INVESTIGATION OF DAILY FOUR MONTH SILDENAFIL ADMINISTRATION ON HETEROZYGOUS CARRIERS OF A PHOSPHODIESTERASE 6 MUTATION IN A CANINE MODEL OF AUTOSOMAL RECESSIVE RETINITIS PIGMENTOSA

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ABSTRACT

INVESTIGATION OF DAILY FOUR MONTH SILDENAFIL ADMINISTRATION ON HETEROZYGOUS CARRIERS OF A PHOSPHODIESTERASE 6 MUTATION IN A CANINE MODEL OF AUTOSOMAL RECESSIVE RETINITIS PIGMENTOSA

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Retinitis pigmentosa (RP) is the most common inherited retinal dystrophy resulting in significant visual deficits and blindness in humans. Autosomal recessive, autosomal dominant, X-linked, maternal, and digenic inheritance patterns encompass the modes of RP inheritance. Phosphodiesterase enzymes hydrolyze intracellular second messengers regulating cell-to-cell interactions. A mutation in the phosphodiesterase type 6 gene, involving the alpha-, beta-, or gamma-subunits, is one of the numerous causes of autosomal recessive retinitis pigmentosa. Several pharmacologic investigations assessing the effect sildenafil, a phosphodiesterase type 5 inhibitor, has on retinal function and vision, as well as genetic investigations in heterozygous individuals for both a phosphodiesterase alpha- and beta-subunit mutation are documented in the literature. We assessed retinal function and vision in dogs heterozygous for a phosphodiesterase type 6 alpha-subunit mutation while receiving sildenafil for four months duration. Low-intensity, dark-adapted, rod-led ERG responses were transiently reduced and a higher threshold response was observed in dogs receiving sildenafil. All ERG alterations were transient and completely reversed at washout. Sildenafil did not have a clinical observed effect on vision. Sildenafil transiently raised the rod-mediated ERG threshold in heterozygous *PDE6A* mutant and control dogs.

DEDICATION

"Let me become dead eyed
Like a fish, -
I'm sure then I'd be wise
For all the wise men I've seen
Have had dead eyes

Let me learn to fit all things
Into law and rule:
I'd be the proper person then
To teach a school"

Wise Men
by Langston Hughes

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2. Autosomal recessive retinitis pigmentosa ……..... RP
3. Calcium ……...…… Ca^{2+}
4. Adenosine 3′, 5′-cyclic monophosphate …….. cAMP
5. Guanosine 3′, 5′-cyclic monophosphate …….. cGMP
6. Electroretinogram ……..….. ERG
7. Erectile dysfunction …….. ED
8. Food and Drug Administration ……... FDA
9. Inner nuclear layer …… INL
10. Inner plexiform layer …… IPL
11. Kilodalton ……...……. kDa
12. Metarhodopsin ……..... R*
13. Nitric oxide ……...…… NO
14. Nonarteritic anterior ischemic optic neuropathy ……..... NAION
15. Optic nerve head ……. ONH
16. Outer nuclear layer ....…….. ONL
17. Outer plexiform layer ……... OPL
18. Phosphodiesterase ……... PDE
19. Phosphodiesterase type 6 alpha-subunit ……...…….…… $PDE6\alpha$
20. Phosphodiesterase type 6 beta-subunit …… $PDE6\beta$
21. Phosphodiesterase type 6 gamma-subunit ……..….. $PDE6\gamma$

22. Postnatal day …….……... P

23. Potassium …….……... K^+

24. Progressive retinal atrophy ……..... PRA

25. Pulmonary arterial hypertension ……. PAH

26. Retinal pigment epithelium ……. RPE

27. Retinitis pigmentosa …….……..… RP

28. Scotopic threshold response ……..... STR

29. Sodium …….……. Na^+

30. Total nerve fiber layer …… Total NFL

31. Total photoreceptor inner and outer segments …….. Total PS

32. Transducin ……..... Tαβγ

CHAPTER 1

LITERATURE REVIEW

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a group of heritable retinal degenerative disorders resulting in vision loss due to progressive dysfunction and death of photoreceptor cells. While originally it was believed the ophthalmoscopic changes associated with disease advancement were due to an inflammatory etiology, hence the *retinitis* nomenclature, distinct genetic mutations have been shown to cause the various forms of RP. Clinical signs can vary depending on whether rod or cone photoreceptors are initially affected. However, the typical clinical characteristics of RP include: progressive visual field loss, a midperipheral or far peripheral "bone-spicule," intraretinal pigment accumulation; retinal arteriolar attenuation; pale optic disk appearance; and night blindness.[1] These ocular changes may exist in isolation (nonsydromic RP) or as part of a complex of abnormalities (syndromic RP) such as occurs with Usher's syndrome and Bardet-Biedl syndrome.[1] Various inheritance patterns including: autosomal recessive (50-60% of cases), autosomal dominant (30-40%), X-linked (5-15%), maternal (mitochondrial), and digenic modes have been reported for RP.[2] The incidence of all forms of RP in the United States is

estimated to be 1 in 3,700 individuals. While the incidence of autosomal recessive RP (the most common form) is estimated at 1 in 4,450 individuals, with the frequency of carriers of recessive forms of RP calculated to be1 in 50.[3] To date mutations causing autosomal RP have been reported in 32 distinct genes (Table 1.1).[4] Many of these genes play a critical role in the phototransduction process occurring in photoreceptor cells.

Retinal Phototransduction

In the dark the rod photoreceptor is in a constant depolarized state as there is a constant K^+ ion influx into the cell through light-insensitive rod inner segment plasma membrane channels, Ca^{2+} removal from rod outer segment Na^+/Ca^{2+}-K^+ exchanger, and Na^+ extrusion by Na^+:K^+ ATPase pumps on the rod inner segment plasma membrane. In this depolarized state there is a constant release of neurotransmitter, glutamate, from the synaptic terminal of the photoreceptor. Upon photoreceptor stimulation by the absorption of a photon of light, the rod photopigment rhodopsin undergoes *cis-trans* isomerization causing a conformational change from 11-*cis*-retinal to all-*trans*-retinal. The active intermediate form of rhodopsin, metarhodopsin (R*), then binds to several hundred heterotrimeric G protein transducin (Tαβγ) molecules causing dissociation of the Tαβγ trimer by exchanging a guanine diphosphate (GDP) to guanine triphosphate (GTP). This GDP-to-GTP exchange causes a dissociation of active Tα (Tα*) from both rhodopsin and the Tβγ dimer. Tα* then binds to one cGMP-PDE6αβγγ protein and removes one inhibitory γ-subunit. Two Tα* are required to remove the two inhibitory γ-subunits of cGMP-PDE6αβγγ, thus fully activating the catalytic α and β sites of this enzyme. It is at

this step when active PDE6αβ** subunits hydrolyze cGMP to 5′-GMP causing decreased intracellular levels of cGMP and inducing closure of cGMP-dependent cation channels of the rod outer segment plasma membrane and hyperpolarization of the cell. Hyperpolarization of the photoreceptor cell causes closure of voltage-gated Ca^{2+} channels, decreased intracellular levels of Ca^{2+} via the Na^{+}/Ca^{2+}-K^{+} exchanger, and reduced photoreceptor synaptic release of glutamate.

The recovery phase of phototransduction is mediated by the inactivation of R*, Tα*, and PDE6αβ**. R* inactivation begins with rhodopsin phosphorylation at its C-terminus, or carboxy-terminus, by rhodopsin kinase. The C-terminus possesses several serine and threonine phosphorylation sites, of which Ser^{334}, Ser^{338}, and Ser^{343} have been shown to be critical residues for R* inactivation.[5] Rhodopsin kinase regulation is governed by recoverin, which is a 23 kDa calcium-binding protein. Inhibition of rhodopsin kinase by recoverin occurs when intracellular concentrations of free Ca^{2+} are high, thus causing prolonged R* activity. Phosphorylated rhodopsin has a higher affinity for arrestin and binds arrestin causing steric hindrance to transducin and a decrease in transducin activation.

Activated transducin is inactivated by its intrinsic GTPase activity, which hydrolyzes GTP to GDP. GDP bound Tα reassociates with Tβγ. Phosducin, a 28-kDa phosphoprotein, binds to Tβγ when it is dephosphorylated. Phosphorylation of phosducin occurs in the dark-adapted state, when intracellular free Ca^{2+} is high, allowing the release of Tβγ and the regeneration of Tαβγ. Lastly, the interaction of Tβγ with the PDE6γ/Tα

complex causes the release of PDE6γ form Tα, binding of PDE6γ with the catalytic PDE6α and β subunits, and further recovery of Tαβγ. Therefore further cGMP hydrolysis is inhibited by the reassociation of PDE6γ with PDE6αβ. Ca^{2+}-dependent guanylyl cyclase restores cGMP to dark-adapted levels when intracellular levels of Ca^{2+} are reduced during light exposure.

Retinal Phosphodiesterase type 6

Function of the retinal enzyme phosphodiesterase type 6 (PDE6) is critical for generation of the photoreceptor membrane potential change required for phototransduction and normal vision. PDE6 was initially localized to the outer segment disc membranes of rod photoreceptor in the frog retina.[6] More recently expression has been documented in the chicken pineal gland.[7] This localized expression is exceptional as other members of the phosphodiesterase family are widely distributed across numerous tissues. Structurally PDE6 is a tetramer composed of one 99-kDa catalytic α-subunit, one 98-kDa catalytic β-subunit, and two 11-kDa inhibitory γ-subunits in rod photoreceptors, and two identical 90-kDa catalytic α′-subunits, and two 13-kDa inhibitory γ′-subunits in cone photoreceptors.[8] The *PDE6A* gene encodes the rod α-subunit[9], the *PDE6B* gene encodes the rod β-subunit[10], the *PDE6C* gene encodes the cone α′-subunit[11,12], the *PDE6G* gene encodes the rod γ-subunit[13], and the *PDE6H* gene encodes the cone γ′-subunit[14,15]. The structure of membrane-bound rod photoreceptor PDE6 is $\alpha\beta\gamma_2$, and $\alpha'_2\gamma'_2$ for cone photoreceptors.[16] It has been estimated that 3 to 4% of autosomal

recessive RP cases are caused by mutations in the gene encoding the alpha subunit of retinal phosphodiesterase enzyme type 6 (PDE6A).[17] Mutations affecting the *PDE6A* gene are located within the cGMP binding domain (Arg102His,[17] Arg102Ser,[17] IVS6+1G→A,[17] and Ser344Arg[18]) and catalytic domain (Trp561Ter,[18] Gln569Lys,[17] Ser573Pro,[17] Tyr583Ter,[18] and Thr706(1-bp del)[19]) of PDE6A.

The Phosphodiesterase enzyme super-family

The phosphodiesterases (PDE) are a super-family of enzymes, which function in the hydrolysis of adenosine 3′, 5′-cyclic monophosphate (cAMP) and/or guanosine 3′, 5′-cyclic monophosphate (cGMP). Both cAMP and cGMP function in intracellular signaling along with intracellular calcium (Ca^{2+}) and inositol trisphosphate (IP3), and are synthesized by adenylyl and guanylyl cyclase, respectively. The molecular targets of cAMP and cGMP include: protein kinase A (PKA), protein kinase G (PKG), exchange protein directly activated by cAMP (EPAC), cyclic nucleotide-gated channels (CNG), and cGMP-binding domains (GAF) of some PDE, specifically PDE2, PDE5, PDE6, PDE10 and PDE11.[20] PDE specifically regulates, via hydrolysis (Figure 1.1), intracellular levels of cAMP and cGMP returning these intracellular second messengers to basal levels and affecting intracellular target molecules of cAMP and cGMP, for example closure of CNG ion channels in rod photoreceptors.[21,22]

Currently 21 PDE genes and greater than 50 total isozymes have been identified and organized[22,23] into eleven different families (Table 1.2), with 1 to 4 genes per PDE family.[22] Gene cloning, protein sequencing, and other molecular biological methods

identifying substrate selectivity and inhibition facilitated the establishment of an official nomenclature of the PDE famlies.[24] The current PDE nomenclature is updated at http://www.depts.washington.edu/pde/Nomenclature.html and described as: "The fist two letters represent the species. The next three letters plus 1 or 2 Arabic numerals designate the cyclic nucleotide phosphodiesterase gene family. The next letter represents the individual gene product within the family. The final Arabic numeral represents the splice variant, and the final letter allows GenBank to assign a unique locus field designation based on when the entry was submitted and also to give different locus names to conflicting or incomplete sequences".

Phosphodiesterase type 5 and the inhibitor Sildenafil

PDE5 was first identified and characterized in rat platelets[25,26] and lung[27,28]. PDE5 was later identified in human, bovine, and rat vascular smooth muscle and characterized as a Ca/calmodin activation-independent, cytosolic isozyme that specifically hydrolyzes cGMP.[29] The functional role of PDE5 is vasorelaxation. Zaprinast, a potent selective PDE5-specific inhibitor, induced an increase in cGMP levels in association with a vasorelaxing effect in rat aortas.[29,30] PDE5 also mediates the nitric oxide (NO)/cGMP-induced relaxing effect of vascular smooth muscle[31] as these vasorelaxing effects were shown to be potentiated selective inhibition with zaprinast.[32,33] These vasodilatory effects of PDE5 inhibition lead to the development of other PDE5

inhibitors derived from zaprinast, such as dipyridamole and sildenafil, to be used as antihypertensive agents or coronary artery vasodilators.

Sildenafil citrate (Viagra®; Pfizer, Inc., New York, NY), a potent and selective cGMP-specific PDE5 inhibitor, was initially developed and investigated for the treatment of angina pectoris. However, the requirement for repeated dosing due to short half-life (~4 hours) and interaction with and equality to nitrate therapy precluded further development of sildenafil for treatment of cardiac disease at that time.[34,35] During the original pharmacokinetic and safety trials penile erection was a commonly reported side effect. In 1994 phase 1 clinical trials showed sildenafil's efficacy in enhancing penile erection and systemic drug tolerance.[36,37] The reported physiological mechanism of penile erection is via the release of NO from cavernous nerve and vascular endothelium of the corpus cavernosum.[38,39] Intracellular cyclic nucleotide/protein kinase messenger systems mediate smooth muscle relaxation and vascular dilation.[40] The enzyme guanylate cyclase, which is activated by NO, increases the levels of cGMP and cGMP-dependent protein kinase I causing a reduction in intracytoplasmic calcium, smooth muscle relaxation, and increased cavernosum blood flow (erection).[20] PDE5 hydrolyzes and reduces cGMP levels within the corpus cavernosum ultimately resulting in a reduction in blood flow and smooth muscle relaxation.[41] Upon sexual stimulation and release of NO higher cGMP levels are achieved and maintained by sildenafil's inhibitory activity on PDE5.[42] Sildenafil gained FDA approval for the treatment of erectile dysfunction (ED) in the United States in 1998. Since then more than 750,000 physicians

have prescribed sildenafil to over 35 million men worldwide, making sildenafil the most widely used treatment for ED.[41,43]

Reported adverse reactions to sildenafil administration in pre-marketing clinical trials were mild to moderate in severity and transient. The reported side effects in over 3,700 patients administered sildenafil include: headache (16%), flushing (10%), dyspepsia (indigestion) (7%), nasal congestion (4%), urinary tract infection (3%), abnormal vision (3%), diarrhea (3%), dizziness (2%), and rash (2%).[44] Commonly reported vision disturbing side effects associated with sildenafil usage are a bluish tinge or haze to vision, or a sense of increased light sensitivity.[45-48] *In vitro* investigations show selectivity of sildenafil for PDE5 to be approximately 80 to over 19,000 times greater than its selectivity for PDEs 1-4 and 2,600 – 8,500 times that of PDEs 7-11.[23,49] Whereas selectivity of sildenafil's for PDE5 is only 10 times greater than for PDE6.[50] The mechanisms of sildenafil-induced visual side effects have not been definitively proven; however, both alterations in blood velocity to ocular tissues and direct binding to photoreceptor PDE6 have been reported.

Sildenafil and ocular blood flow

Commonly ED patients also have multiple cardiovascular-related risk factors and are prescribed sildenafil. Investigations of the effect of sildenafil on ocular blood flow were performed as vision disturbances were reported to occur in patients with ED. Briefly, the vascular supply to the human uveal tract, lamina cribrosa, and optic nerve head is via the long and short posterior ciliary arteries.[51] The surface of the optic nerve

head and retina are perfused by the retinal arterioles, which are branches of the ophthalmic artery.[51] Numerous reports[52-62] assessing the effect sildenafil has on ocular perfusion have documented an overall increase in blood flow velocity within the choroidal and retrobulbar circulation in individuals administered between 50 to 100 mg sildenafil. Little effect was noted within the retinal vasculature.[63] A recent report documented increased choroidal thickness, measured by optical coherence tomography, at 1- and 3-hours after 100 mg sildenafil administration in healthy individuals.[64] The sildenafil-induced ocular perfusion changes are likely mediated associated via vascular innervation, as the choroid is under autonomic nervous system control and the central retinal artery maintains the retinal vasculature.[63] The choroidal choriocapilaris likely responds similarly to the corpus cavernosum as NO activates cGMP release within the vascular smooth muscle.

The development of nonarteritic anterior ischemic optic neuropathy (NAION) is also of concern in sildenafil-users aged between 55 and 70 years.[65] NAION is the most common optic neuropathy in individuals >50 years of age, resulting from obstruction of the short posterior ciliary arteries supplying the anterior portion of the optic nerve at or near the lamina cribrosa.[65] Sudden, usually non-painful, partial vision loss of one eye, characterizes the clinical presentation of NAION.[65] The incidence of NAION in the United States is rare, with estimates ranging from 2.52 and 11.8 in 100,000 individuals $\geq$ 50 years of age for men and between 2.14 and 9.2 in 100,000 for women.[66,67] It is thought that optic nerve ischemia may develop due to crowding of arterioles by retinal

ganglion cell axonal fibers as they pass through the restricted spaces of the lamina cribrosa in the optic nerve head. A small optic nerve head cup-to-disc ratio is a documented risk factor and has been previously described as a "disc at risk".[68,69] Axonal swelling resulting from stasis of axoplasmic flow stasis is thought to perpetuating the condition.[68,69] Although the exact pathogenesis of NAION remains to be determined, numerous risk factors for NAION have been identified and include: diabetes, hypertension, hypercholesterolemia, atherosclerosis, ischemic heart disease, stroke, prothrombotic factors, elevated homocysteine levels, sleep apnea, and nocturnal hypotension.[41] Some of the risk factors associated with NAION are the same risk factors reported in ED patients, including diabetes, hypertension, hyperlipidemia, and smoking.[70-72] Currently 19 cases of NAION have been reported in individuals aged between 36 to 69 years receiving sildenafil at 25 to 100 mg.[41,73] NAION is estimated to occur in 2.8 cases per 100,000 patient-years of exposure to sildenafil,[74] which is similar to the reported incidence in individuals $\geq$ 50 years with NAION.[66,67] Sildenafil does not appear to increase the incidence of NAION; however, the FDA recommends caution with use of sildenafil in individuals at risk for NAION.[44]

Sildenafil affects the retina

A second possible mechanism for sildenafil-induced visual side effects is inhibitory off-target binding of PDE6 within retinal photoreceptors or PDE5 in the inner retina.[50] Inhibition of PDE6 results in persistence of open cGMP-gated cation channels,

excessive Na^+ and Ca^+ ion influx, and the accumulation of elevated levels of cGMP. Persistently raised cGMP concentration is known to be photoreceptor toxic[75-77] and an initiator of photoreceptor apoptosis.[78,79] PDE5 expression in inner retina has been previously shown. Inhibition of retinal PDE5 could potentially alter the electrical signal transferring thorough bipolar or ganglion cells and result in an altered perception of visual stimuli.

Reports of visual adverse events occurred in 3 – 5% of individuals administered 25 or 50 mg doses of sildenafil in flexible-dose phase II/III clinical trials.[44,80,81] An increased incidence of visual complaints up to 11% was reported in individuals administered a 100 mg dose, and nearly 50% in individuals administered a 200 mg dose.[47] Multiple studies have reported variable changes in the ERG of healthy individuals or individuals with ED administered 100 mg sildenafil or higher. These ERG changes consist of significantly reduced dark-adapted a- and b-wave amplitudes,[82,83] prolonged dark-adapted b-wave implicit times without amplitude loss,[84] reduced light-adapted b-wave amplitudes and prolonged b-wave implicit times and 3.3 and 30 Hz flicker responses,[85,86] and prolonged dark and light-adapted ERG implicit times and 33 Hz flicker.[87,88]

During preclinical development it was observed that sildenafil binds retinal PDE6[89]. Although the binding affinity of sildenafil for PDE5 is ten times stronger then PDE6, concerns about possible retinal toxicity arose. Long-term safety studies evaluating

doses of 60 mg/kg in rats for 6 months and 80 mg/kg in dogs for 12 months revealed no histopathological evidence of retinal damage.[90,91] Electroretinogram (ERG) responses in dark-adapted anesthetized dogs administered an increasing intravenous dose at 3.3 – 100 μg/kg/min showed dose-dependent a-wave amplitude reduction and prolongation of the a- and b-wave implicit times.[89] A sildenafil dose of 400 mg, corresponding to 4 times the maximum therapeutic human dose, produced alterations in the ERG rod and cone threshold response, however, all ERG alterations were transient and fully reversible.[89] Contrary to the initial phase I trial reported by Laties et al[92], significant reductions in ERG a- and b-wave amplitude responses, up to 63% and 77% respectively, were reported in an acute preclinical study in 5 healthy men.[82] All ERG alterations occurred concurrently with peak sildenafil plasma levels and completely resolved after 6 hours. Increased dark-adapted b-wave implicit times was documented in men 1 – 2 hours after receiving 100 mg sildenafil.[84] Significantly prolonged implicit times of the dark-adapted a-wave, light-adapted b-wave, and 3.3 Hz-flicker a- and b-wave responses were transient and correlated with peak sildenafil plasma levels in men administered 100 mg.[85] ERG alterations reported in healthy men administered 200 mg sildenafil included: 5% increase in dark-adapted b-wave amplitudes; 9% reduction in light-adapted b-wave amplitudes; prolonged dark-adapted a-wave implicit times by 2.8% and light-adapted b-wave implicit time by 2.4%; and prolonged 30 Hz flicker responses by 6.6%.[86] All of these dose-dependent ERG alterations were transient and reversible with discontinuation of sildenafil administration. Although transient ERG changes were observed in these short-

term post-marketing studies, investigators report that long-term, high-dose animal and human ERG studies have yet to be performed.[81]

Impairment in color vision discrimination in the green-blue to blue-purple range, assessed via the Farnsworth-Munsell 100 hue test, was also reported in individuals administered 100 and 200 mg sildenafil.[92,93] A linear relationship was found between peak plasma sildenafil concentrations and impaired color discrimination.[93] Multiple studies have reported visual changes associated with sildenafil administration in people. Initially, preclinical phase I trials in healthy men receiving 100 or 200 mg sildenafil caused impaired color discrimination in the green-blue to blue-purple range.[93] These color vision alterations were transient, occurred concurrently with peak sildenafil plasma levels, 1 – 2 hours post-administration, and were fully reversible within 5 hours of sildenafil discontinuation.[93] Given the same dose significant effects were not observed in visual field, visual acuity, IOP, pupillometry, and ERG testing in these healthy men.[92,94] Men with early-stage, age-related macular degeneration were also reported without significant color discrimination and visual field sildenafil-induced side effects.[95] Long-term, phase II/III fixed- and flexible-dose trails in men with ED receiving sildenafil for 1 month to 2 years reported no significant visual adverse events.[92,96] However, an acute post-marketing investigation documented an increase in color discrimination errors in 71% of men administered 200 mg sildenafil.[86] Color discriminatory errors were still present 5 hours after sildenafil administration in half of the affected men.[86]

Sildenafil for pulmonary arteriolar hypertension

Subsequent to sildenafil's FDA approval for treatment of ED in 1998, several reports documented clinical and cardiovascular improvement after sildenafil administration in individuals afflicted with pulmonary arterial hypertension (PAH).[97,98] Later the SUPER-1 study[99], a long-term high-dose investigation in PAH individuals, lead to FDA and the European Medicines Agency approval of sildenafil for the treatment of PAH in 2005. Individuals within the SUPER-1 study received sildenafil at 20, 40, or 80 mg orally three times daily for 1 year's duration. All doses significantly reduced mean pulmonary-artery pressure and clinical signs with few side effects, such as flushing, dyspepsia, and diarrhea.[99] However, due to sildenafil's pharmacologic cross-reactivity with retinal PDE6 and ERG alterations, multiple investigators suggest that the first at risk population for toxic effects of sildenafil on visual function over time are those individuals consuming long-term high doses of sildenafil (e.g. individuals afflicted with PAH).[47,89,100,101]

Animal models to study sildenafil-induced retinal toxicity

A recent ex vivo bovine and human sildenafil (3μMol/l) retinal perfusion study reported significantly decreased bovine and human retinal b-wave amplitudes and a 21% reduction in human a-wave amplitudes and prolonged implicit times during the treatment phase.[102] These perfused retinas exhibited an incomplete return to normal for all ERG recordings at washout.[102] The authors documented an affect on outer and inner retinal

function and hypothesize of the potential for retinal toxicity associated with long term high-dose sildenafil administration. Acute safety studies have reported no increased risk or worsening of visual disturbances in individuals with or without ED and preexisting ocular disorders such as open-angle glaucoma,[103,104] diabetic retinopathy,[105] or age-related macular degeneration.[95]

Animal models of heritable retinal degeneration have played a key role in investigations for the characterization and therapeutic assessment of heritable retinal degenerative conditions in humans. A recent investigation documented the affects of sildenafil on heterozygous $Pdeg^{tm1/+}$ knockout mice in comparison to wild-type mice.[101] In this study mice were administered an intraperitoneal injection of sildenafil at 2x and 10x the equivalent human dose for a 70 kg human receiving 100 mg sildenafil. Significant dose-dependent reduction in ERG a- and b-wave amplitudes with concurrent prolongation of implicit times was observed in $Pdeg^{tm1/+}$ mice administered sildenafil compared to wild-type mice that did not exhibit the same ERG alterations. These ERG alterations were reversible at washout. The authors speculated that the heterozygous *PDE6G* mutation probably lead to a decrease in functional PDE6, thus enhancing its susceptibility to the inhibitory effects of sildenafil. Increasing the dose of sildenafil lead to further reduction in PDE6 activity and reduced retinal function in this animal model of retinal degeneration.[101] The authors also propose that long term studies involving animals heterozygous for PDE6 RP causative mutations are warranted as they may be at increased risk of retinal toxicity associated with repeated sildenafil administration and chronically elevated levels of cGMP.[101]

Our laboratory has established a canine model possessing a recessively inherited *PDE6* mutation was identified in the Cardigan Welsh corgi dog. Clinical observations in the Cardigan Welsh corgi dog was first described by Keep as an early onset retinal degeneration beginning by 6 to 16 weeks of age.[106] Early ERG alterations were noted to occur shortly after eyelid opening, measured at 17 days of age in homozygous-affected *Pde6a*$^{-/-}$ puppies.[107] Absent rod photoreceptor function, including reduced dark-adapted ERG amplitudes, absent rod flicker ERG responses, and increased ERG threshold responses, arrested photoreceptor development after 3 weeks of age, and photoreceptor death by 4 weeks of age describe the phenotype.[107] Heterozygous puppies were phenotypically similar to homozygous normal's having no ophthalmoscopic, electroretinographic, or histopathological evidence of retinal degeneration and abnormal retinal function. Normal formation of the β- and γ-subunits was found to be dependent on presence of the PDE α-subunit.[107] This is in contrast to that of the *rcd1* Irish setter dog and *rd* knockout mouse. Genetic investigation in the corgi dog identified a single adenine deletion at codon 616 of exon 15 in the *PDE6A* gene. Translation of this frame shift mutations yields a run of 28 altered amino acids followed by a premature stop codon.[108] The incidence of PDE6A heterozygous corgi dogs was estimated at 6.5%.[108] Autosomal recessive progressive retinal atrophy (PRA) in the Cardigan Welsh corgi dog was identified as rod-cone dysplasia 3 (*rcd3*) and noted to be the first spontaneous animal model of human autosomal recessive RP caused by a *PDE6A* mutation.[108] Our canine model provides an ideal opportunity to investigate the risk for retinal toxicity associated

with high-dose sildenafil administration in individuals heterozygous for a PDE6A mutation.

Table 1.1: Autosomal recessive retinitis pigmentosa gene mutations

Autosomal Recessive Retinitis Pigmentosa	Mapped Loci	Mapped and Identified Genes
	RP22, *RP29*, *RP32*	*ABCA4*, *BEST1*, *C2ORF71*, *CERKL*, *CNGA1*, *CNGB1*, *CRBI*, *EYS*, *FAM161A*, *IDH3B*, *IMPG2*, *LRAT*, *MERTK*, *NR2E3*, *NRL*, *PDE6A*, *PDE6B*, *PDE6G*, *PRCD*, *PROM1*, *RBP3*, *RGR*, *RHO*, *RLBP1*, *RP1*, *RPE65*, *SAG*, *SPATA7*, *TTC8*, *TULP1*, *USH2A*, *ZNF513*

Figure 1.1: Cyclic nucleotide hydrolysis

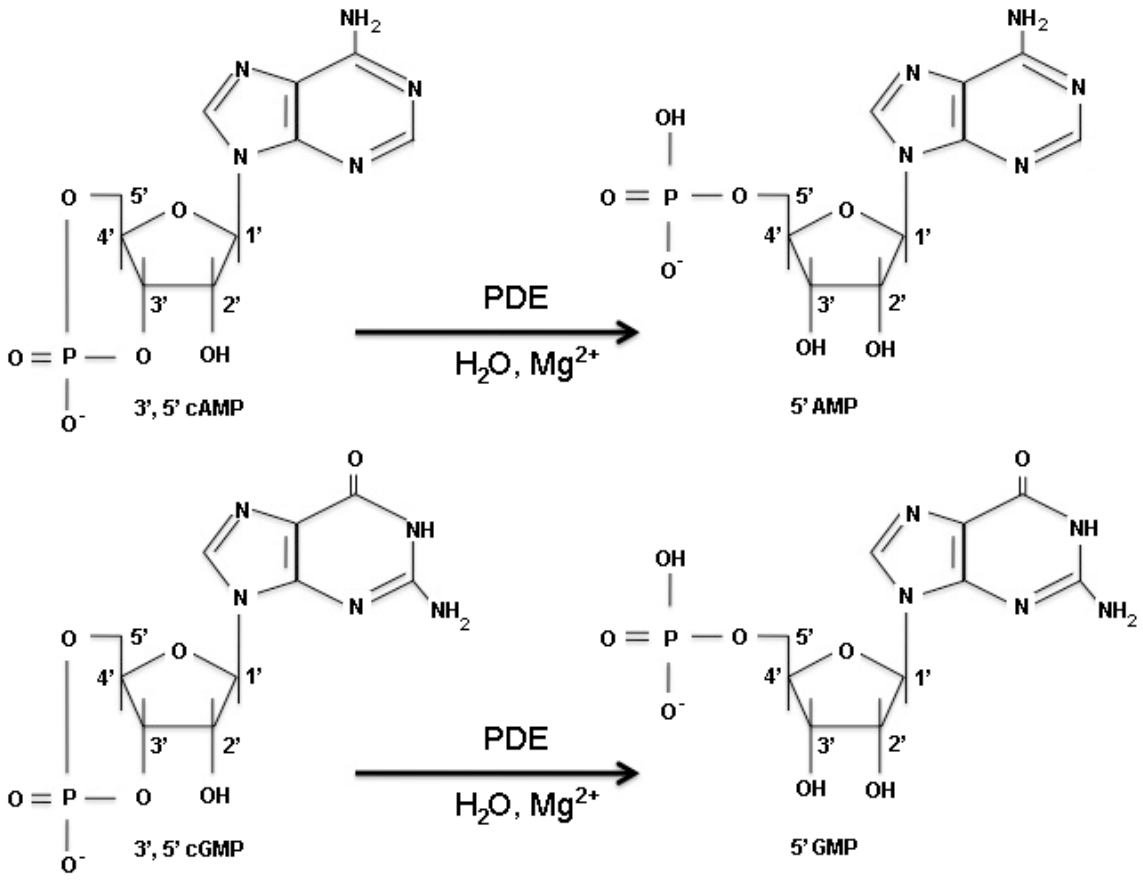


Table 1.2: Phosphodiesterase family classification

PDE Family	Substrate	Property
PDE1	cAMP, cGMP	Ca-Cam-activated
PDE2	cAMP, cGMP	cGMP-activated
PDE3	cAMP, cGMP	cGMP-inhibited
PDE4	cAMP	cGMP-insensitive
PDE5	cGMP	PKA/PKG-phosphorylated
PDE6	cGMP	Transducin-activated
PDE7	cAMP	Rolipram-insensitive
PDE8	cAMP	Rolipram-insensitive IBMX-insensitive
PDE9	cGMP	IBMX-insensitive
PDE10	cAMP, cGMP	Unknown
PDE11	cAMP, cGMP	Unknown

CHAPTER 2

THE ROLE OF PHOSPHODIESTERASE TYPE 5 INHIBITOR, SILDENAFIL, ON REITNAL FUNCTION AND VISION IN A CANINE MODEL OF AUTOSOMAL RECESSIVE RETINITIS PIGMENTOSA

INTRODUCTION

The selective cyclic guanosine monophosphate (cGMP) phosphodiesterase subtype 5 (PDE5) inhibitor sildenafil citrate (Viagra®; Pfizer, Inc., New York, NY) revolutionized management of erectile dysfunction (ED) following final FDA approval in March of 1998.[109] More recently in 2005, sildenafil was approved for treatment of pulmonary arterial hypertension (PAH).[110] Dose-dependent side-effects reported following use of sildenafil for both indications include: skin flush, headache, and alteration of vision.[109,110] The visual disturbances most commonly described by patients include: a bluish tinge to objects, blurred vision, and increased brightness of lights. The incidence of visual alteration is approximately 3% with doses of 25-50 mg of sildenafil, 11% with 100 mg, 50% with 200 mg, and 100% with doses above 600 mg.[46,47] Two general categories of explanatory mechanisms for the visual disturbances have

developed: disruption of ocular perfusion and off-target inhibition of retinal phosphodiesterases.[41,47]

Temporal associations between sildenafil use and vision-threatening ocular perfusion defects such as nonarteritic anterior ischemic optic neuropathy (NAION) and central serous chorioretinopathy (CSC) have been reported.[41,111] However a direct causal relationship has been difficult to confirm since sildenafil users frequently have comorbid diseases which place them at increased risk for development of NAION and CSC.[74] Sildenafil causes dose-dependent changes in color-discrimination, visual sensitivity and electroretinogram (ERG) waveforms.[85,112,113] These changes likely arise from cross-reactivity with cGMP phosphodiesterase subtype 6 (PDE6) in photoreceptor outer segments and/or PDE5 within the inner retina resulting in elevation of intracellular cGMP levels.[47,58] While transient high retinal cGMP concentration may be tolerated, chronic unremitting elevation has been reported to cause photoreceptor degeneration in multiple species.[75,76,114]

A question arises from these observations: could specific patient populations develop retinal toxicity and even blindness following exposure to the elevated levels of cGMP arising from sildenafil use?[47] Significant risk factors contributing to this scenario might include chronic daily high-dose sildenafil use and preexistent compromise of retinal phosphodiesterase function. PDE6 subunit mutations have been shown to cause approximately 8% of autosomal recessive retinitis pigmentosa; however, heterozygous carriers do not typically develop retinal degeneration.[115] Theoretically carriers of PDE6

mutations might be at great risk for sildenafil-induced retinal toxicity as they likely have reduced PDE6 function and may be unaware of their carrier status.[47] Individually each of these risk factors has been preliminarily investigated in short-term studies.

Lüke et al. demonstrated high concentrations of sildenafil in perfusion solutions were able to completely abolish ERG b-wave amplitudes from *ex vivo* human and bovine retinas and only incomplete b-wave recovery could be achieved following 4 hours of reperfusion washout.[102] Sildenafil-induced retinal degeneration was suggested and additional trials to investigate long-term effects were recommended. Recent success treating PAH with sildenafil has created a patient population receiving daily sildenafil with total doses up to 300 mg/day.[99,113] In the only published report to date investigating retinal function in PAH patients managed with sildenafil, Zoumalan et al. noted prolongation of the light-adapted ERG implicit time in a group of 5 individuals receiving daily sildenafil for up to 4 years.[113] While there were no clinically apparent affects on vision, the authors could not rule-out the possibility of permanent sildenafil-induced suppression of photoreceptor function.

Behn et al. explored the second risk factor of preexistent compromise of PDE6 function. Sildenafil was administered to mice heterozygous for a PDE6 gamma-subunit mutation (*Pde6g*).[101] In this murine model of RP, homozygous recessive mice develop photoreceptor degeneration, while heterozygotes retain normal photoreceptor function. Significant reductions in dark-adapted ERG a- and b-wave amplitudes and prolongation of implicit times were observed in the heterozygous mice administered sildenafil

compared to wild-type mice. The effect was reversible at washout; however, long-term follow-up studies were recommended due to the short duration of the study.

We have previously reported on a canine model of autosomal recessive retinitis pigmentosa, which has a functional null-mutation in the PDE6 alpha subunit (*Pde6a*).[107,108] Homozygous affected dogs lack PDE6 activity and develop rapid degeneration of rod photoreceptors with a slower loss of cones. Heterozygous carriers are phenotypically normal; however, we anticipate they are at risk for any adverse effects resulting from sildenafil-induced suppression of PDE6. This canine model provides an ideal system to investigate the combined risk for retinotoxicity associated with chronic high-dose sildenafil administration in carriers of PDE6 mutations. Here we report ERG, vision testing and histopathology results from dogs heterozygous for a *Pde6α* mutation receiving 14.3 mg/kg sildenafil orally once daily for 4 months.

MATERIALS & METHODS

Animals & Ophthalmology Examinations: This study was conducted in accordance with the Association for Research in Vision and Ophthalmology's statement on use of animals in ophthalmic and vision research and approved by the Institutional Animal Care and Use Committee of Michigan State University. Five *Pde6a*$^{+/-}$ dogs and three *Pde6a*$^{+/+}$ dogs were used in this study (mean age: 1.9 ± 0.43). Three *Pde6a*$^{+/-}$ dogs received 14.3 mg/kg sildenafil citrate (equivalent to ten times the dose of a 100 mg tablet taken by a 70 kg human) and two *Pde6a*$^{+/-}$ dogs received placebo once daily for 16

weeks (Tables 2.1 & 2.2). The three *Pde6a*$^{+/+}$ dogs received 14.3 mg/kg of sildenafil once daily for seven days. Sildenafil tablets (Pfizer, Inc., New York, NY) were crushed and filled into opaque capsules by a pharmacist. Placebo capsules were filled with α-lactose monohydrate (Sigma-Aldrich, Inc. St. Louis, MO). Investigators remained masked to treatment status of the dogs during the study. Routine ophthalmic examinations were performed throughout the study (Table 2.1 & 2.2). Ophthalmic examinations included Schirmer Tear Test I measurements (Schering-Plough Animal Health, Kenilworth, NJ), corneal fluorescein staining (Akron, Inc., Buffalo Grove, IL), applanation tonometry (Reichert, Inc., Depew, NY) following application of a topical anesthetic (Falcon Pharmaceuticals, LTD., Fort Worth, TX), slit-lamp biomicroscopy (Kowa Optimed, Inc., Torrance, CA), and indirect ophthalmoscopy (Heine USA, LTD., Dover, NH and Volk Optical, Inc., Mentor, OH) following instillation of a topical mydriatic agent (Tropicamide 1%, Mydriacyl®, Falcon Pharmaceuticals, LTD., Fort Worth, TX). Fundus images were collected using the RetCam II (Clarity Medical Systems Inc., Pleasanton, CA).

General Anesthesia: Dogs were anesthetized by premedication with acepromazine maleate (0.2 mg/kg) intramuscularly, induction with thiopental sodium (10 mg/kg) intravenously, and maintenance with isofluorane (1-2% in oxygen) delivered through an endotracheal tube. Pulse-oxymetery, measuring pulse rate and oxygen saturation, was used to monitor the dogs during the procedure and the initial recovery period. Body temperature was maintained with a temperature regulated water-heating pad.

Electroretinogram: Electroretinograms were performed pre-study, 1 hour after initial dose of sildenafil and regularly throughout the study (Table 2.1 & 2.2). ERGs were conducted using a Utas-E 3000 electrophysiology unit (LKC Technologies, Inc., Gaithersburg, MD) with a Ganzfeld bowl. The bandpass was set at 1 to 500 Hz; gain setting varied from 2 X 10^3 to 4 X 10^4. Dark-adapted intensity series; 5-Hz rod flicker, light-adapted intensity series; and 33-Hz cone flicker ERGs were recorded as previously described[107] except that ERG-Jet lens electrodes (The Electrode Store, Enumclaw, Washington) were used. Briefly, ERG testing began with a dark-adapted intensity series in response to 18 different intensities of white flash (ranging from -3.52 to 2.82 log cdS/m^2) were recorded. Interstimulus intervals were increased from one second at low intensities to 360 seconds at the highest intensity to avoid rod light adaptation. Three to 50 flashes were averaged per intensity. Rod flicker ERG responses at 5 Hz were recorded in response to white flashes -1.6 log cdS/m^2 in intensity with 15 tracings averaged. The dogs were then light adapted for 10 minutes at a white light intensity of 30 cd/m^2. ERG responses were recorded from a series of 13 white flash intensities (ranging from -2.41 to 2.82 log cdS/m^2), superimposed on the same background white light. Interstimulus intervals were one second for intensities -2.41 to 1.36 log cdS/m^2 and 5, 10, and 15 seconds for 1.9, 2.38, and 2.82 log cdS/m^2, respectively. Five to 50 flashes were averaged per intensity. Cone flicker ERG responses were recorded with a white-flash stimulus intensity of 0.39 log cdS/m^2 at 33 Hz. Fifteen cone flicker ERG tracings were

averaged. Cone long-flash ERG responses were recorded with a white-flash intensity of 2.13 log cdS/m^2 over 400 milliseconds. Thirty cone long-flash ERG tracings were averaged.

ERG Analysis: A- and b-wave amplitudes and implicit times were measured, as previously described.[107] ERG amplitudes were plotted as a function of light stimulus. The waveform shapes were compared between pretreatment, treatment, and washout time points. Naka-Rushton[116] fitting was applied to the dark-adapted b-wave intensity-response curve to obtain values for the parameters n, Vbmax, and K. The criterion threshold required to elicit a 20 μV dark-adapted b-wave response was calculated from these parameters. For the flicker responses, amplitude (trough to peak) and implicit times (flash onset to peak amplitude) were measured.

Vision Testing: Vision testing was performed according to a method we have previously described.[117] Briefly, this uses a device consisting of a chamber with 4 exit tunnels. One random tunnel was open for each run of the test. The first choice of exit tunnel and the time taken to exit were recorded. Performance was analyzed by seven repeated trials under eight different lighting intensities (-2.7, -1.7, -0.7, -0.4, 0, 0.9, 1.2, 1.3 Log cd/m^2). A luminometer was used to confirm the illumination level at each tunnel terminus prior to each trial.

Histopathology: Following humane euthanasia of *Pde6a*$^{+/-}$ dogs the globes were rapidly removed, with one processed for plastic embedding and the other for frozen-section immunohistochemistry as previously described.[107] Globes destined for plastic

embedding were initially fixed in 3% gluteraldehyde (Electron Microscopy Sciences, Hatfield, PA) and 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) for 20 minutes. The globes were then removed from the fixative, the anterior segments excised and an anterior vitrectomy performed using a peristaltic vitrectomy unit (American Optisurgical, Inc., Lake Forest, CA), thus leaving the eyecups for adequate retinal fixation. The eyecups were placed in its respective fixative for an additional two hours, washed three times with 0.1 M cacodylate buffer, and stored at 4°C until dehydrated in a graded series of ethanol solutions and infiltrated with semisoluble polymer medium (Electron Microscopy Sciences, Hatfield, PA). After polymerization the block was cut vertically from the superior ora ciliaris retinae through the optic nerve head (ONH) to the inferior ora ciliaris retinae. Three-micrometer sections were stained with toludine blue and hematoxylin and eosin. For measurement of individual retinal layer thickness and photoreceptor nuclei count vertical sections through the ONH from plastic embedded globes were analyzed. Four regions from these retinal sections were evaluated and imaged, two regions located 4 and 8 mm superior to ONH and two regions located 2.5 and 5 mm inferior to ONH. Layer thickness measurements and cell counts from each of the four regions was performed as previously described.[107] Five separate retinal layer thickness measurements were made over adjacent 400-µm lengths of imaged retina at 200x magnification, and the mean of individual retinal layers was calculated. Two adjacent 400x magnified images were captured at each region and the number of outer nuclear layer (ONL) nuclei and cell bodies were counted. Two masked investigators (JB, SPJ) performed the measurements. Similarly prepared sections from four *Pde6a*$^{+/-}$ dogs

that had received no treatment were analyzed as controls (mean age: 79 +/- 22 days). The globes prepared for frozen immunohistochemistry were stained with antibodies for M/L opsin, rod opsin, pKC alpha and activated caspase 3. TUNEL staining was also performed as previously described.[107]

Statistical Analysis: A split-plot ANOVA (SAS ver. 9.1; SAS Institute Inc., Cary, NC) was used to analyze repeated-measures data, including inter- and intra-group ERG responses, Naka-Rushton fitting, criterion threshold, vision testing comparisons, retinal layer thickness measurements, and photoreceptor cell counts. Data was considered significant at $p<0.05$. Post-hoc Bonferoni testing for multiple comparison data, with a critical p-value $p<0.01$, was performed to confirm significance.

RESULTS

Ophthalmology Examinations: Ophthalmology examination findings were within normal limits at all time points in all dogs (Figure 2.1).

ERG Analysis: Sildenafil treatment resulted in an elevation of the dark-adapted b-wave threshold in both *Pde6a*$^{+/-}$ (Figure 2.2) and *Pde6a*$^{+/+}$ dogs. This effect was reversed following washout. The delay in b-wave threshold meant the scotopic threshold response was present up to brighter flash intensities while the dogs were receiving sildenafil compared to pretreatment or following washout. This effect is illustrated by plots of dark-adapted b-wave intensity-response amplitudes that showed a notable rightward curve shift for the first six light intensities plotted in both sildenafil-treated *Pde6a*$^{+/-}$ and *Pde6a*$^{+/+}$ dogs compared to pretreatment and washout, but not placebo-

treated dogs. (Figure 2.3) In the sildenafil-treated *Pde6a*$^{+/-}$ dogs the b-wave amplitudes in response to brighter flashes were not different to pretreatment and washout. The sildenafil-treated *Pde6a*$^{+/+}$ dogs had lower mean b-wave amplitudes at all flash intensities, but this difference was only statistically significant at the lower flash intensities. (Figure 2.3)

Naka-Rushton fits were used to investigate retinal responses. The derived value of rod-mediated scotopic maximum b-wave amplitude (Vbmax) was significantly reduced in *Pde6a*$^{+/+}$ dogs during the treatment phase (142.5 +/- 14 µV) compared to pretreatment (118.0 +/- 14 µV, p=0.01), but was not significantly different in the *Pde6a*$^{+/-}$ dogs. Following washout the Vbmax of *Pde6a*$^{+/+}$ dogs had returned to a value similar to pretreatment. The intensity to elicit one-half maximum rod-mediated b-wave amplitude (k) was not significantly different between groups. The Naka-Rushton parameters were used to extrapolate the stimulus intensities required to generate a 20 µV dark-adapted b-wave response. The stimulus required to elicit this criterion threshold was significantly elevated during the treatment phase in both *Pde6a*$^{+/-}$ (p<0.001) and *Pde6a*$^{+/+}$ dogs (p=0.002) treated with sildenafil compared to the pretreatment value. (Figure 2.4) However, the criterion threshold returned at washout to a level similar to pretreatment.

Sildenafil treatment did not result in any significant differences in dark-adapted b-wave implicit times, a-wave amplitude or implicit times, 5-Hz flicker, light-adapted intensity:response series, or 33-Hz flicker responses at any phase of the study.

Vision Testing: There were no significant differences in first choice of exit tunnel or time taken to exit between groups. (Table 2.3)

Retinal Morphology: Sildenafil–treated *Pde6a*$^{+/-}$ dogs had significantly thinner ONL (24.90 +/-1.88 µm, p=0.004) and significantly lower photoreceptor nuclei counts (273.6 +/-29.3 cells/100 µm, p=0.008) compared to the measurements (35.90 +/-1.63 µm) and counts (391.5 +/-27.0 cells/100 µm) of retinal sections from archived untreated *Pde6a*$^{+/-}$ dogs respectively. (Figure 2.5 – 2.8) However, the differences for the same two measures between placebo-treated and sildenafil-treated *Pde6a*$^{+/-}$ dogs did not reach statistical significance. There were no significant differences in the measured mean thicknesses of the other retinal layers between the groups. (Data shown in Table 2.5)

Immunohistochemical Analysis: There were no significant differences in immunohistochemical labeling or TUNEL staining observed. (Figure 2.9)

DISCUSSION

The current study demonstrates in dogs that orally administered sildenafil, at an equivalent of 10 times the maximum recommended dose for management of erectile dysfunction in men, raises the threshold of the dark-adapted ERG b-wave and reduces its amplitude at light stimulus intensities below -0.8 Log cdS/m^2. This finding is in keeping with previously reported preclinical studies showing reduction in dark-adapted b-wave amplitude in wild-type dogs following intravenous infusion of sildenafil producing plasma concentrations approximately 10 times the typical level achieved with oral

administration in humans.[47] Interestingly we noted this effect of sildenafil administration on the ERG response equally in both wild-type dogs and dogs heterozygous for a *Pde6a* mutation. This finding contrasts with the report by Behn et al. in which mice heterozygous for a *Pde6g* mutation showed a reduction in dark-adapted b-wave amplitudes of 45% and 66% compared to control when administered 2x and 10x the human equivalent dose of sildenafil respectively; whereas wild-type mice showed no significant b-wave effects at either sildenafil dose.[101] Further investigation should help illuminate the roles absorption pharmacokinetics of oral versus intraperitoneal administration routes, species differences in binding efficiency of sildenafil to the PDE6 protein complex, and different gene mutation affects on PDE6 subunit expression levels may play in these disparate study outcomes.

This elevation in dark-adapted b-wave threshold allowed visualization of the scotopic threshold response, which has been previously described in dogs[118] and other species[119,120], at flash intensities where in normal dogs it is typically masked by the rod-mediated b-wave. There are two plausible explanations for the elevated rod-mediated b-wave threshold. In the first, sildenafil might have a direct suppressive effect on PDE6 in rod photoreceptors resulting in greater light stimulation required to get the same degree of rod outer segment hyperpolarization. If this is true, we would expect a similar delay in the dark-adapted a-wave threshold. Although it appeared sildenafil-treated dogs had slightly raised a-wave thresholds this was not a statistically significant difference. If this mechanism is the major cause of the ERG effect, a difference in b-wave threshold between *Pde6a*$^{+/-}$ and *Pde6a*$^{+/+}$ dogs would be expected but we did not find this to be

the case. A second mechanism for the elevated b-wave threshold could be an effect of sildenafil on PDEs in rod bipolar cells. Suppression of PDE activity in bipolar cells could result in a reduction in cation transport across the bipolar cell membrane. The generation of a radial difference in ion concentration due to bipolar cell action is involved in the generation of the rod b-wave.[121] To differentiate between a direct affect on rod photoreceptors as opposed to bipolar cells, additional studies using pharmacological dissection of the ERG could be considered.[122]

Full recovery of normal ERG responses was noted in both *Pde6a*$^{+/-}$ dogs and *Pde6a*$^{+/+}$ dogs treated with sildenafil. No significant differences in objective visual performance were noted between groups during any phase of the study. Our previous studies using *Pde6a*$^{-/-}$ dogs have shown that near complete ablation of dark-adapted ERG responses frequently proceeds substantial deterioration of visual performance in canine models of recessively inherited retinitis pigmentosa.([123] and unpublished data) As dogs possess a rod-dominated retina lacking the clearly delineated anatomical structures of the human macula and fovea, it is uncommon that significant visual deterioration resulting from a retinal-origin lesion occurs without detectable coincident affects on either the rod and/or cone components of the ERG.

Analysis of retinal histology sections seems to clearly show that four months of daily high-dose administration of sildenafil has no significant affect on the thickness measurement of a majority of individual retinal layers in dogs. However, the most striking finding of this study was the significant difference in thickness measurements

and cell counts of the outer nuclear layers of sildenafil-treated *Pde6a*$^{+/-}$ dogs compared to archived samples from untreated *Pde6a*$^{+/-}$ dogs. Although the mean values for both of these measures were lower in the sildenafil-treated compared to the placebo-treated *Pde6a*$^{+/-}$ dogs, this difference was not significant. In an attempt to increase the number of samples we compared the measures to archived retinal sections from *Pde6a*$^{+/-}$ dogs that had been processed in an identical fashion. Our archive is limited to a modest number of *Pde6a*$^{-/-}$ dogs. We chose the samples that were closest in age to the dogs on the current study; however, the average age of dogs from which the archived samples were generated was notably younger than the dogs on the current study. The most likely explanation for the difference in ONL thickness between groups is simply variation of retinal thickness between individuals. Due to the limited size of our current archive of *Pde6a*$^{+/-}$ dogs, we have not yet established ranges for expected variation of retinal layer thicknesses. This remains an ongoing project in our laboratory. We have no evidence that a slowly progressive retinal degeneration occurs in the heterozygous *Pde6a*$^{+/-}$ dogs. Although we have shown the carrier state results in reduced levels of PDE6 within rod outer-segments, the lower expression does not appear to significantly affect phototransduction or cause photoreceptor degeneration. *Pde6a*$^{+/-}$ dogs remain clinically indistinguishable from wild-type *Pde6a*$^{+/+}$ on full-field ERG and objective vision testing throughout life.

Finally we cannot rule-out the possibility that high-dose administration of sildenafil results in a level of PDE6 suppression that photoreceptor toxicity and cell loss does develop in *Pde6a*$^{+/-}$ dogs. Although statistical significance was not reached in our study, the averages of ONL thickness measurements and cell counts were lowest in sildenafil-treated dogs. It is possible the low number of study subjects contributed to a type II statistical error. Subjective assessment of retinal histology sections revealed no evidence of photoreceptor pyknosis, inflammation, or chronic signs of degeneration and there were no positive cells noted on TUNEL staining. However, with the washout phase occurring prior to any retinal morphologic evaluation it is possible the tell-tale signs of slowly progressive toxicity occurring during the treatment phase had resolved and were no longer readily evident. An 18% reduction in photoreceptor cell counts, as noted between placebo-treated *Pde6a*$^{+/-}$ dogs and sildenafil-treated *Pde6a*$^{+/-}$ dogs, may not be extensive enough to cause visual disturbances or to be detectable on ERG following washout.

A major limitation of this study is the low number of dogs in each group contributing to reduced statistical power. This is an unfortunate complication of predicting availability of dogs of a specific genotype resulting from breedings in a closed colony. We believe definitively establishing the site of action of sildenafil to raise the threshold of the rod-driven b-wave responses and ruling-out the possibility of photoreceptor toxicity in *Pde6a*$^{+/-}$ dogs are important reasons to pursue further investigation.

It is estimated that 1 in 3700 individuals world-wide are affected by RP. The population of individuals carrying RP-causative mutations is extensive and a majority of these carriers are unaware of their underlying genotype. Many of these mutations affect expression of critical retinal proteins. The altered expression could theoretically put these individuals at risk for visual side-effects associated with pharmaceutical use for unrelated conditions. 3-4% of autosomal recessive RP of cases are caused by PDE6 mutations. Erectile function is common health concern with prevalence of 52% of men between the ages of 40 and 70. Sildenafil is top selling medication for management of erectile dysfunction and has more recently gained FDA approval for use in treatment of pulmonary arterial hypertension. We feel identification of the risk for vision-loss associated with sildenafil use in individuals affected by or carriers of RP-causative mutations is critical.

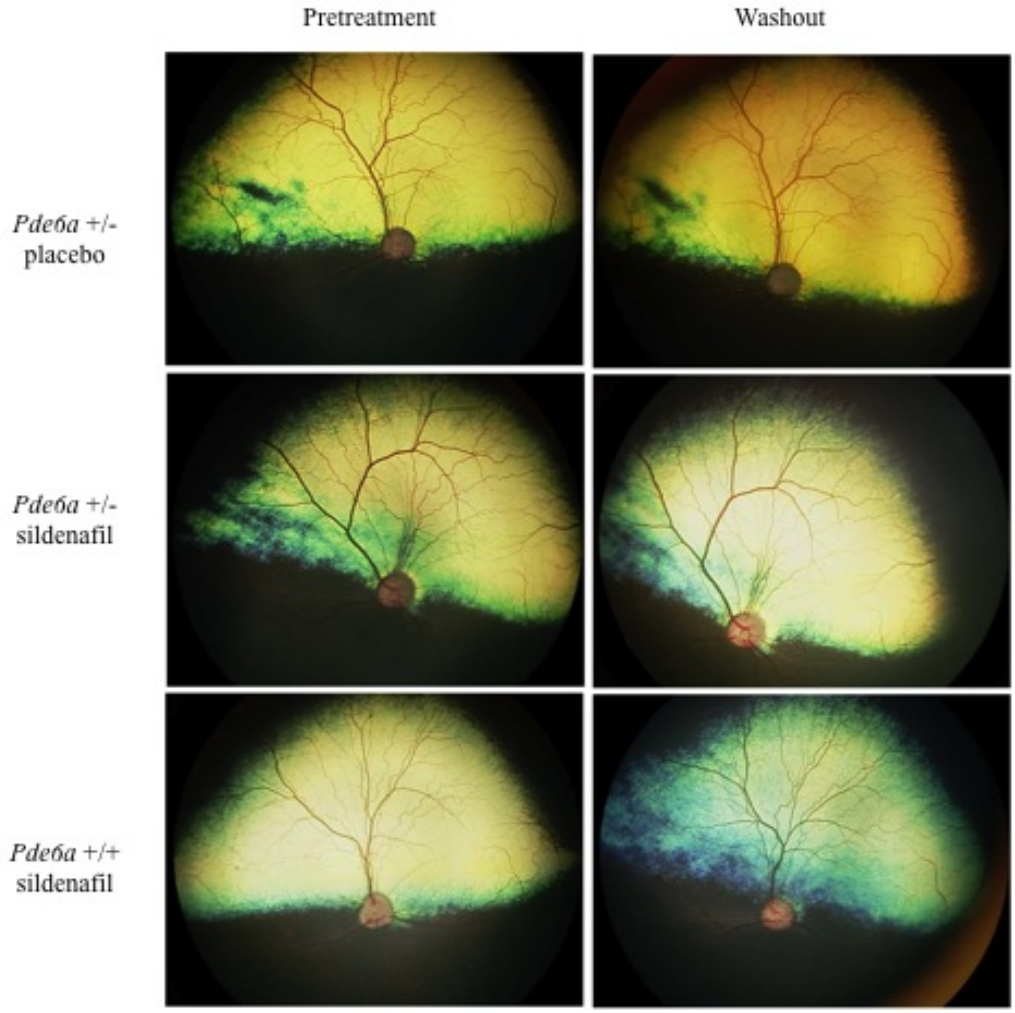
Table 2.1: Acute phase study design

						Examination, ERG & Vision Testing Times			
Dog	Breed	Age (yrs)	Gender	Genotype	Drug	Pretreatment	1st Dose	1st Week	Washout
1	Corgi X	1.42	Female	Pde6a$^{+/-}$	Placebo	X	X		X
2	Corgi X	1.90	Female	Pde6a$^{+/-}$	Placebo	X	X		X
3	Corgi X	1.86	Female	Pde6a$^{+/-}$	Sildenafil	X	X		X
4	Corgi X	1.27	Female	Pde6a$^{+/-}$	Sildenafil	X	X		X
5	Corgi X	1.86	Female	Pde6a$^{+/-}$	Sildenafil	X	X		X
6	Beagle	2.39	Male	Pde6a$^{+/+}$	Sildenafil	X	X	X	X
7	Beagle	2.35	Male	Pde6a$^{+/+}$	Sildenafil	X	X	X	X
8	Beagle	2.36	Male	Pde6a$^{+/+}$	Sildenafil	X	X	X	X

Table 2.2: Chronic phase study design

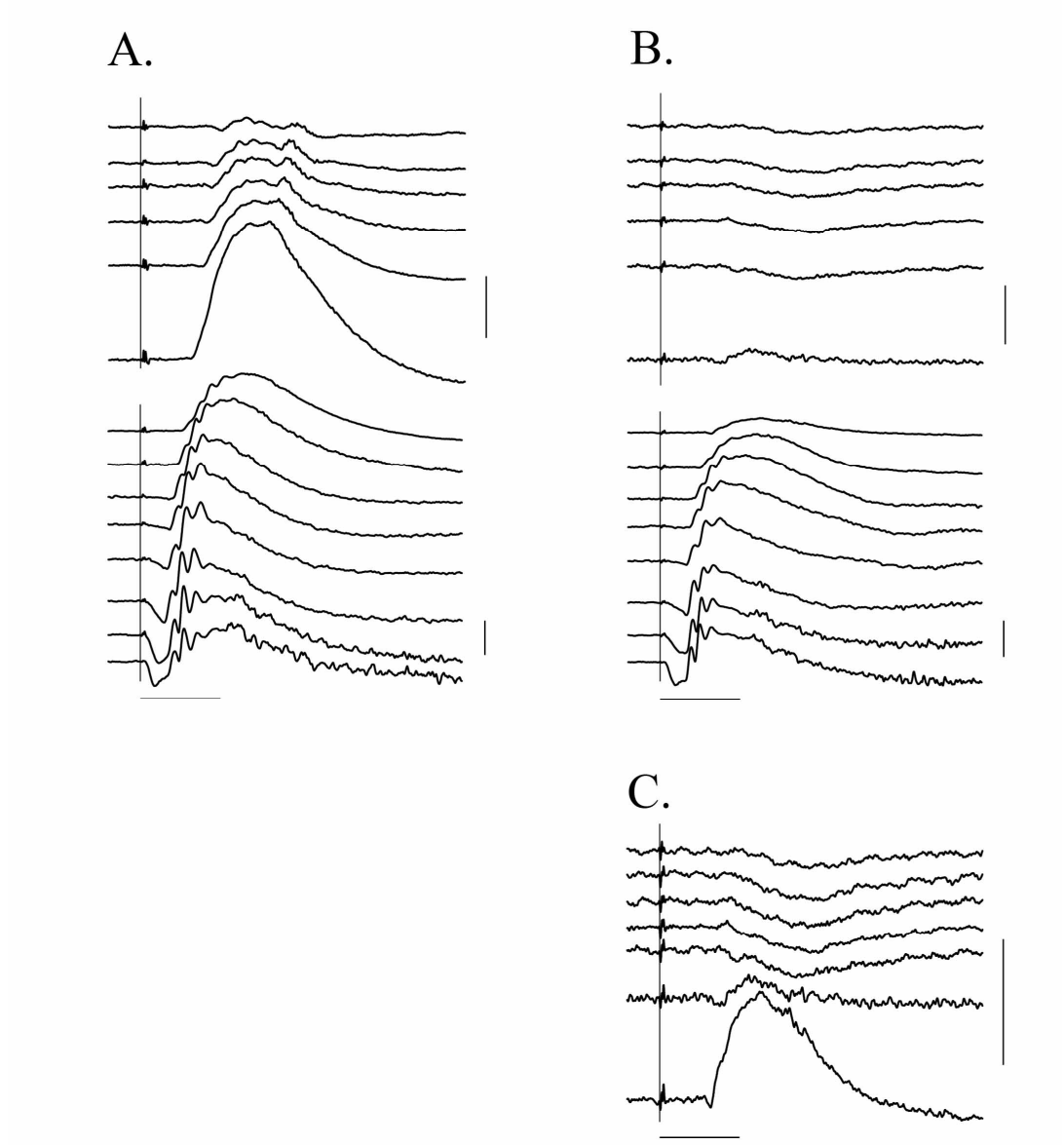
				Examination, ERG & Vision Testing Times						
Dog	Breed	Genotype	Drug	Pretreatment	1st Month	2nd Month	3rd Month	4th Month	Washout	Histopathology
1	Corgi X	Pde6a$^{+/-}$	Placebo	X	X	X	X	X	X	X
2	Corgi X	Pde6a$^{+/-}$	Placebo	X	X	X	X	X	X	X
3	Corgi X	Pde6a$^{+/-}$	Sildenafil	X	X	X	X	X	X	X
4	Corgi X	Pde6a$^{+/-}$	Sildenafil	X	X	X	X	X	X	X
5	Corgi X	Pde6a$^{+/-}$	Sildenafil	X	X	X	X	X	X	X
6	Corgi X	Pde6a$^{+/-}$	Untreated							X
7	Corgi X	Pde6a$^{+/-}$	Untreated							X
8	Corgi X	Pde6a$^{+/-}$	Untreated							X
9	Corgi X	Pde6a$^{+/-}$	Untreated							X

Figure 2.1: Fundus montage



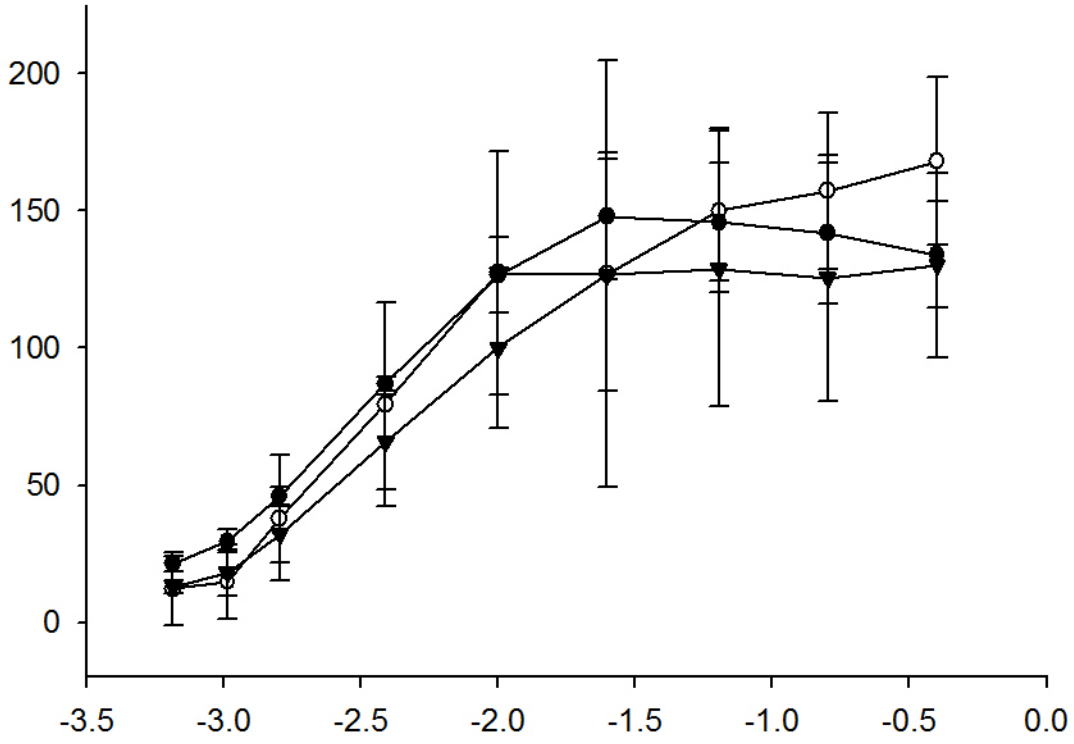
Representative funduscopic images of the left eye from a dog of each group during the pretreatment and washout time points. No ophthalmoscopic evidence of retinal degeneration was observed. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

Figure 2.2: Representative raw dark-adapted ERG waveforms



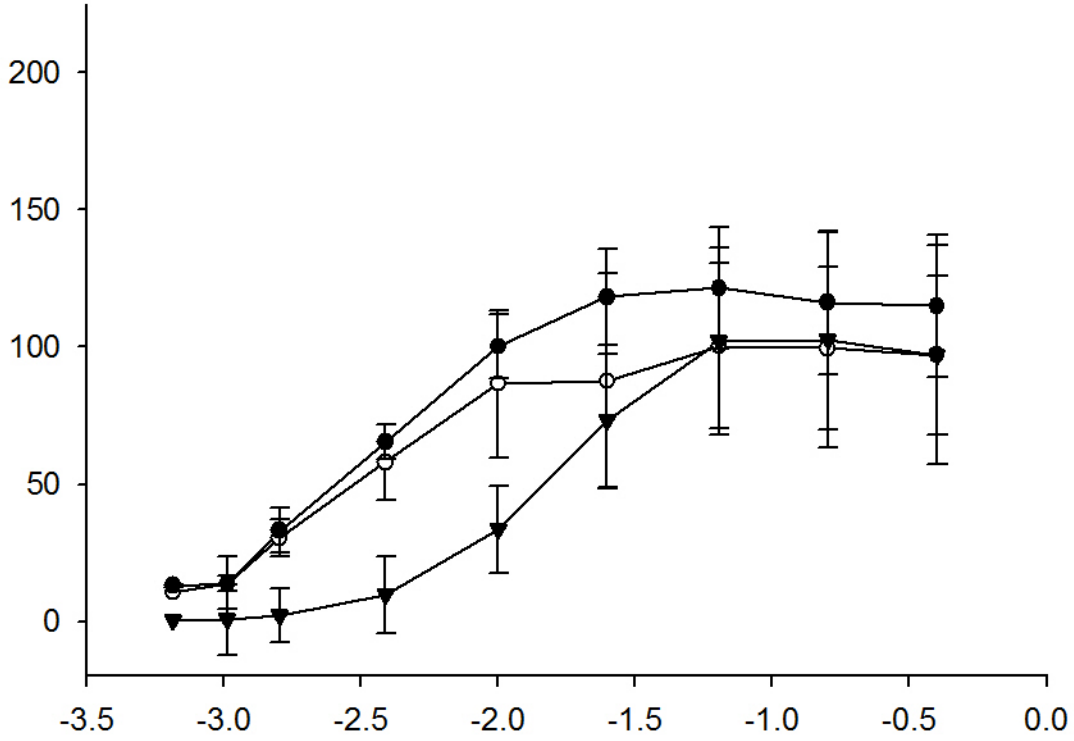
Representative Pde6a$^{+/-}$ sildenafil treated dog ERG waveforms from a dark-adapted intensity series. A. While not receiving Sildenafil. B. While on Sildenafil and C. A more magnified view of the first 7 responses from B. Vertical line through tracings indicates flash. Vertical size bars = 50µV. Horizontal size bars = 50 mSec. Flash intensity from top to bottom

Figure 2.3: Mean group dark-adapted b-wave intensity:response curves



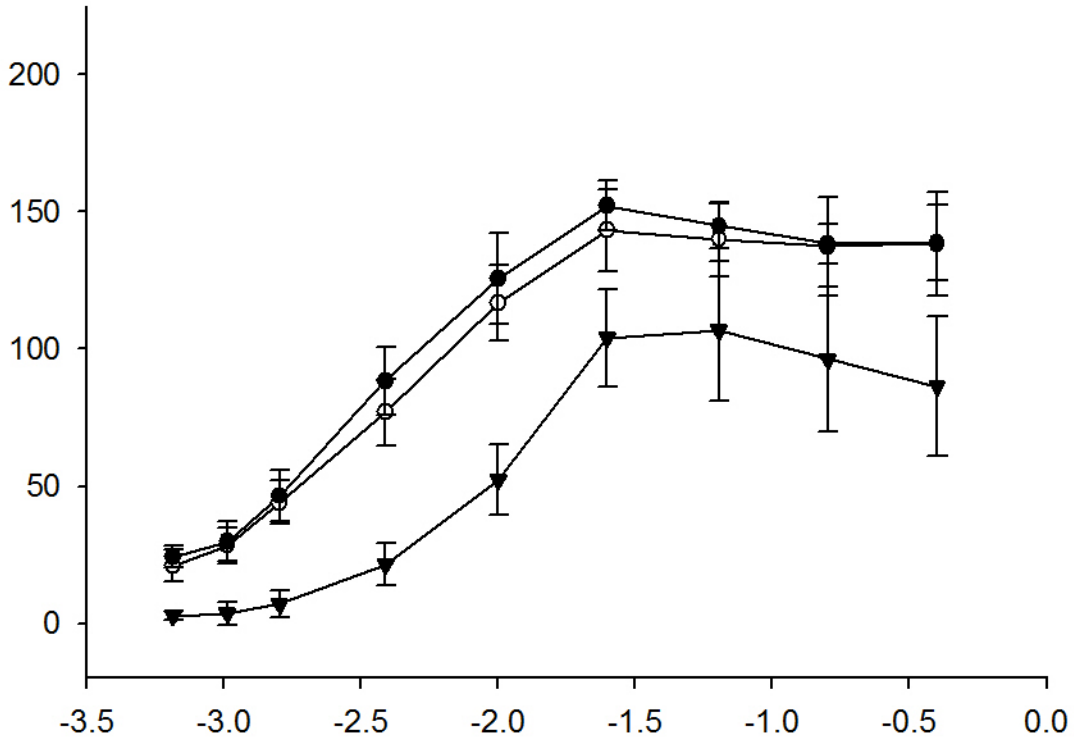
Pde6a$^{+/-}$ placebo group; X-axis: light intensity; Y-axis: amplitude response in microvolts; pretreatment phase = open circle; treatment phase = black triangle; washout phase = black circle; No significant difference was observed at all light intensities.

Figure 2.3 (cont'd): Mean group dark-adapted b-wave intensity:response curves



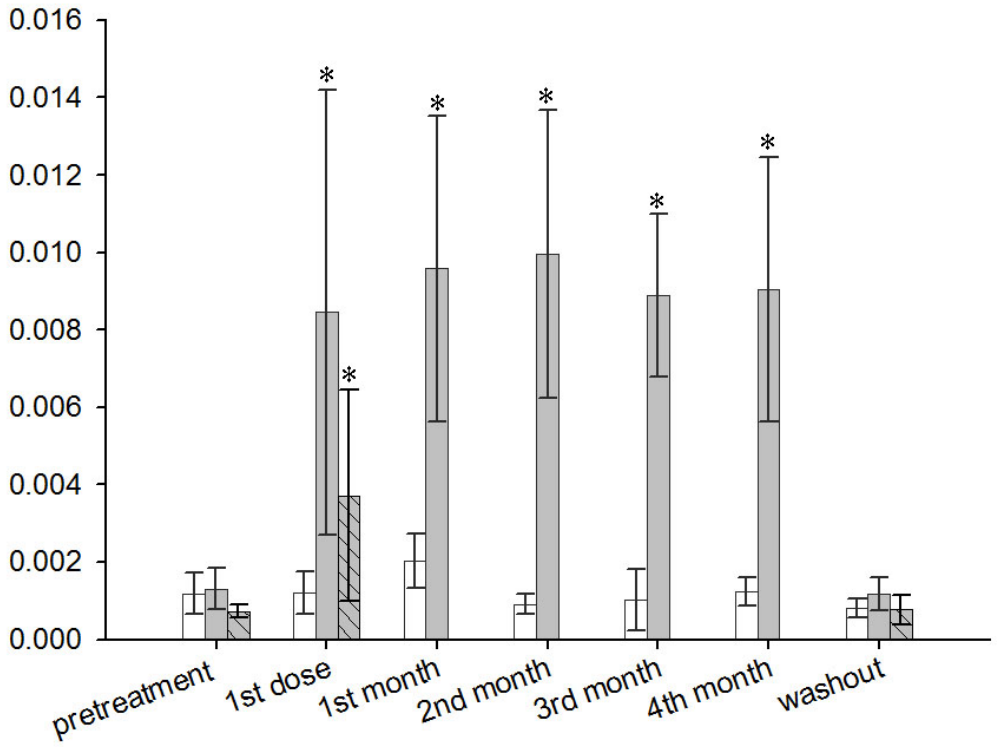
Pde6a$^{+/-}$ sildenafil treated group; X-axis: light intensity; Y-axis: amplitude response in microvolts; pretreatment phase = open circle; treatment phase = black triangle; washout phase = black circle; Note the rightward shift in response to the five lowest light intensities during the treatment phase.

Figure 2.3 (cont'd): Mean group dark-adapted b-wave intensity:response curves



Pde6a$^{+/+}$ wild-type sildenafil treated group; X-axis: light intensity; Y-axis: amplitude response in microvolts; pretreatment phase = open circle; treatment phase = black triangle; washout phase = black circle; Note the statistically significant rightward shift in response to the six lowest light intensities during the treatment phase.

Figure 2.4: Mean 20-microvolt dark-adapted b-wave criterion threshold responses



X-axis: study phase; Y-axis: light intensity (Log cdS/m^2) at which a 20-microvolt b-wave response is generated. Asterisks (*) indicate the time of significant difference compared to placebo controls. White bar = $Pde6a^{+/-}$ placebo; gray bar = $Pde6a^{+/-}$ sildenafil treated; stripped gray bar = $Pde6a^{+/+}$ wild-type sildenafil treated

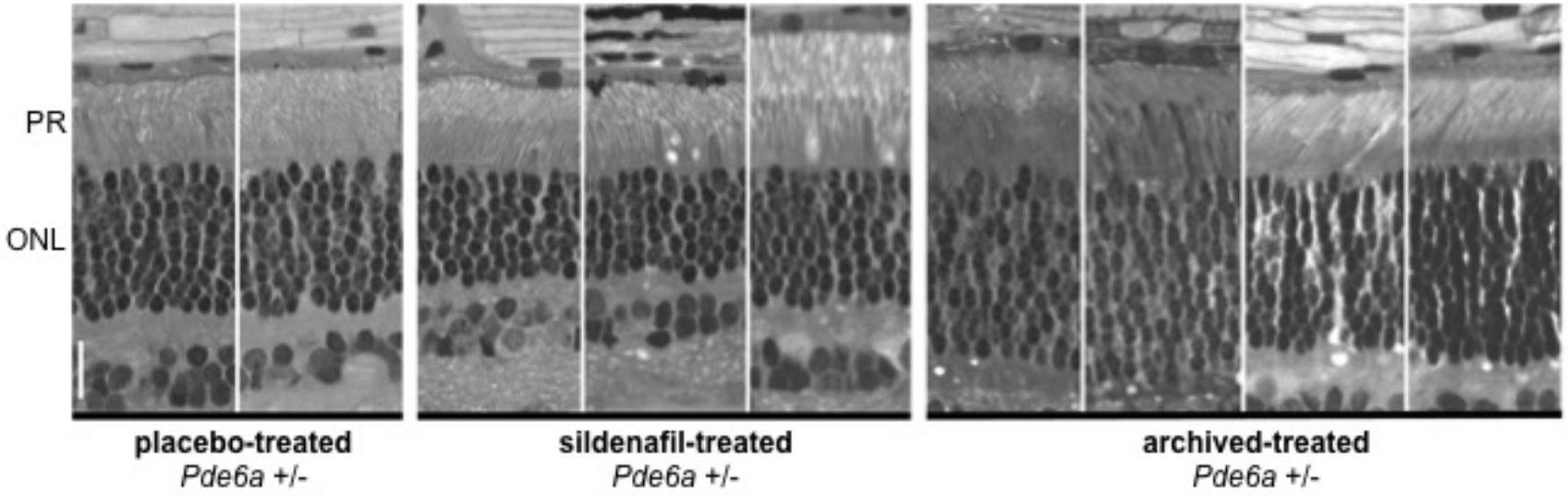
Table 2.3: Mean vision testing tunnel exit times and percentage correct tunnel choice per study phase.

Light Intensity (Log cdS/m2)	**Pde6a$^{+/-}$ Placebo**			**Pde6a$^{+/-}$ Sildenafil**			**Pde6a$^{+/+}$ Sildenafil**		
	Mean exit (sec)	(+/- SD)	% correct choice	Mean exit (sec)	(+/- SD)	% correct choice	Mean exit (sec)	(+/- SD)	% correct choice
Pretreatment Phase									
-2.7	10.13	14.87	93.33	5.62	3.97	100.00	9.00	6.31	100.00
-1.7	4.12	3.74	94.12	3.09	1.51	100.00	3.90	4.21	100.00
-0.7	5.21	2.39	100.00	3.19	1.13	100.00	4.10	3.80	100.00
-0.4	3.14	1.16	100.00	9.50	15.85	100.00	2.60	1.29	100.00
0	4.00	3.59	92.86	2.86	2.07	100.00	3.70	5.98	100.00
0.9	3.14	2.16	100.00	4.42	2.85	83.33	3.30	3.34	100.00
1.2	5.50	2.37	85.71	3.67	1.56	100.00	3.13	1.81	100.00
1.3	3.43	2.25	100.00	4.00	6.09	100.00	3.97	2.87	100.00
Treatment Phase									
-2.7	3.60	1.59	98.75	7.85	7.83	90.91	16.70	17.95	72.73
-1.7	3.49	2.37	98.77	3.88	3.10	100.00	2.23	1.02	100.00
-0.7	3.18	2.29	100.00	3.59	3.82	100.00	2.40	1.21	100.00
-0.4	2.89	1.62	100.00	3.12	1.74	100.00	3.00	1.45	100.00
0	3.34	1.82	100.00	3.02	1.93	100.00	2.29	1.17	100.00
0.9	2.40	1.73	100.00	2.56	1.69	100.00	2.63	1.28	100.00
1.2	2.73	1.30	100.00	2.72	1.71	100.00	2.20	1.05	100.00
1.3	2.87	1.86	100.00	2.54	1.53	100.00	2.90	1.51	100.00

Table 2.3 (cont'd): Mean vision testing tunnel exit times and percentage correct tunnel choice per study phase.

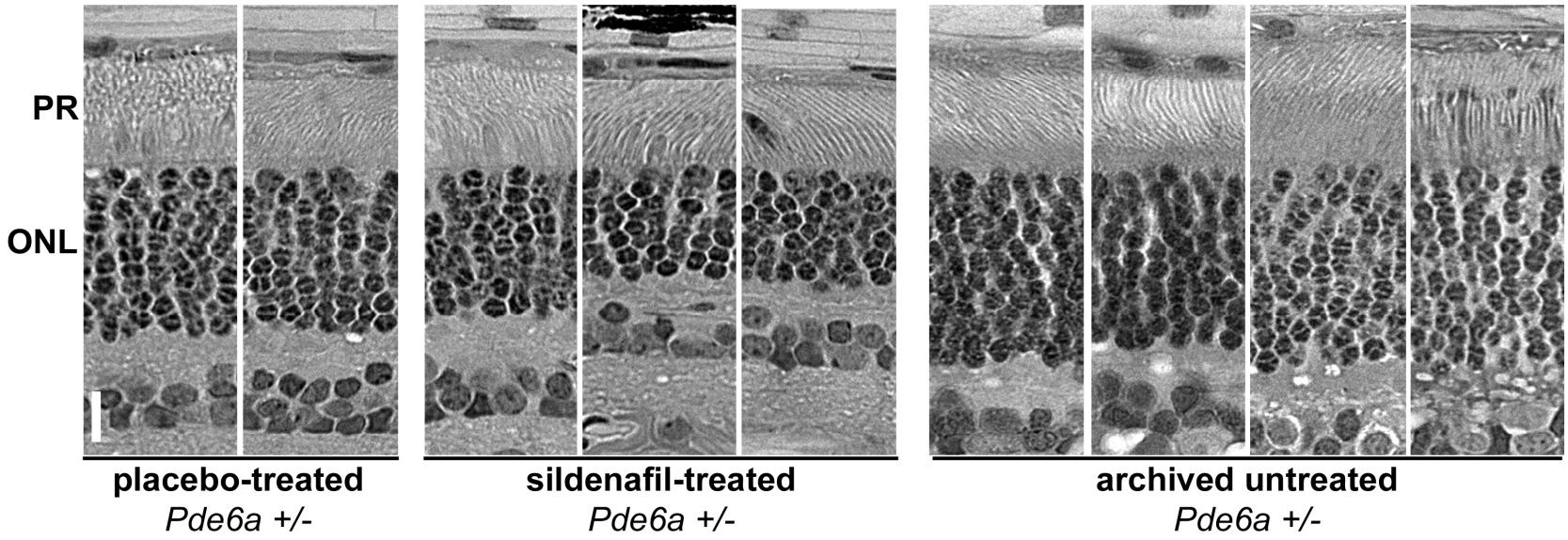
Light Intensity (Log cdS/m2)	**Pde6a$^{+/-}$ Placebo**			**Pde6a$^{+/-}$ Sildenafil**			**Pde6a$^{+/+}$ Sildenafil**		
	Mean exit (sec)	(+/- SD)	% correct choice	Mean exit (sec)	(+/- SD)	% correct choice	Mean exit (sec)	(+/- SD)	% correct choice
Washout Phase									
-2.7	3.50	1.75	100.00	4.17	2.21	100.00	5.47	7.66	100.00
-1.7	2.65	0.96	100.00	4.37	2.48	100.00	2.63	1.28	100.00
-0.7	1.85	0.77	100.00	4.27	2.60	100.00	1.50	0.63	100.00
-0.4	2.19	1.01	95.24	2.83	1.11	100.00	2.27	1.06	100.00
0	1.75	0.85	100.00	3.90	1.97	100.00	2.07	1.44	100.00
0.9	1.35	0.48	100.00	3.97	1.44	100.00	2.60	1.46	100.00
1.2	1.50	0.51	100.00	2.83	1.82	100.00	1.87	0.86	100.00
1.3	1.44	0.61	100.00	2.37	1.31	100.00	2.00	1.02	100.00

Figure 2.5: Distal tapetal retina 400x photomicrograph montage



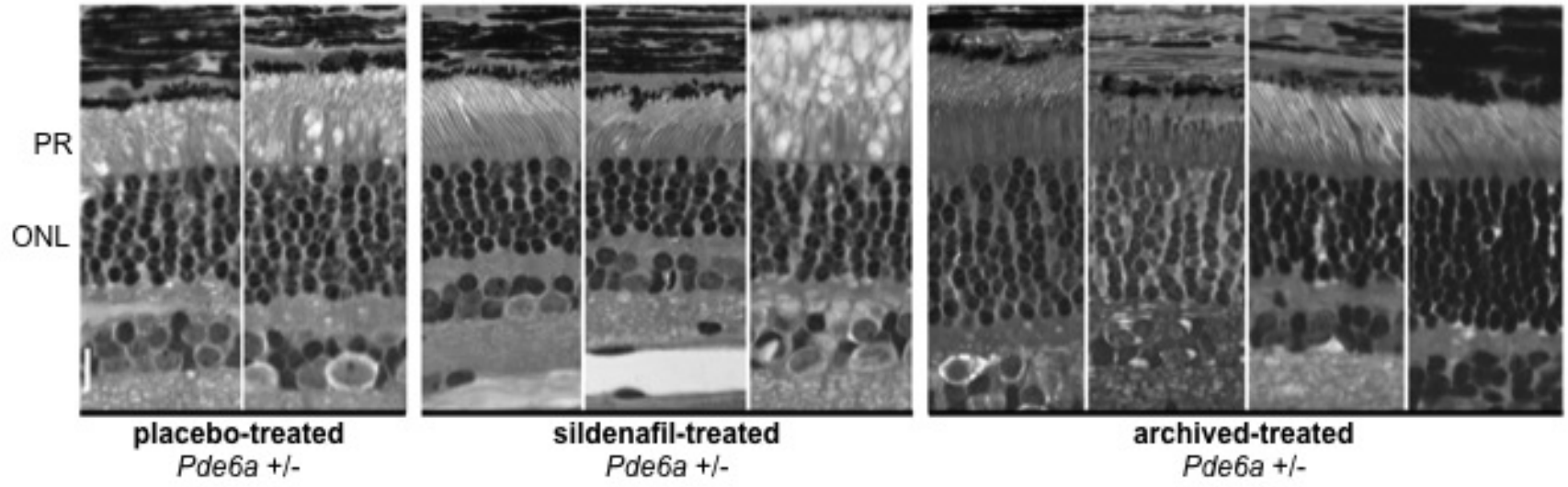
Variation in retinal thickness measurements and photoreceptor outer nuclear layer cells counts per group. White bar = 10 micrometers

Figure 2.6: Proximal tapetal retina 400x photomicrograph montage



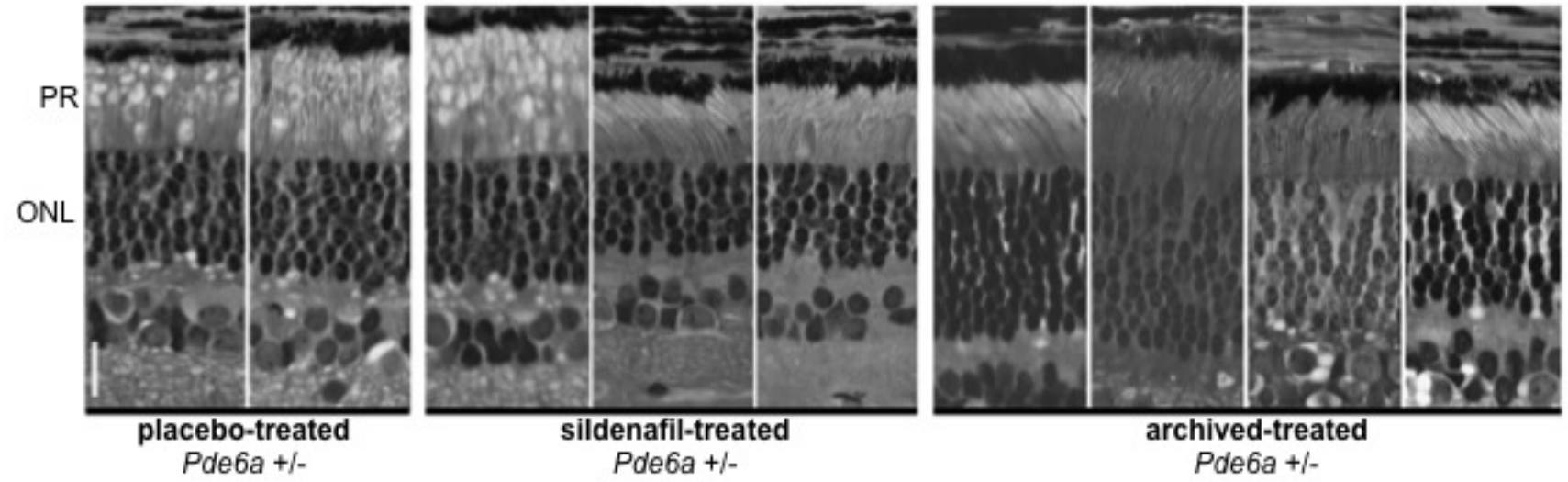
Variation in retinal thickness measurements and photoreceptor outer nuclear layer cells counts per group. White bar = 10 micrometers

Figure 2.7: Proximal nontapetal retina 400x photomicrograph montage



Variation in retinal thickness measurements and photoreceptor outer nuclear layer cells counts per group. White bar = 10 micrometers

Figure 2.8: Distal nontapetal retinal 400x photomicrograph montage



Variation in retinal thickness measurements and photoreceptor outer nuclear layer cells counts per group. White bar = 10 micrometers

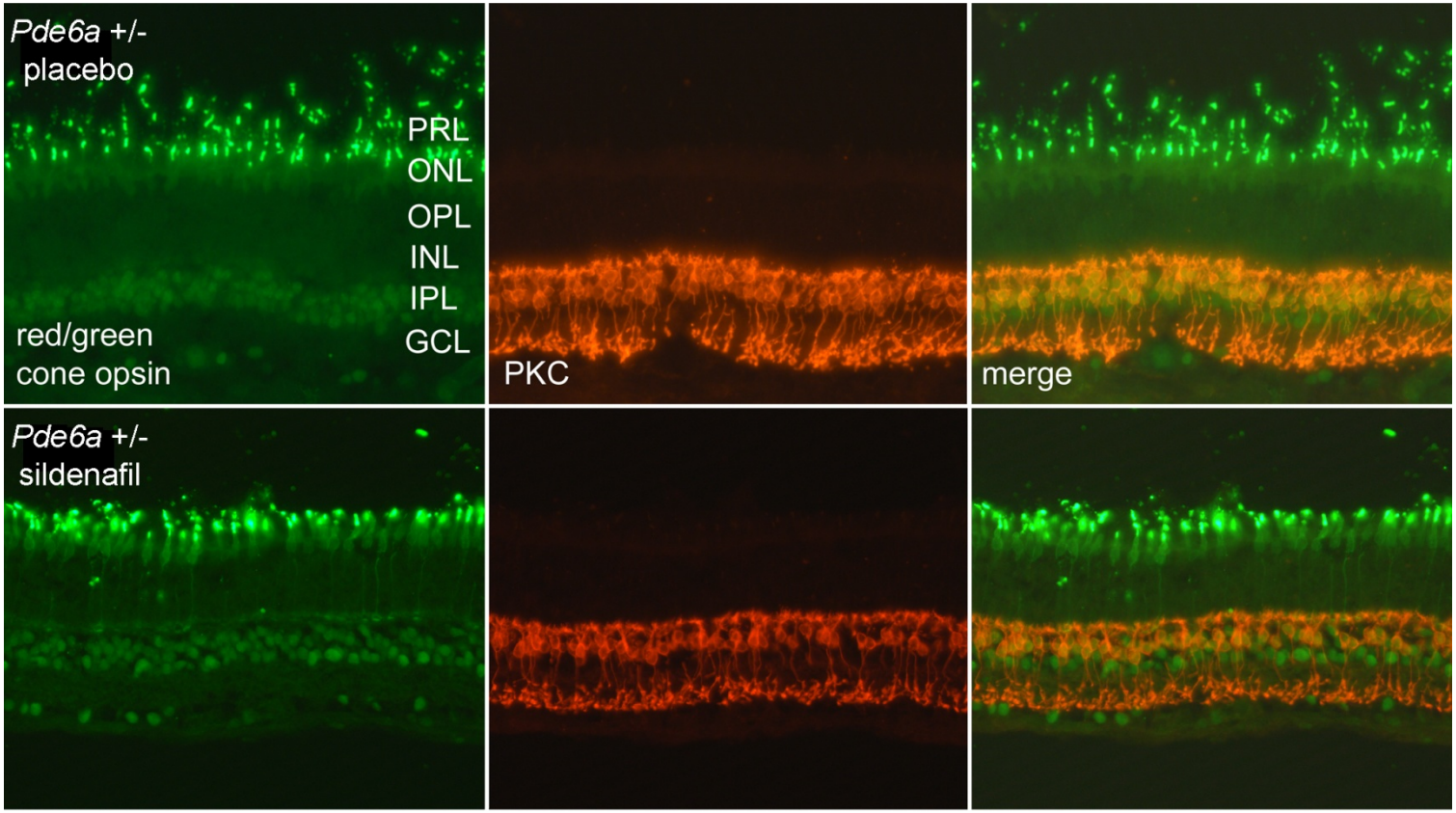
Table 2.4: Outer nuclear layer cell counts

Location	Pde6a$^{+/-}$ Placebo		Pde6a$^{+/-}$ Sildenafil		Untreated Pde6a$^{+/-}$	
	Mean (μm)	+/- SD	Mean (μm)	+/- SD	Mean (μm)	+/- SD
Distal Tapetum	350.25	36.00	274.33	22.15	393.84	66.00
Proximal Tapetum	387.38	30.54	288.67	22.92	389.81	66.54
Proximal Nontapetum	312.13	29.05	280.75	21.22	380.66	56.51
Distal Nontapetum	282.75	22.95	250.67	25.78	384.64	62.86

Table 2.5: Mean retinal thickness measurements

Location	Pde6a$^{+/-}$ Placebo		Pde6a$^{+/-}$ Sildenafil		Pde6a$^{+/+}$ Untreated	
Distal Tapetum	Mean (μm)	+/- SD	Mean (μm)	+/- SD	Mean (μm)	+/- SD
RPE	4.59	0.96	4.62	0.94	5.07	0.96
Total PS	20.77	2.70	19.02	2.82	19.17	3.48
ONL	32.05	1.68	24.68	3.42	37.12	2.65
OPL	6.54	1.06	5.70	1.24	7.11	1.40
INL	11.37	1.58	10.53	1.36	16.10	2.70
IPL	11.48	1.72	10.73	2.14	11.77	2.93
Total NFL	5.92	1.74	5.96	2.35	7.27	2.41
Proximal Tapetum						
RPE	4.44	0.95	4.85	0.72	5.67	1.32
Total PS	19.17	2.04	18.98	3.63	21.76	4.40
ONL	34.44	1.19	26.61	3.07	40.51	2.56
OPL	7.66	1.25	5.92	0.69	7.49	1.88
INL	13.40	1.31	12.84	1.38	17.59	2.86
IPL	12.55	2.27	12.03	2.99	14.79	3.23
Total NFL	7.69	2.60	8.82	4.47	9.58	3.42
Proximal Nontapetum						
RPE	5.44	0.65	5.52	0.91	6.05	1.40
Total PS	19.53	1.80	19.61	5.92	19.17	3.72
ONL	30.36	2.92	25.31	2.98	34.20	5.54
OPL	6.15	1.08	6.04	1.21	5.92	1.33
INL	13.67	1.53	14.30	1.96	15.52	4.51
IPL	11.95	2.15	11.62	2.45	13.28	4.27
Total NFL	7.46	3.28	7.00	1.64	7.58	2.69
Distal Nontapetum						
RPE	5.64	1.31	6.23	1.36	6.23	1.36
Total PS	20.23	2.12	18.40	5.22	17.70	4.37
ONL	26.51	2.67	23.02	3.48	31.77	3.93
OPL	5.52	1.29	5.23	1.37	5.92	1.51
INL	12.31	1.67	10.99	2.11	15.36	3.89
IPL	12.01	1.57	10.68	2.33	12.00	4.71
Total NFL	7.16	2.22	5.17	1.68	6.36	1.71

Figure 2.9: Representative immunohistochemical staining from a *Pde6a* +/- placebo and sildenafil treated dog



Red/green (M/L) cone opsin; protein kinase C alpha (PKC); photoreceptor layer (PRL); outer nuclear layer (ONL); outer plexiform layer (OPL); inner nuclear layer (INL); inner plexiform layer (IPL); ganglion cell layer (GCL). No significant difference in immunohistochemical labeling between the *Pde6a*$^{+/-}$ placebo and sildenafil treated groups.

CHAPTER 3

FUTURE DIRECTIONS

The dark-adapted ERG amplitude reductions observed in *Pde6a*$^{+/-}$ and *Pde6a*$^{+/+}$ dogs receiving 14.3 mg/kg sildenafil are consistent with previously reported ERG alterations in men on elevated doses.[82,83] Cross-binding of sildenafil to PDE6 within the photoreceptor outer segment discs is one mechanism suspected to contribute to the reported visual disturbances. Sildenafil readily crosses the blood-retinal barrier but has not been shown to cross the blood-brain barrier. Sildenafil administered both orally and intravenously is pharmacologically well-tolerated in healthy men 45 to 58 years of age.[124] A 50 mg oral dose of sildenafil is rapidly and completely metabolized by the gastrointestinal and liver cytochrome P450 enzyme system via N-demethylation, oxidation, and aliphatic dehydroxylation.[124] First pass metabolism results in 38 - 41% bioavailability, of which 92% is completely absorbed. A single 25 mg intravenous sildenafil dose accounts for 60% of the total plasma sildenafil level compared to 32% plasma levels after oral administration.[124] A clinically insignificant 29% reduction in sildenafil absorption and ~1 hr delay in drug onset occurs when sildenafil is administered

with a meal.[125] In dogs sildenafil has a similar level of systemic absorption but lower plasma protein binding affinity compared to humans, 84% vs. 95%, respectively.[34] Lower plasma protein binding results in a wider volume of distribution in dogs (5.21/kg) compared to rats and humans (1 – 2.1/kg).[34] Investigations comparing intraperitoneal pharmacokinetics of sildenafil in a larger animal model are limited in the literature. It is possible the observed ERG alterations in both *Pde6a*$^{+/-}$ and *Pde6a*$^{+/+}$ dogs treated with sildenafil may be due to the higher fraction of freely circulating sildenafil as compared to freely circulating fraction reported in rats and humans. This could potentially explain why a similar dose-dependent response was not observed in wild-type rats administered sildenafil.[101] An improvement on the study reported here would be regular collection and analysis of blood samples from treated dogs to determine accurately the serum concentration of sildenafil achieved during the study and determine if a significant correlation exists between the serum levels achieved and the alteration in ERG responses detected.

Expression levels of rod photoreceptor cGMP-PDE α- and β-subunits have been determined in mouse retina.[126] Rod PDEα mRNA levels were lower compared to PDEβ mRNA levels, at ~1.5 x 10^8 and 7.5 x 10^8 copies/μg respectively, in normal mice.[126] However, more efficient post-transcriptional regulation of PDEα protein synthesis and/or trafficking resulted in equimolar cellular protein concentrations.[126] Interestingly cGMP-PDE α- and β-subunit mRNA expression levels differed according to genotype in the

retinal degeneration (*rd1*) mouse.[127] The *rd1* mouse possesses a recessively inherited spontaneous nonsense ochre mutation in codon 347 within exon 7 of the *rd PDE6B* gene.[128] This nonsense mutation causes a TAC → TAA transversion or substitution of a cytosine (pyrimidine) to an adenine (purine) resulting in the stop codon TAA. This chain termination truncates more than half of the normal peptide chain, including the β–subunit catalytic domain.[128] Homozygous affected *rd1/rd1* mutant mice phenotypically exhibit a rapid, early onset of rod-led photoreceptor degeneration beginning at postnatal day 8 (P8).[129] Unlike the homozygous affected *rd1* mice, heterozygous *rd1/+* mice develop histologically normal rod outer segments by P21and have no evidence of retinal degeneration up to 26 months of age.[129] Equimolar PDEα mRNA levels were observed in +/+, *rd1*/+, and *rd1/rd1* mice, however carrier *rd1/+* mice over expressed PDEβ protein apparently to compensate for the mutant allele.[127] Currently the level of PDEα mRNA transcription in dogs heterozygous for a gene mutation encoding the α-subunit of cGMP-PDE are unknown. The *rcd3* Cardigan Welsh corgi dog would be an ideal animal model to investigate PDEα transcription and translation and the effects sildenafil might have on PDE6α activity. The question arises, do heterozygous *Pde6a*$^{+/-}$ dogs over express PDEα protein in a manner similar to the *rd1* mice when compared to *Pde6a*$^{+/+}$ and *Pde6a*$^{-/-}$? And if so, is the difference in ERG alterations observed in *Pde6a*$^{+/-}$ dogs associated with more abundant PDEα protein level compared to the significantly reduced ERG alteration observed in *Pde6a*$^{+/+}$ dogs, which in comparison may have lower PDEα

protein levels? If *Pde6a*$^{+/+}$ dogs have lower PDEα protein levels it may be possible that sildenafil has a greater inhibitory effect on PDE6 in these dogs compared to the over compensating *Pde6a*$^{+/-}$ dogs. This could account for the mild rightward shift in the dark-adapted b-wave responses observed at the lowest six light intensities in *Pde6a*$^{+/-}$ dogs. Quantification of PDEα mRNA and subsequent protein expression would be similar to that performed by Phelan et al[127], and include reverse transcriptase polymerase chain reaction, *in situ* hybridization and analytical quantification, and western blot analyses techniques from retinas of *Pde6a* +/+, +/-, and -/- dogs. Further characterization of PDEα expression within *Pde6a*$^{+/-}$ dogs may elucidate a mechanism by which altered functional visual disturbances manifest in association with sildenafil use.

Recent immunohistochemical localization of PDE5 within the inner retina has raised the second question: do the visual disturbances associated with sildenafil use occur due to inhibition of PDE5 within bipolar and ganglion cells alone, or does it occur in combination with PDE6 inhibition in photoreceptors?[58] Pharmacological ERG dissection during times of peak sildenafil plasma levels (1 – 2 hours post sildenafil administration) would be helpful in isolating the precise location sildenafil interacts within the retina. Origination of the ERG a-wave has been extensively researched with the use of pharmacologic agents such as L-2-amino-4-phosphonobutyric acid (APB or AP4), cis-2, 3-piperidine dicarboxylic acid (PDA), and kynurenic acid (KYN).[122,130] APB, a metabotropic glutamate receptor (mGluR6) agonist, blocks the light-induced responses of depolarizing ON bipolar cells as well as more proximal ON pathway contributions.[130]

Both PDA and KYN are ionotropic glutamate receptor (iGluR) antagonists that block signal transmission to hyperpolarizing OFF bipolar cells and horizontal cells, as well as amacrine and ganglion cells in both ON and OFF pathways.[122] Intravitreal administration of APB has no effect on the a-wave amplitude[131], thus resulting in a negative-going photoreceptor generated waveform. The combination of APB + PDA will generate a pure photoreceptor driven response; however, the a-wave is slightly reduced in amplitude with the addition of PDA.[131] If sildenafil directly inhibits retinal PDE5 in bipolar and ganglion cells, the combination of APB + PDA should result in no change in the isolated a-wave slope compared to controls. However, a reduction in a-wave amplitude and prolongation in latency, depicted by a rightward shift in the a-wave slope, may occur if its action affects photoreceptor PDE6 function. Further differentiation of rod- vs. cone-driven isolated a-wave response under the influence of sildenafil inhibition could be determined by subtracting the pure cone-driven light-adapted response from the mixed dark-adapted rod-cone a-wave response. This will determine if sildenafil's PDE6 inhibition is exclusively rod-mediated, cone-mediated, or mixed.

Intravitreally administered Ba^{2+} reportedly blocks inward-rectifying K^{+} channels in Müller cells and blocks slow PIII as well as the M-wave and the STR.[132-134] A pure b-wave amplitude waveform can be isolated under dark-adapted conditions with intravitreal Ba^{2+}. The physiological effect sildenafil has on bipolar cells can be determined by investigations of dark-adapted b-wave isolated responses in groups with or without sildenafil administration. A significant reduction in amplitude and prolongation

in implicit time would be anticipated with sildenafil's direct inhibition of PDE5 occurs in bipolar cells. These pharmacological investigations would provide insight into the mechanism by which sildenafil induces visual disturbances during high-dose treatment.

In preclinical safety studies, morphometric retinal cell layer counts and thickness measurements in *Pde6a*$^{+/+}$ dogs administered 50 mg/kg sildenafil for 6- to 12-months were not different from untreated controls.[45] Our findings of regional differences in retinal layer thickness and ONL photoreceptor nuclei counts between carrier dogs were inconsistent with the preclinical histopathological safety investigations. Variations observed between carrier groups may be associated with the low subject number and the random assortment of subjects with lower ONL cell counts and thinner retinal layers into the same group. This random assortment of subjects may have resulted in significant differences due to low subject numbers. In order to achieve a statistical power of $\geq$ 80% a minimum of 41 dogs would be required to rule out retinal thickness and ONL cell count variation between groups. The observed histopathological differences in dogs administered sildenafil could also be explained by a slowly progressive retinal degeneration resulting from chronic drug exposure. It is unknown if transient photoreceptor stress occurs during peak sildenafil plasma levels or if chronic usage will result in gradual photoreceptor loss due to intermittent periods of photoreceptor stress. A proposed method of investigating this would be a long-term (for example 2+ years), high-dose (between 10 to 15 mg/kg *per os* daily) study utilizing *Pde6a*$^{+/-}$ and *Pde6a*$^{+/+}$ dogs. This investigation would include placebo- and sildenafil-treated groups for each genotype. The groups could be arranged with five dogs per group where two groups,

placebo and sildenafil-treated groups, per genotype are serially sacrificed every 6 months until study completion. The same premortem and antimortem recordings would be performed as in this pharmacologic investigation, except testing would coincide with the time of reported peak sildenafil plasma levels. This will accurately identify active photoreceptor stress at the time of visual assessment, retinal functional analyses, and euthanasia and globe fixation. Serial retinal thickness measurement via optical coherence tomography or adaptive optics can also facilitate accurate and repeated monitoring of retina layer thickness changes during sildenafil treatment. An additional factor that can be investigated is the expression levels of retinal PDE6 between *Pde6a*$^{+/-}$ and *Pde6a*$^{+/+}$ groups with chronic sildenafil usage. Continued investigation of the effect of sildenafil on carriers of PDE mutations will contribute significantly to an understanding of the risk for vision compromise in individuals associated with long-term use of PDE-inhibitors for treatment of cardiovascular diseases.

APPENDIX

Appendix Table 1: Scotopic a-wave ANOVA table for acute study phase.

Effect	DF	DF	F Value	Pr > F
Group	2	5	14.01	0.0089
Time	2	10	16.3	0.0007
Group*Time	4	10	6.51	0.0076
Intensity	9	45	363.05	<.0001
Group*Intensity	18	45	10.69	<.0001
Intensity*Time	18	90	7.56	<.0001
Group*Intensity*Time	36	90	4.91	<.0001

Appendix Table 2: Scotopic a-wave ANOVA table for chronic study phase.

Effect	DF	DF	F Value	Pr > F
Group	1	3	15.64	0.0288
Time	6	17	11.43	<.0001
Group*Time	6	17	4.46	0.0069
Intensity	9	27	405.71	<.0001
Group*Intensity	9	27	6.53	<.0001
Intensity*Time	54	153	5.88	<.0001
Group*Intensity*Time	54	153	2.95	<.0001

Appendix Table 3: Scotopic b-wave ANOVA table for acute study phase.

Effect	DF	DF	F Value	Pr > F
Group	2	5	3.91	0.0949
Time	2	10	7.66	0.0096
Group*Time	4	10	1.82	0.2022
Intensity	13	65	179.4	<.0001
Group*Intensity	26	65	3.22	<.0001
Intensity*Time	26	130	1.51	0.069
Group*Intensity*Time	52	130	1.49	0.0367

Appendix Table 4: Scotopic b-wave ANOVA table for chronic study phase.

Effect	DF	DF	F Value	Pr > F
Group	1	3	6.19	0.0886
Time	6	17	3.13	0.0296
Group*Time	6	17	1.54	0.2251
Intensity	13	39	194.49	<.0001
Group*Intensity	13	39	2.7	0.0082
Intensity*Time	78	221	1.33	0.0553
Group*Intensity*Time	78	221	1.68	0.0017

Appendix Table 5: Criterion threshold ANOVA table for acute study phase.

Effect	**DF**	**DF**	**F Value**	**Pr > F**
Group	2	5	1.68	0.2763
Phase	3	12	15.48	0.0002
Group*Phase	4	12	4.69	0.0164

Appendix Table 6: Criterion threshold ANOVA table for chronic study phase.

Effect	**DF**	**DF**	**F Value**	**Pr > F**
Group	1	3	88.04	0.0026
Phase	6	17	5.58	0.0023
Group*Phase	6	17	4.84	0.0047

Appendix Table 7: ONL nuclei counts ANOVA table

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23910.338	2	11955.169	7.542	0.023
Within Groups	9510.585	6	1585.097		
Total	33420.923	8			

Appendix Table 8: Retinal thickness measurements ANOVA table

		Sum of Squares	df	Mean Square	F	Sig.
RPE	Between Groups	0.793	2	0.397	1.431	0.31
	Within Groups	1.663	6	0.277		
	Total	2.456	8			
PR	Between Groups	1.043	2	0.521	0.037	0.964
	Within Groups	83.806	6	13.968		
	Total	84.849	8			
ONL	Between Groups	207.426	2	103.713	9.799	0.013
	Within Groups	63.503	6	10.584		
	Total	270.929	8			
OPL	Between Groups	1.448	2	0.724	0.897	0.456
	Within Groups	4.84	6	0.807		
	Total	6.288	8			
INL	Between Groups	31.91	2	15.955	2.902	0.131
	Within Groups	32.988	6	5.498		
	Total	64.897	8			
IPL	Between Groups	5.015	2	2.508	0.299	0.752
	Within Groups	50.243	6	8.374		
	Total	55.259	8			

Appendix Table 8 (cont'd): Retinal thickness measurements ANOVA table

		Sum of Squares	df	Mean Square	F	Sig.
GCL	Between Groups	1.669	2	0.834	0.862	0.469
	Within Groups	5.807	6	0.968		
	Total	7.476	8			
NFL	Between Groups	141.709	2	70.855	2.197	0.192
	Within Groups	193.5	6	32.25		
	Total	335.209	8			
TOTAL NFL	Between Groups	171.468	2	85.734	2.552	0.158
	Within Groups	201.537	6	33.59		
	Total	373.005	8			

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