	35	35.2
	IT4	42	39.8	40.8	39.6	41
HT	OP1	42	31.7	45.4	50.7	58.2
	OP2	56.6	71.9	79.4	76.6	94.3
	OP3	40.5	46.6	60.5	68.3	53
	OP4	21.3	20.1	24.4	9.9	25.5
	IT1	20	20.8	19.6	19.6	19.2
	IT2	28.4	27.6	27.4	26.6	27.4
	IT3	34.2	33.8	33.8	33.4	33.6
	IT4	41.2	42.2	42.6	39	43.2
JW	OP1	41.6	24.4	0	30.4	0
	OP2	45.2	27.2	0	19.0	0
	OP3	34.7	35.3	0	23.8	0
	OP4	30.4	16.7	0	0	0
	IT1	18.6	19.2	0	18.8	0
	IT2	28.8	28.6	0	26.6	0
	IT3	33.2	34	0	29.6	0
	IT4	40.6	40.4	0	0	0
AT	OP1	44	43.5	56.5	49.2	64.1
	OP2	96.8	97.2	103.1	121.2	132
	OP3	78.8	62.7	68.1	84.1	73.4
	OP4	34.8	29.5	31.5	44.7	35.1
	IT1	18.4	18.6	19.2	19	18.6
	IT2	27.8	27.4	27.6	27	26.6
	IT3	33	33.4	33	33	33

	IT4	40.4	40.4	39.2	40	40.4
SW	OP1	31.1	34	28.8	25.9	26.9
	OP2	42.7	51.1	35.3	60	44.9
	OP3	24.5	43	28.6	87.2	30.4
	OP4	18.5	15.2	10.7	37.2	13.1
	IT1	20	19.8	19.8	15.8	20.2
	IT2	26.8	26.2	27.2	19.4	27.6
	IT3	32.4	33	32.8	31.8	33.8
	IT4	40.6	40.6	38.8	39	40.4
WP	OP1	16.0	18.7	18.3	25.7	14.2
	OP2	29.5	33.7	26.6	33.8	43.0
	OP3	19.9	20.1	19.1	19.0	19.2
	OP4	7.0	6.4	10.3	14.3	13
	IT1	20.4	19	19.6	20.2	19.6
	IT2	29.4	29.2	29	29	29.2
	IT3	33	33.6	34	33.8	33.2
	IT4	41.4	40.6	42.6	41	40.4
RY	OP1	36.4	36.5	21.5	19.0	43.3
	OP2	35.5	44.6	42.2	31.8	40.5
	OP3	27.5	19.0	36	27.4	21.6
	OP4	27.5	26.3	18.3	5.1	18.4
	IT1	19.6	19.4	20.8	19	20.4
	IT2	30.2	30.2	28	29.6	30
	IT3	34.6	36.4	33.6	35.6	34.4
	IT4	45	40.8	40.8	43.8	41.8
RC	OP1	41.3	37.2	26.1	43.3	39.7
	OP2	101.1	49.2	60.6	92.4	91
	OP3	78.7	41.8	72.7	92.7	84.6
	OP4	35.4	33.5	35.6	41.3	38.7
	IT1	19	19.4	19	18.4	19.4
	IT2	25.4	26	26.4	25.4	25.6
	IT3	32.4	32	32.4	32.2	32.2
	IT4	39.8	39.4	38.8	38.4	39.6

Table A2.5 NDR subject individual OP amplitudes (μV) and implicit times (ms) with 5 minutes of O_2 inhalation.

2f: BDR Individual OP Amplitudes and Implicit Times

Subject ID	Parameter	Baseline	During O_2	2min After	7min After	12min After
GL	OP1	24.6	25.1	30.9	28.7	33.5
	OP2	26.3	26.0	32.3	35.5	37.4
	OP3	23.9	25.8	28.2	22.4	37.6
	OP4	6.4	13.5	19.0	15.4	24
	IT1	19.2	19.2	19.2	19.2	19.4
	IT2	26.4	27.2	26.2	25.8	29.4
	IT3	32.2	32.2	32.2	32	32.8
	IT4	40.2	40.2	39.6	37.8	40
DJ3	OP1	30	36.4	32.7	23.7	39.5
	OP2	64.2	62.1	63.4	41.4	40.4
	OP3	49.8	29.0	33.3	23.0	29.4
	OP4	32.6	21.8	20.1	10.5	22.2
	IT1	20.4	20.2	20.4	20.8	20.2
	IT2	29.2	29.6	30.8	30.2	31.2
	IT3	34	34.4	34.2	34.6	34
	IT4	41.4	41.4	42.4	41.4	42.8
RG	OP1	24.1	12.6	21.9	31.4	33.8
	OP2	29.2	22.3	49.2	71.3	83.1
	OP3	43.9	15.8	32	67.5	79.6
	OP4	27	9.3	21.7	31.3	32
	IT1	20	20.8	19.4	19.4	21.2
	IT2	28.8	29.8	28.6	28.2	27.4
	IT3	34	34.6	33.8	34.2	33.4
	IT4	40.2	40.4	40.4	41	41
DJ4	OP1	29	28.9	32.2	29.1	24.1
	OP2	42.4	37.3	54	49.4	38.2
	OP3	26.7	25.3	34.3	31.7	25
	OP4	17.4	16.1	21.1	15.1	12.9
	IT1	19.4	19.6	20.6	20.8	21
	IT2	27	26.8	27.6	27.6	27.6
	IT3	32.4	33.4	33.2	33.2	33
	IT4	38	39	39.8	39.4	38.6

WM	OP1	19.1	35.6	42	31	30.0
	OP2	57.9	40.2	74.9	58.9	65.7
	OP3	27.9	34.1	46.9	38.3	46.7
	OP4	11.4	10.3	13.2	20.7	20.7
	IT1	21.2	21.4	21	21	21.2
	IT2	28.2	28	28.2	28.4	28.8
	IT3	35.4	35	35.4	35	35.8
	IT4	43	45	43.2	45.2	43.6
RF2	OP1	28.3	46.9	34	35.1	40.1
	OP2	72.6	65.9	93.6	91.9	110.1
	OP3	65.5	47.1	81.5	70.5	72.2
	OP4	32.7	28.2	45.6	39.1	27.6
	IT1	19.6	19.4	20.4	19.6	19
	IT2	27.2	27.4	27	26.6	26.4
	IT3	32.6	32.4	32.4	32.4	32.2
	IT4	39.4	39.4	39	39	39.2
CD	OP1	7.9	11.1	16.1	15.6	15.4
	OP2	13.1	7.7	13.3	12.4	15.6
	OP3	14.8	9.6	27.6	5.4	18.5
	OP4	3.8	4.1	9.5	7.9	22
	IT1	29.4	29.8	27.4	28.8	27
	IT2	34	33.2	31.4	35.4	34.2
	IT3	38	36.8	35.8	37.4	37.8
	IT4	41.4	39.6	43	42.8	43.8
RS	OP1	21.0	23.3	28.8	24.1	44.5
	OP2	34.7	41.0	51.7	40.8	66.1
	OP3	22.8	27.8	49.1	37.8	43.2
	OP4	14.0	21.3	21.2	15.0	19.9
	IT1	21	19.6	19.6	21	19.8
	IT2	30	29.6	28.6	29.6	29.2
	IT3	34.2	34.6	33.4	34.4	34.8
	IT4	44.2	39.4	40.8	41.4	40.8
KB	OP1	25.0	30.6	36.1	28.3	23.0
	OP2	40	39.1	50.4	56.7	62.7
	OP3	33	34.4	26.4	33.9	4.4
	OP4	22.8	16	16.7	11.6	30.8
	IT1	19.8	20.4	20.2	21.2	21.6
	IT2	27.4	27	27.2	27.2	28.2
	IT3	33.2	33.4	33.8	33.8	33

	IT4	39.8	39.8	39	39.8	40.8
TJ	OP1	30.5	30	29.6	44	35.3
	OP2	40.7	53.5	57.3	73.2	66.5
	OP3	46.5	66.2	70.6	76.7	71.6
	OP4	27.7	40.2	37.1	38.3	33.1
	IT1	20.2	19.8	19.8	20	20.6
	IT2	27.2	27.2	27.6	26.6	27.2
	IT3	33	33.2	33.2	33.4	33.4
	IT4	40	40.4	40.4	41	40.4
GH	OP1	6.8	14.2	14.2	15.7	14.0
	OP2	23.3	30.6	34.5	29.3	36.6
	OP3	18.5	18.1	10.0	16.5	17.5
	OP4	13.8	11.4	8.4	9.6	11.1
	IT1	19.2	20.6	20	21.2	21.4
	IT2	28.8	29.8	29.2	29.6	29.2
	IT3	34.6	33.6	32	34.4	24.6
	IT4	41.4	42.2	40.6	41.6	40.2
RJ	OP1	13.7	43.8	25	33.3	39
	OP2	25.8	51.1	49.1	59.5	57.8
	OP3	22.1	36.4	35.8	29.4	30.9
	OP4	19.7	26.9	20.9	24.5	22.6
	IT1	20.4	20.2	20.4	20.8	20
	IT2	27.4	27.4	28	28.2	28.2
	IT3	35.4	34.2	33.8	34	33.2
	IT4	42.8	40.8	39.4	42	40.6
VB	OP1	12.5	18.1	15.9	15.2	18.6
	OP2	22.1	27.7	24.9	34.7	14.5
	OP3	25.0	39.5	21.2	22.7	16.5
	OP4	10.2	30	10.5	11.2	21.3
	IT1	22.8	18.6	21.4	20.4	26
	IT2	28.4	26.4	33.4	32.4	29
	IT3	34	30.6	37.4	39.2	34.8
	IT4	40.8	40.8	43.2	42.2	39.8
KW	OP1	33.3	47.7	24.2	22.3	17.8
	OP2	71.3	78.5	55.3	32.1	49.7
	OP3	60.1	56.5	49	42.7	42.9
	OP4	26.1	25.2	29.9	31	38.9
	IT1	19.6	19.8	21	21.4	21.4
	IT2	25.4	25	25.8	26.4	25.8

	IT3	32.8	32.6	33	32.8	33.6
	IT4	40.6	40.2	41.4	41	42.2

Table A2.6 BDR subject individual OP amplitudes (μV) and implicit times (ms) with 5 minutes of O_2 inhalation.

Appendix 3

OP Measurements With 15 minutes O_2

3a: Control and NDR Group Characteristics

Group	No. of Subjects	Gender	Age (years)	Disease Duration (years)
Control	7	3 ♀ 4 ♂	Range 39-71 Mean 54.4 (±10.2SD)	-
NDR	7	3 ♀ 4 ♂	Range 56-73 Mean 65.1 (±6.0)	Range 2-19 Mean 8.9 (±5.5SD)

Table A3.1 Control and NDR group characteristics

3b: Summed OP Amplitudes with 5 minutes and 15 minutes of O_2 Inhalation

Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	116.5	129.9			133.4	147.1	148.1
	SD	*36.0*	*42.4*			*31.4*	*52.8*	*51.2*
	SE	*13.6*	*16.0*			*11.9*	*20.0*	*19.4*
15min O_2	Mean	119.3	129.6	127.4	131.5	128.7	137.4	141.7
	SD	*40.5*	*61.1*	*53.1*	*51.9*	*49.3*	*51.8*	*63.4*
	SE	*15.3*	*23.1*	*20.1*	*19.6*	*18.6*	*19.6*	*24.0.*
p		0.892	0.990			0.834	0.736	0.839
NDR Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	174.6	166.6			188.3	196.7	204.7
	SD	*62.4*	*55.5*			*51.3*	*62.2*	*67.0*
	SE	*23.6*	*21.0*			*19.4*	*23.5*	*25.3*
15min O_2	Mean	162.8	158.9	170.1	165.9	178.4	186.1	196.6
	SD	*54.6*	*65.5*	*53.7*	*38.6*	*48.6*	*56.0*	*45.5*
	SE	*20.6*	*24.8*	*20.3*	*14.6*	*18.4*	*21.1*	*17.2*
p		0.714	0.817			0.719	0.745	0.794

Table A3.2 Summed OP amplitudes across time with 5 minutes and 15 minutes of O_2 inhalation.
(p-values shown for Bonferroni pairwise comparisons between 5 minutes of O_2 inhalation results and 15 minutes of O_2 inhalation results).

3c: Percentage Change in Summed OP Amplitudes with 5 minutes and 15 minutes of O_2 Inhalation

Control Subjects							
		During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	+11.2			+18.9	+27.0	+28.6
	SD	*12.9*			*29.5*	*22.9*	*25.6*
	SE	*4.9*			*11.2*	*8.6*	*9.7*
15min O_2	Mean	+5.7	+5.4	+9.9	+7.9	+14.9	+16.5
	SD	*27.0*	*19.1*	*19.3*	*15.6*	*11.1*	*17.6*
	SE	*10.2*	*7.2*	*7.3*	*5.9*	*4.2*	*6.6*
p		0.638			0.402	0.235	0.320
NDR Subjects							
		During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	-1.9			+12.7	+15.5	+20.3
	SD	*16.3*			*23.0*	*15.5*	*20.0*
	SE	*6.2*			*8.7*	*5.9*	*7.6*
15min O_2	Mean	-4.1	+5.2	+6.2	+14.7	+20.2	+29.1
	SD	*12.6*	*12.5*	*18.1*	*25.3*	*32.4*	*40.3*
	SE	*4.8*	*4.7*	*6.9*	*9.5*	*12.2*	*15.2*
p		0.784			0.882	0.740	0.615

Table A3.3 Percentage change in summed OP amplitudes across time with 5 minutes and 15 minutes of O_2 inhalation.
(p-values shown for Bonferroni pairwise comparisons between 5 minutes of O_2 inhalation results and 15 minutes of O_2 inhalation results).

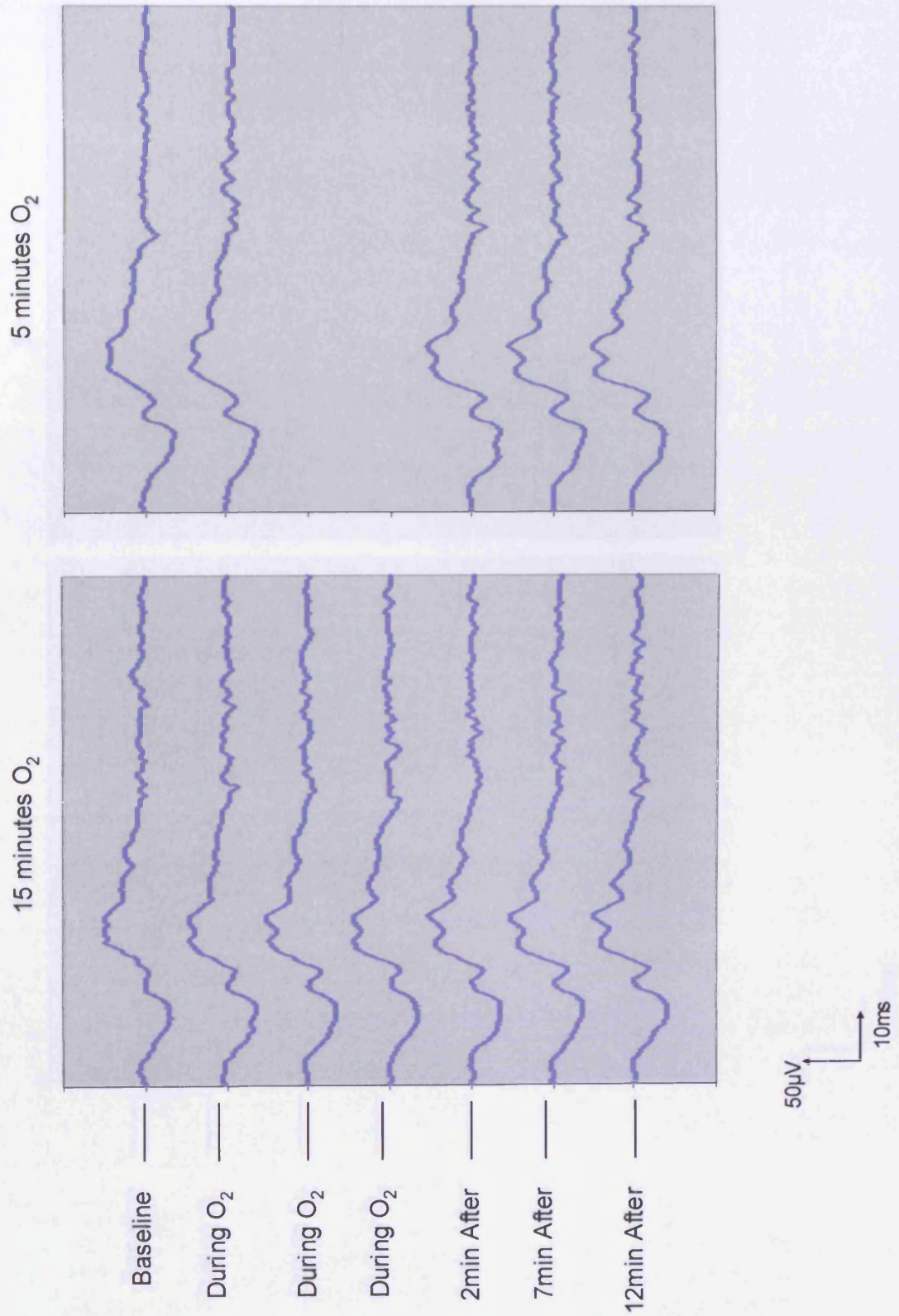

Figure A3.1 Control group averaged OPs with 15 minutes and 5 minutes of O_2 inhalation

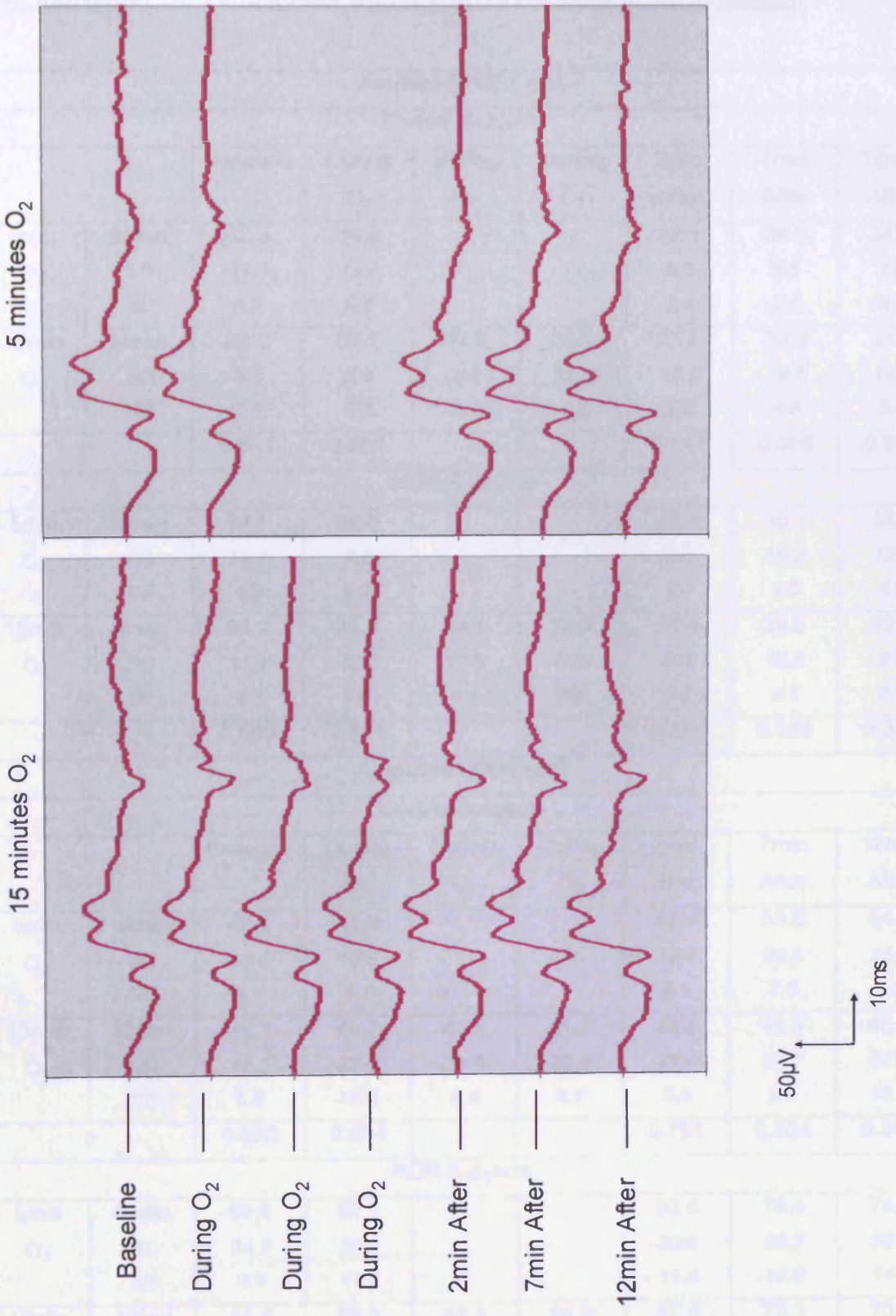

Figure A3.2 NDR group averaged OPs with 15 minutes and 5 minutes O_2 inhalation

3d: Individual OP Amplitudes with 5 and 15 minutes of O_2 Inhalation

Amplitude OP1 (μV)								
Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	32.3	34.8			32.1	36.3	34.7
	SD	*11.4*	*13.8*			*6.8*	*9.6*	*7.8*
	SE	*4.3*	*5.2*			*2.6*	*3.6*	*2.9*
15min O_2	Mean	29.2	31.5	32.2	28.5	27.7	30.0	31.3
	SD	*5.8*	*9.3*	*9.8*	*12.8*	*12.7*	*12.6*	*14.5*
	SE	*2.2*	*3.5*	*3.7*	*4.8*	*4.8*	*4.8*	*5.5*
p		0.541	0.610			0.441	0.309	0.594
NDR Subjects								
5min O_2	Mean	34.1	32.6			38.6	36.1	38.3
	SD	*11.1*	*7.5*			*9.0*	*10.5*	*12.9*
	SE	*4.2*	*2.8*			*3.4*	*4.0*	*4.9*
15min O_2	Mean	34.2	31.3	33.4	32.9	33.4	34.9	32.2
	SD	*11.1*	*12.2*	*10.8*	*10.2*	*9.9*	*10.9*	*9.8*
	SE	*4.2*	*4.6*	*4.1*	*3.8*	*3.7*	*4.1*	*3.7*
p		0.993	0.811			0.329	0.838	0.337
Amplitude OP2 (μV)								
Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	42.4	41.2			47.1	53.5	54.7
	SD	*11.8*	*13.6*			*13.4*	*20.6*	*25.9*
	SE	*4.4*	*5.1*			*5.1*	*7.8*	*9.8*
15min O_2	Mean	37.1	40.7	43.3	41.9	43.8	45.3	45.9
	SD	*18.3*	*27.2*	*24.9*	*23.6*	*23.4*	*24.1*	*27.4*
	SE	*6.9*	*10.3*	*9.4*	*8.9*	*8.8*	*9.1*	*10.3*
p		0.528	0.964			0.751	0.504	0.549
NDR Subjects								
5min O_2	Mean	66.6	55.7			63.6	70.4	74.0
	SD	*24.5*	*30.2*			*30.6*	*33.2*	*39.1*
	SE	*9.3*	*11.4*			*11.5*	*12.5*	*14.8*
15min O_2	Mean	58.9	58.3	62.7	61.3	67.9	72.1	73.8
	SD	*22.2*	*32.2*	*26.4*	*18.6*	*22.0*	*26.7*	*22.8*
	SE	*8.4*	*12.1*	*10.0*	*7.0*	*8.3*	*10.1*	*8.6*
p		0.549	0.881			0.766	0.919	0.990
Amplitude OP3 (μV)								

Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	27.4	29.6			31.1	35.5	35.2
	SD	*15.3*	*17.3*			*15.5*	*22.0*	*20.8*
	SE	*5.8*	*6.5*			*5.8*	*8.3*	*7.9*
15min O_2	Mean	34.9	38.7	33.5	37.0	38.6	39.4	39.8
	SD	*21.2*	*26.2*	*18.9*	*25.2*	*14.4*	*17.1*	*21.4*
	SE	*8.0*	*9.9*	*7.2*	*9.5*	*5.5*	*6.5*	*8.1*
p		0.462	0.457			0.368	0.719	0.686
NDR Subjects								
5min O_2	Mean	49.4	51.0			54.0	57.0	61.5
	SD	*24.8*	*19.2*			*17.0*	*19.1*	*19.8*
	SE	*9.4*	*7.3*			*6.4*	*7.2*	*7.5*
15min O_2	Mean	45.3	45.7	48.8	46.7	50.5	50.7	57.2
	SD	*17.5*	*16.5*	*15.4*	*12.1*	*13.8*	*15.0*	*12.5*
	SE	*6.6*	*6.2*	*5.8*	*4.6*	*5.2*	*5.7*	*4.7*
p		0.726	0.592			0.680	0.504	0.641
Amplitude OP4 (μV)								
Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	16.9	24.4			19.3	24.6	22.2
	SD	*6.4*	*7.8*			*7.1*	*10.4*	*7.6*
	SE	*2.4*	*3.0*			*2.7*	*3.9*	*2.9*
15min O_2	Mean	19.1	18.6	19.9	24.0	18.5	22.8	24.5
	SD	*8.2*	*10.3*	*6.5*	*6.0*	*8.6*	*6.8*	*9.0*
	SE	*3.1*	*3.9*	*2.5*	*2.3*	*3.3*	*2.6*	*3.4*
p		0.581	0.265			0.858	0.705	0.606
5min O_2	Mean	23.8	24.1			28.5	30.0	27.3
	SD	*11.7*	*5.7*			*5.4*	*7.9*	*7.7*
	SE	*4.4*	*2.2*			*2.1*	*3.0*	*2.9*
15min O_2	Mean	24.9	23.5	24.0	24.9	26.6	28.4	33.3
	SD	*10.2*	*7.0*	*6.8*	*5.3*	*7.8*	*6.9*	*5.3*
	SE	*3.9*	*2.6*	*2.6*	*2.0*	*2.9*	*2.6*	*2.0*
p		0.856	0.881			0.601	0.697	0.113

Table A3.4 Individual OP amplitudes across time with 5 minutes and 15 minutes of O_2 inhalation.
(p-values shown for Bonferroni pairwise comparisons between 5 minutes of O_2 inhalation results and 15 minutes of O_2 inhalation results).

3e: Individual Control Subject Summed OP Amplitudes with 15 minutes of O_2 Inhalation

Subject ID	Baseline (μV)	During O_2 (μV)	During O_2 (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
JH	83.5	96.1	105.6	97.1	106.1	107.8	103.4
MB	140.8	202.4	181.7	164.6	155.8	148.5	169.5
BV	184.5	208.8	210.3	226.9	224.5	238.4	264.6
TH	96.8	106.8	100	116.5	113.5	111.8	117.8
JC	88.5	47.1	67.0	67.8	81.7	100.7	99.6
PM	86.0	89.3	83.4	110.0	87.4	100.7	99.6
PG2	155.1	156.5	143.8	137.7	131.8	164.0	159.3

Table A3.5 Control subject summed OP amplitudes with 15 minutes of O_2 inhalation.

3f: Individual NDR Subject Summed OP Amplitudes with 15 minutes of O_2 Inhalation

Subject ID	Baseline (μV)	During O_2 (μV)	During O_2 (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
TD	153	152.5	181.2	180.1	173.2	198.7	198.2
AD	113.5	124.0	110.3	114.7	134.8	138.9	142.6
PG3	199.5	183.7	196.8	193.2	199.7	213.9	239.6
CA	172.1	147.5	217.4	165.5	155.6	144.9	163.1
RF	177.2	162.8	179.6	181.9	178.1	165.6	187.8
AT	245	279.2	223.5	213.5	274.5	295.2	273
SP	79.4	62.5	82.1	112.4	133.0	145.7	171.7

Table A3.6 NDR subject summed OP amplitudes with 15 minutes of O_2 inhalation.

3g: Individual Control Subject Individual OP Amplitudes with 15 minutes of O_2 Inhalation

Subject ID	Baseline (μV)	During O_2 (μV)	During O_2 (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
OP1 Amplitude (μV)							
JH	35.3	32.6	31.6	34.5	8.1	9.2	8.2
MB	22.3	36	27.5	37.3	21.8	26.8	28.4
BV	36.6	40.1	42.5	40.9	44	42.7	47.5
TH	33.1	39.9	36.2	37.8	42.3	41.7	42
JC	24.2	13.1	13.3	17.1	20.9	21.1	16.9
PM	28.4	31	33.0	25.6	24.9	27	33.5
PG2	24.8	28.1	41.1	6.5	32.2	41.3	42.9
OP2 Amplitude (μV)							
JH	29.8	34.8	41.6	33.6	38.8	34.0	32.5
MB	59.8	70.4	66.2	56.8	58.1	48.2	63.3
BV	66.5	84.9	84	87.3	85.5	94.9	99.7
TH	18.9	23.4	23.5	37.7	25	26.0	29.0
JC	28.8	9.8	16.3	15.0	24.9	27.6	18.9
PM	26.1	23.4	23.5	27.9	21.3	33.8	37.2
PG2	29.7	37.9	47.8	35.3	53.1	52.8	41.0
OP3 Amplitude (μV)							
JH	8.7	11.5	10.0	4.9	41.8	46.2	44.3
MB	38.7	57.3	58.2	45.2	45.5	44.6	53.3
BV	51.8	58.1	58.6	67.7	65.3	71.5	74.3
TH	33.8	34.1	29.9	28.1	31.2	23.3	26.3
JC	18.0	8.7	19.4	15.2	25.2	25.2	23.1
PM	22.8	23.8	22.0	27.3	23	25.7	10.6
PG2	70.7	77.6	36.3	70.5	38.1	39.2	47
OP4 Amplitude (μV)							
JH	16.9	17.2	22.4	23.9	17.5	18.5	18.5
MB	20.1	38.7	29.8	25.3	30.4	28.9	24.5
BV	29.6	25.7	25.2	31	29.7	29.3	43.1
TH	11.1	9.3	10.4	12.9	15.0	20.8	20.4
JC	17.5	15.6	18.0	20.6	10.7	17.0	18.6
PM	8.7	11.2	14.9	29.1	18.2	14.2	18.3

PG2	29.9	12.9	18.6	25.4	8.3	30.7	28.4

Table A3.7 Control Individual OP amplitudes with 15 minutes of O_2 inhalation.

3h: Individual NDR Subject Individual OP Amplitudes with 15 minutes of O_2 Inhalation

OP1 Amplitude (μV)							
Subject ID	**Baseline (μV)**	**During O_2 (μV)**	**During O_2 (μV)**	**During O_2 (μV)**	**2min After (μV)**	**7min After (μV)**	**12min After (μV)**
TD	31.1	31.4	33.1	36.4	33.9	38.4	37.9
AD	24.7	23.3	26.2	22.9	25.6	34	20
PG3	36.1	32.2	40.6	32.9	26.3	31.9	37.9
CA	48.7	34.2	41.1	39.1	33.6	31.7	28.2
RF	26.6	30.9	33.7	35.3	33.7	29.8	26
AT	49.6	53.7	45.6	47	54.2	56.9	48.8
SP	22.5	13.6	13.6	16.7	26.7	21.9	26.4
OP2 Amplitude (μV)							
TD	59.6	55.7	75.7	72.6	73.9	74.4	82.5
AD	33.7	36.6	23.7	38.8	46.8	53.6	43.7
PG3	78.8	74.9	77.7	85.9	78.6	90.1	94
CA	60.9	44.6	84.5	55.6	55.9	50.8	56.4
RF	55.8	55.2	58.6	61.5	65.8	57	65.7
AT	92.5	120.2	88.8	77.3	108.6	123.6	109.5
SP	30.8	20.7	29.6	37.7	45.7	55.2	64.9
OP3 Amplitude (μV)							
TD	40.9	47.2	51.7	49.4	48.5	59.8	53.6
AD	34.8	39.2	36.3	33.7	41.9	33.4	50.6
PG3	60.5	53.3	58.8	57	63.6	61.7	69.1
CA	42.3	44.7	58.9	41	41.2	35.8	42.8
RF	58.5	50.7	56.2	54.7	54.6	47.9	59.4
AT	65.1	69.8	60.4	61.6	71.7	74	77.8
SP	15.1	15.3	19.3	29.8	32.2	42.1	47.4
OP4 Amplitude (μV)							
TD	21.4	18.2	20.7	21.7	16.9	26.1	24.2
AD	20.3	24.9	15.3	19.3	20.5	17.9	28.3
PG3	24.1	23.3	19.7	17.4	31.2	30.2	38.6

CA	20.2	24.1	32.9	29.8	24.9	26.6	35.7
RF	39.3	26	31.1	30.4	24.0	30.9	36.7
AT	37.8	35.5	28.7	27.6	40	40.7	36.9
SP	10.9	12.9	19.7	28.2	28.4	26.5	33

Table A3.8 NDR Individual OP amplitudes with 15 minutes of O_2 inhalation.

3i: Individual Control Subject Individual OP Implicit Times with 15 minutes of O_2 Inhalation

OP1 Implicit Time (ms)							
Subject ID	**Baseline (μV)**	**During O_2 (μV)**	**During O_2 (μV)**	**During O_2 (μV)**	**2min After (μV)**	**7min After (μV)**	**12min After (μV)**
JH	20.2	19.6	20.2	20.8	12.8	12.2	14.4
MB	19	19	18.8	19.4	20	19.6	19.2
BV	19.2	19.4	20	19.4	19.8	19.8	20
TH	20	19.2	19.6	19.6	19.2	19.6	19.8
JC	21.4	23.4	20.8	21.6	20.2	19.8	21.2
PM	21	19.8	20	20.6	21	20	20
PG2	18.4	11.8	19.2	13.4	18.8	19.8	19.6
OP2 Implicit Time (ms)							
JH	26.2	26.6	26.4	26.8	20.2	18.8	19.6
MB	28.8	28	28.4	28.4	28.4	28.6	27.8
BV	27.6	27.4	27.6	27.4	27.4	27.2	27.4
TH	24	24	24.6	24.8	24.4	26.6	26
JC	30.2	32.4	28.8	29	28.6	28.4	29.6
PM	26.8	26.4	27.6	27.2	27.6	26.6	28.2
PG2	23.6	18.8	26.8	19.2	27.2	25.8	25.2
OP3 Implicit Time (ms)							
JH	28.2	28	28.4	28.6	25.8	27	26.6
MB	34.4	33.2	33.2	33.4	33.2	33.8	33.2
BV	33	32.6	32.6	32.4	32.8	32.4	32.6
TH	30.8	30	28.6	31	30.8	28.8	31.2
JC	34.2	36.2	33.6	34	33.2	33.4	33.6
PM	31.4	32.6	33.2	32.8	33.8	32.2	34
PG2	31.2	31	33.4	28.8	34.2	32.4	32.4
OP4 Implicit Time (ms)							

JH	30.4	31.8	31.2	30.8	31.2	30.8	31
MB	40.8	40.8	40.6	41.6	41.2	41.6	41.4
BV	38.8	38.4	38	39	38.4	39	39.2
TH	38.2	38.4	36	38	38.4	38.2	38.6
JC	41.2	45.2	39.4	39.6	39.8	39.6	39.8
PM	38.6	38	39.6	38.2	38.6	39.8	38.4
PG2	45.6	50.6	46.4	40.8	42.8	39.8	39.6

Table A3.9 Control Individual OP Implicit Times with 15 minutes of O_2 inhalation.

3j: Individual NDR Subject Individual OP Implicit Times with 15 minutes of O_2 Inhalation

OP1 Implicit Time (ms)							
Subject ID	**Baseline (μV)**	**During O_2 (μV)**	**During O_2 (μV)**	**During O_2 (μV)**	**2min After (μV)**	**7min After (μV)**	**12min After (μV)**
TD	19.6	20.2	20.8	20	20	19.6	19.4
AD	18.8	19.4	19	20	19.4	19.8	19
PG3	19.8	20.2	20	19.8	20.2	20.2	19.4
CA	19.2	19.4	18.8	19.6	19.4	19.6	19.4
RF	20	19.2	19.2	19.2	19.2	19.4	20
AT	18.6	18.2	19	19	19	18.8	18.6
SP	19.6	19.6	19.8	19.4	20	19.6	19.8
OP2 Implicit Time (ms)							
TD	28.2	28.4	28.2	27.8	28.2	28.2	27.8
AD	25.8	25.4	24.6	26.8	26.4	25.6	24.6
PG3	27	26.8	27	27	27	26.8	26.4
CA	27	28.4	27	28.4	28.6	28.4	27.2
RF	26	26	26.2	26.2	26.2	26	25.8
AT	26.8	26.6	27.2	27	27	26.4	26.4
SP	28.6	29.8	29	28.4	28	27.8	27.6
OP3 Implicit Time (ms)							
TD	34.8	34.6	34.4	34.2	34	34.4	34.2
AD	31	31.2	31.2	31.4	31.2	31.2	31.2
PG3	32.4	32.2	32.2	32.2	32.2	32.4	32.2
CA	32.8	32.8	32.6	33.6	33.2	33	32.8

RF	31.4	31.2	31.6	31.6	31.4	31.8	31.4
AT	32.8	32.2	32.8	33.4	33	32.6	32.6
SP	34.4	33.8	34.2	34.2	34	34	34
OP4 Implicit Time (ms)							
TD	41.8	42.6	42.2	41.8	42.8	42	42.2
AD	37.2	37.2	37.2	37.4	37.2	37.6	37.2
PG3	39.8	39.6	39.6	39.4	39.2	40	40.4
CA	39.4	38.6	39	39.2	39.6	39.6	39.4
RF	37.4	37.2	37.6	37.6	38.2	38.2	37.2
AT	39.6	38.8	39.8	39.6	40	39.2	39
SP	39.2	41.4	41.4	41.8	41.4	42.2	41.2

Table A3.10 NDR Individual OP Implicit Times with 15 minutes of O_2 inhalation.

Appendix 4

B-wave Measurements

4a: Control Subject B-wave Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
JH	134	146	158	164	161
MG	93.7	85.4	100	85.7	94.8
GO	186	262	241	235	270
CG	139	152	135	141	234
DJ	84.3	85.6	96.7	93.2	101
JC	85.7	41.3	75.8	103	103
HC	38.9	24.8	29	49	54.9
MC	184	145	130	219	177
RW	119	135	125	119	103
BA	113	106	122	113	112
BV	111	96.9	104	97.7	108
PH	71.2	44.2	63	57.4	53.6
RB	115	133	124	125	90.5
CS	85.9	51.5	95.6	86.6	60.4
RC	85.8	104	97.7	117	126
TH	109	110	136	110	119
MB	79.8	77.3	82.1	73.1	79.8
BH	119	105	108	111	102
PG	133	118	142	124	138
RM	125	186	117	104	96.8
PG2	89.5	112	93	142	93.4
DJ2	130	129	99.1	110	113
LH	155	179	172	196	160
PM	93	68.4	86.9	82	83.1

Table A4.1 Control subject b-wave amplitudes with 5 minutes of O_2 inhalation.

4b: NDR Subject B-wave Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
JG	132	105	153	172	201
HP	120	134	142	130	126
CA	150	136	176	162	184
AB	168	190	178	179	184
AD	29.9	36.6	36.3	45.3	26.9
TD	82	78.2	71.4	78.5	76.4
RF	115	135	132	148	129
CJ	158	151	128	125	113
PG3	153	188	188	181	173
PD	76.8	93	82.7	87.2	89.8
EC	117	111	128	124	126
AJ	79.6	69.3	84.8	109	86.9
SP	122	143	132	134	144
PM2	96.7	67.9	75.3	77	53.9
GM	94.8	96.6	86.1	106	84.9
PM3	98.1	117	117	115	129
JC2	57.9	41.8	49.5	60.8	69.3
CP	118	108	106	113	103
HT	57.3	22.7	63.4	77.3	97.4
JW	74.5	78	84.5	61.7	87.5
AT	170	200	183	201	199
SW	120	99.3	114	109	114
WP	84.1	97.9	97.2	85.4	81.7
RY	114	72.4	100	101	89
RC	112	140	153	154	141

Table A4.2 NDR subject b-wave amplitudes with 5 minutes of O_2 inhalation.

4c: BDR Subject B-wave Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
GL	67.1	90.5	77.4	86.6	80.1
DJ3	134	102	104	107	110
RG	76.6	81.2	90.9	97.2	89
DJ4	98.1	90.7	104	90.4	93.9
WM	79.5	91.4	103	141	119
RF2	122	134	166	151	176
JD	82.2	85.5	99.6	92.5	102
CD	44.1	62.7	66.6	75.3	72.6
RS	95.2	90.9	101	102	112
KB	57.5	57.4	81.1	68.1	79.2
TJ	99.1	102	120	107	103
RJ	102	122	116	117	109
VB	56.7	37.2	34.7	45.5	76.7
KW	151	156	164	175	153

Table A4.3 BDR subject b-wave amplitudes with 5 minutes of O_2 inhalation.

4d: Control Subject B-wave Implicit Times

Subject ID	Baseline (ms)	During O_2 (ms)	2min After (ms)	7min After (ms)	12min After (ms)
JH	45	47.5	48	41.5	48
MG	45.5	52.5	48.5	49	52
GO	46	45.5	45.5	43	44
CG	57.5	55.5	54	56.5	52
DJ	50.5	48	45	49	48
JC	52.5	53	55	51.5	54
HC	59	60	65	57.5	53
MC	44	47.5	43.5	44.5	44
RW	52.5	54.5	51.5	51.5	54.5
BA	50	47.5	51.5	54	47.5
BV	55	54	53.5	53.5	54
PH	47.5	55.5	51	48.5	58.5
RB	53.5	50.5	51	51.5	51
CS	47	49	49.5	46	54.5
RC	49.5	46.5	50.5	49	51.5
TH	46.5	45	45.5	47	46
MB	53.5	54.5	54.5	56.5	54
BH	50	49	50	48.5	48
PG	49.5	50	49.5	48.5	49
RM	47	41	34.5	45	46.5
PG2	43	48.5	47.5	52	38
DJ2	51	50.5	48.5	49.5	49
LH	45	42.5	43.5	44.5	43
PM	59.5	46	39.5	50.5	55.5

Table A4.4 Control subject b-wave implicit times with 5 minutes of O_2 inhalation.

4e: NDR Subject B-wave Implicit Times

Subject ID	Baseline (ms)	During O_2 (ms)	2min After (ms)	7min After (ms)	12min After (ms)
JG	50.5	48.5	57.5	55	54.5
HP	50.5	44	36	43	47
CA	50.5	54.5	51.5	50	50.5
AB	52	51	53	52.5	53
AD	43.5	53	52.5	48	44
TD	51.5	52	51	52	55
RF	50.5	48.5	49	48.5	49
CJ	52	47	48.5	49	49.5
PG3	48	45	46	45.5	46.5
PD	53.5	55	54.5	53.5	54.5
EC	54.5	50.5	54.5	53	53
AJ	42.5	42.5	44	49.5	48
SP	51	53.5	54	51.5	52
PM2	49.5	46	53	59.5	52.5
GM	39	45.5	51	55	53
PM3	49	48	50	49.5	49.5
JC2	56.5	39.5	59.5	57	55
CP	60	56.5	57	56	56
HT	52	66	53.5	54.5	52
JW	35.5	47	49.5	36	60.5
AT	47	46.5	46	45.5	45.5
SW	51	49.5	50.5	43	49.5
WP	51	49.5	50.5	43	49.5
RY	58	55.5	52	56.5	59
RC	47	46	47.5	47	49.5

Table A4.5 NDR subject b-wave implicit times with 5 minutes of O_2 inhalation.

4f: BDR Subject B-wave Implicit Times

Subject ID	Baseline (ms)	During O_2 (ms)	2min After (ms)	7min After (ms)	12min After (ms)
GL	54.5	51.5	48.5	47.5	53
DJ3	58	54	53.5	53.5	55.5
RG	52.5	46.5	51	52	51
DJ4	52	50	51	50.5	51
WM	41	43.5	37.5	38	43
RF2	40	48	51.5	44.5	50
JD	51	53	54.5	57	54
CD	40.5	58.5	57.5	58	57.5
RS	45	40.5	49.5	54.5	48.5
KB	55	52.5	52.5	54	52
TJ	38.5	41.5	40	47	53.5
RJ	55.5	54	54	54.5	54
VB	44	60.5	50.5	64.5	53
KW	44.5	42	42	42.5	43.5

Table A4.6 BDR subject b-wave implicit times with 5 minutes of O_2 inhalation.

4g: Group-averaged B-wave Implicit Times and Percentage Change Across Time

Group		B-wave Implicit time (ms)				
		Baseline	During O_2	2min After	7min After	12min After
Control	Mean	50.0	49.8	49.0	49.5	49.8
	SD	*4.7*	*4.5*	*5.9*	*4.3*	*4.8*
	SE	*1.0*	*0.9*	*1.2*	*0.9*	*1.0*
NDR	Mean	49.7	49.5	50.8	50.4	51.6
	SD	*5.5*	*5.5*	*4.8*	*5.3*	*4.0*
	SE	*1.1*	*1.1*	*1.0*	*1.1*	*0.8*
BDR	Mean	48.0	49.7	49.5	51.3	51.4
	SD	*6.7*	*6.2*	*5.8*	*6.9*	*4.1*
	SE	*1.8*	*1.7*	*1.5*	*1.8*	*1.1*
Group		% Change B-wave Implicit time From Baseline				
		Baseline	During O_2	2min After	7min After	12min After
Control	Mean		-0.1	-1.7	-0.7	-0.1
	SD	NA	*8.5*	*10.2*	*6.6*	*6.6*
	SE		*1.7*	*2.1*	*1.4*	*1.3*
NDR	Mean		+0.6	+3.2	+2.2	+5.2
	SD	NA	*13.0*	*13.0*	*11.4*	*16.1*
	SE		*2.6*	*2.6*	*2.3*	*3.2*
BDR	Mean		+4.9	+4.4	+8.3	+8.8
	SD	NA	*17.4*	*15.3*	*18.8*	*16.3*
	SE		*4.7*	*4.1*	*5.0*	*4.4*

Table A4.7 B-wave implicit times and percentage change in b-wave implicit times across time with O_2 inhalation.

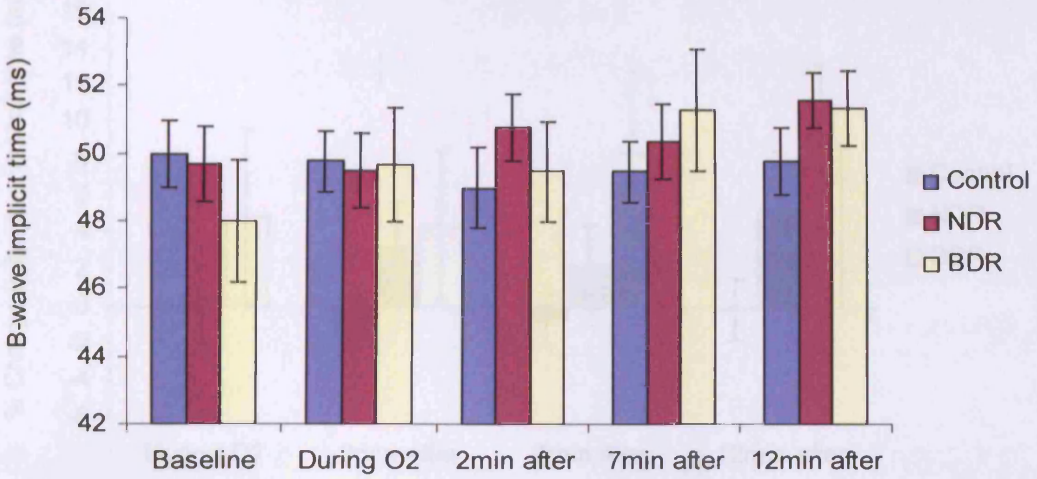

Figure A4.1 B-wave implicit times across time with O_2 inhalation.

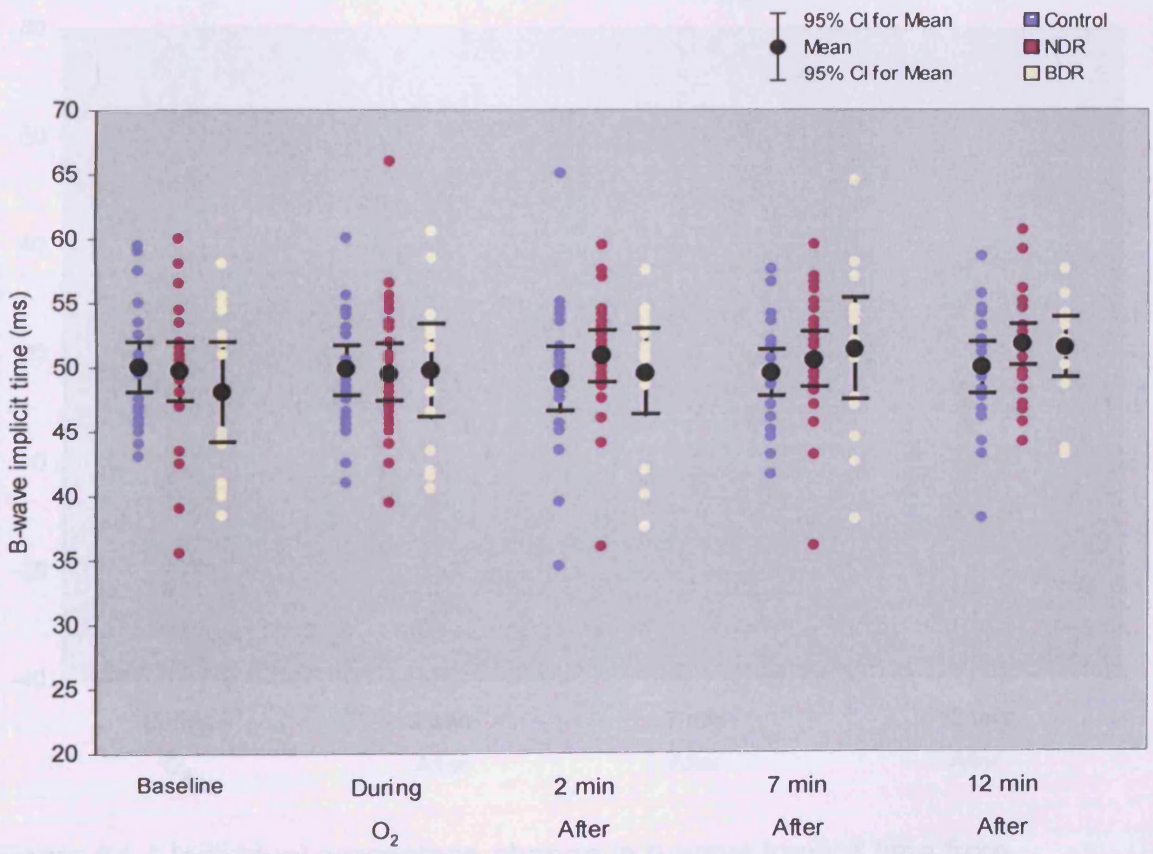

Figure A4.2 Individual b-wave implicit times.
(Group mean and 95% confidence intervals for mean shown).

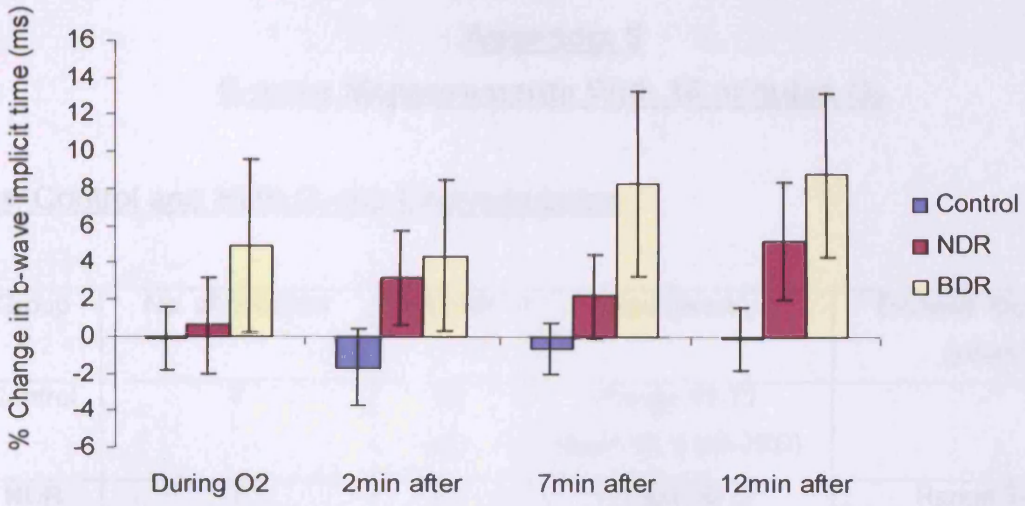

Figure A4.3 Percentage change in b-wave implicit time from baseline with O_2 inhalation.

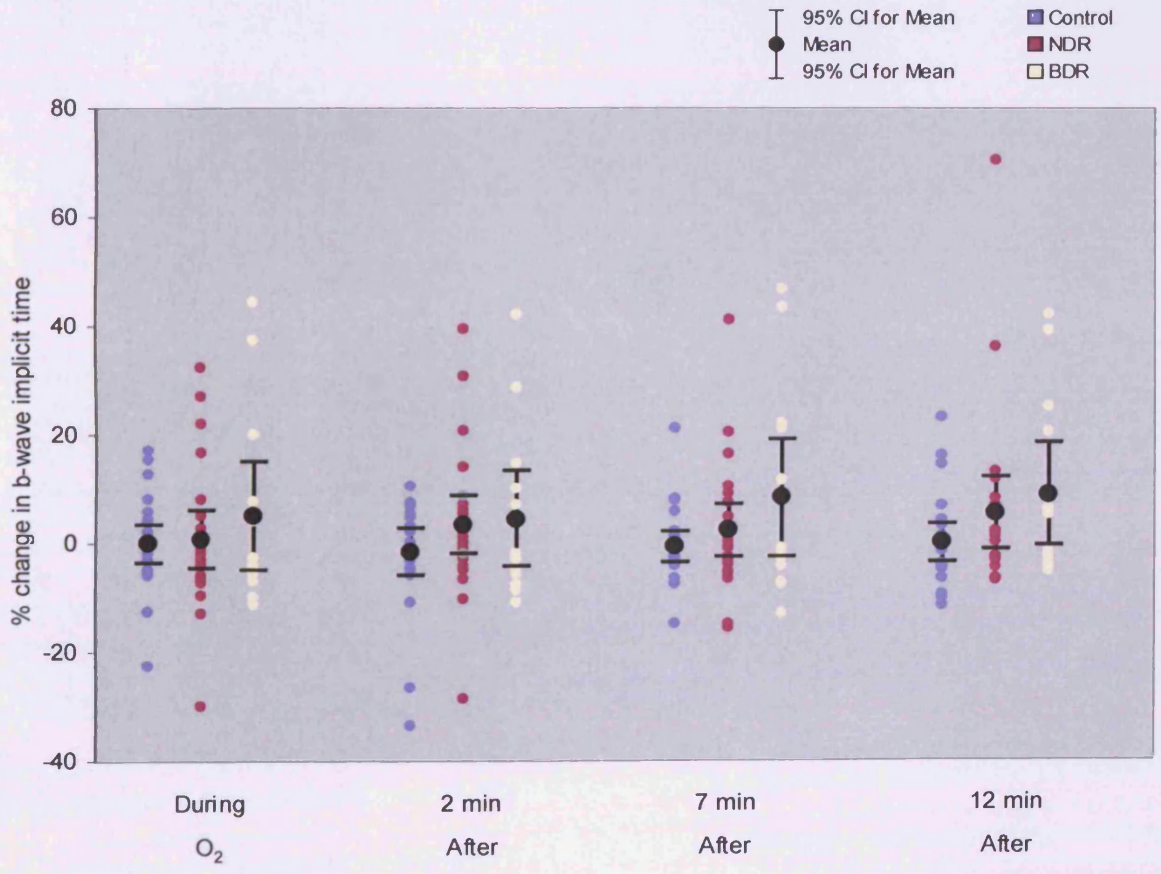

Figure A4.4 Individual percentage change in b-wave implicit time from baseline.

(Group mean and 95% confidence intervals for mean shown)

Appendix 5

B-wave Measurements With 15 minutes O_2

5a: Control and NDR Group Characteristics

Group	No. of Subjects	Gender	Age (years)	Disease Duration (years)
Control	7	1 ♀ 6 ♂	Range 52-72 Mean 61.9 (±8.2SD)	-
NDR	7	3 ♀ 4 ♂	Range 55-72 Mean 64.9 (±6.9)	Range 3-12 Mean 6.3 (±3.1SD)

Table A5.1 Control and NDR group characteristics

5b: B-wave Amplitudes with 5 minutes and 15 minutes of O_2 Inhalation

Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	106.0	108.1			110.9	115.9	124.2
	SD	*21.1*	*37.1*			*21.1*	*15.4*	*49.6*
	SE	*8.0*	*14.0*			*8.0*	*5.8*	*18.8*
15min O_2	Mean	87.0	92.6	85.6	85.0	90.3	71.7	85.2
	SD	*26.5*	*24.2*	*27.3*	*27.5*	*31.5*	*30.8*	*20.5*
	SE	*10.0*	*9.2*	*10.3*	*10.4*	*11.9*	*11.6*	*7.8*
p		0.165	0.372			0.177	**0.005**	0.079
NDR Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	111.6	110.4			127.3	132.5	136.3
	SD	*32.4*	*53.3*			*41.8*	*35.6*	*40.3*
	SE	*12.3*	*20.1*			*15.8*	*13.4*	*15.2*
15min O_2	Mean	123.7	126.0	129.5	131.6	143.2	138.0	148.3
	SD	*52.3*	*65.2*	*63.3*	*65.8*	*72.5*	*63.2*	*69.5*
	SE	*19.8*	*24.6*	*23.9*	*24.9*	*27.4*	*23.9*	*26.3*
p		0.612	0.634			0.625	0.843	0.701

Table A5.2 B-wave amplitudes across time with 5 minutes and 15 minutes of O_2 inhalation.

(p-values shown for Bonferroni pairwise comparisons between 5 minutes of O_2 inhalation results and 15 minutes of O_2 inhalation results).

5c: Percentage Change in B-wave Amplitude with 5 minutes and 15 minutes of O_2 Inhalation

Control Subjects							
		During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	+0.5			+5.0	+11.0	+17.1
	SD	*24.8*			*9.4*	*13.3*	*32.3*
	SE	*9.4*			*3.5*	*5.0*	*12.2*
15min O_2	Mean	+7.7	+1.7	+3.4	+11.1	-14.0	+3.5
	SD	*9.1*	*29.3*	*35.7*	*40.0*	*32.0*	*29.7*
	SE	*3.4*	*11.1*	*13.5*	*15.1*	*12.1*	*11.2*
p		0.557			0.365	0.086	0.212
NDR Subjects							
		During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	-6.7			+13.1	+20.7	+25.0
	SD	*28.6*			*6.0*	*13.2*	*25.5*
	SE	*10.8*			*2.3*	*5.0*	*9.6*
15min O_2	Mean	-0.9	+3.7	+6.0	+13.1	+11.5	+19.4
	SD	*21.6*	*17.6*	*19.2*	*11.7*	*19.2*	*25.4*
	SE	*8.2*	*6.7*	*7.3*	*4.4*	*7.3*	*9.6*
p		0.803			0.957	0.283	0.787

Table A5.3 Percentage change in b-wave amplitudes across time with 5 minutes and 15 minutes of O_2 inhalation.
(p-values shown for Bonferroni pairwise comparisons between 5 minutes of O_2 inhalation results and 15 minutes of O_2 inhalation results).

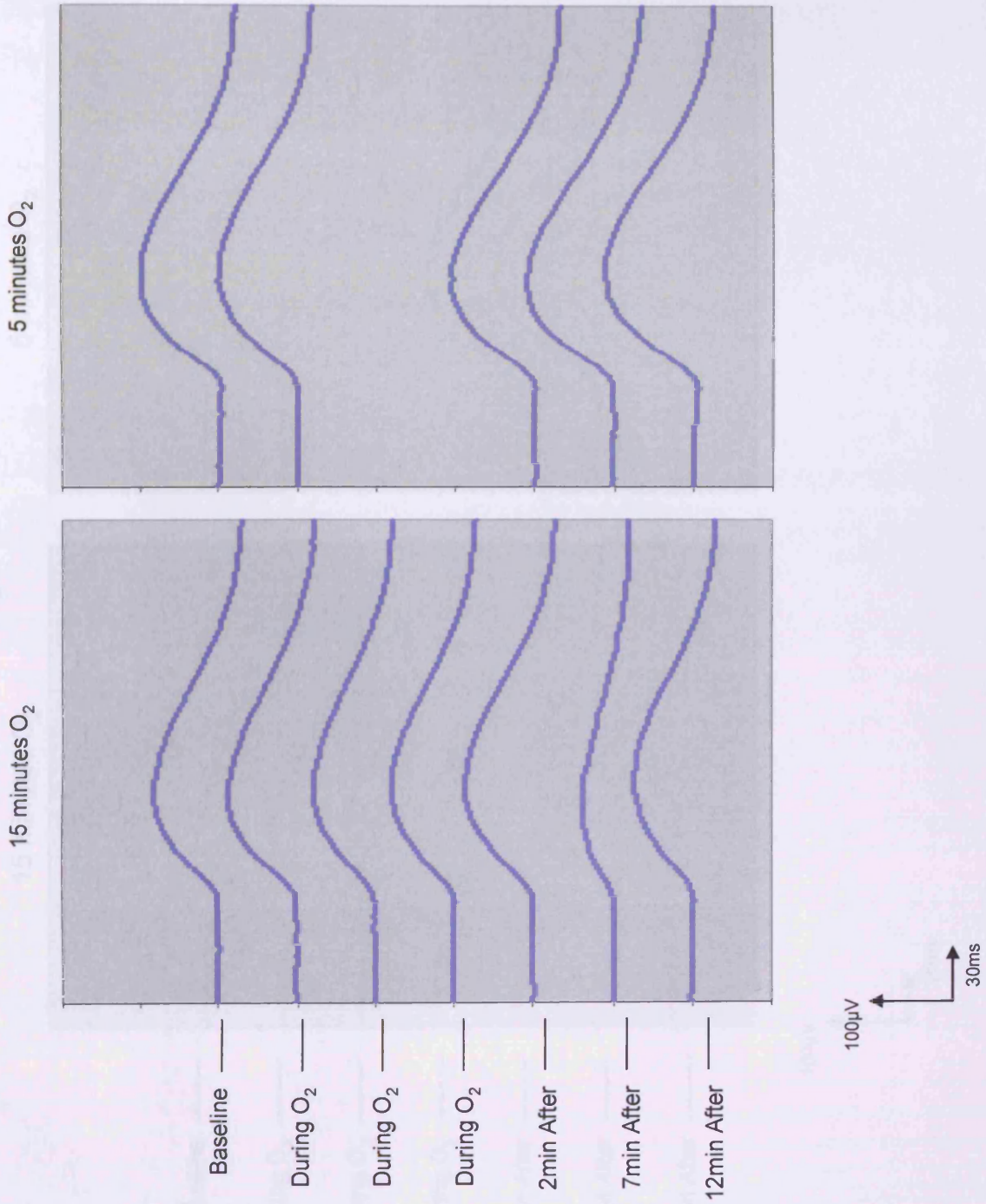

Figure A5.1 Control group-averaged b-wave traces with 15 minutes and 5 minutes of O_2 inhalation

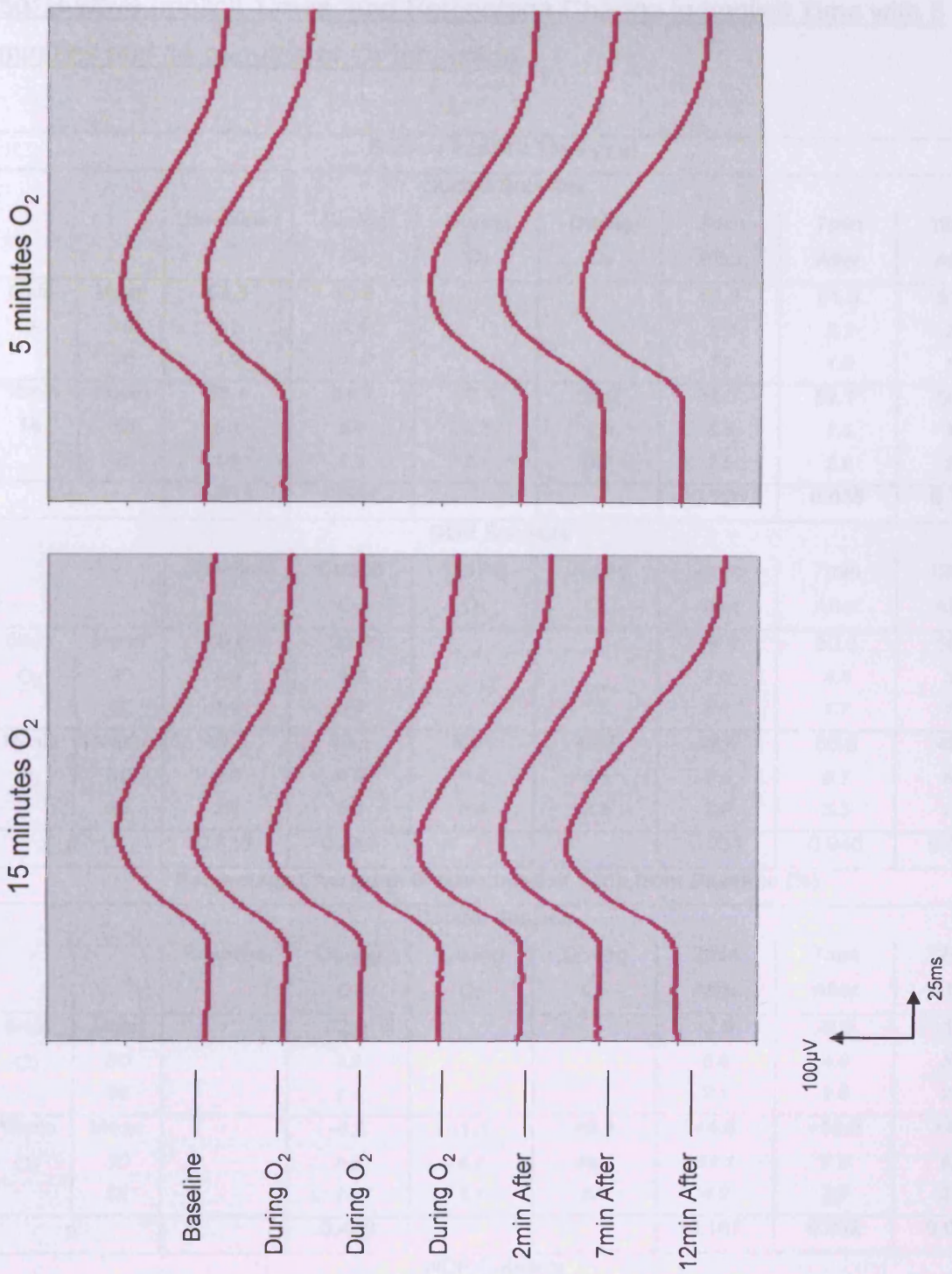

Figure A5.2 NDR group-averaged b-wave traces with 15 minutes and 5 minutes of O_2 inhalation

5d: B-wave Implicit Times, and Percentage Change in Implicit Time with 5 minutes and 15 minutes of O_2 Inhalation

B-wave Implicit Time (ms)								
Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	52.3	50.8			51.2	51.9	51.2
	SD	*2.7*	*3.6*			*3.2*	*2.7*	*2.7*
	SE	*1.0*	*1.4*			*1.2*	*1.0*	*1.0*
15min O_2	Mean	53.4	51.1	53.1	56.0	55.7	**59.1***	56.1
	SD	*5.1*	*6.0*	*8.2*	*8.0*	*6.5*	*7.5*	*7.4*
	SE	*1.9*	*2.3*	*3.1*	*3.0*	*2.5*	*2.9*	*2.8*
p		0.611	0.894			0.126	**0.035**	0.128
NDR Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	49.9	50.0			49.4	50.3	50.4
	SD	*3.8*	*8.0*			*7.6*	*4.6*	*3.2*
	SE	*1.4*	*3.0*			*2.9*	*1.7*	*1.2*
15min O_2	Mean	49.3	49.1	50.4	49.1	49.0	50.0	49.1
	SD	*6.2*	*9.0*	*6.8*	*6.6*	*7.5*	*8.7*	*6.9*
	SE	*2.3*	*3.4*	*2.5*	*2.5*	*2.8*	*3.3*	*2.6*
p		0.838	0.842			0.931	0.940	0.644
Percentage Change in B-wave Implicit Time from Baseline (%)								
Control Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	-	-2.9			-2.0	-0.8	-1.9
	SD		*3.8*			*5.6*	*4.0*	*5.4*
	SE		*1.4*			*2.1*	*1.5*	*2.0*
15min O_2	Mean	-	-4.4	-1.1	+5.1	+4.6	+10.5	+4.7
	SD		*4.9*	*8.1*	*13.4*	*11.1*	*7.9*	*6.2*
	SE		*1.9*	*3.1*	*5.1*	*4.2*	*3.0*	*2.4*
p			0.488			0.167	**0.002**	0.093
NDR Subjects								
		Baseline	During O_2	During O_2	During O_2	2min After	7min After	12min After
5min O_2	Mean	-	+0.2			-1.0	+1.2	+1.4
	SD		*13.0*			*13.4*	*10.2*	*6.9*
	SE		*5.0*			*5.1*	*3.8*	*2.6*
15min O_2	Mean	-	-0.5	+2.3	-0.4	-0.9	+1.1	-0.3
	SD		*11.6*	*2.6*	*3.8*	*5.0*	*7.6*	*7.9*
	SE		*4.4*	*1.0*	*1.4*	*1.9*	*2.9*	*3.0*
p			0.915			0.987	0.978	0.677

Table A5.4 Control and NDR group-averaged b-wave implicit times, and percentage change in implicit time, across time with O_2 inhalation. (p-values shown for Bonferroni pairwise comparisons between 5 minutes of O_2 inhalation results and 15 minutes of O_2 inhalation results).

5e: Individual Control Subject B-wave Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	During O_2 (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
BA	122	128	124	116	126	119	109
RC	67.1	66.9	44.6	99.7	94	61.6	63.1
RW	83.2	95.6	94.1	76.7	91.3	53.2	83.1
DJ	87.2	97.6	108	112	114	96.3	103
CG	72.3	85.9	93.9	91.7	109	90.8	106
RB	54.4	60.2	66.1	51	62	46	67
JC	123	114	68.4	48.2	35.9	34.7	65.4

Table A5.5 Control subject b-wave amplitudes with 15 minutes of O_2 inhalation.

5f: Individual NDR Subject B-wave Amplitudes

Subject ID	Baseline (μV)	During O_2 (μV)	During O_2 (μV)	During O_2 (μV)	2min After (μV)	7min After (μV)	12min After (μV)
JG	64.1	42.2	56.9	62.3	68.5	65.5	58.8
EC	128	110	130	131	128	130	105
SP	172	163	161	142	231	181	219
AJ	101	94.5	83.5	85.7	105	83.7	154
HP	105	140	113	126	117	144	123
HT	82.6	87.1	110	107	93.6	110	118
PG3	213	245	252	267	259	252	260

Table A5.6 NDR subject b-wave amplitudes with 15 minutes of O_2 inhalation.

5g: Individual Control Subject B-wave Implicit Times

Subject ID	Baseline (ms)	During O_2 (ms)	During O_2 (ms)	During O_2 (ms)	2min After (ms)	7min After (ms)	12min After (ms)
BA	43	39.5	37	51	50.5	49	44.5
RC	56.5	49.5	56	46	51.5	64.5	63
RW	54	53	56	55.5	54	63.5	55
DJ	52	49	50	51.5	50	50	51
CG	53.5	55	53.5	54.5	57	55	52.5
RB	58.5	57.5	55.5	65.5	58.5	67	62
JC	56.5	54.5	63.5	68	68.5	64.5	64.5

Table A5.7 Control subject b-wave implicit times with 15 minutes of O_2 inhalation.

5h: Individual NDR Subject B-wave Implicit Times

Subject ID	Baseline (ms)	During O_2 (ms)	During O_2 (ms)	During O_2 (ms)	2min After (ms)	7min After (ms)	12min After (ms)
JG	54	63.5	55.5	57.5	56.5	63	60.5
EC	55.5	53	59	54	53.5	56.5	54
SP	51.5	54	53.5	52	52.5	52.5	51.5
AJ	50	39.5	50.5	47.5	52	47	44.5
HP	38	39	39	38.5	34.5	36	41
HT	52	51.5	51	51	50	50.5	49
PG3	44	43	44.5	43	44	44.5	43

Table A5.8 NDR subject b-wave implicit times with 15 minutes of O_2 inhalation.

Appendix 6
OPs and POBF During Light and Dark Adaptation

6a: Photopic and Scotopic Summed OP Amplitudes During Light and Dark Adaptation

Control Subjects			
Subject ID	**Photopic OPs (μV)**	**Scotopic OPs (μV)**	**Photopic OPs (μV)**
CG	115.1	107.3	119.6
PG	128.6	177.0	130.7
RE	146.9	60.9	91.6
RN	113.8	84.9	141
BV	119.3	135.1	88.4
BA	146.7	216.7	135.9
SH	135.6	159.9	146.9
NDR Subjects			
JG	116.7	109.5	156.8
PM3	178.1	193	119.1
PG3	119.1	160.7	97.9
AB	132.0	196.1	93.9
SP	95.3	88.4	89.5
AD	116.6	103.8	61.3
JC2	90.1	207.9	122.2
TD	83.9	109.8	67.3
RF	149.1	155.6	106.5

Table A6.1 Summed OP amplitudes during light and dark adaptation

6b: POBF During Light and Dark Adaptation

Control Subjects			
Subject ID	**POBF Light (μl/min)**	**POBF Dark (μl/min)**	**POBF Light (μl/min)**
CG	794	923	857
PG	962	926	835
RE	543	588	630
RN	1225	1124	1277
BV	726	850	1015
BA	492	558	545
SH	703	974	906
NDR Subjects			
JG	864	1086	1083
PM3	-	-	-
PG3	899	763	664
AB	-	-	-
SP	584	602	777
AD	555	910	541
JC2	-	-	-
TD	1321	1209	1268
RF	1068	1298	926

Table A6.2 POBF during light and dark adaptation

Appendix 7

Supporting Publications

7a: Poster presentation at the British Congress of Optometry and Vision Science, Aston University, Birmingham, UK, 2003

Design of an Electrophysiological Technique Able to Investigate the Optimum Light Level Required to Suppress the Rod System.

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Purpose: It has been hypothesised by Arden et al (1998) that retinal hypoxia due to the high oxygen consumption of rod photoreceptors during dark adaptation may be a significant factor in the development of diabetic retinopathy. It has therefore been suggested that in order to reduce the oxygen demand to the retina subjects with diabetes may benefit from sleeping with the lights on. We report the development of an electrophysiological technique, employing the simultaneous cone-rod electroretinogram, to investigate further the optimum light level required to suppress the rods.

Methods: Electroretinograms were recorded monocularly using DTL fibres from 7 healthy controls, mean age 24yrs (range 21-48). Signals were amplified, averaged and band-pass filtered using a Medelec Sapphire^{II} 4E system. To obtain optimum separation of the cone and rod responses, a red (peak 655nm) 5msec flash, was presented at 1.3Hz over a range of stimulus intensities (0.022-0.264cdsm^{-2}), following 20mins dark adaptation and with pupil dilation. In order to mimic conditions of sleep, this was repeated through closed eyelids and without pupil dilation. Finally, background illumination was increased in order to determine the optimum level required to saturate the rod response.

Results: Our technique elicits a clear response from the cone and rod systems simultaneously in the dark adapted eyes of a group of control subjects. Dim ambient illumination suppressed the rod response, whilst the cone response remained intact.

Conclusions: From our preliminary data it appears that levels of 30lux or less saturate or significantly reduce the rod response, which is presumed to be associated with a marked reduction in oxygen demand.

7b: Poster presentation at the ISCEV meeting, Glasgow, UK, 2005

Electrophysiological Investigation Into the Effects of Light and Dark on the Retinal Blood Flow in Diabetes Mellitus.

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[2]School of Medicine, Cardiff University, Cardiff, UK

Purpose:
Retinal blood flow increases during periods of darkness, presumably due to the high oxygen demand from the photoreceptors. However it is not known whether this occurs in subjects with diabetes mellitus (DM), where there are known to be changes in autoregulation of the retinal blood vessels. Therefore the aim of this study is to investigate the retinal blood flow directly using pulsatile ocular blood flowmeter (POBF), and the Heidelberg Retinal Flowmeter (HRF), and indirectly by recording oscillatory potentials (OPs) in the dark adapted diabetic eye.

Methods:
Scotopic OPs are to be recorded in a group of 8 control subjects, and 8 subjects with Type II DM with no retinopathy (NDR), after a period of 20 minutes dark adaptation. Signals will be amplified, averaged and band-pass filtered (100-1000Hz) using a Medelec Sapphire^{II} 4E system. They will be recorded to 6 white flashes, 3ms duration, intensity 3cdsm^{-2} (ISCEV standard), at 15s intervals with the first 2 treated as conditioning flashes. Photopic OPs will then be recorded after 10 minutes light adaptation to a white background, intensity 3cdsm^{-2}, to 4 white flashes of 3ms duration and intensity 3cdsm^{-2} at 1.5s intervals (ISCEV standard). Ocular and retinal blood flow will be assessed under photopic and scotopic conditions by means of the POBF (OBF Labs, UK), and the HRF (Heidelberg Engineering GmbH, Germany), respectively.

Preliminary results are still to be collected and will be presented in full at the meeting.

7c: Poster presentation at the ARVO annual general meeting, Fort Lauderdale, Florida, USA, 2005

Electrophysiological Investigation of the Effects of Systemic Oxygen Inhalation on the Oscillatory Potentials and Scotopic B-Wave in Diabetes Mellitus.

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Purpose: The oscillatory potentials (OPs) are known to reflect inner retinal function and have been found to be reduced in subjects with diabetes mellitus (DM). The scotopic b-wave is thought to reflect primarily the activity of the rod bipolars and has also been found to be reduced in DM. Since retinal hypoxia has been implicated in abnormalities of visual function we investigated the effect of 5 minutes of systemic oxygen (O_2) inhalation on the OPs and scotopic b-wave in subjects with DM.

Methods: Fifteen subjects with Type II DM, mean disease duration 8yrs, with no diabetic retinopathy (NDR), mean age 62yrs (SD 6.0), were compared to a group of 15 age-matched controls, mean age 62yrs (SD 10.6). OPs were recorded monocularly after 20mins dark adaptation to 6 white flashes, intensity 3cdsm^{-2} (ISCEV standard), 3ms duration at 15s intervals (first two flashes treated as conditioning flashes). This was then repeated: a) after 2mins of 100% O_2 inhalation through a 60% Ventimask, b) 2mins, c) 7 mins and d) 12mins after removal of the mask. The scotopic b-wave was recorded monocularly (n =17 NDR, n=18 controls), after 20mins dark adaptation at the same time points to a 5ms green flash (peak 515nm), intensity 0.0012cdsm^{-2} (ISCEV standard), at 0.5Hz.

Results:O_2 inhalation increased the summed OP amplitude with a significant increase of 12% and 19% from baseline at 7 and 12mins after removal of the mask respectively using Bonferroni pairwise comparisons ($p<0.001$ RM ANOVA). No significant change in summed amplitude was found in the controls ($p=0.154$). An increase in amplitude was also observed for the scotopic b-wave, of 10% and 8% in the NDR group at these respective time points, though this did not reach significance using the RM ANOVA.

Conclusion:O_2 inhalation significantly increased the summed amplitude of the OPs in NDR subjects while control subject amplitudes remained stable. The scotopic b-wave amplitude increased for both groups though not significantly in this experiment. The increase in summed OP amplitude with O_2 inhalation supports the suggestion of impaired retinal autoregulation in subjects with DM even when no retinopathy is apparent, and suggests that tissue hypoxia may be present in the surface layers of the retina in these subjects.

7d: Poster presentation at the ISCEV meeting, Fontevraude, France, 2006

The Effects of Hyperoxia on the Scotopic ERG in Patients With Diabetes Mellitus.

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PURPOSE: Both the oscillatory potentials (OPs) and scotopic b-wave have been found to be reduced in diabetes mellitus (DM) with retinal hypoxia implicated as a possible cause. We investigated the effects of systemic hyperoxia on these in subjects with and without diabetic retinopathy.
METHODS: OPs: 22 Type 2 DM subjects with no visible retinopathy (NDR), mean age 62yrs (SD10.6), 11 Type 2 DM with background retinopathy (BDR), mean age 65yrs (SD 7.0) and 21controls, mean age 60yrs (SD10.8), were recruited. OPs were recorded after 20mins dark-adaptation to ISCEV standards. Subjects inhaled 100% oxygen (O_2) for 5mins through a 60% Ventimask. This procedure was repeated: a) after 2mins of O_2 inhalation and b) 2mins, c) 7mins and d) 12mins after mask removal.
B-wave: The b-wave was recorded in 24 NDR subjects, mean age 62yrs (SD 6.0), 11 BDR subjects, mean age 63yrs (SD 6.5), and in 24 controls mean age 60 yrs (SD 10.6), after 20mins dark-adaptation to ISCEV standards. It was then recorded both during and after 5mins of O_2 inhalation as described above.
RESULTS:OPs: Hyperoxia increased summed OP amplitudes in the BDR group with a significant increase of 40.9% 12min after mask removal (Bonferroni pairwise comparisons, $p<0.001$ RM ANOVA). Non significant increases in amplitude were found in both the control ($p=0.08$), and the NDR group ($p=0.57$).
B-wave: A significant increase in b-wave amplitude was also observed for the BDR group ($p<0.001$ RM ANOVA), though increases of 20.3%, 22.4% and 23.2% at 2, 7 and 12min after mask removal respectively did not reach significance with Bonferroni pairwise comparisons. Non-significant increases were found in the control ($p=0.33$), and the NDR group ($p=0.10$).
COMMENTS: Hyperoxia significantly increased summed OP and scotopic b-wave amplitudes in BDR subjects, which supports the suggestion of impaired retinal autoregulation and the presence of tissue hypoxia in the inner retinal layers of these subjects.