Evaluation of retinal function and structure in a Parkinson's disease murine model

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Evaluation of retinal function and structure in a Parkinson's disease murine model

by

Courtenay M Brines

A thesis submitted to the graduate faculty

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MASTER OF SCIENCE

Major: Veterinary Clinical Sciences

Program of Study Committee:
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Iowa State University

Ames, Iowa

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DEDICATION

I dedicate my thesis work to my father. Ever since I was a little girl he has supported and encouraged my desire to learn about nature and the world’s creatures -- I thank him from the bottom of my heart.
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I would like to thank my committee chair, Gil Ben-Shlomo, and my committee members, Mary Greenlee and Albert Jergens for their guidance and support throughout the course of this research.

In addition, I would also like to thank my friends, colleagues, the department faculty and staff for making my time at Iowa State University a wonderful experience. I would also like to thank Louisiana State University’s Statistician Michael Kearney for his expertise. Finally, thanks to my family for their encouragement and for their hours of patience, respect and love.
Parkinson’s disease (PD) is characterized by a progressive failure of mitochondrial respiratory function and subsequent loss of dopaminergic neurons, which results in severe neuromuscular deficits. Additionally, patients with PD may have visual function abnormalities. The MitoPark (MP) mouse is an experimental model of PD created by disrupting mitochondrial function in dopamine-producing neurons. MitoPark mice develop motor symptoms of PD, but it is currently unknown whether abnormalities in retinal function and structure exist. The objective of this study was to determine whether the retina of MP mice demonstrates any visual system abnormalities.

Retinal function of 5 symptomatic MP mice, and 5 age-matched wild type (WT) mice was evaluated by full field electroretinography (ERG) at varying light intensities following both dark (scotopic) and light (photopic) adaptation. Comparison between the full field ERG a- and b-wave components of MP and WT mice were performed. Animals were subsequently euthanized and the eyes were prepared for standard histopathologic and immunohistochemical analyses. Immunohistochemistry was performed on retinal tissues using antibodies against Glial Fibrillary Acidic Protein (GFAP) and Tyrosine Hydroxylase (TH), which are markers of retinal stress and dopaminergic cells respectively.
Minor alterations of retinal function were observed in the onset, or implicit time, of b-wave formation in the MP mouse, most notably at the highest intensity.
of light tested. Further, the MitoPark mouse had smaller wave amplitudes than WT mice, but these observations did not reach statistical significance. The histological analyses show that although retinal thickness tended to be decreased in the MP mouse, there were no obvious differences in retinal architecture or in GFAP and TH staining between the two groups. Using a multivariate linear mixed model which included retinal thickness, implicit time and mouse type (i.e., either MP or WT), MP mice exhibited a significantly shorter b-wave implicit time at the highest light intensity tested (10 cd·s/m²) in the scotopic recordings. These results suggest that there are changes in the retinal synaptic architecture of the MP mouse which is both reflected in the retinal thickness and electrical signaling of the retina under conditions which stimulate the rod and cone photoreceptors. It would be beneficial to evaluate more mice to strengthen the general trends observed in this study.

The following chapter will briefly discuss ocular anatomy and electroretinography that serves as the foundation of my thesis.
CHAPTER I. GENERAL INTRODUCTION

Structure of the Eye

The eye is a forward extension of the central nervous system which allows an individual to monitor its surround by utilizing the emission and reflection of light energy (Figure I). The basic structure of the eye in vertebrates is similar across species. The eye, or the globe, is composed of three basic layers. The outer coat is a fibrous tunic which is comprised of the sclera and the cornea. The fibrous sclera gives the globe a constant shape and form and also provides physical protection and attachment points for the extra-ocular muscles to allow for movement of the globe. The cornea, which is the most anterior aspect of the fibrous tunic is transparent which allows light to pass through. The cornea has a high refractive index which focuses light rays centrally towards the visual axis of the eye. The second layer of the eye is the vascular tunic, or uveal tract, which is a highly vascularized structure. The uveal tract is further divided into the choroid, ciliary body and iris. The iris is comprised of vascular connective tissue which provides nourishment, waste removal and functions to modify the amount of light, by indirect change in pupil diameter, allowed into the back of the eye. The lens further refracts light and alteration of the lens curvature via the ciliary body allows for the focusing in near and far fields. The central layer or tunic of the eye is the neural tunic and is comprised of the retina and associated optic nerve. The retina transduces the light into electrical...
signals, a process termed photo transduction, which is then propagated through the neuroretina to ultimately terminate in higher visual centers (i.e., the visual cortex in the brain). The three tunics surround the transparent media within the eye: the aqueous humor, lens and vitreous humor. The transparent media within the eye collectively function to transmit and refract light onto the retina. The transparent media also provides an internal pressure that helps maintain the shape of the globe.

Anatomy and Physiology of the Retina

The retina is a multi-layer organ and includes five main types of nerve cells, two varieties of non-neuronal cells, and two principal synaptic layers. Figure II is a representative diagram of the important retinal cells for this discussion. The five main types of nerve cells of the retina include photoreceptors (rods and cones), horizontal, bipolar, amacrine, and retinal ganglion cells (RGCs). The inner and outer plexiform layers are synaptic layers where information passes from one retinal cell to the next. As such, they are sandwiched in between the retinal cell layers (nuclear layers).

Incident light transverses the many retinal layers and unmyelinated fibers before striking the light-sensitive photoreceptors, called the rods and cones. The visual impulse initiated in the photoreceptor cells is carried to the visual centers by a pathway of two additional radially oriented neurons: the bipolar cells and the RGCs. The axons of the RGCs form the optic
nerve. The bipolar cells are located within the inner nuclear layer (INL) and are deemed to be an intraretinal interneuron. There are three types of bipolar cells, and they can be distinguished by their connections with the photoreceptor cells (1). Briefly, there are depolarizing bipolar cells (ON-response) which depolarize under stimulation of light. ON-response bipolar cells receive input from either rod or cone photoreceptors. There are also hyperpolarizing bipolar cells (OFF-response) which do not propagate an electrical signal in the presence of light.

Horizontal or lateral neurotransmission mediated by neurons in the OPL and INL play a critical role in shaping the temporal and spatial qualities of scotopic (dark) and photopic (light) vision. The horizontal cells are at the first synapse in the OPL and modify the response between the photoreceptor and bipolar cells. Horizontal cells form triads, composed of a presynaptic photoreceptor axon terminal and three postsynaptic dendrites: one of a BP cell and two of horizontal cells. Two subtypes of horizontal cells have been described (1). Horizontal cells form a network, coupled laterally by cell junctions which allows for a rapid exchange of electrical current and small molecules (1).

Another major cell responsible for lateral neurotransmission is the amacrine cell and a subtype of cells called the interplexiform cells (IPcs). The amacrine cells reside in the second synapse, the INL, and provide a connection between bipolar and RGCs. Amacrine cell processes form dyads composed of one presynaptic bipolar axon terminal and two
postsynaptic dendrites to one ganglion cell and one fellow amacrine cell (1). There are several classification schemes which have been used to describe the different types of amacrine cells (1, 2). Functionally, amacrine cells serve to increase the sensitivity of ganglion cells to changes in illumination, thus contributing to motion, direction, and contrast discrimination. The interplexiform amacrine cells are a subtype of amacrine cells that constitute a group of long-range feedback neurons that modulate information. This specialized neuron has its soma within the INL and forms a feedback pathway, mainly presynaptic, on horizontal cells (3). This regulatory loop acts on horizontal cell regulating the diameter of receptive fields and subsequently, the bipolar cells (1).

Glutamate, an excitatory neurotransmitter, serves as the principal vertical neurotransmitter of the retina (4, 5). Glutamate plays an important role in vertical transmission of electrical signal from the photoreceptors, bipolar cells to the RGCs. Under dark conditions, photoreceptors are continuously depolarizing and releasing glutamate. Briefly, when the photoreceptors perceive light stimulus, glutamate release from these cells decreases, which prompts transmission of the signal up to the second-order retinal neurons as post synaptic glutamate receptors of BP cells are inhibitory.

The main inhibitory transmitter in the retina is gamma Amino Butyric Acid (GABA) (6). Gamma Amino Butyric Acid is utilized by both horizontal and amacrine cells for lateral processing of the signal which has an
inhibitory function in the OPL and IPL, respectively. Together with glycine, GABA acts to inhibit ganglion cells by opening Cl⁻ channels on retinal neurons (7).

Dopamine, an abundant catecholamine in the retina, is contained within the amacrine and interplexiform cells (1, 8). Dopamine containing cells have been identified in the rodent and human retina using an antibody directed to tyrosine hydroxylase (TH), the biosynthetic enzyme which produces dopamine (DA) (1, 9). Dopamine has been established to function as a major neurotransmitter and neuromodulator in vertebrate retina (1, 10). Dopamine decreases horizontal cell-gap junction permeability and consequently narrows the receptive field which aids in visual contrast sensitivity while GABA increases permeability and widens the receptive field of horizontal cells (1). Modulation of information occurs by the interplexiform cells occurs via glycine, dopamine or gamma-aminobutyric (GABA) (11).

The Full Field Electroretinogram

Electrodiagnostic testing provides a unique, noninvasive opportunity for researchers to objectively evaluate the visual system. An electroretinogram (ERG) is a recording of the cumulative electric potentials of the retina generated to a standardized light stimulus (12). An ERG is a compound electrical wave arising from the activity of different cells within the neuronal layers of the retina. The wave form of the ERG is affected not
only by the intensity and duration of the stimulus but also by wavelength.
The ERG can be recorded in scotopic (dark adapted) conditions to assess the rod or mixed rod-cone functions and in photopic condition (light adapted) to investigate the cone system (12). Measurement of the amplitude and implicit time generated from retinal responses to visual stimuli can help determine the integrity of the functional retina. The ERG provides objective results and assists in characterizing the function of specific cell types within the retina.

Full field ERG tracings are typically comprised of two major components. Values reported are the implicit times (milliseconds) and amplitude (microvolts) for the major components (See figure III). The first wave is the a-wave. It is the first negative deflection, or hyperpolarization, representing mainly the intracellular charge of the photoreceptors caused by light-evoked closure of the sodium channels on the cell membranes (13, 14). The amplitude of the a-wave is measured from the baseline to the trough of the a-wave. The a-wave implicit time refers to the time between stimulus onset and the trough of the a-wave. The hyperpolarization diminishes the release of neurotransmitter, which in turn activates bipolar cells.

The b-wave is the positive deflection of the fERG and is primarily generated by bipolar cells (13, 15, 16). The amplitude of the b-wave is measured from the trough of the a-wave to the peak of the b-wave. Implicit time of the b-wave is measured from the onset of the stimulus to the peak
of the b-wave. On the ascending portion of the b-wave, a series of high-frequency wavelets, called oscillatory potentials (OPs), can be observed. The exact origin of these wavelets is still unclear, but it is thought that they arise from the interaction between amacrine and bipolar cells (17, 18). The flash ERG is used to assess mainly the function of the photoreceptors and bipolar cells, or the outer and mid-retinal function of the retina (18, 19).

Different stimuli and recording paradigms will allow separation of responses contributed by different types of cells in the retina. The International Society for Clinical Electrophysiology of Vision (ISCEV) provides recommendations for standardized protocols so that comparable ERGs could be recorded throughout the world (20). In their recommended standards, the following responses, named according to conditions of adaptation and stimulus (flash strength in cd·s/m²) are as follows: Dark-adapted 0.01 ERG (“rod response”), Dark-adapted 3.0 ERG (“maximal or standard combined rod-cone response”), Dark-adapted 3.0 Oscillatory potentials, Light-adapted 3.0 ERG (“single-flash cone response”), Light-adapted 3.0 flicker ERG, and recommended additional response testing of either Dark-adapted 10.0 ERG or Dark-adapted 30.0 ERG. Although the separation of different components is usually not complete, enhancing responses driven by one system and suppressing responses from other systems in the retina helps to increase the diagnostic
value of the FERG. Varying the stimulus and background light wavelengths and intensities, stimulus duration and frequency enhances different components of the FERG (20).

Thesis Organization

In chapter II this thesis provides an introduction to Parkinson’s disease and summarizes relevant animal models, including the MitoPark mouse which was used in this study to evaluate retinal function and structure. Next, chapter III describes the observed retinal structure and function of MP mice exhibiting motor symptoms of PD. Lastly, chapter IV summarizes the findings and concludes with recommendations for future research.
Figure I. General anatomy of the eye.
Figure II. Cross-section of the retina demonstrating the principal cell types involved in retinal signaling.

Key: RPE = Retinal Pigmented Epithelium; PRs = Photoreceptors; ONL = Outer Nuclear Layer; OPL = Outer Plexiform Layer; INL = Inner Nuclear Layer; IPL = Inner Plexiform Layer; RGCL = Retinal Ganglion Cell Layer.
Figure III. Examples of photopic and scotopic ERG recordings.
Figure III represents an example of scotopic ERG trace in a mouse. The red arrow indicate flash onset. The a-wave amplitude (first blue solid arrow bar) is measured from the pre-stimulus, or baseline, to the trough of the a-wave, and the b-wave amplitude (second solid black arrow bar) is measured from the trough of the a-wave to the peak of the b-wave. The oscillatory potentials can be observed on the ascending part of the b-wave. The implicit time of the a-wave (first dashed blue arrow bar) refers to the time between stimulus onset and the trough of the a-wave whereas the b-wave implicit time (second dashed black arrow bar) is measured from the onset of the stimulus to the peak of the b-wave.
CHAPTER II. LITERATURE REVIEW

An Introduction to Parkinson’s Disease

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, behind Alzheimer’s, with a prevalence of 0.3% and has an estimated incidence of 8-18 per 100,000 cases per year (2, 21). The prevalence of PD increases with age, with approximately 1% in the over 60 (22, 23) and 4% in the over 80 year old population being affected (21). Parkinson’s disease is characterized by selective and progressive degeneration of mesencephalic dopaminergic (DA) neurons and neurotransmitter in the substantia nigra compacta (SNpc) and striatal dopamine depletion (24, 25). Like many neurodegenerative diseases, the cause of PD is not known, but it is thought to be due to a complex interaction among multiple predisposing genes and environmental contributions (26). Most cases of PD are sporadic, or idiopathic; however there are a few rare familial forms that have been described (27). The first report of PD, described by James Parkinson in 1817, focused on the cardinal signs of motor dysfunction in patients, including resting tremors, bradykinesia, impaired balance and rigidity (28). The basal ganglia, located within the brain, are affected in individuals with PD. The basal ganglia are
associated with a variety of functions including the control of voluntary movement, procedural learning, routine behaviors, eye movements, cognition and emotion.

Since PD was first described, neural circuits that extend beyond the basal ganglia have been identified as part of the PD neurodegenerative disorder, and therefore PD is now considered to be a multi-system disease (2). There are many non-motor symptoms associated with PD that can appear well before the onset of the motor phenotype (29-31). Non-motor symptoms, similar to the classically described motor symptoms, can also play a significant role in quality of life for PD patients. Common non-motor symptoms of PD can include depression, apathy, hyposomnia, impaired cognition and autonomic failure including gastrointestinal and sensory dysfunction (2, 29-33). Sensory dysfunction may include loss of smell, auditory issues, ‘restless leg’ syndrome, and visual dysfunction.

The mitochondrial theory of aging remains one of the most prominent hypotheses for the gradual decline of an organism’s fitness with age (34) and may help explain the changes seen in patients with PD. Animal models of mitochondrial dysfunction have been successful in replicating a number of the phenotypic traits associated with normal aging such as weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anemia, reduced fertility and heart enlargement (35). Specific to retinal disease, studies have indicated that increased mitochondrial DNA damage is associated with age related macular degeneration (36, 37), late stage
retinitis pigmentosa (38) and degeneration of retinal ganglion cells in certain disease conditions (39). These findings indicate a potential link between mitochondrial dysfunction and progression of retinal diseases (34).

Evidence for Visual Dysfunction in Parkinson’s Disease

The physiologic abnormalities resulting from DA neuron degeneration in the central nervous system extends to the visual pathways in PD (24). Visual dysfunction is thought to occur at several levels of the visual pathway, supported by evidence of psychophysical, electrophysiological, and morphologic disruption of retinal structure and function (2). For example, in some PD patients there is evidence to support electrophysiological deficits in visual information processing from the retina to the primary cortex (33, 40). Perceptual defects like abnormal vision-evoked potentials can be recognized in PD patients and in the monkey model (41, 42). Electrophysiological as well as spatial and temporal contrast sensitivity measurements suggest that visual dysfunction is caused by a degeneration of DA neurons (42). Post-mortem assays for DA in the retina showed severe degeneration of the foveal DA innervation in PD patients (24).

Common visual symptoms in patients with PD can range from complaints of dry eye (43), visual acuity loss (44-47), reduced color discrimination (48, 49), perceptual disturbances (i.e., orientation motion
perception (50), facial perception (51) and complex visual hallucinations (47, 52) and have been studied extensively (2, 32, 46, 53-55). Such visual symptoms are a considerable cause of morbidity in PD (56), and with respect to visual hallucinations, are an important predictor of cognitive decline as well as institutional care and mortality (57-60). Animal models have been developed in an effort to study the multi-system nature of PD, as understanding this may help achieve early diagnosis of disease and may help understand the pathophysiology of the disease (61).

Animal Models of Parkinson’s Disease

Parkinson’s disease patients have been observed to possess frequent mitochondrial defects in the affected DA neurons (62, 63). There is a higher susceptibility of DA neurons to mitochondrial dysfunction and the resultant generation of reactive oxygen species when compared to other neurons (27). Toxins used to create PD animal models, such as 6-hydroxydopamine (6-OHDA) and 1 methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), inhibit mitochondrial function, which in turn impairs cellular energy generation and subsequently causes acute damage (i.e., death) to midbrain DA neurons and severe motor impairment (63). This results in work-related cellular injury occurring from the generation of reactive oxygen species which occur when ATP supply does not meet demand.
The classical preclinical toxin based models of PD use 6-OHDA and MPTP to induce selective loss of DA neurons in the SNpc (25, 63), and produce similar biochemical and neuropathological defects as those in PD patients. Both of these toxic compounds cause acute damage to midbrain DA neurons, severe motor impairment and a sudden decrease in striatal DA levels (27, 63). Both MPTP and 6-OHDA have been shown to quickly induce degeneration of retinal DA amacrine interneurons in various animal models (64-69). Since there are near instantaneous effects of MPTP and 6-OHDA on rodent models, high inter-animal variability and potential for systemic toxicity (62), there is a limit to their use in studies focused on the development and progression of PD.

Genetic linkage studies of PD in humans have also led to the development of transgenic mouse models for the study of PD. In brief, mouse models have provided insight into the pathogenic role of specific genes, such as \(\alpha\)-synuclein, leucine-rich repeat kinase 2 (LRRK2) and parkin within the context of familial forms of PD (27, 70). Knockout mice with deletion of parkin, Dj-1 or PINK1 genes have been generated (71-73), as have mice overexpressing mutant forms of human \(\alpha\)-synuclein (74). These genetic models result in only minor motor impairment with little or no nigrostriatal DA degeneration. Nevertheless, mouse models based on inactivation or mutations in known PD genes provide valuable information.
on the causes and mechanisms of PD development, as disease etiology and progression is expected to be a combination of genetic and environmental factors (27).

In studies examining PD patients versus age-matched controls, higher numbers of respiratory chain deficient dopamine neurons were found in patients with PD than in their age-matched counterparts (75). In the MitoPark (MP) mouse model (DAT^{+/cre} –Tfam^{loxP/loxP}), DA neurons are rendered respiratory chain deficient by cell type-specific inactivation of mitochondrial transcription factor A (Tfam), which is a protein essential for mitochondrial DNA expression and maintenance (76-80). Targeting the genetic lesion to dopaminergic neurons alone is accomplished by utilizing the dopamine transporter gene locus which is activated only in DA cells. The severe respiratory chain deficiency which results is demonstrated by evidence of decreased mitochondrial DNA (mtDNA) encoded cytochrome c oxidase (COX) subunit I transcripts and reduced COX enzyme activity (81) in midbrain DA neurons in this model.

The MP mouse model demonstrates clinical, or phenotypical, features of PD including adult onset of neurodegeneration, progressive phenotypic decline in motor function, presence of intraneuronal inclusions, earlier onset and more extensive cell death in the SNpc than in the VTA and altered responsiveness to levodopa (L-DOPA) treatment dependent upon the disease stage (81-83).
The MP mouse model provides researchers with a more than a 30 week time window during which the process of nigrostriatal DA neuron loss and related secondary changes in neurocircuitry can be studied. MitoPark mice appear normal at birth with no increased embryonic or neonatal lethality and show no PD like symptoms at weaning or as young adults (82). Locomotion deficits progress and at 20 weeks the mice start to display more evident phenotypic PD manifestations such as tremor, twitching and abnormal gait. By 30 weeks there are severe motor deficits. MitoPark mice are usually euthanized around 45 weeks because of their poor general condition (81, 82).

Conclusions

The MP mouse model demonstrates phenotypical features of PD including adult onset neurodegeneration and progressive phenotypic decline in motor function evident by tremors, twitching and abnormal gait. Visual function abnormalities similar to those reported in humans have not been evaluated in the MP mouse model. It would be beneficial to determine, using flash electroretinogram recordings, whether retinal function abnormalities exist in a mouse model of PD that is displaying phenotypical signs of motor dysfunction, and if so, whether these are similar to the human disease. Since the available evidence suggests that humans with PD often exhibit decreased amplitudes and prolonged implicit times of fERG
b- waves, we anticipate that a mouse model developed to reflect the dopaminergic motor dysfunction of humans will display similar non-motor dopaminergic dysfunction.

Furthermore, we are interested in relating potential alterations in retinal structure to functional changes in this murine model. Correlating retinal structure and functional changes may help clarify the potential trophic role of dopamine in maintaining retinal structure and function.
REFERENCES


CHAPTER III. EVALUATION OF RETINAL FUNCTION AND STRUCTURE IN A PARKINSON’S DISEASE MURINE MODEL

A paper to be submitted to Journal of Investigative Ophthalmology and Vision Science

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Abstract:

Objective. Parkinson’s disease (PD) is characterized by a progressive failure of mitochondrial respiratory function and subsequent loss of dopaminergic neurons, which results in severe neuromuscular deficits. Additionally, PD patients often have abnormalities in visual function. The MitoPark (MP) mouse is a PD model created by targeted disruption of mitochondrial function in dopamine-producing neurons. MitoPark mice develop motor symptoms similar to human PD, but it is currently unknown whether abnormalities occur in retinal function and structure. The objective of this study is to evaluate functional and structural changes to the retina of MP mice.

Methods. Retinal function of 5 symptomatic MP and 5 age-matched wild type mice was evaluated following overnight dark adaptation using full field electroretinography (ERG) with intensities of 0.0003, 0.01, 1, 3 and 10 cd·s/m².

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as well as at 3 cd·s/m² following 10 minutes of light adaptation. Retinal staining, supplemented by immunohistochemistry for glial acid fibrillary protein (GFAP) and tyrosine hydroxylase (TH). Comparison between the full field ERG a- and b-wave components of MP and WT mice and retinal measurements was performed using a mixed model linear regression.

**Results.** The results of the scotopic and photopic full field ERG showed no statistically significant changes in a- and b-wave amplitudes; however, the b-wave amplitude tended to be lower in MP mice compared to WT at all tested light intensity levels. The a-wave implicit time was not different between the groups.

In contrast, the MP mice exhibited tendency towards both an increased b-wave implicit time at 0.0003 cd·s/m² illumination (p =0.06) and a shorter implicit time at 10 cd·s/m² illumination (p=0.05) compared to WT. No obvious difference in retinal architecture was observed between MP and WT mice, although MP mice tended to have thinner retinas. Immunohistochemistry revealed strong TH staining in the inner plexiform layer in both mouse types. No GFAP staining was observed in either group. When retinal thickness was included as a variable in multivariate linear analysis, MP mice had significantly shorter implicit times (p=0.03) when compared to WT mice at the highest light intensity tested under scotopic conditions.

**Conclusions.** The retinal functional changes observed in MP mice are consistent with a delay of the initiation of electrical transmission vertically
through the outer retina at low light intensities and without significant effects on the ultimate b-wave amplitudes generated. In contrast, b-wave implicit time is decreased at the highest light intensity tested, but without effects on the ultimate wave amplitude generated. The MP model does not show significant retinal neuronal losses when motor symptoms of Parkinson’s disease are manifest. However, there is a trend for reduced retinal thickness in the MP mouse which may be the result of subtle retinal cell losses. The shortened implicit time of b-wave formation at 10 cd·s/m² suggests that the retinal neurons affected in the MP mouse are inhibitory neurons within the retina. Further studies with increased number of mice are needed to clarify the physiologic significance of the differences observed between the groups.

Key Words: MitoPark mouse model, retinal degeneration, Parkinson’s disease, amacrine cell, interplexiform cell, flash electroretinogram

Introduction:

Parkinson's disease (PD) is characterized by striatal dopamine depletion caused by a progressive degeneration of mesencephalic dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) (1, 2). Like many neurodegenerative diseases, the etiology of PD is unknown, but is hypothesized to arise from a combination of genetic and environmental factors that result in neuronal mitochondrial dysfunction (3).
This metabolic abnormality ultimately results in DA neuron degeneration in the central nervous system, and also occurs in the retina, as electrophysiological and functional testing have demonstrated perceptual deficits in PD patients and in experimental animal models (4, 5). Further, the observed abnormal processing of visual information has been correlated to decreased retinal levels of dopamine and its rate-limiting synthesizing enzyme tyrosine hydroxylase in dopaminergic nerve processes in parkinsonian humans (6-8) and toxin-based (e.g., 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine, or MPTP) in animal models (9, 10). Retinal DA concentration has also been documented to be decreased in post-mortem tissue from PD patients(7). As a result of neuronal losses, optical coherence tomography has shown retinal thinning of the macular region and inner retina of human PD cases (11). Additionally, electrophysiological retinal measurements (e.g., full field electroretinography), in addition to spatial and temporal contrast sensitivity measurements, help support the hypothesis that visual dysfunction in PD is related to the degeneration of DA neurons (5). The flash electroretinogram (fERG) is a tool utilized to evaluate the functionality of the outer retina, mainly photoreceptors and bipolar cells. In PD fERG amplitudes in human patients have predominantly been documented to be decreased (12-16) and have prolonged implicit, i.e., wave onset, times.

Since TH is the rate limiting enzyme in the synthesis of DA, TH staining can be used to identify dopaminergic neurons within the retina. In
mammalian retinas, TH immunoreactive DA neurons include a subtype of amacrine (AII) and the interplexiform cells (6, 17). Dopaminergic amacrine cells modulate both rod- and cone-mediated vertical pathways, with dopamine playing a crucial role as a chemical messenger in light-to-dark adaptation (12, 13). Dopaminergic interplexiform cells send processes to both the inner and outer plexiform layers and regulate horizontal cell coupling which, in turn, modulates the diameter of horizontal cell receptive fields (6).

Mutations in mitochondrial DNA are known to cause neurodegeneration and may help explain, at least in part, PD pathophysiology (3, 18). Gradual failure of mitochondrial function leads to work-related neuronal injury and ultimately to cell death. The MitoPark (MP) mouse model, generated by mitochondrial gene disruption targeted to dopamine-producing cells by use of the dopamine transporter gene (18), recapitulates many of the phenotypic motor features of human PD, including bradykinesia, resting tremor, rigidity and impaired motor balance. Progressively declining motor deficits seen in the MP mice are directly related to declining numbers of TH-positive cells and fibers which becomes severe by 30 weeks of age (18). The MP mouse model is based on direct genetic impairment of respiratory chain function in dopamine neurons, and therefore may closely resemble the human disease.

To our knowledge retinal function and structure have not been evaluated in the MitoPark murine model. The aim of this study was to
characterize the retinal function and structure of symptomatic MP mice by means of electroretinography and histopathology, respectively.

**Materials and Methods:**

All experiments were performed in accordance with the guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Iowa State University Animal Care Committee #1-12-7281- M (Ames, Iowa, USA).

Generation of MitoPark mice (DAT<sup>+/cre</sup>–Tfam<sup>loxP/loxP</sup>) has been described previously (18). The MitoPark mice were obtained from Dr. Anumantha Kanthasamy (Department of Biomedical Sciences; Parkinson’s Disorder Research Laboratory, Iowa Center for Neurotoxicology; College of Veterinary Medicine, Iowa State University; Ames, IA USA). Respective wild type (WT) mice of C57bl/6 background (Charles River Laboratories, Wilmington, Massachusetts) were used as age- and sex-matched controls (n=5).

A total of five MP mice were used in this study. The females (n=2) were between 20-25 weeks of age while male mice (n=3) were 30-35 weeks of age. Ages of mice used in this study were determined based on when the mice were phenotypically displaying signs of dyskinesia and motor incoordination. All MP mice were deemed to be displaying clinical signs of motor dysfunction. Mice were housed in standard conditions: 1-3/cage in a
humidity controlled (relative, 30%) room and constant temperature (22 +/- 1°C) and a 12-hour light/dark cycle with ad libitum access to food and water.

**In vivo Electroretinography**

For *in vivo* full field electroretinography (fERG), animals were dark-adapted for at least 12 hours. All preparations were performed under a dim red light. Anesthesia was induced with isoflurane gas and maintained with an intraperitoneal injection of ketamine (0.08 mg/1g; Bioniche Teoranta, Inverin, Co. Galway, Ireland) and xylazine (0.015 mg/1 g; Lloyd, Shenandoah, Iowa, USA) mixture. Animals were then positioned in sternal position on a heated table (Roland Consult, Brandenburg, Germany) maintained at a constant temperature 37 +/- 1°C. Topical application of 1% tropicamide (Bausch and Lomb, Tampa, Florida, USA) was used as a mydriatic agent prior to full field ERG recordings.

For fERG recordings a 2.5 mm gold ring active electrode (Acrivet Veterinary Division, Hennigsdorf, Germany) was placed concentrically on both corneas after the application of 2.5% Hyromellose Ophthalmic Solution (Goniovisc™, HUB Pharmaceuticals Rancho Cucamonga, California, USA) to each eye to ensure good contact with the electrodes and to prevent desiccation of the corneas. Subdermal needle electrodes (Roland Consult, Brandenburg, Germany) placed in the ipsilateral scruff and at the base of the tail served as reference and ground electrodes, respectively. The RETIanimal software RETIport (Roland Consult,
Brandenburg, Germany) generated and controlled the light stimulus. Brief single flash stimuli were delivered in a Ganzfeld Q450 dome shielded by a custom-made Faraday cage.

For scotopic recordings, stimuli were elicited by brief flashes of LED white light. The ERG was recorded in response to the following stimuli: 0.0003 cd·s/m², 0.01 cd·s/m², 3 cd·s/m² and 10 cd·s/m². For each 0.003 cd·s/m² intensity tested, 40 responses were averaged. Five responses were averaged for 0.01, 3.0 and 10 cd·s/m² in order to reduce noise. An interstimulus interval of 5 seconds was used for the dimmest intensity (0.0003 cd·s/m²). For 0.01 cd·s/m² stimuli an interstimulus interval of 2.1 was used. An interstimulus interval of 10.5, seconds was used for 3.0 and 10.0 cd·s/m² intensities. For photopic recordings, animals were light adapted to bright LED white light (30 cd·s/m²) for 10 minutes and the ERG was recorded in response to bright flashes of 3 cd·s/m². Five responses were averaged for photopic recordings and there was an interstimulus interval of 1.6 seconds between flashes of light with a background intensity of 25 cd·s/m².

**ERG Data Analysis**

Electroretinogram recordings were analyzed in accordance to the International Society for Clinical Electrophysiology of Vision (ISCEV) guidelines (19). The a-wave amplitude and implicit time were measured
from the baseline to the first negative trough. The b-wave implicit time was measured from the baseline to the first positive peak, and the amplitude was measured from the first negative trough (the a-wave) to the first positive peak.

**Histologic and Morphometric Analysis of the Retina**

While still under anesthesia at the end of the ERG recordings, the mice were humanely euthanized via intraperitoneal injection of 0.1 ml pentobarbital/phenytoin sodium solution (Beuthanasia-D Special, Intervet/Merck Animal Health). The eyes were collected and then embedded and sectioned for histological analysis. Retinal sections were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. Digital photographs of the retina were taken using a photomicroscope (Olympus BX41 Microscope, Japan, and Olympus DP 20 Camera, Japan). ImageJ software (National Institutes of Health) was used for morphometric evaluation of the different retinal layers from the photographs.

Five measurements, at approximately 50 um intervals, were performed to measure the IPL, INL, ONL along 250 um of retina located near the optic nerve. The entire thickness of the retina, from outer to inner limiting membrane was also determined.
Statistical Analysis

The SAS® (Version 9.4, SAS Institute, Cary, NC) Mixed Procedure was used to conduct an analysis of variance using a mixed effects model. Fixed effects in the first set of analyses included eye, type (MP or WT), light intensity, and the two- and three-way interactions, with models analyzed by wave form. Fixed effects in the second set of analyses included Type, Light Intensity, full retinal thickness, and the two- and three-way interactions, with models analyzed by waveforms. In both analyses, animal was a random effect. When overall significance was found, post hoc comparisons were conducted with pairwise t-tests of the least-squares means. Differences in retinal thickness were assessed using a student’s t-test for independent samples. All analyses were considered significant at \( p \leq 0.05 \) (two tailed).

Results

Electroretinography

Values for all parameters are tabulated in Appendix B. The a-wave implicit time values were similar for both groups of mice under scotopic conditions at 3.0 and 10.0 cd·s/m² and photopic conditions at 3.0 cd·s/m²
(p>0.05). Figure I shows the adjusted least square means for scotopic recordings of a-wave implicit times at 3 cd·s/m², 10 cd·s/m² and photopic recordings at 3.0 cd·s/m².

The a-wave amplitude values were similar for both groups of mice under scotopic conditions of 3.0 and 10.0 cd·s/m² and photopic recordings at 3.0 cd·s/m² (p>0.05). There is a general trend of delay of the MitoPark mouse response at all levels tested. Figure II shows the adjusted least square means for scotopic recordings of a-wave amplitude at 3 cd·s/m², 10 cd·s/m² and photopic recording at 3.0 cd·s/m².

The b-wave implicit time approached significant differences between groups at 10.0 cd·s/m² (p = 0.05) and at 0.0003 cd·s/m² (p=0.06). (Fig. III). For the scotopic recordings, the general trend was shortening of implicit time (p≥0.05) as stimulus intensity increased except for the WT mouse group at 10.0 cd·s/m². The slopes of the implicit times of each group are parallel until higher stimulus intensities, in which the MP mice have a shorter implicit time than the WT. The b-wave implicit time for intensity 3 cd·s/m² under photopic conditions was not significant (See figure V.A)

B-wave amplitudes did not significantly differ (p> 0.05) between groups, although there was a general trend that MP mice had lower b-wave amplitudes compared to WT at all light intensities tested under scotopic conditions which included 0.0003 cd·s/m², 3.0 cd·s/m² and 10.0 cd·s/m².
(See figure IV). The mean b-wave amplitude formation of intensity 3 cd·s/m² tested under photopic conditions did not differ between mouse groups (See figure V.B).

**Retinal Histology**

Representative retina histology images are depicted in Figure VI-A & B. The average retinal thickness (± standard deviation) although less in the MP mouse, did not differ significantly between mouse group (See Table I and Figure VII). The total thickness of the retina was 126.94 (±20.61) µm for the WT mice group and 112.63 (±22.44) µm for the MP mouse group. Reported thickness for the INL was 27.34 (±9.62) µm and 23.17 (±4.48) µm for the WT and MP mice, respectively. Measurements for IPL were 43.61 (±11.57) µm and 34.41 (±7.33) µm for the WT and MP, respectively. Lastly, measurements for ONL were 42.08 (±9.66) µm and 37.55 (±6.08) µm, respectively. The immunohistologic staining for GFAP revealed little or no detectable GFAP in any of the retinal sections (Data not shown). Tyrosine hydroxylase staining revealed no appreciable differences between mouse groups (Figure VIII A, B).
**Combination of b-wave Implicit Time and Full Thickness Retinal Values**

When full retinal thickness was incorporated into the linear model, significant differences between mouse groups was seen at the highest light intensity tested ($p = 0.03$) (Figure IX).

**Evaluation of b-wave implicit time at 10 cd·s/m$^2$**

Given the significance of b-wave implicit time and full retinal thickness at the highest light intensity tested, further evaluation of the data for that individual intensity was evaluated using an unpaired t-test. Significant differences between mouse groups were seen ($p=0.008$) (Figure X).

**Discussion**

To the best of our knowledge, this is the first study which has evaluated retinal function and structure of the retina in the MitoPark mouse model. We characterized the full field ERG response in MitoPark mice at an age in which they were phenotypically displaying signs consistent with Parkinson’s disease- like symptoms, such as obvious resting tremors, rigidity and impaired motor balance. There were no appreciable differences in amplitude of the a- or b- waves, indicating that photoreceptor and bipolar neuron response, respectively, to the stimulus were not overtly affected.
However, the shorter implicit time of the b-wave at the highest light intensity tested (10 cd·s/m²) in the MP group compared to WT group suggests that there are alterations in the retinal synaptic interconnectivity, e.g., dopaminergic interplexiform neurons, in the MP mouse model that affects the function outer retina.

Although MP animals were studied when they exhibited clear neurological symptoms consistent with the loss of dopamine neurons modulating the neuromuscular system, no clear dropout of retinal dopaminergic neurons was appreciated when evaluating TH staining in the MP compared to WT mouse groups. Furthermore, a lack of GFAP immunoreactivity, a marker of glial response to injury(20), is consistent with a lack of retinal injury in the MP mouse. In sum, these results show that although the MP model has a targeted injury of mitochondria in dopaminergic neurons, there is no major anatomical lesion within the retina comparable to the massive injury in the motor system. This is surprising as retinal neurons are generally have a high metabolic rate and ATP production, which would lead rapidly to cellular injury due to metabolic work-related injury from free radical production (21, 22). This reasoning suggests that the dopaminergic neurons within the retina do not have an inherently high metabolic rate which would account for the lack of injury in this model. This concept is supported by the observation that chemically-induced mitochondrial dysfunction which leads to loss of dopaminergic neurons in
the striatum and produces parkinsonian symptoms, e.g., MTPT, does not have any observable effect on dopamine producing cells in the retina (2).

Another potential explanation is that the MP mice used in the study were not old enough to have evident changes such as changes in GFAP or TH immunoreactivity of amacrine cells. MitoPark mice typically have to be sacrificed around 45 weeks of age due to weight loss and poor general condition (18). This is in contrast to the motor neurons of the basal ganglia which succumb quickly, degenerate, and cause obvious phenotypical motor impairment in the MP mouse (18, 23). As similarly seen in toxin-based animal models for PD, discrepancies between the presence or absence in amacrine cell death appeared to be the result of different model paradigms: long term applications of systemic MPTP over 2 years resulted in dramatic decrease in TH-expression and DA amacrine cell loss (24) compared to one-time local application of 6-OHDA and short term intraperitoneal injections of MPTP which did not result in loss TH immunoreactivity (2).

However, the slightly reduced mean retinal thickness in the MP group could arise from the loss of a small number of dopaminergic neurons and/or their processes which would not be detectable on gross examination. This hypothesis is supported by the finding that retinal thickness was in fact a significant independent variable when included in the mixed linear model with mouse type, individual mouse, and light intensity as covariants. The results show that at low light intensities there was no significant difference between the groups. However, under scotopic conditions of higher light
intensity (10 cd·s/m²), in which both the rod and cone system are stimulated, the implicit time of the b-wave was significantly reduced. This could imply that a loss of inhibitory dopaminergic neurons which ordinarily are involved in the processing of retinal electrical information under brighter illumination.

Alternatively, the impairment in mitochondrial energy production due to insufficient cytochrome c oxidase activity (and subsequent ATP generation) produced by the gene targeting of the MP model may provide a functional defect in selected dopamine-containing amacrine cells under a high level of photic stimulation. Future research should evaluate whether synaptic or neurochemical remodeling occurs in the MP model in response to sustained high levels of neuronal activity and subsequent metabolic stress within the retina.

Conclusions

In summary, the MP mouse does not demonstrate obvious dopamine-containing amacrine cell losses and thus fails to reproduce fERG changes that have been reported in human patients and chronic animal models. However, this model does bring to the forefront the possibility of inhibitory dopaminergic interneurons of the retina are affected and therefore, in turn, the implicit time b-wave formation is affected. Perhaps the mice are not old enough to have as severe dopamine damage evident in the retina as in the mesencephalon. The amacrine cells are protected in some fashion, and do
not succumb to degeneration seen with other cells that are comprised of dopamine in the midbrain. Since it is thought that PD in humans is a combination of genetic and environmental factors, a single animal model will probably never be ideal for the study of the pathophysiology of human PD. Instead, insight will likely emanate from a combination of different approaches and preclinical models, toxin-based as well as genetic, to study various aspects of the disease etiology.

Conflict of Interest:

The authors declare no conflict of interest.

REFERENCES


Figures for Chapter III.

**Figure I** - MitoPark and Wild Type mice have similar a-wave implicit times under the scotopic and photopic conditions tested. LMS = Least Mean Squares from the mixed model analysis. ($p > 0.05$).

**Figure II** - MitoPark and Wild Type mice have similar a-wave amplitudes in the scotopic and photopic conditions tested. LMS = Least Mean Squares from the mixed model analysis. ($p > 0.05$).
Figure III: There is a general trend (although not significant) of MP mice having prolonged implicit times of b-wave formation for most time points when compared to WT mice except at the highest light intensity evaluated under scotopic conditions. Interestingly, B-wave implicit time showed a tendency of shortening in MP mice at the highest light intensity measured (10 cd·s/m²) (p=0.05), while the WT mice exhibited longer implicit times.

Figure IV: There was no statistically significant difference (p>0.05) between the mean b-wave amplitudes of the MP and WT mice, when recorded in response to all stimulus light intensities, under scotopic conditions.
**Figure V** - The graphs represent implicit time (A) and amplitude (B) for b-wave formation at 3 cd·s/m² tested under photopic conditions. LMS = Least Mean Squares from the mixed model analysis. (p>0.05).

**Figure VI A & B** - Histological analysis of the mice groups: Representative Slides of Mice Retina for WT (A) and MP (B) Mouse Groups at 40X. The slides were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy, 40X.
Table I- Histological measurements of mice retinas (µm).
The results are tabulated as the average (standard deviation) of each mouse group (5 measurements each animal). Although mean retinal thickness was less in the MP mouse, the difference was not statistically different (p>0.05).

<table>
<thead>
<tr>
<th></th>
<th>Wild Type Mice</th>
<th>MitoPark Mice</th>
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<tbody>
<tr>
<td>Retina Thickness</td>
<td>126.94 (±20.61)</td>
<td>112.63 (±22.44)</td>
</tr>
<tr>
<td>INL Thickness</td>
<td>27.34 (±9.62)</td>
<td>23.17 (±4.48)</td>
</tr>
<tr>
<td>IPL Thickness</td>
<td>43.61 (±11.57)</td>
<td>34.41 (±7.33)</td>
</tr>
<tr>
<td>ONL Thickness</td>
<td>42.08 (±9.66)</td>
<td>37.55 (±6.08)</td>
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Figure VII- Summary of the structural changes in retinal layers from WT and MP mice. The measured retinal layers are designed as Whole Retinal Thickness (Full Thickness), inner nuclear layer thickness (INL), inner plexiform layer thickness (IPL), outer nuclear layer thickness (ONL). Refer to table 1 for measurement values.
Figure VIII- Representative Slides of Mouse Retinas for WT (A) and MP (B) mice using Tyrosine Hydroxylase Immunoreactivity. (40x) There were no differences appreciated between TH staining between mouse groups.

Figure IX- There is a general trend (although not significant) of MP mice having prolonged implicit times of b-wave formation for most time points when compared to WT mice except at the highest light intensity evaluated under scotopic conditions. B-wave implicit time showed significant shortening in the MP mice at the highest light intensity measured (10 cd·s/m²) (p=0.03) compared to WT when retinal thickness was included as a covariate in the linear mixed model.
Figure X. There is a significant difference in mean implicit time between mouse groups at the highest light intensity tested, when evaluated using a t-test.
CHAPTER IV. GENERAL CONCLUSIONS

Summary and Recommendations for Further Research

Parkinson’s disease is currently regarded as the second most common degenerative disorder of the aging brain after Alzheimer’s disease. Although most often associated with its classic phenotypical motor symptoms such as tremor at rest, bradykinesia and postural instability, research over the past thirty years has investigated the presence of non-motor manifestations that go beyond the classic neurodegenerative disease description. The non-motor manifestations of PD can cause significant disability in human patients and can complicate disease therapies.

Early detection of PD may be beneficial as early recognition may slow down progression of disease development by implementation of medical therapies. Electroretinography and even certain imaging modalities such as optical coherence tomography is minimally invasive for patients. Such diagnostic equipment could aid in reaching diagnoses and also be used to evaluate response to medical therapy (like Levopa), as an investigator could evaluate changes in implicit time or amplitude of retinal waves during therapy trials. A better appreciation of how structural and physiological components of the eye are affected in PD patients is imperative to aid in improvement in the quality of life in PD patients.
There are many areas in which this initial study could be expanded upon. For example, further studies may have more definitive results if more mice are included in each group (i.e., wild type and MitoPark mouse groups). The anesthesia protocol should be reevaluated, as improvements in the anesthetic protocol may aid in the ability to perform electroretinography in older MP mice with a decreased risk of anesthetic complications. Furthermore, testing and assessment of the inner retina, through the use of pattern electroretinogram protocols could be investigated, as pattern ERGs give us information about contrast sensitivity and visual acuity more so than the flash ERG.

**Concluding remarks**

We have described PD and common associated visual symptoms in humans with the neurodegenerative disease. Furthermore we characterized the flash electroretinogram and retinal histology and found alterations in implicit time of the b-wave formation in the MP mouse. This allows us to move forward with similar investigations in this mouse model, as well as other mouse models, to improve the knowledge of PD animal models.